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ORIGINAL ARTICLE *Embryology*

Circulating maternal prorenin and oocyte and preimplantation embryo development: the Rotterdam Periconception Cohort

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STUDY QUESTION: Could circulating maternal prorenin serve as a proxy for oocyte and preimplantation embryo development, assessed by time-lapse parameters and clinical treatment outcomes?

SUMMARY ANSWER: High circulating maternal prorenin concentrations after ovarian stimulation associate with a larger oocyte area, faster cleavage divisions from the five-cell stage onwards and increased chance of successful implantation.

WHAT IS KNOWN ALREADY: After ovarian stimulation, circulating prorenin (renin's precursor), is largely ovary-derived. Prorenin may contribute to ovarian angiotensin synthesis, which is relevant in reproduction given its role in follicular development and oocyte maturation.

STUDY DESIGN, SIZE, DURATION: Prospective observational cohort study including couples requiring fertility treatment from May 2017 as a subcohort of the ongoing Rotterdam Periconception Cohort conducted in a tertiary referral hospital.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Between May 2017 and July 2020, 309 couples with an indication for IVF treatment or ICSI were included. Resulting embryos (n = 1024) were submitted to time-lapse embryo culture. Time of fertilization (t0), pronuclear appearance (tPNa), and fading (tPNf) as well as the exact timing of reaching the two- to eight-cell stage (t2–t8), the start of blastulation (tSB), reaching the full (tB), and expanded blastocyst (tEB) were retrospectively recorded. Oocyte area was measured at t0, tPNa, and tPNf. Prorenin was determined at the day of embryo transfer.

MAIN RESULTS AND THE ROLE OF CHANCE: After adjustment for patient- and treatment-related factors, linear mixed modeling showed that higher prorenin concentrations associate with a larger oocyte area at tPNa (β 64.45 μm^2, 95% CI 3.26; 125.64, $P = 0.04$), and faster progression from five-cell stage onwards (e.g. $\beta_{8\text{-cell}}$ −1.37 h, 95% CI −2.48; −0.26, $P = 0.02$). Prorenin associated positively with pre-transfer outcomes (e.g. $\beta_{\text{fertilized oocytes}}$ 2.09, 95% CI 1.43; 2.75, $P < 0.001$) and implantation (odds $\text{ratio}_{+\beta\text{-hCG-test}}$: 1.79, 95% CI 1.06; 3.08, $P = 0.03$), but not with live birth.

LIMITATIONS, REASONS FOR CAUTION: This prospective observational study provides associations and therefore residual confounding cannot be excluded and causality has to be shown in intervention studies.

WIDER IMPLICATIONS OF THE FINDINGS: Theca cell-derived factors, such as prorenin, may help to clarify the underlying endocrine mechanism of oocyte maturation and embryo development, with a special focus on the (patho)physiological reproductive role of prorenin and the identification of factors influencing its secretion and activity, which is of great added value for improving embryo selection and predicting implantation and pregnancy outcomes. This will bring us to investigate which determinants of oocyte quality and embryo development should take center stage in developing preconception care strategies.

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Key words: prorenin / renin–angiotensin–aldosterone system / *in vitro* fertilization / oocyte size / embryo development

Introduction

The ovaries as endocrine glands for secreting hormones are the key drivers of human reproduction. Essential for the establishment of pregnancy is adequate follicular development and maturation, ovulation, fertilization, and early embryonic development, which involves significant endocrine alterations. The granulosa- and theca cells, which transform after ovulation into lutein cells, are prominent endocrine cells during reproduction, as they are involved in the production of progesterone, androgens, and estrogens. Remarkably, the circulating plasma levels of prorenin, the biosynthetic precursor of renin, also increase substantially during the luteal phase of the menstrual cycle, and subsequently in pregnancy (Sealey *et al.*, 1985; Derkx *et al.*, 1987b). The ovaries contribute greatly to this rise in prorenin, with the theca (-lutein) cells being the main prorenin-synthesizing cells following their stimulation with gonadotropins (Itskovitz and Sealey, 1987; Derkx *et al.*, 1987a; Do *et al.*, 1988). In pregnancies without a corpus luteum, this rise in prorenin was absent (Derkx *et al.*, 1987a; Wiegel *et al.*, 2020). Follicular prorenin levels are ~10-fold higher than those in plasma (Glorioso *et al.*, 1986). However, the significance of ovarian prorenin in reproductive physiology remains unclear.

In order to display enzymatic activity, prorenin needs to be converted to renin. This implies that its prosegment (covering the active site) is cleaved off. However, under physiological conditions (pH 7.4, 37°C) a small fraction of prorenin (≈1% or less) occurs in a so-called 'open' conformation, i.e. a conformation where the prosegment has moved out of the enzymatic cleft (Schalekamp *et al.*, 2008), yet without being cleaved. Open prorenin displays the same enzymatic activity as renin, and contributes to cardiovascular and hemodynamic adaptations to pregnancy by activating the renin–angiotensin system (RAS). Renin cleaves angiotensin (Ang) I from angiotensinogen, which is subsequently converted by angiotensin-converting enzyme into Ang II, a potent vasoconstrictor and growth factor that stimulates the release of aldosterone for sodium-retention (August, 1990). Therefore, if prorenin levels are sufficiently high, even 1% of the prorenin molecules displaying Ang I-generating activity would be enough to result in substantial Ang II generation (Schalekamp *et al.*, 2008).

Given its local synthesis, one further option is that prorenin exerts effects in the ovaries themselves, i.e. that it is part of an independent ovarian RAS. In line with this view, ovarian Ang II has been linked to follicular development, steroidogenesis, oocyte maturation, ovulation, and follicle atresia (Lightman *et al.*, 1987; Yoshimura *et al.*, 1992, 1996). Ang II might also display vascular effects in the follicular microcirculation (affecting blood flow) and facilitate the process of neovascularization (Fernandez *et al.*, 1985; Stirling *et al.*, 1990). The pro-angiogenic activity of Ang II is predominantly mediated by stimulation of the Ang II type 1 receptor, by promoting endothelial cell proliferation (Delforce *et al.*, 2019). Since Ang II is the main effector protein of the RAS, most attention went to this peptide rather than prorenin. However, Ang II has a short half-life, and its detection is notoriously difficult. We propose that prorenin might serve as a proxy for local ovarian Ang II activity. It is already known that circulating prorenin levels during gestation correlate directly with the number of corpora lutea and are affected by the ovarian stimulation protocol used during IVF treatment and maternal characteristics (Itskovitz *et al.*, 1987; Itskovitz and Sealey, 1987; Wiegel *et al.*, 2020). Since circulating prorenin levels after ovarian stimulation are derived primarily from the ovaries (Itskovitz *et al.*, 1987; Derkx *et al.*, 1987a), these levels may serve as a proxy for the local environment in the ovary during follicle development and oocyte maturation.

Based on these considerations, we hypothesize that periconceptional circulating maternal prorenin concentrations play a role during follicle development and oocyte maturation, for instance by affecting steroidogenic or angiogenic activity and as such impact IVF treatment outcomes. To investigate this, we examined in a prospective periconception cohort whether circulating maternal prorenin measured after ovarian stimulation at embryo transfer is associated with oocyte size, preimplantation embryo development, as determined by developmental time-lapse parameters, and clinical IVF treatment outcomes.

