Starting and resulting testosterone levels after androgen supplementation determine at all ages in vitro fertilization (IVF) pregnancy rates in women with diminished ovarian reserve (DOR)

Norbert Gleicher · Ann Kim · Andrea Weghofer ·
Aya Shohat-Tal · Emanuela Lazzaroni · Ho-Joon Lee ·
David H. Barad

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Abstract

Purpose To investigate whether androgen conversion rates after supplementation with dehydroepiandrosterone (DHEA) differ, and whether differences between patients with diminished ovarian reserve (DOR) are predictive of pregnancy chances in association with in vitro fertilization (IVF).

Capsule This study suggests a strong statistical association between improving androgen levels and IVF pregnancy chances.

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N. Gleicher · A. Kim · A. Weghofer · A. Shohat-Tal ·
E. Lazzaroni · H.-J. Lee · D. H. Barad
Center for Human Reproduction,
New York, NY 10021, USA

N. Gleicher · D. H. Barad
Foundation for Reproductive Medicine,
New York, NY 10021, USA

A. Weghofer
Department of Gynecologic Endocrinology and Reproductive
Medicine, Medical University Vienna,
1090 Vienna, Austria

N. Gleicher (✉)
The CHR, 21 East 69th Street,
New York, NY 10021, USA
e-mail: ngleicher@thechr.com

Methods In a prospective cohort study we investigated 213 women with DOR, stratified for age (≤ 38 or >38 years) and ovarian *FMRI* genotypes/sub-genotypes. All women were for at least 6 weeks supplemented with 75 mg of DHEA daily prior to IVF, between initial presentation and start of 1st IVF cycles. Levels of DHEA, DHEA-sulfate (DHEAS), total T (TT) and free T (FT) at baseline (_{BL}) and IVF cycle start (_{CS}) were then compared between conception and non-conception cycles.

Results Mean age for the study population was 41.5 ± 4.4 years. Forty-seven IVF cycles (22.1 %) resulted in clinical pregnancy. Benefits of DHEA on pregnancy rates were statistically associated with efficiency of androgen conversion from DHEA to T and amplitude of T gain. Younger women converted significantly more efficiently than older females, and selected *FMRI* genotypes/sub-genotypes converted better than others. FSH/androgen and AMH/androgen ratios represent promising new predictors of IVF pregnancy chances in women with DOR.

Conclusions DOR at all ages appears to represent an androgen-deficient state, benefitting from androgen supplementation. Efficacy of androgen supplementation with DHEA, however, varies depending on female age and *FMRI* genotype/sub-genotype. Further clarification of *FMRI* effects should lead to better individualization of androgen supplementation, whether via DHEA or other androgenic compounds.

Keywords Diminished ovarian reserve · Androgens · Androgen deficiency · Androgen supplementation · Dehydroepiandrosterone (DHEA) · Testosterone · *FMRI* gene · Premature ovarian aging · Follicle stimulating hormone (FSH) · Anti-Müllerian hormone (AMH) · Pregnancy rates · In vitro fertilization (IVF) · Adrenal insufficiency

Abbreviations

AMH	Anti-Müllerian hormone
AMHR	Anti-Müllerian hormone receptor
AR	Androgen receptor
BL	Baseline
CS	Cycle start
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DOR	Diminished functional ovarian reserve
<i>FMRI</i>	Fragile X mental retardation 1
FOR	Functional ovarian reserve
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
<i>het</i>	Heterozygous
IVF	In vitro fertilization
<i>hom</i>	Homozygous
<i>norm</i>	Normal
PCO	Polycystic ovary
PCOS	Polycystic ovary syndrome
POA	Premature ovarian aging
T	Testosterone
FT	Free testosterone
TT	Total testosterone
Δ	Change

Introduction

While androgen effects on follicle maturation in humans have remained controversial [1], experiments in rodents strongly support an essential androgen function, mediated by the androgen receptor (AR) on granulosa cells, especially at early stages of follicle maturation, up to preantral stages [2].

Direct studies of androgen effects on human follicle maturation do not exist. Androgens, indeed, for the longest time, have been considered detrimental to normal follicle maturation [1]. Similarities of receptor expression in mouse and human follicles, however, suggest that small growing human follicles may be as much androgen-dependent as mouse follicles [1].

