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Association of high fetuin-B concentrations in serum with fertilization rate in IVF: a cross-sectional pilot study

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STUDY QUESTION: Is serum fetuin-B associated with the fertilization rate in *in vitro* fertilization (IVF)?

SUMMARY ANSWER: Serum fetuin-B increased during IVF cycles when oocytes could be fertilized while remained unchanged in fertilization failure.

WHAT IS KNOWN ALREADY: Fetuin-B deficiency in mice causes premature zona pellucida hardening mediated by the zona protease ovastacin. Thus fetuin-B deficiency renders females infertile.

STUDY DESIGN, SIZE, DURATION: We determined the human serum fetuin-B reference range, studying longitudinally, over the course of one month, five male and seven female volunteers without hormone treatment and four female volunteers on varying hormonal contraception. We sampled blood and determined serum fetuin-B, luteinizing hormone (LH), estradiol (E2) and progesterone (P4). In addition, we determined serum fetuin-B and estradiol in eight women undergoing intracytoplasmatic sperm injection (ICSI, nine ICSI cycles) and 19 women undergoing IVF (21 IVF cycles) after ovarian stimulation with recombinant human follicular stimulating hormone (rFSH) and/or a combined medication of FSH and LH. At least three blood samples were analyzed in each cycle. We compared serum fetuin-B and follicular fluid fetuin-B in nine patients by measuring follicular fetuin-B in pooled follicular fluid, and in fluid obtained from individual follicles. Samples were drawn from January 2012 to March 2014.

PARTICIPANTS/MATERIALS, SETTING, METHOD: All volunteers and patients gave informed consent. Fetuin-B was measured employing a commercial sandwich enzyme-linked immunosorbent assay. Serum fetuin-B was determined as duplicates in 5 male (34 ± 14.6 years) and 11 female volunteers (29.4 ± 4.1 years) as well as in female volunteers on hormonal contraception (30.0 ± 6.5 years). The duplicate standard deviation was 4.0 ± 2.3%. The contraceptive drugs were mono or combined preparations containing 0–0.03 mg ethinyl estradiol, and 0.15–3.0 mg of various progestins. In addition, serum fetuin-B was determined as triplicates in 27 female patients undergoing conventional IVF (19) or ICSI (8). The triplicate standard deviation was 3.3 ± 1.8%. IVF was declared as 'successful', if at least one oocyte was fertilized, and 'unsuccessful', if no oocyte could be fertilized. Patient age was 34.4 ± 4.4 years in successful IVF, and 35.4 ± 3.3 years in unsuccessful IVF. Serum and follicular fluid of patients undergoing controlled ovarian hyperstimulation were analyzed. Serum was drawn at the day of follicle aspiration.

MAIN RESULTS AND THE ROLE OF CHANCE: Serum fetuin-B and follicular fluid fetuin-B were not significantly different in six out of nine patients suggesting, in principle, free exchange of fetuin-B between serum and follicular fluid. Thus serum fetuin-B may be used as a proxy of follicular fluid fetuin-B. Serum fetuin-B increased during successful IVF cycles ($n = 15$, $P < 0.0001$), but did not change in unsuccessful IVF cycles ($n = 6$, $P = 0.118$) despite increased estradiol levels ($P = 0.0019$ and $P = 0.0254$, respectively).

LIMITATIONS, REASONS FOR CAUTION: The female volunteers self-reported their respective hormone medication. Medication was verified by serum estradiol, LH and progesterone measurements. For oocyte harvesting, the vaginal wall was punctured once only to minimize co-morbidity. Low grade cross-contamination of individual follicular fluid aspirates and contamination of the follicular fluid with small amounts of blood were inevitable. Samples were routinely checked for the presence of hemoglobin that would suggest blood contamination. Only samples containing <250 erythrocyte equivalents/μl were used for analysis.

WIDER IMPLICATIONS OF THE FINDING: Serum fetuin-B may be used as a marker to predict the fertilization success in IVF. Fetuin-B levels attained during IVF stimulation may help to make an informed decision whether oocytes should be fertilized by IVF or by ICSI to overcome the zona pellucida as a barrier.

STUDY FUNDING/COMPETING INTEREST(S): The research was supported by a grant from Deutsche Forschungsgemeinschaft and by the START program of the Medical Faculty of RWTH Aachen University. J.F., E.D., J.N., B.R. and W.J.-D. declare that they are named inventors on the RWTH Aachen University patent application EP 13157317.2, 'Use of fetuin-B for culture of oocytes', applied for by RWTH Aachen University.