Materials and methods

Study design

The data used for this study were collected between May 2017 and July 2020 in the Virtual EmbryoScope study, which is a subcohort embedded in the Rotterdam Periconceptional Cohort (Predict Study) (Steegers-Theunissen *et al.*, 2015). The Predict study is an ongoing observational open prospective tertiary hospital-based cohort embedded in the outpatient clinic of the Department of Obstetrics and Gynecology at the Erasmus MC, University Medical Center Rotterdam, the Netherlands. The Predict study is designed to focus on interactions between determinants and underlying mechanisms of periconceptional parental health affecting reproductive performance, pregnancy course and outcomes, and health of the offspring up to 1 year of age. For the Virtual EmbryoScope study, subfertile couples, with an indication for IVF or ICSI treatment with ejaculated or surgical retrieved sperm were enrolled. Furthermore, inclusion criteria were 18 years of age or older, adequate understanding, and ability to speak and read the Dutch language.

For this study, couples were excluded if (i) no fertilization occurred and no embryos were available; (ii) the embryos were not cultured in the EmbryoScope; (iii) IVF/ICSI treatment was performed with donated or vitrified oocytes; (iv) no maternal blood withdrawal was possible on the day of embryo transfer; or (v) measurement of prorenin and renin could not be performed.

Oocyte stimulation, oocyte retrieval, and IVF procedures

Standard protocols were used for controlled ovarian stimulation, oocyte retrieval, the IVF/ICSI procedure, and the assessment of embryo morphology (Hohmann et al., 2003; Heijnen et al., 2007). Women received ovarian stimulation with either Menopur, Ferring, St. Prex, Switzerland, a highly purified, urine-derived, hMG (containing FSH, LH, and recombinant hCG) or recombinant human (rh)FSH (Bemfola, Gedeon Richter, Belgium; Gonal-F, Merck Serono, Switzerland; or Rekovelle, Ferring, St. Prex, Switzerland) with GnRH agonist (Lucrin, Abbott, Hoofddorp, the Netherlands; or Decapeptyl, Ferring St. Prex, Switzerland) or GnRH-antagonist (Orgalutran, MSD, Belgium; Cetrotide, Merck Serono, Switzerland; Fyremadel, Ferring St. Prex, Switzerland) co-treatment. The dosage of rhFSH was based on female age, BMI, antral follicle count, and anti-Müllerian hormone (AMH) level. Final oocyte maturation and ovulation are induced by a subcutaneous injection with hCG or a GnRH-agonist. Oocytes were collected 35 h later and placed in fertilization medium (G-IVF, Vitrolife, Göteborg, Sweden). Ejaculated sperm was washed in commercially available discontinuous two-layer density gradient (45–90%, SpermGrad, Vitrolife) and testicular sperm was retrieved and frozen-thawed as previously described (van Marion et al., 2021). In a standard volume of 0.5 ml of washed sperm, a total motile count of <1.5 million was an indication for ICSI; otherwise, IVF was performed. Oocytes were subsequently fertilized conform routine IVF, ICSI with ejaculated sperm, or testicular sperm extraction procedures as described previously (van Marion et al., 2021).

After sperm injection, ICSI oocytes were denuded and transferred to an EmbryoSlide (Vitrolife, Göteborg, Sweden) for culture in the EmbryoScope time-lapse incubator (Vitrolife). After insemination, IVF oocytes were cultured overnight and after the appearance of both pronuclei, only fertilized dipronucleate oocytes were transferred to an EmbryoSlide and cultured in the EmbryoScope. Embryos were cultured in culture medium SAGE 1-step (Origio/Cooper Surgical™, Denmark) between June 2017 and December 2019 and Vitrolife G-TL (Vitrolife, Göteborg, Sweden) between December 2019 and July 2020, with a temperature setting of 36.8°C and in an atmosphere containing 7% oxygen and 5% or 6% carbon dioxide, respectively (van Marion et al., 2022). Until 1 April 2019, embryo transfer was performed on day 3 after fertilization ($n=222$, $n=731$ embryos) and thereafter on day 5 after fertilization ($n=87$, $n=293$ embryos). Embryo evaluation and selection for transfer was performed on day 3 or 5 after oocyte retrieval, based on developmental stage and morphology as assessed on the last time-lapse recording. Morphological evaluation of the embryos was based on the number of blastomeres, fragmentation, size equality, and signs of early compaction. The best-ranked embryos consisted of eight equally sized blastomeres with little ($<10\%$) or no fragmentation. Single embryo transfer (SET) is standard care conforming with the protocols of our clinic. Double embryo transfer (DET) is an exception and was considered only for women ≥ 38 years of age without medical contra-indications or women who previously underwent two or more unsuccessful IVF or ICSI cycles.

Study parameters

Participants completed detailed self-administered questionnaires containing data on demographic and lifestyle information. At study entry, a researcher verified all data and performed standardized anthropometric measurements including parental blood pressure, weight, and height. Subfertility diagnosis, treatment, and clinical measurement of quantitative AMH in serum, which was performed prior to the assessment of rhFSH dosage were obtained from medical records. In case of oligo- or anovulation, women were stratified according to the World Health Organization classification of anovulation (Dhont, 2005). Women with polycystic ovary syndrome (PCOS) were diagnosed according to the Rotterdam 2003 consensus criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004).

Time-lapse imaging and assessment

The EmbryoScope records every 10 min embryo images automatically in seven focal planes. Time-lapse parameters were annotated manually according to the definitions and guidelines of the ESHRE consensus for dynamic monitoring of human preimplantation development (Ciray et al., 2014). The time of insemination or injection of the last oocyte for IVF and ICSI embryos respectively, was defined as $t=0$ (t0). The total procedure takes approximately 20–50 min, depending on the number of oocytes. The fresh and cryopreserved embryos were individually, manually retrospectively annotated including time of fertilization (t0), the time of pronuclear appearance (tPNa), number of pronuclei (PN), the first frame where both pronuclei faded (tPNf), the timing of reaching the two-, three-, four-, five-, six-, seven-, and eight-cell stage (t2, t3, t4, t5, t6, t7, and t8), the start of blastulation (tSB), when the blastocoel cavity is filled up and the embryo starts to expand (tB) and when the blastocyst is expanded and the zona pellucida is thin (tEB) (Fig. 1).

Oocytes were termed 'used' if they were fertilized and embryo development resulted in an embryo that was cryopreserved or transferred. Fertilized oocytes that failed to develop into an embryo of sufficient quality for transfer or cryopreservation were classified as 'discarded'. Oocyte size was determined by using the images of the EmbryoScope incubator and measuring the oocyte area, excluding the zona pellucida, longitudinally at t0, tPNa, and tPNf by using the ellipse tool of the EmbryoViewer software (Vitrolife) (Fig. 2). Out of the standard 7 focal plane pictures, the focal plane with the clearest ooplasm perimeter was selected for measuring. IVF embryos were transferred to an EmbryoSlide after the appearance of both pronuclei 18–20 h after insemination, whereas for ICSI, oocytes were transferred directly after sperm injection. The oocyte area measurements at t0 and tPNa could therefore only be performed for oocytes fertilized after ICSI.