Such a conclusion is also supported by clinical evidence: Supplementation with dehydroepiandrosterone (DHEA), in women with objectively determined DOR improves IVF cycle outcomes and pregnancy chances in general (reviewed in reference 3). These investigations were initiated after a 43-year-old patient with severe DOR developed a typical polycystic ovary (PCO) phenotype over consecutive in vitro fertilization (IVF) cycles after self-medicating with DHEA [3]. DHEA has been utilized to induce PCO-like ovarian phenotypes in rats [4], and mice [5]. From subsequent observational studies we then concluded that DHEA significantly and beneficially affects functional ovarian reserve (FOR), egg and embryo quality and overall pregnancy chances in women with

DOR [6]. How DHEA supplementation causes these effects has, however, remained undetermined.

In women DHEA in a majority converts to testosterone (T), and to a lesser degree to estradiol [7]. The hormone, therefore, can affect processes through AR or estrogen receptors [1]. That a minimum of 6 weeks of DHEA supplementation are required to see significant beneficial effects [6, 8], supports a DHEA effect on relatively early follicle maturation stages, coinciding with presence of ARs, likely only present at small growing follicle stages, up to early preantral follicles [1]. Beneficially affected follicles, therefore, still will require weeks to months to reach gonadotropin-sensitive stages, where they become clinically relevant, explaining the need for at least 6 weeks of supplementation prior to IVF cycle starts [6, 8]. During this time period DHEA conversion dynamics into other androgens offer a potential model to investigate androgen effects on follicle maturation.

We recently reported preliminary clinical data, suggesting that T levels after DHEA supplementation are statistically associated with IVF pregnancy chances [9]. Increasing androgen levels have previously been associated with increasing AMH levels, and, therefore, better FOR [10, 11]. Better androgen levels have also been associated with higher IVF pregnancy chances [12], though data have remained conflicted [13].

Ovarian genotypes and sub-genotypes of the fragile X mental retardation 1 gene (*FMRI*) have recently been demonstrated to affect ovarian aging [14] and IVF pregnancy chances [15]. They differ from classical expansive *FMRI* genotypes of premutation and mutation ranges, traditionally used to predict neuro-psychiatric risks, including the fragile X syndrome. Because of their effects on female reproduction, we suggested that fertility studies should now be stratified for these *FMRI* genotypes and sub-genotypes [16]. This was done in this study.

Here presented study offers a systemic investigation of androgen effects on IVF and, by doing so, offers insights into the pathophysiology of DOR, androgen effects on pregnancy chances, differences at younger and older ages and of *FMRI* effects. Here presented data also offer evidence for the importance of androgens to normal follicle maturation and female fertility in humans.

Materials and methods

Patients

This study involves a cohort of 213 women with primary or secondary infertility and a diagnosis of DOR. A DOR diagnosis was reached based on abnormal FSH [17] and/or AMH levels [18], outside of the 95 % confidence interval (CI) for age and/or because of age above 40 years [19]. To

prevent confounding biases from repeat cycles, only first IVF cycles, which offered a complete data set as described below, were included.

Androgen supplementation

DOR patients at our center, prior to IVF cycle start, are for at least 6 weeks routinely supplemented with 25 mg of micronized pharmaceutical grade DHEA, p. o., TID [6].

Assessments of androgen levels

Androgen profiles, including DHEA, DHEA-sulfate (DHEAS), total T (TT) and free T (FT) were serially evaluated monthly, at DHEA supplementation start (i.e., “baseline”), tagged with the subscript “_{BL}”, and at IVF cycle start, with subscript “_{CS}”. All androgens were measured by routine commercial assays.

Ovarian stimulation for IVF

With first menses, after at least 6 weeks of DHEA supplementation, women with DOR were started in a microdose agonist ovarian stimulation cycle (leuprolide acetate, 50 µg, s.c., BID, Lupron®, Abbot Laboratories, North Chicago, Illinois), with daily gonadotropin dosage of 450–600 IU, all FSH but 150 IU, given as human menopausal gonadotropin (hMG) (gonadotropin manufacturers varied, based on patient preference/insurance). In DOR patients our center performs oocyte retrieval whenever at least one mature follicle of ≥ 20 mm mean diameter is seen on ultrasound.

Definition of clinical pregnancy

A pregnancy was defined as clinical if at least one gestational sac and fetal heartbeat were confirmed on vaginal ultrasound.