Key words: fetuin-B / infertility / IVF fertilization rate / zona pellucida hardening / follicular fluid / early embryo development / sperm attachment / embryo hatching

Introduction

One critical factor for mammalian fertilization is the penetration of the zona pellucida by sperm. The zona pellucida is a glycoprotein matrix that surrounds the oocyte (Zhu *et al.*, 2015). Zona pellucida proteins control sperm attachment, penetration and thus fertilization success. The human zona pellucida contains four zona proteins (ZP1–ZP4) (Bauskin *et al.*, 1999; Hughes and Barratt, 1999; Lefièvre *et al.*, 2004), the mouse zona pellucida is composed of three proteins (ZP1–ZP3) (Wassarman, 1988), and an additional silent pseudo gene for ZP4 (Lefièvre *et al.*, 2004). Overall, the zona pellucida structure is highly conserved in mammals (Wassarman, 2008) rendering mice an appropriate model to study the role of zona pellucida components in fertilization. Published data suggest that the microscopic appearance of the zona pellucida is a useful marker for successful pregnancies after *in vitro* fertilization (IVF). The zona pellucida thickness is associated with the fertilization success (Palmstierna *et al.*, 1998). The thickness of the zona pellucida is generally believed to indicate the level of zona pellucida hardening (ZPH). ZPH physiologically occurs after fertilization and involves the cleavage of zona pellucida proteins by proteases released from the cortical granules of oocytes, an event triggered by sperm entry. The metalloprotease ovastacin, which cleaves ZP2 to the fragmented ZP2f thus triggers definitive ZPH (Burkart *et al.*, 2012). ZPH can be monitored by analyzing the cleavage of ZP2 (Bleil *et al.*, 1981; Bauskin *et al.*, 1999), the non-attachment of sperm (Braden *et al.*, 1954) as well as a longer digestion time of the zona pellucida using various experimental protocols (Inoue and Wolf, 1974; Drobnis *et al.*, 1988).

Premature ZPH, the hardening of the ZP before fertilization, is a hitherto unspecified complication of IVF both in humans (ICD N98.9) and animals that drastically reduces fertilization success (Schiewe *et al.*, 1995). Recently, we showed that premature ZPH can occur *in vivo*. Female fetuin-B deficient mice were completely infertile because of premature ZPH (Dietzel *et al.*, 2013). Fetuin-B is a member of the cystatin superfamily of protease inhibitors (Lee *et al.*, 2009). Fetuin-B is made in the liver and secreted into the circulation supplying peripheral tissues (Denecke *et al.*, 2003). The fetuin-B gene is well conserved between mammals with 61% sequence homology in mice rats and humans (Olivier *et al.*, 2000). Therefore, we determined serum and follicular fluid fetuin-B levels in healthy volunteers and in patients attending our infertility clinic, in an attempt to study the role of fetuin-B for the first time in human female reproductive biology.

Materials and Methods

Study population and design

We measured longitudinally serum fetuin-B and hormones luteinizing hormone (LH), estradiol (E2) and progesterone (P4) in five healthy male volunteers (34.3 ± 14.6 years), in seven female volunteers with menstrual cycle (29.4 ± 4.1 years) and in four female volunteers on hormonal contraception (30.0 ± 6.5 years). The female volunteers took either no contraceptive drugs or mono or combined preparations containing 0–0.03 mg ethinyl estradiol, and 0.15–3.0 mg of various progestins as specified in the figure legends.

Serum fetuin-B and sex hormones were also measured in 25 patients undergoing assisted reproduction technique (ART) with either gonadotrophin stimulated IVF cycles ($n = 21$, 34.7 ± 4.2 years) or intracytoplasmatic sperm injection (ICSI) cycles ($n = 9$, 37.3 ± 4.8 years). Four patients had one IVF cycle followed by one ICSI cycle, respectively; one patient had two ICSI cycles. At least three blood samples of each IVF cycle were analyzed for the correlative analysis of serum fetuin-B, sex hormones and fertilization rate. IVF was successful (oocytes fertilized) in fifteen patients (34.4 ± 4.4 years), of which eight became pregnant; six patients (35.4 ± 3.3 years) failed IVF with no fertilized oocyte.

Fetuin-B and albumin serum levels were also measured in patient-matched follicular fluids from nine patients undergoing oocyte aspiration (35.3 ± 4.0 years). Albumin content was taken as a proxy of overall protein concentration in the follicular fluid. The fetuin-B–albumin ratio was calculated in matched serum and follicular fluid samples to judge the volume distribution of these major plasma proteins. Pooled follicular fluid was obtained from five patients, and individual follicular fluids from altogether 69 follicles was obtained from another five patients. Low grade cross-contamination of individual follicular fluid aspirates and contamination of the follicular fluid with small amounts of blood was controlled by routinely checking for the presence of hemoglobin, which would suggest blood contamination. Only samples with a hemoglobin content representing <250 erythrocytes/μl (Combur-Test, Roche Diagnostics, Mannheim, Germany) were used for the analysis.