Clinical treatment outcomes

Pre-transfer clinical treatment outcomes included the number of follicles, total follicle diameter (determined on the day of ovulation triggering and representing the sum of the diameters of all follicles per patient), number of oocytes, metaphase II oocytes (MII), fertilized oocytes, usable embryos, fertilization rate, and usable embryo rate. Fertilization rate was calculated by dividing the number of fertilized oocytes by the total number of retrieved oocytes for IVF and total number of MII oocytes for ICSI. Embryo usage rate was calculated by dividing the total number of usable embryos per patient, i.e. all embryos transferred or cryopreserved, by the number of fertilized

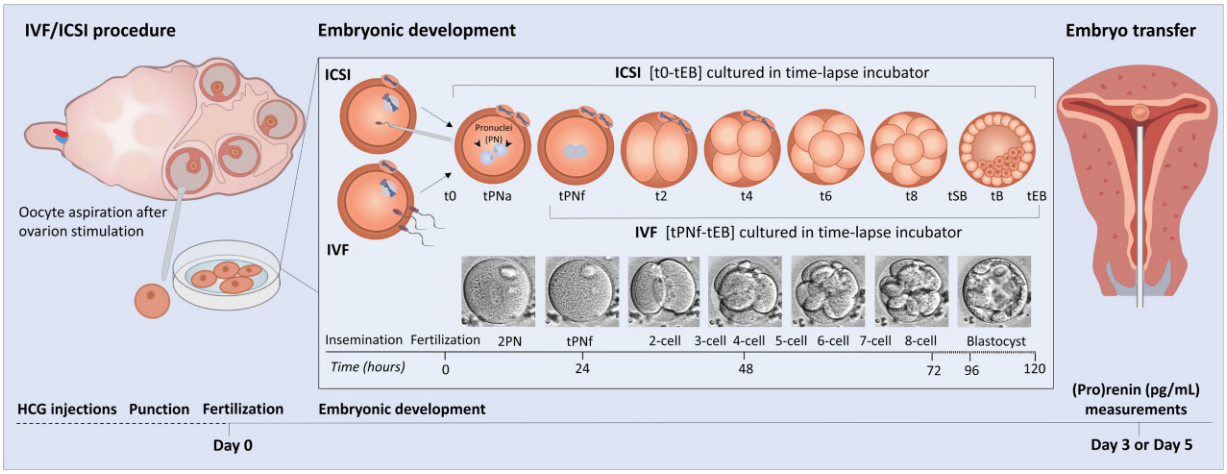

Figure 1. Schematic presentation of the study parameters including embryo developmental events after fertilization up to the blastocyst stage (embryonic day 1 till day 5) with the corresponding time points. The morphokinetic events annotated in this study are indicated. t0, time of fertilization; tPNa, time interval between the time of injection of spermatozoa into the oocyte and appearance of the two pronuclei (PN); tPNf, fading of the PN; t2, time of cleavage of the embryo to the two-cell stage; t3, time of cleavage of the embryo to the three-cell stage; t4, time of cleavage of the embryo to the four-cell stage; t5, time of cleavage of the embryo to the five-cell stage; t6, time of cleavage of the embryo to the six-cell stage; t7, time of cleavage of the embryo to the seven-cell stage; t8, time of cleavage of the embryo to the eight-cell stage; tSB, the start of blastulation; tB, reaching the full blastocyst stage; tEB, expanded blastocyst stage.

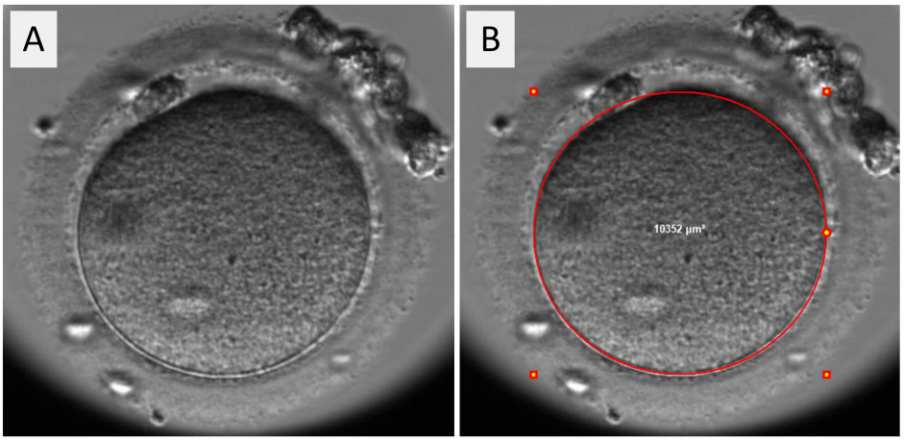

Figure 2. Oocyte area measurement performed with the drawing tools of the Embryoviewer software at t0. The red circle delineates the outermost perimeter of the ooplasm, excluding the zona pellucida and the perivitelline space. (**A**) Before and (**B**) after oocyte area measurement.

oocytes. Clinical and biochemical parameters were used to predict and diagnose mild or severe ovarian hyperstimulation syndrome (OHSS). Post-transfer clinical treatment outcomes included a positive β-hCG test after 2 weeks of embryo transfer, ongoing pregnancy, which was confirmed by a fetal heartbeat during ultrasound at 12 weeks of gestation, live birth, and cumulative pregnancy rate, which was defined as an ongoing pregnancy resulting from either fresh of cryopreserved embryos from the studied treatment cycle. Clinical treatment outcomes were retrieved from medical records.

Biochemical measurements

Non-fasting venous blood samples (750 µl) were obtained at the day of embryo transfer (either day 3 (n = 222) or day 5 (n = 87) after

fertilization) and collected in vacutainer EDTA tubes, processed, and stored at −80°C until analysis. Blood samples were measured at the Internal Medicine Department of the Erasmus MC. Renin was measured by the immunoradiometric assay (Cisbio, Saclay, France: detection limit 1 pg/ml, interassay variability 4%) using an active site-directed radiolabeled antibody that recognizes renin only. Total renin was measured with the same assay, after activating prorenin with 10 µmol/l aliskiren at 4°C for 48 h. The amount of prorenin was calculated by subtracting renin from total renin.

Statistical analyses

Baseline characteristics, treatment factors, and outcomes of the total study population, stratified for included and excluded participants, were analyzed by using mean and standard deviation for normal distributed data or median and interquartile range (IQR) for skewed continuous data, and frequencies with proportions for categorical data. To test for difference, the Student's *t*-test, Kruskal–Wallis test, or the Chi-squared test were used, respectively. Prior to analyses, longitudinal study measurements of oocyte size, and preimplantation embryo morphokinetics were presented crude. To obtain a normal distribution, prorenin concentrations were transformed to the natural logarithmic scale and associations were studied with plasma prorenin concentrations as a continuous variable.

Analyses on oocyte size were performed on both the used and discarded oocytes, while analyses regarding the developmental time points of reaching the different cell stages up to the eight-cell stage and the blastocyst stage were performed on solely the transferred and cryopreserved embryos.

Since couples often have multiple embryos and those embryos are likely to exhibit comparable developmental patterns, we applied linear mixed models with oocyte area (at t0, tPNa, and tPNf) and time-lapse parameters (tPNa, tPNf, t2–t8, tSB, tB, and tEB) as response variables, and maternal prorenin concentration as independent variable. To investigate the effect of prorenin on the dynamics of oocyte area we used a two-step approach. Each oocyte follows its own developmental pattern resulting in a different timing of pronulcei appearance and fading. Therefore, we calculated the shrinking rate of oocyte area by using a linear regression model to summarize the individual trajectory of oocyte area (at t0, tPNa, and tPNf) per oocyte, with timing of t0, tPNa, and tPNf as independent variable. Subsequently, a linear mixed model analysis was performed to analyze the effect of prorenin on the coefficients generated from the linear regression, representing oocyte area shrinking. These regression coefficients were used as response variables and maternal prorenin as independent variable in a linear mixed model to analyze the effect of prorenin on oocyte area shrinking rate.