FMRI genotypes and sub-genotypes

FMRI genotypes and sub-genotypes are based on a normal range of 26 to 34 (median 30) CGG triple nucleotides (CGG $n=26-34$). Women with both alleles in normal range are considered to have a normal genotype (*norm*), those with one outside range as heterozygous (*het*) and those with both alleles outside range as homozygous (*hom*). *Het* and *hom* genotypes, depending on whether abnormal alleles are above (*high*) or below (*low*) normal range, can then be further subdivided into sub-genotypes [14–16].

Statistical analyses

Where appropriate, statistical analyses were carried out by using Mann–Whitney U tests, t-tests or ANOVAs. All post

hoc procedures utilized the Holm–Sidak test but were confirmed with independent t-tests for significance. Differences in distributions between categorical data were compared using Chi-Square tests. All tests were 2-tailed, with $P < 0.05$ considered statistically significant.

A series of logistic regressions was performed to determine whether various androgen levels affected clinical potential for pregnancy in association with IVF. In interactions between two contributing factors the effect of the response variable depends on the value of the other factor. For multi-factor ANOVAs interaction terms of α were set at 0.05 in regards to individual contributions (at $\alpha \leq 0.05$ the 95 % CI indicates that factors of interaction terms are significant) but at 0.10 for interaction terms.

The first series of logistic regression models were adjusted (in weeks) for duration of DHEA supplementation from DHEA start (_{BL}) to IVF cycle start (_{CS}). When examining changes in various androgen levels, models were adjusted (in weeks) for time intervals between blood draw _{BL} and blood draw _{CS}. To correctly assess potential effects of androgens and potential effects of the inter-conversions between androgens on pregnancy odds, models were, where indicated, further adjusted for length of DHEA supplementation, oocyte yields and patient age. (Pearson product moment correlation coefficients confirmed that there were significant negative correlations between age and DHEA_{CS}, $r=0.274$, $P \leq 0.001$; age and TT_{CS}, $r=0.291$, $P=0.002$; age and FT_{CS}, $r=0.381$, $P \leq 0.001$; and age and oocyte yields, $r=0.258$, $P \leq 0.001$. There was also a significant positive correlation between oocyte yields and FT_{CS}, $r=0.204$, $P=0.035$.)

To correctly assess the differences between low, normal and high DHEA levels at cycle start, DHEA_{BL} and DHEA_{CS} were binned, and re-coded into categorical variables. To determine whether there was change from androgen _{BL} to androgen _{CS}, the same grouping paradigm was used at both time points. To offer a fair comparison, optimal cut offs were calculated, based on percentiles to yield equal sample sizes. Low, normal and high groups were assessed independently to examine the effects of androgens _{BL} and androgens _{CS} on pregnancy odds. They were for DHEA_{BL} and DHEA_{CS}, respectively: for low DHEA levels: <461 ng/dL ($n_{BL}=113$, $n_{CS}=70$); for normal $461-661$ ng/dL ($n_{BL}=45$, $n_{CS}=69$); and for high >661 ng/dL ($n_{BL}=45$, $n_{CS}=69$). Five patients missed cycle DHEA data but did have DHEAS data.

To assess pregnancy odds, factor increases or decreases in pregnancy odds were calculated as e^{β} for increases and $1/e^{\beta}$ for decreases, using the β coefficients derived from the logistic regression. To directly assess an androgen conversion index from DHEA to T, we created the following

formula: Ratio = % change T/absolute value of % change in DHEA. The actual formula is:

$$\text{Ratio} = \frac{\frac{T_{CS} - T_{BL}}{T_{BL}}}{\frac{DHEA_{CS} - DHEA_{BL}}{1 + \frac{DHEA_{BL}}{DHEA_{CS}}}} = \frac{\% \Delta T}{1 + \% \Delta DHEA}$$

The same formula is potentially applicable to conversion calculations for any androgen. This is a unit-less formula because a unit of DHEA is not necessarily converted into a unit of T (i.e., the molar ratio of DHEA to T is not 1:1).

Institutional review board (IRB)

Here presented data only involved the retrospective review of an anonymized electronic research database. Such studies at our center only require expedited IRB reviews since patients at initial consultation are asked to sign a universal informed consent, which allows for medical record reviews for research purposes if the patient's medical record remains confidential and her identity protected. These conditions were met, and the study, therefore, underwent only expedited review. Patients also signed a specific consent for *FMRI* testing. The center's clinical and research staffs are, in accordance with federal HIPAA rules, in writing committed to confidentiality of medical records and privacy of individual patients.