Serum and follicular fluid sampling

Blood was collected into S-Monovette serum tubes (Sarstedt, Nümbrecht, Germany) following venous puncture. After 1 h clotting time, serum was separated by centrifugation (1500 × *g*, 10 min, 4°C). Serum was transferred to fresh microtubes, snap-frozen in liquid nitrogen and stored at −20°C. The follicular fluid was aspirated during routine oocyte harvest by transvaginal ultrasound-guided follicular puncture and was cleared by centrifugation (200 × *g*, 5 min, 4°C). The clear supernatant was snap-frozen in liquid nitrogen and stored at −20°C.

Protein and hormone measurements

Fetuin-B was assayed in triplicates using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (human fetuin B DuoSet; R&D Systems, Minneapolis, USA), following the manufacturer's protocol. E2, P4 and LH were measured by electrochemiluminescence immunoassay (ECLIA; Cobas Roche Diagnostics, Mannheim, Germany), human serum albumin was measured using a colorimetric assay with bromocresol green (Cobas Roche Diagnostics).

Statistical analysis

Data were analyzed using GraphPad Prism 5.0c (GraphPad Software, San Diego, CA, USA) as detailed in the respective figure legends. Serum fetuin-B was analyzed by column statistics as mean ± SD. We employed the Tukey method of plotting outliers, which are defined as separated more than 1.5 interquartile distances IQR from the 75 percentiles. Coefficient of variation, CV, equals the standard deviation divided by the mean expressed as a percent. The Student *t*-test was used to compare fetuin-B levels in serum and follicular fluid. The Spearman correlation test was used to study the correlation between fetuin-B and estradiol. Serum fetuin-B levels are represented as measured with the exception of Fig. 5, where serum fetuin-B is represented as a linear regression of measured values. A $P < 0.05$ was regarded as significant.

Ethical approval

This study was approved by the local ethics committee of the RWTH Aachen University Clinics in accordance with the World Medical Association Helsinki Declaration on Ethical Principles for Medical Research Involving Human Subjects. Informed written consent was obtained from all participating volunteers and patients.

Results

Serum fetuin-B in humans

We measured the serum fetuin-B levels in humans attained over the course of 1 month. Figure 1 shows that the serum fetuin-B levels varied between 2 and 5 μg/ml with an overall CV of 19.4% in women (mean ± SD, 3.6 ± 0.7 μg/ml) and 33.3% in men (3.6 ± 1.2 μg/ml) (Fig. 1A and B, respectively). One male measuring at 6 μg/ml serum fetuin-B was identified as an outlier. The remaining four males had serum fetuin-B levels of 3.0 ± 0.4 (CV, 13.3%). Intra-individual serum fetuin-B CV over the course of 1 month ranged from 7.1 to 24.0% in women, and from 4.9 to 13.0% in men. Collectively the data indicate a more constant hepatic expression of fetuin-B in men than in women.

Fetuin-B expression is stimulated by ethinyl estradiol

Figure 2 illustrates that serum fetuin-B was elevated in women on hormonal contraception. In women on medication with combined 17α-ethinyl estradiol and progestin; 0.03 mg EE_2 and 0.15 mg Levonorgestrel (Fig. 2A), 0.03 mg EE_2 and 2 mg Dienogest (Fig. 2B) or 0.03 mg EE_2 and 3 mg Drospirenon (Fig. 2C) serum fetuin-B levels increased, and rapidly decreased during the treatment-free interval suggesting steroid-dependent hepatic gene expression of fetuin-B. This steroid-induced increase in serum fetuin-B was observed in all women, irrespective of the basal serum fetuin-B level. Maximum serum fetuin-B levels reached 15 μg/ml (Fig. 2C) and thus up to 4-fold normal serum levels. Progestin alone (2 mg Dienogest, Fig. 2D) had no stimulatory effect, but a treatment change to an ethinyl estradiol containing pill in the same volunteer stimulated serum fetuin-B expression (Fig. 2E).

Fetuin-B levels in serum and follicular fluid are tightly associated

We previously showed that fetuin-B is predominately made in the liver (Denecke *et al.*, 2003). For fetuin-B to exert its activity onto oocytes, it must therefore traverse the follicle wall. We never measured fetuin-B content in mouse follicular fluid because of experimental difficulties. Copious amounts of follicular fluid can however, be harvested during follicle puncture and oocyte isolation in IVF patients. We compared the fetuin-B level in follicular fluid to serum fetuin-B. Figure 3A illustrates the results of such a comparison in nine patients, demonstrating that serum fetuin-B and follicular fluid fetuin-B are tightly associated in two-thirds of individuals tested. Generally, the tight association of follicular fluid fetuin-B with serum fetuin-B supports the view that serum fetuin-B is the source of follicular fluid fetuin-B. Three out of nine patients had significantly lower follicular fluid fetuin-B than serum fetuin-B. Nevertheless, the results suggest that serum fetuin-B passes relatively freely into the follicular fluid. Therefore, serum fetuin-B can be taken as a proxy of follicular fluid fetuin-B. Both serum and follicular fluid fetuin-B levels did, however, vary considerably between individual patients undergoing hormone treatment for ART. Figure 3B shows that follicular fluid isolated from several individual follicles derived from one donor all contained comparable amounts of fetuin-B. Patient 48 had one outlier follicle that contained substantially lower follicular fluid fetuin-B. Figure 3C shows that this difference vanished when the ratio of fetuin-B–albumin was determined in each follicular fluid. These values clustered even closer than the fetuin-B concentrations, suggesting