Spearman ρ rank correlation coefficient (*R*) was used to evaluate correlations between prorenin concentrations and pre-transfer treatment outcomes, such as number of follicles, retrieved oocytes on the date of the punction after ovum retrieval, and total follicle diameter (determined on the day of ovulation triggering, and representing the sum of the diameters of all follicles). Associations between prorenin and continuous treatment outcomes (e.g. number of follicles and fertilization rate) were analyzed using linear regression. For associations between prorenin and dichotomous outcomes, such as a positive β-hCG test, logistic regression was applied. First, the models were performed without adjustments (crude model). The second model was adjusted for couple and treatment characteristics depending on the outcome analyzed, such as maternal and paternal age, fertilization method, type of ovarian stimulation, and sperm retrieval method (adjusted model). Relevant confounders were defined by literature search, and a correlation matrix was used to decide which confounders would be applied to analyses. Prorenin concentrations generally increase after hCG administration and this rise is directly related to the number of ovarian follicles (Itskovitz *et al.*, 1987). To take into account theca cell activity influencing prorenin concentrations, adjustments were also made for number of aspirated oocytes, as this is the most accurate measure. Given that prorenin is measured on the day of embryo transfer (day 3 or 5), which may affect the concentrations, we additionally adjusted the models for day of blood collection.

Two-sided *P*-values <0.05 were considered statistically significant. All analyses were performed in R (R for Windows, version 3.5; R Core Team).

Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the Medical Ethical Institutional Review Board of the Erasmus, University Medical Centre, Rotterdam, the Netherlands. Written informed consent was obtained from all participants at enrollment.

Results

Baseline characteristics

The Virtual EmbryoScope study included 453 participants between May 2017 and July 2020. Exclusion occurred in 144 cases because (i) no fertilization had occurred or no embryos were available (n = 14); (ii) the embryos were not cultured in the EmbryoScope (n = 52); (iii) IVF/ICSI treatment was performed with donated or vitrified oocytes (n = 9); (iv) no maternal blood was drawn on the day of embryo transfer (n = 68); or (v) measurement of prorenin and renin was unsuccessful (n = 1). This resulted in a total of 309 women (n = 1024 embryos) that could be included for analysis (Fig. 3). Of these, 95 (embryos: n = 366) underwent IVF treatment and 214 (embryos: n = 658) ICSI treatment with ejaculated or surgically retrieved sperm. SET was performed in 270 cycles, DET in 22 cycles, and no embryo transfer (in case of OHSS or medical/personal reasons) in 17 cycles. Baseline characteristics and treatment outcomes of the total study population were stratified for included and excluded participants and are shown in Table I. Baseline characteristics were comparable between both groups. Comparison of treatment factors and outcomes demonstrated more favorable treatment outcomes in the included cases, mainly due to the large proportion of total fertilization failure and no blood withdrawal on day of transfer. The included participants had a mean maternal age of 33.8 years and an average BMI of 24.7. Most couples were subfertile due to male subfertility (n = 142, 46%). Of the subfertile women, the majority (n = 64; n = 236 embryos) were clinically diagnosed with PCOS according to the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004).

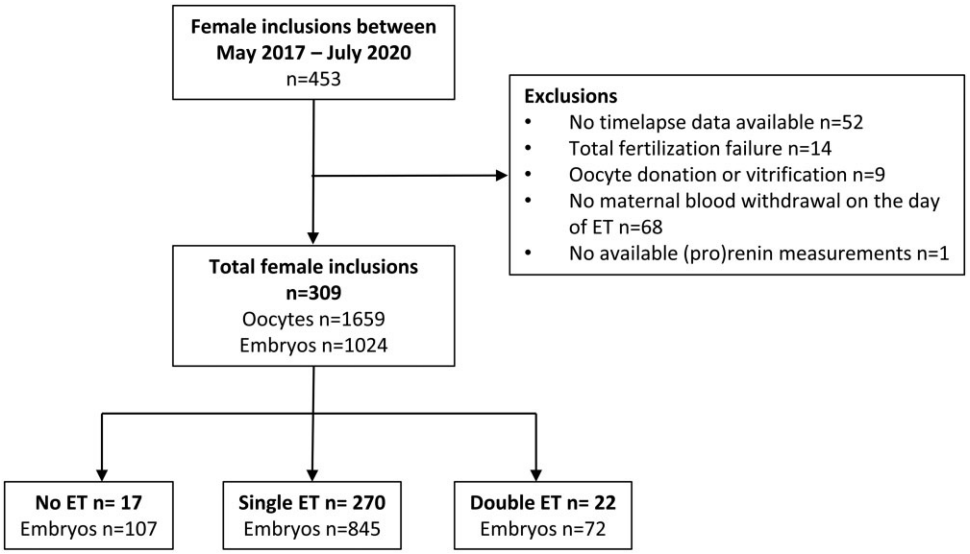

Figure 3. Flowchart of in- and exclusions of the total study population. ET, embryo transfer.

Prorenin and renin measurements

A total of 309 blood samples were analyzed. The maternal plasma concentrations of prorenin (pg/ml) (median: 198.4 (IQR: 129.2–282.7)) and renin (pg/ml) (median: 36.0 (IQR: 22.9–51.1)) were highly correlated ($R = 0.62$, $P < 0.001$) (Table I). Prorenin and renin concentrations were significantly increased in plasma of women without embryo transfer compared to SET or DET (e.g. prorenin (pg/ml), median: 287.05 (IQR: 212.01–370.94), median: 198.4 (IQR: 130.3–280.9) and median; 145.1 (99.2–207.2), respectively). In addition, of the 309 blood samples, 222 samples were measured 3 days after oocyte retrieval and 87 samples 5 days after oocyte retrieval. Prorenin and renin concentrations differed significantly between these moments, with higher prorenin levels at embryo transfer Day 3 and higher renin concentrations at embryo transfer day 5 (e.g. prorenin (pg/ml), day 3 ($n = 222$) median: 217.14 (IQR: 137.99, 297.17) and day 5 ($n = 87$) median: 168.57 (IQR: 116.68, 240.36), $P < 0.01$, renin (pg/ml), day 3 ($n = 222$) median: 34.02 (IQR: 22.10, 49.73) and day 5 ($n = 87$) median: 37.80 (IQR: 28.59, 52.98), $P = 0.04$). Therefore, all analyses are adjusted for the day of blood collection. Given the fact that it is prorenin rather than renin which is released from the ovaries into the circulation, all analyses below were performed with prorenin. Nevertheless, when repeating the analysis with renin, similar (albeit less significant) trends were observed (data not shown).