Results

Table 1 summarizes patient and IVF cycle characteristics as well as primary infertility diagnoses for the study cohort. The 213 women (IVF cycles) had a mean age of 41.5 ± 4.4 years and a mean BMI (kg/m^2) of 24.4 ± 5.0 . Out of 213 women, 140 (65.7 %) carried a primary infertility diagnosis of DOR, with remaining patients having DOR as secondary diagnosis or being above age 40. The cohort's mean FSH (12.2 ± 9.4 mIU/mL) and AMH levels (1.0 ± 1.3 ng/mL) are confirmatory of the diagnosis of DOR.

Younger (≤ 38 years) and older women (> 38 years), of course, differed in mean age ($P \leq 0.001$), but also AMH_{BL} ($P \leq 0.001$), gonadotropin dosage ($P \leq 0.001$), oocyte yield ($P = 0.003$) and number of embryos cryopreserved ($P = 0.013$). Younger women were less likely to have a primary diagnosis of DOR ($P = 0.004$).

A total of 47 clinical pregnancies were established (22.1 %). Younger women (≤ 38 years) conceived more frequently [14/41, 34.1 % vs. 33/172, 19.2 %; χ^2 (1, $N = 213$) = 4.31, $P = 0.038$]. Pregnancy rates did not differ between *FMRI* genotypes and sub-genotypes and between races.

Androgen conversion rates by age and *FMRI*

Two-way ANOVA for pregnancy outcome and age group demonstrated significant interactions [$F(1, 58) = 5.32$,

$P = 0.025$]. Figure 1a demonstrates a significant difference between pregnant ($M = 1.30$, $SD = 2.25$) and non-pregnant groups ($M = 0.33$, $SD = 0.86$; $P = 0.015$), which, independently, was primarily observed amongst younger women (pregnant, $M = 2.96$, $SD = 3.01$; not pregnant, $M = 1.05$, $SD = 1.44$; $P = 0.007$), favoring better conversion in women who achieved pregnancy. Amongst pregnant patients, there was also a significant difference between younger women with better conversions ($M = 2.96$, $SD = 3.01$) and older women with lower conversion rates ($M = 0.31$, $SD = 0.69$; $P < 0.001$).

Figure 1b demonstrates that the conversion rates from DHEA to FT significantly differed between *FMRI* genotypes and sub-genotypes [$F(3, 54) = 3.52$, $P = 0.021$] and between younger and older patients [$F(1, 54) = 9.55$, $P = 0.003$]. The interaction term between *FMRI* genotypes/sub-genotypes and age also almost reached significance ($P = 0.057$).

A post-hoc comparison (Holm-Sidak method) confirmed significant differences in conversion rates between *het-norm/high* ($M = 1.38$, $SD = 2.60$) and *het-norm/low* *FMRI* sub-genotypes ($M = 0.01$, $SD = 0.64$; $P = 0.003$), with the former converting more efficiently than the latter; and between younger ($M = 2.01$, $SD = 2.46$) and older patients ($M = 0.24$, $SD = 0.70$; $P = 0.003$).

FSH_{BL} and AMH_{BL} comparison with FSH_{CS} and AMH_{CS}, and FSH/androgen and AMH/androgen ratios

FSH_{BL} and AMH_{BL} differed significantly between pregnant and non-pregnant patients (both $P < 0.001$), but with cycle start only AMH_{CS} had improved ($P < 0.001$) (Fig. 2a). Comparing androgens at baseline with androgens at cycle start, only FT_{CS} was significantly increased, comparing pregnant and non-pregnant women ($P = 0.006$) (Fig. 2b).

Since, especially at small follicle sizes, FSH and androgens are believed to act synergistically [20], we investigated ratios of FSH over the various androgens. Significant differences were noted between pregnant and non-pregnant women in: FSH_{BL}/DHEA_{BL} ($P = 0.039$), FSH_{BL}/DHEAS_{BL} ($P = 0.040$), FSH_{BL}/DHEA_{CS} ($P = 0.013$), FSH_{BL}/DHEAS_{CS} ($P = 0.009$) and FSH_{BL}/FT_{CS} ($P = 0.014$). FSH_{BL}/TT_{CS} almost reached significance ($P = 0.051$) (Fig. 2C1 and C2). Analyses of FSH_{CS} over cycle start androgen levels, proved all not significant (data not shown).