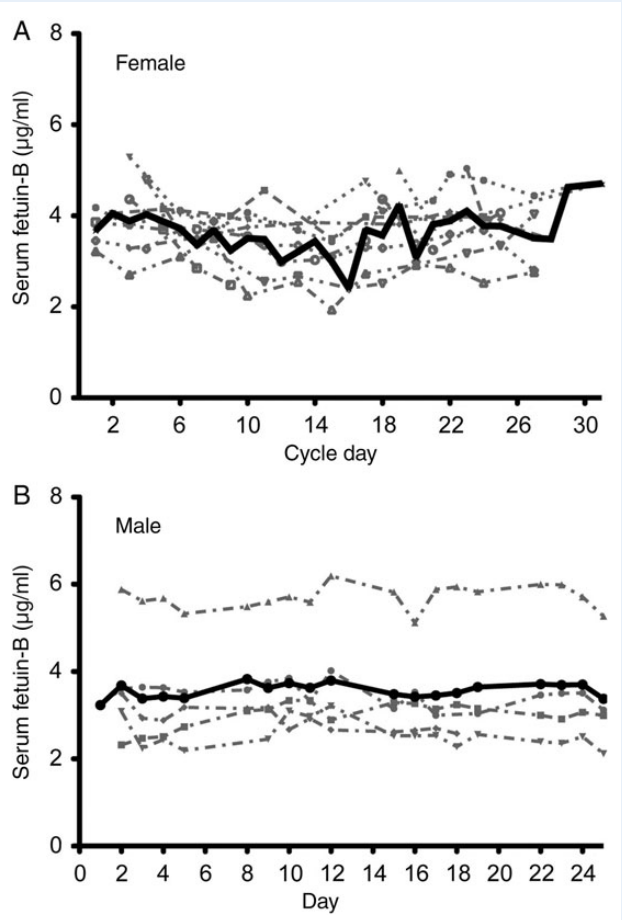

Figure 1 Serum fetuin-B level variation over the course of 1 month (**A**) in female menstrual cycles ($n = 8$, seven individuals) and (**B**) in males ($n = 5$). Female cycle day 1 in (A) corresponds to day 1 of menstruation. Each curve represents one individual. The black lines represent the mean of all individuals.