Follicular and luteal phase characteristics

Table I demonstrates the pre-transfer treatment outcomes, with a median number of eight retrieved oocytes and a subsequent average fertilization rate of 80%. We observed positive correlations between prorenin concentrations and the follicle and luteal phase characteristics of the total study population, such as total follicle count ($R = 0.49$, $P < 0.001$) and diameter ($R = 0.44$, $P < 0.001$), number of oocytes ($R = 0.47$, $P < 0.001$), MII oocytes ($R = 0.41$, $P < 0.001$), fertilized oocytes ($R = 0.34$, $P < 0.001$), and usable embryos ($R = 0.28$, $P < 0.001$) (Fig. 4). Consistent with the results of the correlation analysis, the linear regression outcomes also showed a positive association (Table II). After adjustment for maternal age, type of ovarian stimulation, and day of blood collection, linear regression demonstrated significant positive associations between prorenin and total follicle number and diameter, number of oocytes, and MII oocytes (Table II). After additional adjustment for paternal age, method of fertilization, and sperm retrieval, prorenin was also significantly positively associated with the number of fertilized oocytes and usable embryos. It is well known that AMH is an important predictor of ovarian response (Popovic-Todorovic *et al.*, 2003), while circulating prorenin in pregnancy largely originates from the ovary (i.e. theca cell-derived). Thus, a further possibility could be that the above-described correlations with prorenin indirectly represent AMH. Indeed, circulating AMH and prorenin are highly correlated ($R = 0.45$, $P < 0.001$, Supplementary Fig. S1). However, when adjusting for AMH instead of the number of aspirated oocytes, all correlations with prorenin remained unaltered (data not shown). This finding strongly suggests that the observed associations for prorenin are independent of AMH.

Oocyte area

Mean oocyte area significantly decreased from 10 476 μm^2 at t0 to 9802 μm^2 at tPNf in both used and discarded oocytes (Table III). Since larger oocytes at t0 shrink faster to tPNf than smaller oocytes (Wiegel *et al.*, unpublished observations), Fig. 5 shows the oocyte area trajectories relative to the first measurement at t0. Additionally, we adjusted the mixed modeling analyses for the oocyte area at t0. Linear mixed model analysis revealed a significant positive association between circulating maternal prorenin concentrations and oocyte area measured at

Table I Baseline characteristics of the in- and exclusions of the Virtual EmbryoScope study population (n = 453).

	Included study participants n = 309[1] Embryos = 1024	Excluded study participants n = 144[1] Embryos = 282	*P*-value
Maternal characteristics			
Maternal age, years	33.8 ± 4.9	33.8 ± 5.0	0.97
Nulliparous	230 (74.4)	109 (75.7)	0.86
Geographic origin			0.85
Dutch	242 (79.3)	108 (80.0)	
Western	15 (4.9)	5 (3.7)	
Non-Western	48 (15.7)	22 (16.3)	
Education			0.84
Low	16 (5.2)	9 (6.6)	
Middle	122 (39.9)	54 (39.7)	
High	168 (54.9)	73 (53.7)	
Maternal BMI (kg/m^2)	24.68 [21.73, 28.15]	24.62 [22.37, 27.37]	0.76
BMI >25	143 (46.4)	65 (45.5)	0.93
BMI >30	57 (18.5)	18 (12.6)	0.15
Mean arterial pressure, *at study entry*	86.67 [79.33, 94.00]	86.00 [80.00, 93.33]	0.48
Folic acid supplement use	287 (94.1)	126 (92.0)	0.28
Multivitamin supplement use	151 (53.2)	71 (57.3)	0.51
Alcohol use	128 (42.1)	60 (44.4)	0.28
Smoking	40 (13.2)	22 (16.3)	0.22
Treatment factors			
Diagnosis			0.21
Combined	67 (21.7)	24 (16.7)	
Only male factor	142 (46.0)	58 (40.3)	
Only female factor	67 (21.7)	40 (27.8)	
Unexplained	33 (10.7)	21 (14.6)	
PCOS	64 (20.7)	24 (16.7)	0.38
ICSI, yes	214 (69.4)	66 (59.5)	0.07
ICSI with ejaculated sperm	118 (38.2)	115 (79.9)	
ICSI with surgical retrieved sperm	96 (31.1)	29 (20.1)	
Ovarian stimulation, GnRH-agonist	85 (27.7)	22 (21.8)	0.30
No. of embryos transferred			<0.001*
No ET	17 (5.5)	36 (32.4)	
Single ET	270 (87.4)	70 (63.1)	
Double ET	22 (7.1)	5 (4.5)	
Days of culture, *3 days*			0.026*
3 days	222 (71.8)	72 (50.0)	
5 days	87 (28.2)	45 (31.3)	
Culture media			<0.001*
SAGE 1-Step	282 (91.3)	104 (72.2)	
GTL Vitrolife	27 (8.7)	29 (21.8)	
Treatment outcomes			
Oocytes aspirated	8 [5, 13]	9 [5, 12]	0.58
Total fertilized oocytes	5 [3, 7]	4 [2, 7]	0.20
Fertilization rate	0.80 [0.61, 1.00]	0.80 [0.60, 1.00]	0.69
Total usable embryos	3 [2, 5]	2 [1, 4]	0.14
Usage rate	0.67 [0.50, 0.88]	0.60 [0.50, 0.88]	0.55

(continued)

Table I Continued

	Included study participants n = 309[1] Embryos = 1024	Excluded study participants n = 144[1] Embryos = 282	*P*-value
OHSS	15 (4.9)	1 (1.3)	<0.001*
hCG +	132 (42.7)	22 (28.6)	0.01*
GS +	119 (38.5)	20 (26.0)	0.022*
Fetal heartbeat +	109 (35.3)	14 (18.2)	0.002*
Live births	101 (33.0)	12 (15.8)	0.002*
Cumulative pregnancy	157 (52.0)	18 (27.3)	0.008*
Biochemical measurements			
Prorenin (pg/ml)	198.4 [129.2, 282.7]	257.9 [147.6, 338.7]	0.607
Renin (pg/ml)	36.0 [22.9, 51.1]	27.3 [23.0, 32.2]	0.398
AMH	2.80 [1.50, 5.20]	4.45 [1.55, 8.88]	0.112

Data are presented as mean ± standard deviation, median [interquartile range], or number of individuals (percentage).
*Significant differences with a $P < 0.05$.
[1]Missing data rates by variable were reported and excluded from the total sum of participants or the denominator during analyses.
AMH, anti-Müllerian hormone; ET, embryo transfer; OHSS, ovarian hyperstimulation syndrome; GS, gestational sac.