Similarly, the following AMH/androgen ratios were found to differ between pregnant and non-pregnant women: AMH_{BL}/DHEA_{BL} ($P < 0.001$), AMH_{BL}/DHEAS_{BL} ($P = 0.002$), AMH_{BL}/DHEA_{CS} ($P < 0.001$) and AMH_{BL}/DHEAS_{CS} ($P = 0.002$). AMH_{BL}/TT_{CS} ($P = 0.005$). In contrast to FSH_{CS}, which had not shown significant ratios, AMH_{CS} did demonstrate one significant ratio, AMH_{CS}/DHEAS_{CS} ($P = 0.038$) (Fig. 2d).

A series of logistic regressions, adjusting for age eliminated significance in all previously significant ratios, except

Table 1 Patient, IVF cycle characteristics and primary infertility diagnoses

Characteristic	Results		
	Total (<i>n</i> =213)	Younger patients (<i>n</i> =41)	Older patients (<i>n</i> =172)
Age (years)	41.5±4.4	34.6±2.4 ¹	43.2±3.0 ¹
BMI (kg/m ²)	24.4±5.0	23.4±3.7	24.6±5.2
Estradiol (pg/mL)	57.3±38.0	60.3±36.5	56.6±38.4
FSH _{BL} (mIU/mL)	12.2±9.4	10.8±6.0	12.5±10.0
AMH _{BL} (ng/mL)	1.0±1.3	1.7±1.6 ²	0.8±1.1 ²
Gonadotropin dosage/IVF cycle (IU)	6011.8±2330.8	4840.9±1925.8 ³	6304.5±2336.4 ³
Oocyte yield (n)	6.2±5.6	9.0±6.4 ⁴	5.6±5.2 ⁴
Embryos Transferred (n)	2.0±1.4	1.8±1.2	2.0±1.5
Embryos Cryopreserved (m_	0.9±2.2	2.0±3.3 ⁵	0.6±1.7 ⁵
Race [n (%)]			
Caucasian	159 (74.6)	31 (75.6)	128 (74.4)
Asian	31 (14.6)	8 (19.5)	23 (13.4)
African American	23 (10.8)	2 (4.9)	21 (12.2)
<i>FMRI</i> [n (%)]			
<i>norm</i>	106 (49.8)	22 (53.7)	84 (48.8)
<i>het-norm/high</i>	38 (17.8)	7 (17.1)	31 (18.0)
<i>het-norm/low</i>	58 (27.2)	8 (19.5)	50 (29.1)
<i>hom</i>	11 (5.2)	4 (9.8)	7 (4.1)
Primary infertility diagnoses [n (%)] ^a			
Diminished ovarian reserve	140 (65.7)	19 (46.3) ⁶	121 (70.3) ⁶
Male factor	48 (22.5)	12 (29.3)	36 (20.9)
Endometriosis	9 (4.2)	3 (7.3)	6 (3.5)
Uterine factors	17 (8.0)	4 (9.8)	13 (7.6)
Polycystic Ovary Syndrome	11 (5.2)	4 (9.8)	7 (4.1)
Clinical Pregnancy [n (%)]			
Per IVF Cycle	47 (22.1)	14 (34.1) ⁷	33 (19.2) ⁷

^aSome patients had more than one infertility diagnosis. Only 39 of 213 cycles ended up with cryopreserved embryos. Values are presented as means ± SD. Subscripts denote significant differences at $P \leq 0.05$ (Chi-square and t-tests). ¹ $P \leq 0.001$; ² $P \leq 0.001$; ³ $P \leq 0.001$; ⁴ $P = 0.003$; ⁵ $P = 0.013$; ⁶ $P = 0.004$; ⁷ $P = 0.038$

for AMH_{CS}/DHEA_{CS} ($\beta = -0.499$, $SE = 0.240$, $P = 0.048$). For every unit increase in ratio, clinical pregnancy potential decreased by a factor of 1.7.

Evaluation of pregnancy chances per DHEA, binned low, normal or high

Assessments of pregnancy chances, based on binned DHEA_{BL} (or other androgens) did not affect pregnancy chances, either based on low, normal or high DHEA levels or based on *FMRI* genotypes and sub-genotypes (data not shown).