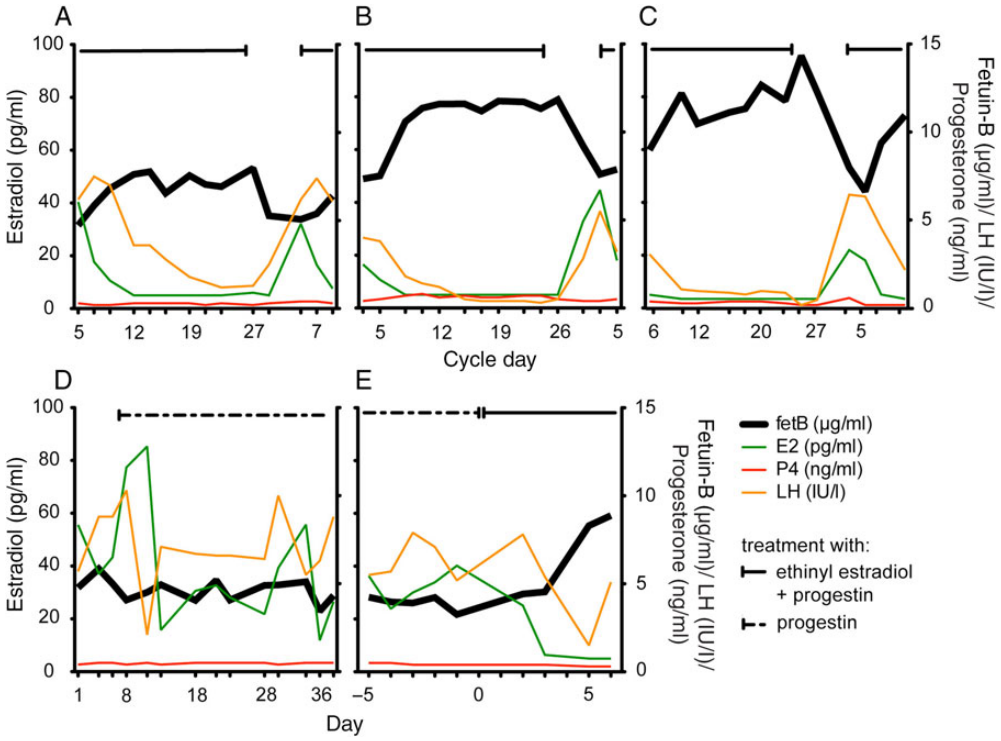

Figure 2 Serum fetuin-B in women on contraceptive medication. Women received combinations of ethinyl estradiol and progestin, (**A**) 0.03 mg EE_2 and 0.15 mg levonorgestrel, (**B**) 0.03 mg EE_2 and 2 mg Dienogest (**C**), 0.03 mg EE_2 and 3 mg Drospirenon. The woman in (**D**) took 2 mg Dienogest alone, followed by (**E**) 0.03 mg ethinyl estradiol and 2 mg Dienogest. Combined ethinyl estradiol and progestin stimulated serum fetuin-B expression, and serum levels dropped during the treatment-free interval. Progestin alone did not stimulate fetuin-B serum expression suggesting that the up-regulation fetuin-B expression by a combination contraceptive drug is caused by ethinyl estradiol. Serum estradiol (E2), progesterone (P4) and LH followed the expected patterns given the respective medication.

that variations in follicular fluid fetuin-B reflect overall protein content which is known to increase with follicle maturation (Spitzer *et al.*, 1996). Thus the outlier follicle from Patient 48 was most likely an immature follicle.

Fetuin-B expression is stimulated by high endogenous estradiol

The synthetic high potency estrogen 17α-ethinyl estradiol stimulated serum fetuin-B (Fig. 2). We asked whether high endogenous estradiol would likewise stimulate fetuin-B expression. Linear regression of matched serum estradiol and serum fetuin-B in women undergoing spontaneous menstrual cycling showed no correlation ($n = 99$, seven individuals; $r = 0.176$; $r^2 = 0.015$; $P = 0.08$) (Fig. 4A). In contrast, the correlation between serum estradiol and serum fetuin-B became statistically significant ($n = 142$, 25 individuals; $r = 0.414$; $r^2 = 0.249$; $P < 0.0001$) (Fig. 4B) at very high endogenous estradiol, like the ones attained upon hormonal treatment before IVF or ICSI, respectively. Unlike serum estrogen, serum progesterone or LH were not associated with serum fetuin-B concentration (data not shown).

Serum fetuin-B is associated with fertilization rate in IVF

Knowing that the lack of fetuin-B leads to premature ZPH in mice and thus to blocked fertilization, we asked whether a similar correlation also existed in human patients undergoing IVF. To this end, we correlated serum fetuin-B in patients undergoing ovarian stimulation with the fertilization rate in IVF. Successful IVF was defined if at least one oocyte fertilized during the respective cycle. Successful IVF procedures were associated with increasing serum fetuin-B ($n = 15$, $P < 0.0001$) during the ovarian stimulation (Fig. 5A). In contrast, fertilization failure (unsuccessful IVF = no oocyte fertilized) showed on average no increase of serum fetuin-B ($n = 6$, $P = 0.118$) (Fig. 5B). Thus increased serum fetuin-B may be predictive of fertilization rate in IVF. Endogenous estradiol were routinely checked for the ovarian response to controlled hormonal ovarian hyperstimulation and increased in both IVF with fertilized oocytes and fertilization failure, respectively (Fig. 5C and D). Here, we studied fertilization rate in IVF only. We did not correlate serum fetuin-B in IVF stimulation with clinical pregnancy, because our work in mice showed that fetuin-B is essential for fertilization, but not for later stages of pregnancy.

Discussion

Fetuin-B, a potent ovastacin inhibitor, prevents ZPH before fertilization and thus maintains oocytes in a fertilizable state. The state of the zona pellucida is essential for IVF success both in humans and animals (Braden *et al.*, 1954; Dell'Aquila *et al.*, 1999; Primakoff and Myles, 2002). ZPH occurs naturally following fertilization but also spontaneously during