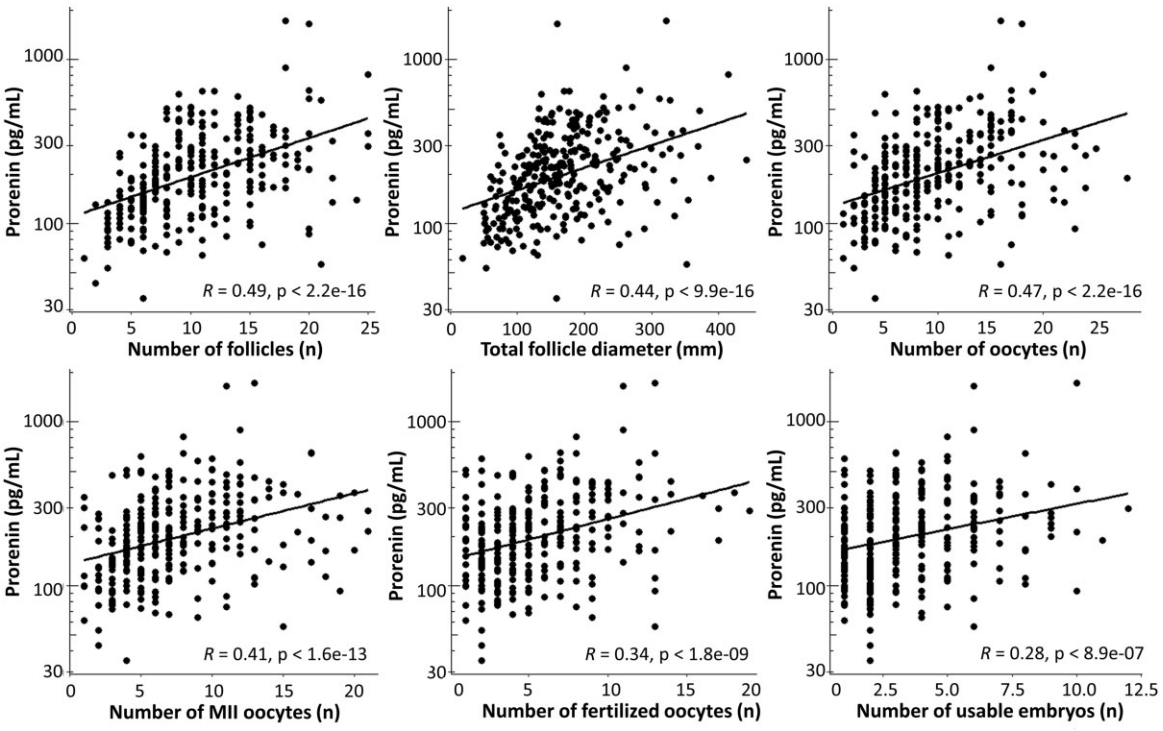

Figure 4. Spearman correlations (*R*) between periconceptional maternal prorenin plasma concentrations and follicular and luteal phase characteristics from 309 patients after controlled ovarian stimulation treatment. MII, metaphase II oocytes.

tPNa (β_{tPNa} 64.45 μm^2 (95% CI 3.26, 125.64), $P = 0.039$) (Table IV). Analyzing oocyte area longitudinally showed that higher prorenin concentrations were also significantly associated with a higher shrinking rate of the oocyte area, but only in ICSI cases since IVF oocytes were transferred to the EmbryoSlide after fertilization. Correction for AMH levels did not alter this outcome (data not shown).

Table II The impact of maternal prorenin plasma concentrations on pre- and post-transfer clinical treatment outcome parameters in the total study population.

	[log] Prorenin (pg/ml)			
	Crude		Adjusted	
Pre-transfer[1]	**Beta (95% CI)**	***P*-value**	**Beta (95% CI)**	***P*-value**
No. of follicles (n)	3.85 (3.00, 4.71)	<0.001*	3.74 (2.89, 4.59)	<0.001*
Total follicle diameter (mm)	51.38 (38.11, 64.65)	<0.001*	48.80 (35.49, 62.10)	<0.001*
No. of oocytes (n)	3.77 (2.84, 4.69)	<0.001*	3.83 (2.90, 4.75)	<0.001*
Metaphase II oocytes (n)	2.52 (1.76, 3.27)	<0.001*	2.61 (1.84, 3.37)	<0.001*
Total fertilized oocytes (n)[2]	2.03 (1.40, 2.66)	<0.001*	2.09 (1.43, 2.75)	<0.001*
Fertilization rate (n)[3]	−0.55 (−5.06, 3.96)	0.81	0.65 (−4.19, 5.49)	0.79
Total usable embryos (n)[2]	1.03 (0.61, 1.44)	<0.001*	0.98 (0.54, 1.41)	<0.001*
Usage rate (n)[3]	−0.04 (−0.9, 0.01)	0.09	0.64 (−4.69, 5.97)	0.81
	Crude		**Adjusted**	
Post-transfer[3]	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**
Positive β-hCG-test n = 132	1.58 (1.04, 2.42)	0.03*	1.79 (1.06, 3.08)	0.03*
Gestational sac n = 119	1.45 (0.95, 2.23)	0.08	1.65 (0.98, 2.83)	0.06
Fetal heartbeat n = 109	1.42 (0.93, 2.20)	0.11	1.68 (0.99, 2.91)	0.06
Live birth[a] n = 101	1.36 (0.88, 2.11)	0.17	1.57 (0.92, 2.72)	0.10
Cumulative pregnancy[b] n = 157	1.56 (1.03, 2.39)	0.04*	1.25 (0.75, 2.11)	0.39

[1]Adjusted for maternal age, type of ovarian stimulation and day of blood collection.
[2]Adjusted for maternal age, type of ovarian stimulation, sperm retrieval method, fertilization method, paternal age and day of blood collection.
[3]Adjusted for maternal age, type of ovarian stimulation, sperm retrieval method, fertilization method, paternal age, day of blood collection and number of aspirated oocytes.
[a]Missing n = 3.
[b]missing n = 7.
*Significant differences with a $P < 0.05$.
OR, odds ratio.

Morphokinetic parameters

Linear mixed model analyses showed negative regression coefficients for all morphokinetic parameters, indicating faster cleavage divisions up to the eight-cell stage (embryos n = 858) and from the morula stage to the blastocyst stage (embryos n = 260) for increased prorenin concentrations (Table V). This association was significant from the five-cell up to the eight-cell stage, after adjustment for maternal age, fertilization method, type of ovarian stimulation, sperm retrieval method, day of blood collection, number of retrieved oocytes, and oocyte area at t0 (e.g. β_{t8} −1.369 h (95% CI −2.482, −0.257), $P = 0.016$). Interestingly, linear mixed model analyses with embryos incubated until day 5 after fertilization showed significant associations between prorenin and the morphokinetic parameters from the morula stage onwards (i.e. tSB, tB, and tEB) in the crude and adjusted model (e.g. tSB $\beta_{adjusted}$ −2.659 h (95% CI −4.850, −0.469), $P = 0.018$). Additional adjustment for AMH did not alter this outcome (data not shown). Retransformation of the betas to the original values showed that the embryos from women with an increase of 50 pg/ml in circulating prorenin reached the time-point start of blastulation (tSB) 0.56 h earlier.

Post-transfer clinical treatment outcomes

Crude and adjusted logistic regression were applied to investigate the associations between circulating maternal prorenin at the day of embryo transfer and the post-transfer clinical treatment outcomes. Results indicated that women with increased plasma prorenin concentrations were significantly more likely to achieve implantation of the embryo (+β-hCG-test odds ratio: 1.79, 95% CI 1.06, 3.08, $P = 0.03$), independent of the number of retrieved oocytes. No significant association with live birth was observed (Table II). Correction for AMH did not alter this outcome (data not shown).

Discussion

Summary

In this prospective cohort study, we demonstrate that, following ovarian stimulation and IVF/ICSI treatment, elevated periconceptional maternal plasma prorenin concentrations at the moment of embryo transfer are associated with *in vitro* measured larger oocytes at tPNa,

Table III Longitudinal study measurement of oocyte size and preimplantation embryo morphokinetics of the total study population of the Virtual EmbryoScope study (n = 309).

	Total study participants n = 309
Oocyte area (μm^2)	Mean ± SD **Oocytes = 1659**
t0	10476 ± 651
tPNa	9848 ± 649
tPNf	9802± 650
Shrinking rate (μm^2/h)	26.14 ± 17.8
Embryo morphokinetics (h)	Median [IQR] **Embryos = 1024**
tPNa	7.46 [6.22, 8.78]
tPNf	24.11 [22.22, 26.37]
t2	26.54 [24.69, 29.07]
t3	37.39 [34.54, 40.33]
t4	38.62 [35.93, 42.03]
t5	50.46 [46.33, 55.25]
t6	52.57 [48.62, 57.14]
t7	54.21 [50.29, 59.61]
t8	56.50 [52.04, 62.38]
tSB	97.50 [93.35, 102.20]
tB	102.70 [98.27, 107.53]
tEB	103.30 [98.53, 108.02]

Data are presented as mean ± standard deviation or median [interquartile range]. IQR, interquartile range; t0, time of fertilization; tPNa, time of pronuclei appearance; tPNf, time of pronuclei fading; t2–t8, time of cleavage of the embryo to the two-cell till eight-cell stage; tSB, the start of blastulation; tB, reaching the full blastocyst stage; tEB, expanded blastocyst stage.