DHEA_{CS}, however, did: In presence of low DHEA_{CS}, odds of achieving pregnancy were significantly influenced by FT_{CS} ($\beta = 0.948$, $SE \pm 0.361$, $P = 0.009$), increasing pregnancy odds 2.6-fold. In presence of normal DHEA_{CS} levels, FT_{CS} missed significance ($\beta = 0.456$, $SE \pm 0.255$, $P = 0.074$). With high DHEA_{CS} levels, odds of achieving clinical pregnancy were significantly influenced only by DHEA_{CS} ($\beta =$

0.607, $SE \pm 0.193$, $P = 0.002$), increasing odds 1.8-fold (Fig. 3a). With normal levels of DHEA_{CS}, change of FT_{BL} to FT_{CS} was almost significant in affecting pregnancy chances ($\beta = 1.662$, $SE \pm 0.858$, $P = 0.053$). With high DHEA_{CS}, the ratio of DHEA_{CS}/DHEA_{BL} also significantly affected pregnancy chances ($\beta = 0.040$, $SE \pm 0.014$, $P = 0.005$) (Fig. 3b), increasing pregnancy odds 1.04-fold.

After adjusting for age, oocyte yields and length of DHEA treatment (time between DHEA_{BL} and DHEA_{CS}), FT_{CS} was, in presence of low DHEA_{CS}, significantly associated with increased odds of pregnancy ($\beta = 0.882$, $SE \pm 0.401$, $P = 0.028$) by a factor of 2.4. In presence of high DHEA_{CS}, increasing DHEA_{CS} ($\beta = 0.568$, $SE \pm 0.196$, $P = 0.004$) and the ratio of DHEA_{CS}/DHEA_{BL} ($\beta = 0.040$, $SE \pm 0.015$, $P = 0.006$) also increased odds of pregnancy by factors of 1.8 and 1.04, respectively (Fig. 3c).

Fig. 1 Androgen conversion rates by age and *FMR1* genotypes and sub-genotypes. Two-way ANOVA for pregnancy outcome and age group demonstrated significant interaction ($P=0.025$). **a** There was also a significant interaction between pregnant and non-pregnant women ($P=0.015$), independently observed in younger women only ($P=0.007$). Amongst pregnant women, older women also demonstrated a significantly lower androgen conversion rate from DHEA to T ($P<0.001$). **b** demonstrates that androgen conversion rates differ significantly between *FMR1* genotypes and sub-genotypes ($P=0.021$), between younger and older patients ($P=0.003$), and in the interaction term between *FMR1* and age ($P=0.057$). *Post-hoc comparison (Holm-Sidak) demonstrated that sub-genotype *het-norm/high* converts androgens significantly more efficiently than *het-norm/low* ($P=0.003$), and younger convert androgens more efficiently than older women ($P=0.003$)

Pregnancy chances based on *FMR1* genotypes and sub-genotypes

As Fig. 4a demonstrates, in women with *het-norm/high* *FMR1* sub-genotype, TT_{CS} was associated with greater odds of achieving pregnancy ($\beta=0.106$, $SE\pm 0.052$, $P=0.043$), increasing pregnancy odds, though only 1.1-fold. In contrast, *norm* and *het-norm/low* women demonstrated only a trend towards significance (data not shown). Even collapsed *hom* patients were too few in number to allow for valid statistical analyses. FT_{CS} almost reached significance in increasing odds of pregnancy in women with *het-norm/low* sub-genotype ($\beta=0.502$, $SE\pm 0.267$, $P=0.060$), and a similar trend was observed in *norm* and *het-norm/high* women (data not shown).

Moreover, in *het-norm/low* patients, interaction between $DHEA_{CS}$ and FT_{CS} actually decreased odds of clinical pregnancy 1.3-fold ($\beta=-0.295$, $SE\pm 0.155$, $P=0.056$). Adjusted for oocyte yields, patient age, and time between $DHEA_{BL}$ and $DHEA_{CS}$, the interaction between $DHEA_{CS}$ and FT_{CS} significantly affected clinical pregnancy chances in women with *het-norm/low* *FMR1* sub-genotypes ($\beta=0.333$, $SE\pm 0.180$, $P=0.064$), actually decreasing odds of pregnancy 1.4-fold (Fig. 4b). A similar trend, not reaching significance was also

observed for *het-norm/high* but not for women with *norm* genotypes (data not shown). *Norm* women, however, demonstrated almost statistically significant odds of increased pregnancy rates based on changes from $DHEA_{BL}$ to $DHEA_{CS}$ ($\beta=0.206$, $SE\pm 0.111$, $P=0.062$) (Fig. 4b).