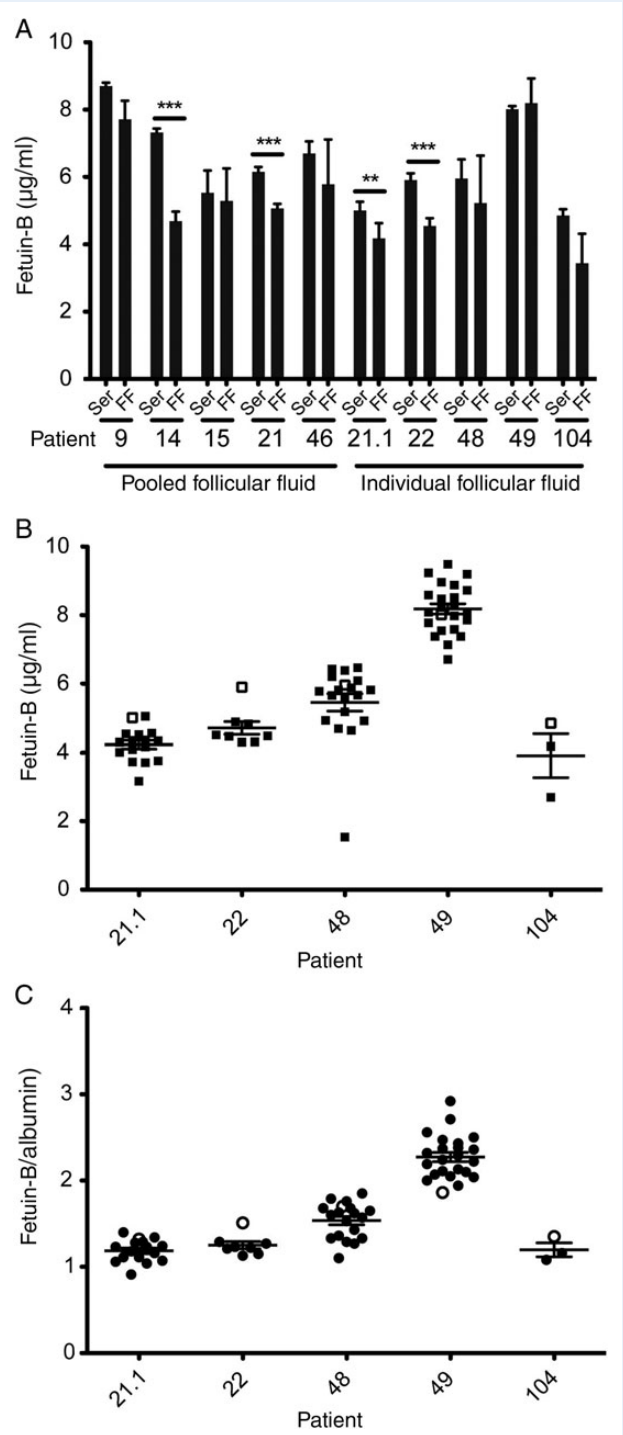

Figure 3 Serum and follicular fluid fetuin-B. (**A**) The intra-individual serum (Ser) and follicular fluid fetuin-B ratios were not significantly different in six out of nine patients. (A, left) The follicular fluid of patients 9, 14, 15, 21, 46 were pooled during oocytes aspiration. (A, right) The follicular fluid of patients 21.1, 22, 48, 49 and 104 were aspirated individually. The fetuin-B concentration of pooled and individual follicular fluid was measured, and the mean value was calculated. (**B**) Fetuin-B concentration of individual follicular fluid (black squares) and sera (white squares). (**C**) Fetuin-B over albumin ratio corrected the outliers shown in (B). Two tailed Student's *t*-test; $^{**}P < 0.01$, $^{***}P < 0.001$.

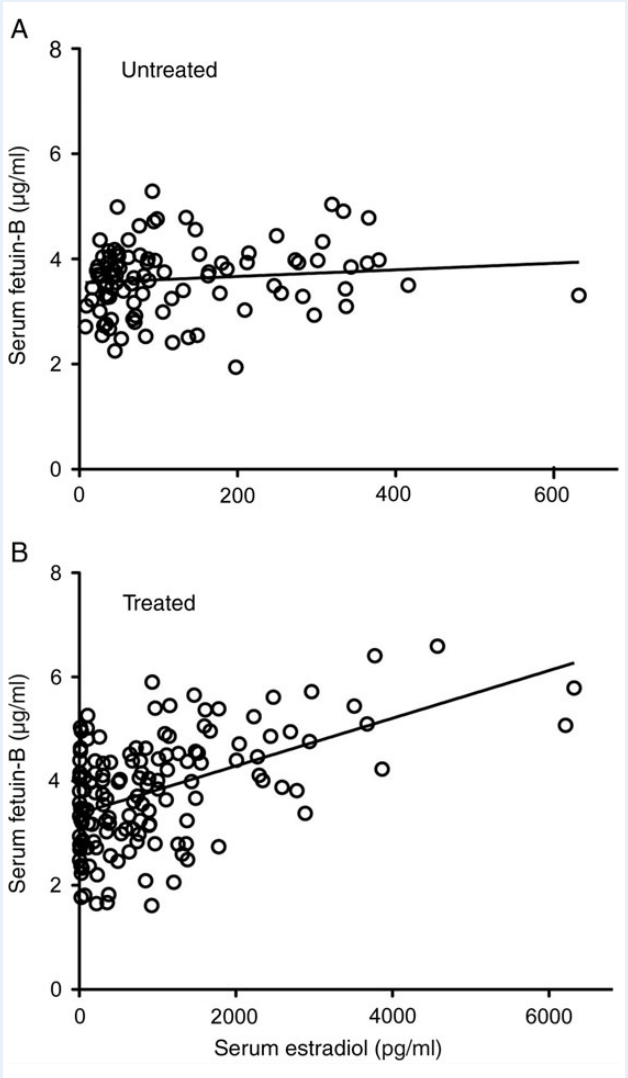

Figure 4 Association of serum fetuin-B and serum estradiol in women during menstrual cycle and in women undergoing hormonal ovarian stimulation. Each data point represents matched serum fetuin-B and estradiol concentrations measured during spontaneous menstrual cycle of untreated individuals ($n = 99$, 7 individuals) and during treatment cycles of fertility clinic patients ($n = 142$, 25 individuals). The solid lines depict Spearman linear regression (**A**) $r = 0.176$; $r^2 = 0.015$; $P = 0.08$; (**B**) $r = 0.414$; $r^2 = 0.249$; $P < 0.0001$).