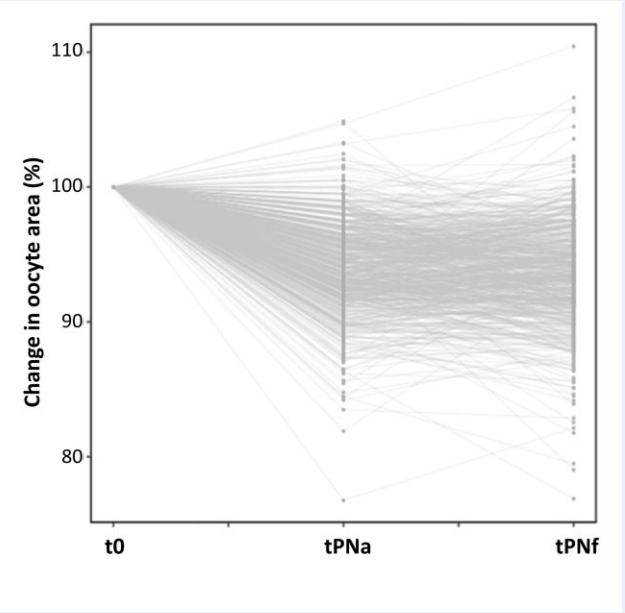

Figure 5. Longitudinal oocyte area (μm^2) trajectories relative to the first measurement in oocytes used for ICSI treatment (n = 807). t0, time of fertilization; tPNa, time of pronuclei appearance; tPNf, time of pronuclei fading.

faster shrinking of the oocyte area, and faster cleavage divisions of the preimplantation embryo from the five-cell stage onwards, and an increased chance of successful implantation. Moreover, we found that plasma prorenin is directly related to theca (-lutein) cell activity, measured as the total number and size of ovarian follicles, retrieved oocytes, metaphase II oocytes, and usable embryos. These latter results are in line with previous studies, which showed a significant positive association between circulating prorenin concentrations and the number of ovarian follicles (Itskovitz *et al.*, 1987; Wiegel *et al.*, 2020). However, we observed no impact of prorenin on fertilization potential, as no associations were found with fertilization and embryo usage rate.

Interpretation

The positive correlation between circulating prorenin and follicle number not only confirms the concept that circulating prorenin is of ovarian origin (Itskovitz *et al.*, 1987; Derkx *et al.*, 1987a) but also raises the possibility that circulating prorenin levels may serve as a proxy for ovarian prorenin levels. Indeed, following hCG injection, prorenin peaks between days 4 and 6, i.e., around the day of embryo transfer (3 or 5 days after oocyte retrieval) (Itskovitz *et al.*, 1987). The half-life of prorenin is 8 h (Krop *et al.*, 2008), and the prorenin peak obviously is the result of ongoing stimulation and degradation. Our data demonstrate that the prorenin levels on day 3 were higher than on day 5. This suggests that the prorenin peak in plasma is likely closer to day 3. We therefore corrected all analyses for the day of blood collection.

Oocytes resulting in a live birth display higher follicular prorenin levels than discarded oocytes or empty follicles (Cornwallis *et al.*, 1990). Given that increased theca (-lutein) cell activity associates with successful implantation (Verberg *et al.*, 2009; van Loendersloot *et al.*, 2010), it may not be surprising that this is also true for high circulating prorenin levels, since these are the consequence of increased theca cell activity. The relationship between the number of follicles and successful implantation has been known for years (Verberg *et al.*, 2009; van Loendersloot *et al.*, 2010). However, our data now demonstrate that circulating prorenin at embryo transfer also associates with oocyte size, preimplantation embryonic development, and implantation after adjustment for the number of oocytes. This implies that it may have a direct modulatory role, independently of the fact that it simply reflects theca cell activity. Indeed, it has already been shown that follicular prorenin levels correlate positively with follicular development, oocyte–cumulus complex maturity, and oocyte viability (Itskovitz *et al.*, 1987; Cornwallis *et al.*, 1990). By using strict morphological criteria to determine stages of follicle development and oocyte maturation, Itskovitz *et al.* (1991) were able to show that immature follicles contain lower levels of follicular prorenin than mature follicles. To what degree these effects involve Ang II formation remains to be shown. Previous evidence supports that Ang II contributes to steroidogenesis, the development and vascularization of the follicle, as well as cell proliferation

Table IV The association between maternal prorenin concentrations on oocyte area measurements in oocytes used for ICSI treatment at t0, tPNa, and tPNf and oocytes used for ICSI and IVF treatment at tPNf time points.

Oocyte area (μm²)		[log] Prorenin (pg/ml)			
		Crude		Adjusted	
	No. of oocytes	Beta (95% CI) μm²	*P*-value	Beta (95% CI) μm²	*P*-value
Total group (n = 309)					
t0	1048	18.74 (−114.55, 152.04)	0.782	64.49 (−86.22, 215.20)	0.397
tPNa	1077	51.79 (−70.55, 174.13)	0.405	64.45 (3.26, 125.64)	0.039*
tPNf	1613	5.22 (−89.81, 100.24)	0.914	57.12 (−53.35, 167.59)	0.309
Shrinking rate (μm²/h)	807	4.94 (2.08, 7.81)	<0.001*	6.20 (1.70, 10.71)	0.007*

Oocytes include both used oocytes that were fertilized and embryo development resulted in an embryo that was cryopreserved or transferred as well as the discarded undeveloped fertilized oocytes (used: t0 n = 627, tPNa n = 655, tPNf n = 1000 and discarded: t0 n = 421, tPNa n = 422, tPNf n = 613). Shrinking rate is only calculated for ICSI oocytes with two or more measurements of oocyte area (used n = 493, discarded n = 314). Adjusted model is adjusted for maternal age, type of ovarian stimulation, sperm retrieval method, day of blood collection, and number of aspirated oocytes. For shrinking rate the model is additionally adjusted for the first measurements of oocyte area (t0).
*Significant differences with a $P < 0.05$.
t0, time of fertilization; tPNa, time of pronuclei appearance; tPNf, time of pronuclei fading.

Table V The association between maternal prorenin and morphokinetic parameters until day 5 after fertilization in the total study population.