Androgens in *FMR1* genotypes and sub-genotypes, stratified by age

In younger women (≤ 38 years), TT significantly changed between TT_{BL} and TT_{CS} assessments for the various *FMR1* genotypes and sub-genotypes [$F(2,10)=5.53$, $P=0.024$]. Post-hoc comparisons (Holm-Sidak test) suggested the mean TT change to be significantly lower for *het-norm/low* ($M=-116.69$, $SD=152.29$) than for *het-norm/high* *FMR1* sub-genotypes ($M=28.00$, $SD=57.98$; $P=0.028$) and *norm* genotypes ($M=26.38$, $SD=25.59$; $P=0.009$) (Fig. 5a). As the figure suggests, *het-norm/low* women at younger ages demonstrate higher mean TT_{BL} than TT_{CS} .

None of the mean ratio values between androgens showed significant differences for different *FMR1* genotypes and sub-genotypes between baseline and cycle start.

Fig. 2 Comparison of FSH at baseline and cycle start, and ratios between FSH and AMH with androgens. **a** Pregnant and non-pregnant women differed significantly in FSH_{BL} ($P = 0.006$) and AMH_{BL} ($P = 0.006$) but only in AMH_{CS} ($P < 0.001$). **b** In comparing androgens at baseline and cycle start only FT_{CS} differed significantly between pregnant and non-pregnant women ($P = 0.006$). **C1** and **C2** demonstrate the large number of FSH_{BL}/androgen ratios that

demonstrated significant or almost significant differences between pregnant and non-pregnant women (for detail, see text). None of the FSH_{CS}/androgen ratios proved different. **D1** and **D2** demonstrate a similarly large number of significant AMH_{BL}/androgen ratios, and only one AMH_{CS}/androgen ratio (for detail see text). Adjustment for age eliminated all significant ratios, except for AMH_{CS}/DHEAS_{CS}, pointing at the age-dependency of most ratios

Fig. 3 Pregnancy chances per low, normal and high DHEA values. Predicted probabilities of pregnancy odds are presented as means and 1 SE from the mean. Different levels of DHEA_{BL} or of other baseline androgen levels did not affect pregnancy chances. As **a** demonstrates, in presence of low DHEA_{CS}, FT_{CS}, however, significantly influenced odds of pregnancy ($P=0.009$). With increasing DHEA_{CS}, the significance of FT_{CS}, declines (normal DHEA_{CS}, $P=0.074$). With high DHEA_{CS}, odds of achieving pregnancy were only significantly affected by DHEA_{CS} ($P=0.002$; data not shown). **b** summarizes significant changes in hormones between baseline and cycle start: With normal

DHEA_{CS}, Δ FT_{BL} to FT_{CS} almost reached significance in affecting pregnancy chances ($P=0.053$). With high DHEA_{CS}, the ratio of DHEA_{CS}/DHEA_{CS} also affected pregnancy chances significantly ($P=0.005$), though pregnancy odds barely changed (data not shown). **c** demonstrates associations after adjustments for age, oocyte numbers and length of DHEA supplementation: With low DHEA_{CS}, FT_{CS} was still significantly associated with increasing odds of pregnancy ($P=0.028$), and the same applied to rising DHEA_{CS} ($P=0.004$) and DHEA_{CS}/DHEA_{CS} ratio ($P=0.006$) in presence of high DHEA_{CS}, though pregnancy odds barely changed

Older women (>38 years) demonstrated a different pattern: They showed significant differences in mean DHEA_{CS} between *FMRI* genotypes and sub-genotypes [F (3,168)=3.17, $P=0.026$]. Holm-Sidak confirmed that with *hom FMRI* genotypes (because of small numbers,

hom sub-genotypes were not evaluated separately) DHEA_{CS} was significantly higher ($M=578.29$, $SD=475.85$) than in *het-norm/high* ($M=334.96$, $SD=182.53$; $P=0.004$) and in *het-norm/low* women ($M=349.06$, $SD=188.51$; $P=0.005$) (Fig. 5b).