in vitro oocyte culture in mouse (DeFelici and Siracusa, 1982), rats (Zhang *et al.*, 1991), horses (Dell'Aquila *et al.*, 1999) and humans (Schiewe *et al.*, 1995). Recently, we described that premature ZPH also occurs *in vivo* before fertilization in fetuin-B deficient mice (Dietzel *et al.*, 2013). To determine the role of fetuin-B in human female reproductive biology, we measured the fetuin-B level in serum and follicular fluid. Male and female serum fetuin-B levels in healthy volunteers remained constant over the course of 1 month. In females, serum fetuin-B was unaffected by the menstrual cycle and its associated hormonal changes. Ethinyl estradiol in healthy female volunteers on hormonal contraception, and very high estrogen levels as shown for exceeding 600 pg/ml in IVF and ICSI patients undergoing hormonal stimulation were, however, associated

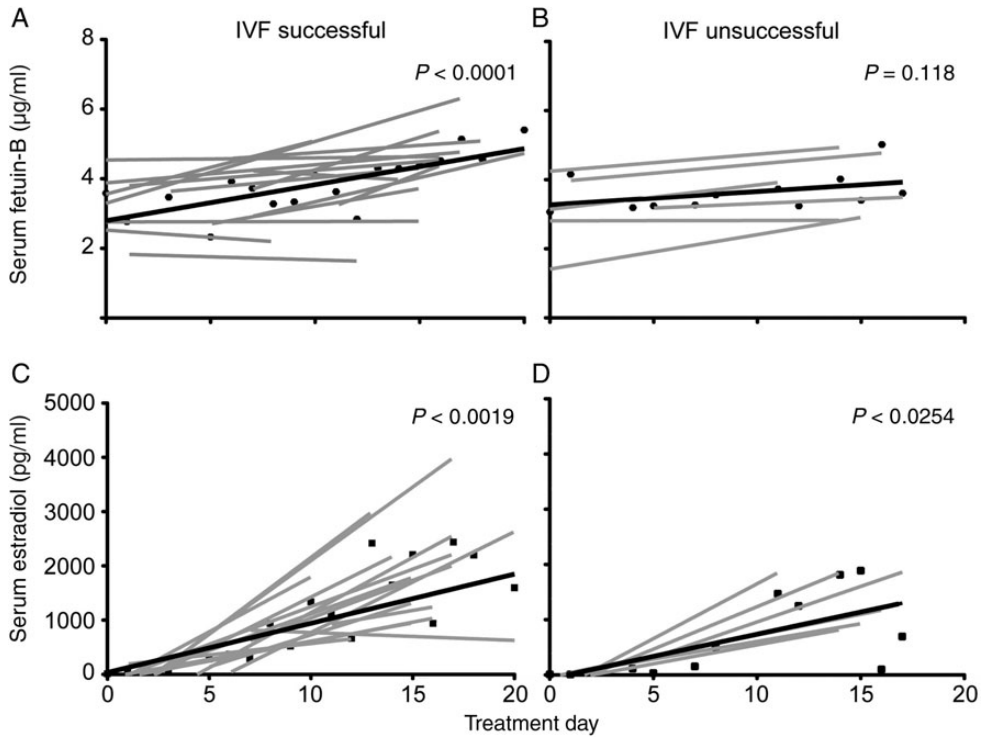

Figure 5 Association of serum fetuin-B and fertilization rate in IVF. (**A**) Serum fetuin-B increased upon ovarian stimulation in successful (at least one oocyte was fertilized) IVF cycles ($n = 15$, $P < 0.0001$), but remained unchanged (**B**) in unsuccessful (no oocyte fertilized) IVF cycles ($n = 6$, $P = 0.118$). Endogenous estradiol increased in both successful (**C**) and unsuccessful (**D**) IVF cycles. Gray lines represented individual IVF cycles. Black lines depict the linear regression curves of mean serum fetuin-B and estradiol, respectively.