Morphokinetic parameters			[log] Prorenin (pg/ml)			
			Crude		Adjusted	
	No. of patients	No. of embryos	Beta (95% CI) hours	*P*-value	Beta (95% CI) hours	*P*-value
tPNa	208	632[a]	−0.034 (−0.647, 0.578)	0.910	−0.196 (−0.649, 0.257)	0.394
tPNf	302	1003	−0.159 (−0.713, 0.394)	0.571	−0.330 (−0.867, 0.207)	0.227
t2	302	1018	−0.258 (−0.848, 0.332)	0.390	−0.467 (−0.921, 0.192)	0.110
t3	302	1016	−0.326 (−1.102, 0.449)	0.408	−0.580 (−1.359, 0.199)	0.144
t4	302	1013	−0.612 (−1.412, 0.187)	0.133	−0.807 (−1.627, 0.013)	0.054
t5	296	1000	−0.799 (−1.895, 0.296)	0.152	−1.178 (−2.310, −0.047)	0.041*
t6	293	982	−0.758 (−1.812, 0.296)	0.158	−1.013 (−2.109, 0.083)	0.070
t7	286	948	−0.908 (−2.016. 0.200)	0.108	−1.191 (−2.301, −0.081)	0.036*
t8	269	858	−1.059 (−2.168, 0.049)	0.060	−1.369 (−2.482, −0.257)	0.016*
tSB	82	260[b]	−2.536 (−4.614, −0.457)	0.018*	−2.659 (−4.850, −0.469)	0.018*
tB	78	234[b]	−2.616 (−4.754, −0.477)	0.017*	−2.745 (−4.995, −0.496)	0.018*
tEB	51	154[b]	−3.844 (−6.668, −1.020)	0.009*	−3.733 (−6.840, −0.626)	0.019*

Adjusted for maternal age, fertilization method, type of ovarian stimulation, sperm retrieval method, day of blood collection, number of retrieved oocytes, and oocyte area at t0.
[a]In cases of regular IVF, embryos are only transferred to the EmbryoScope after PN inspection, thus tPNa cannot be observed.
[b]Until 1 April 2019, embryo transfer was performed on day 3 after fertilization, thereafter embryos are incubated till day 5 after oocyte retrieval and tSB, tB, and tEB parameters could be assessed.
*Significant differences with a $P < 0.05$.
t0, time of fertilization; tPNa, time interval between the time of injection of spermatozoa into the oocyte and appearance of the two pronuclei (PN); tPNf, fading of the PN; t2–t9, time of cleavage of the embryo to the two- till eight-cell stage; tSB, the start of blastulation; tB, reaching the full blastocyst stage; tEB, and expanded blastocyst stage.

during corpus luteum formation (Fernandez *et al.*, 1985; Cornwallis *et al.*, 1990; Stirling *et al.*, 1990; Itskovitz *et al.*, 1991).

Studies by Baltz *et al.* have shown that oocyte volume decreases progressively after the initiation of ovulation, triggered by hCG injection. This process is followed by meiotic maturation (Baltz and Tartia, 2010; Baltz and Zhou, 2012). If cell volume regulation is disrupted, fertilized oocytes fail to develop into blastocysts (Baltz and Tartia, 2010). Ang II induces meiotic maturation of the oocyte (Yoshimura *et al.*, 1992, 1996), and may thus contribute to the oocyte shrinkage that precedes this process. This would explain why high prorenin levels associate with faster shrinking of the oocyte area, as well as faster cleavage divisions of the preimplantation embryo from the five-cell

stage onwards. Importantly, timing of cleavage to the five-cell stage of an embryo is the main morphokinetic time-point for prediction of implantation in IVF cycles (Meseguer *et al.*, 2011).

Frequently investigated predictive factors for a high ovarian response and favorable clinical outcomes after IVF/ICSI include age, BMI, PCOS, levels of AMH, and number of total antral follicles (Popovic-Todorovic *et al.*, 2003). Interestingly, all these parameters are also independent factors that influence the secretion of prorenin (Wiegel *et al.*, 2020). Recently, we confirmed that age, BMI, AMH, and PCOS are linked to prorenin levels during the first trimester of pregnancy (Wiegel *et al.*, 2020). These associations were also observed in this study population following ovarian stimulation treatment, with data showing that prorenin concentrations decrease with increased age, BMI, mean arterial pressure, and low levels of AMH (Supplementary Fig. S1). When analyzing these predictive factors of ovarian response in a multiple regression model, only AMH and prorenin remained independent determinants of the number of oocytes, i.e. ovarian response. Therefore, analyses were additionally adjusted for AMH levels instead of the number of aspirated oocytes. Since this did not affect the outcome, it is likely that the observed associations with prorenin are independent of AMH. Future studies should therefore investigate to what degree circulating prorenin, like AMH, might be used as clinical biomarker for the prediction of quantitative and qualitative aspects in assisted reproduction treatment.

Strengths and limitations

To the best of our knowledge, our study is the first reporting prorenin concentrations in a study population of this size with blood collection at a fixed time point after ovarian stimulation and at the moment of embryo transfer with complete longitudinal *in vitro* measurements of the preimplantation embryo and extended follow-up of clinical outcomes. The strength of this study is the periconceptional design, involving detailed standardized measurements and questionnaires prior to subfertility treatment, resulting in the ability to correct for variability in fertility treatment, subfertility diagnosis, and parental characteristics. This allowed us also to investigate effects of maternal prorenin independent of paternal effects (e.g. paternal age, BMI, and sperm retrieval method) on preimplantation embryo development. Another strength is the statistical application of linear mixed model analyses, which takes the clustering effects of multiple embryos from one couple into account. Furthermore, the unique measuring method of the embryo developmental stages *in vitro* allows us to study only prorenin effects on the maternal gamete present till oocyte retrieval without interference of the uterine environment.

This study was conducted mainly in a time period when embryo culture until day 3 was routine clinical practice, which changed mid-2019 to embryo culture until day 5. This change allowed us to study preimplantation embryo development after culturing until day 5, i.e. blastocyst quality. Unfortunately, the group of examined embryos up to day 5 is modest. We realize that hCG injection is the main stimulator of prorenin, and that the peak in circulating prorenin is reached between days 3 and 5 (Itskovitz *et al.*, 1987). In our model, we therefore corrected for the day of embryo transfer, i.e. the day of blood sampling. Another limitation is the diverse study population. It included both IVF and ICSI treatments, different ovarian stimulation protocols, and subfertility diagnoses, therefore residual confounding cannot be excluded. Nonetheless, this might also be seen as a strength, as it increases the generalizability of the results.

Conclusions

In conclusion, elevated circulating maternal prorenin concentrations after ovarian stimulation associate with larger oocyte size, faster cleavage divisions up to the blastocyst stage, and increased chance of successful implantation, but not with live birth. Whether prorenin results in better embryos being transferred and or exerts direct endometrial effects (or both), and to what degree this involves Ang II, cannot be concluded from this study.

The main goal of IVF is to obtain high quality, developmentally competent embryos, which increase live birth rates. Our findings may help to clarify the underlying endocrine mechanism of oocyte maturation and embryo development, with a special focus on the (patho)physiological reproductive role of prorenin and the identification of factors influencing its secretion and activity, which is of great added value for improving embryo selection and predicting implantation and pregnancy outcomes. This will bring us to investigate which determinants of oocyte quality and embryo development should take center stage in developing preconception care strategies.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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Authors' roles

Under supervision of A.H.J.D. and R.P.M.S.-T., R.E.W. collected, analyzed, and interpreted data and drafted the first manuscript. L.v.D. and E.B.B. were involved in preparation and interpretation of the data. S.P.W. supervised the statistical procedures of the manuscript. E.A.P.S. and J.S.E.L. contributed to the interpretation of the data and inference with literature. A.H.J.D. was responsible for the renin and prorenin measurements in the laboratory, interpretation of the data, inference with the literature and provided expertise on the RAAS during pregnancy. R.P.M.S.-T. is principal investigator of The Rotterdam Periconceptional Cohort (Predict Study). All authors contributed to the writing and the critical revisions of the manuscript and all authors approved the final version of the manuscript and authorized the submitted version.

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Conflict of interest

The authors have nothing to declare.

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