with increased serum fetuin-B. This suggested an indirect estrogen-mediated regulation of hepatic fetuin-B expression. The male subject having up to 6 µg/ml serum fetuin-B had however, no conspicuous changes in estradiol, progesterone and LH arguing against exclusive estrogen regulation of fetuin-B expression. An estrogen receptor-binding site (GGTCANNNTGACC), which could potentially mediate the estrogen induction of fetuin-B, is present between the genetic loci of α2-Heremans-Schmid glycoprotein (AHSG) and fetuin-B on human chromosome 3 (Fullwood *et al.*, 2010). However, the estrogen receptor-binding site is located ~8000 bp upstream of the fetuin-B gene, suggesting that major DNA conformational changes must occur for this site to exert a direct influence on the fetuin-B synthesis. Accordingly, fetuin-B expression was affected by exceedingly high endogenous estradiol only. In summary, a direct stimulation by estrogen of fetuin-B gene expression is unlikely. Instead very high estradiol concentrations, and the potent synthetic estrogen ethinyl estradiol may lead to signaling cross-talk, and the activation transcription factors other than the estrogen receptor. Studies in cultured human hepatoma cells indeed demonstrated that fetuin-B expression is induced by the farnesoid X receptor (Murakami *et al.*, 2007). Gene reporter studies involving estrogen and farnesoid X receptor agonists will tell if serum fetuin-B can be elevated, and if this rescues female infertility.

Recombinant mouse fetuin-B inhibits mouse ovastacin with an IC_{50} of 75 nM (Dietzel *et al.*, 2013), and we assume a similar IC_{50} for human fetuin-B and ovastacin. Given the molecular weight of fetuin-B (~50 kDa) and the respective serum concentrations in mice (150 µg/ml equivalent to 3000 nM, 5 µg/ml equivalent to 100 nM in human) fetuin-B is expected to act as potent ovastacin inhibitor *in vivo* as well. These numbers illustrate that the prevention of premature ZPH discovered in mice may be even more relevant to human reproductive biology. During the maturation process of human oocytes serum fetuin-B must be maintained at a level decidedly higher than the IC_{50} to ensure that follicular fluid fetuin-B keeps the oocyte fertilizable. We hypothesize that slight changes in gene expression in fetuin-B will be functionally significant in humans, because the serum levels are close to the IC_{50} value and therefore fetuin-B might easily become a limiting factor.

Serum fetuin-B is a useful value in this respect because serum and follicular fluid are closely related suggesting that fetuin-B can freely diffuse from the blood into the follicles to act as an ovastacin inhibitor. This finding corroborates previous studies demonstrating that the overall protein concentration in serum and follicular fluid is comparable (Andersen *et al.*, 1976; Spitzer *et al.*, 1996), and that most molecules up to 220–500 kDa can freely pass the blood–follicle barrier (Legge, 1995; Powers *et al.*, 1995; Hess *et al.*, 1998; Schweigert *et al.*, 2006). Several studies reported that the composition of the follicular fluid indicated the maturation stage of the follicle (Spitzer *et al.*, 1996; Schweigert *et al.*, 2006). The association of serum and follicular fluid fetuin-B support this view and renders serum fetuin-B a proxy of the fetuin-B concentration surrounding the oocyte.

Further we showed that serum fetuin-B increased during the hormone treatment of women undergoing IVF with fertilized oocytes, while it remained unchanged in patients with fertilization failure. Serum estradiol increased in both groups indicating an ovarian response to controlled ovarian stimulation. The endogenous estradiol concentration in

fertilization failure increased generally less than in IVF cycles with fertilized oocytes. Our pilot study population is small and therefore the results need further confirmation. The fertilization rate of 29% (6/21 IVF cycles) in our patients was well within range of published fertilization rates of 17–49% (Zhu *et al.*, 2015). Nevertheless, fetuin-B behaved fundamentally different from its closest relative, fetuin-A, which was recently studied regarding its possible role in reproductive biology of women undergoing IVF (Bódis *et al.*, 2014). Similar to fetuin-B, fetuin-A concentration was high in follicular fluid of patients undergoing IVF. However, unlike fetuin-B, fetuin-A was not associated with fertilization success. This is in full agreement with the finding that fetuin-A deficient mice are fully fertile (Jahnen-Dechent *et al.*, 1997) and that fetuin-B, but not fetuin-A, in commercial 'fetuin' preparations was responsible for the inhibition of ZPH (Schroeder *et al.*, 1990; George and Johnson, 1993; Kalab *et al.*, 1993; Dietzel *et al.*, 2013).

In summary, we propose that serum fetuin-B may be useful in predicting the fertilization success in IVF. Fetuin-B levels attained during IVF stimulation may help to make an informed decision whether oocytes should be fertilized by IVF or by ICSI to overcome the zona pellucida as a barrier.

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Authors' roles

J.F., E.D., W.J-D., B.R. and J.N. designed the study. J.F., B.R. and U.W collected the data. J.F., E.D., B.R and W.J-D. analyzed the data. J.F. and W.J-D drafted and revised the manuscript. E.D., B.R., U.W. and J.N. revised the manuscript. All authors have approved the final version of the article.

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Conflict of interest

J.F., E.D., J.N., B.R. and W.J.-D. declare that they are named inventors on the RWTH Aachen University patent application EP 13157317.2, 'Use of fetuin-B for culture of oocytes', applied for by RWTH Aachen University.

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