Fig. 4 Pregnancy chances based on *FMR1* genotypes and sub-genotypes. Predicted probabilities of pregnancy odds are presented as means and 1 SE of the mean. **a** demonstrates that increasing TT_{CS} in women with *het-norm/high* *FMR1* sub-genotype was associated with greater odds of pregnancy ($P=0.043$). The collapsed *hom* genotype, overall, behaved differently from other *FMR1* genotypes/sub-genotypes: Predicted probabilities for pregnant and non-pregnant groups in *hom* women were very similar, while other genotype and sub-genotypes demonstrated statistical trends. Rising FT_{CS} also almost

reached significance in *het-norm/low* ($P=0.060$) and so, though to lesser degree, did other genotypes and sub-genotypes. As **b** demonstrates, in *het-norm/low* sub-genotypes, interaction between $DHEA_{CS}$ and FT_{CS} decreased odds of pregnancy ($P=0.056$) and this conclusion remained valid after adjustments for oocyte numbers, age and length of DHEA supplementation ($P=0.064$). *Het-norm-high* demonstrated a similar trend but not women with *norm* and *hom* genotype. Odds of pregnancy, however, did demonstrate a trend in *norm* women, based on $\Delta DHEAS_{BL}$ to $DHEAS_{CS}$ ($P=0.062$)

Moreover, older women also demonstrated significant differences in androgen ratios: Specifically, $DHEAS_{CS}/DHEA_{CS}$ differed between *FMR1* genotypes and sub-genotypes [$F(3,164)=3.22$; $P=0.024$]. Holm-Sidak post-hoc test suggests that mean $DHEAS_{CS}/DHEA_{CS}$ for women with *hom* genotype ($M=126.4$, $SD=126.2$) was significantly higher than in women with *het-norm/high* ($M=63.5\%$, $SD=39.6$; $P=0.003$) and *het-norm/low* *FMR1* sub-genotypes ($M=69.4\%$, $SD=42.7$; $P=0.005$) and with *norm* genotype ($M=76.8\%$, $SD=47.5$; $P=0.013$) (Fig. 5c). As the figure

demonstrates, this may suggest lower $DHEA_{CS}$ in women with *hom* genotypes.

The change between $DHEAS_{BL}$ and $DHEAS_{CS}$, in itself, indeed occurred significantly differently between *FMR1* genotypes and sub-genotypes [$F(3,165)=2.98$; $P=0.033$]. Holm-Sidak indicates that in *hom* women the increase in mean levels from $DHEAS_{BL}$ to $DHEAS_{CS}$ was significantly larger ($M=330.71$, $SD=382.85$) than in either *het-norm/high* ($M=57.49$, $SD=210.99$; $P=0.003$) or *norm* women ($M=103.76$, $SD=209.53$; $P=0.009$).

Fig. 5 *FMR1* genotypes and sub-genotypes stratified by age. **a** demonstrates that in women ≤ 38 years ΔTT_{BL} to TT_{CS} *FMR1* genotypes and sub-genotypes, overall significantly changed ($P=0.024$). Post-hoc comparisons further demonstrated that this change in Δ of mean TT was significantly smaller for *het-norm/low* than *het-norm/high* ($P=0.028$) or *norm* ($P=0.009$) but mean ratio values between androgens did not differ. As **b** demonstrates, older women > 38 years behaved differently: DHEAS_{CS} differed significantly between *FMR1* genotypes and sub-genotypes ($P=0.026$), and post-hoc analysis confirmed that

DHEAS_{CS} was significantly higher in *hom* than *het-norm/high* ($P=0.003$) and *het-norm/low* women ($P=0.005$). Older women, however, also demonstrated significant differences in mean androgen ratios: **c** demonstrates that DHEAS_{CS}/DHEA_{CS} differed significantly between *FMR1* genotypes and sub-genotypes ($P=0.024$), and post-hoc analysis suggested that *hom* women had a significantly higher ratio than *het-norm/high* ($P=0.003$), *het-norm/low* ($P=0.005$) and *norm* women ($P=0.013$)

Discussion

Androgen conversion rates by age and *FMR1*

Women who conceived had better conversion rates than those who did not, and this was particularly apparent amongst younger patients (Fig. 1a). These observations are

of significance because they suggest that: (a) better conversion from DHEA to T improves pregnancy chances in women with DOR; (b) younger women convert androgen more effectively than older women; (c) that older women may, therefore, potentially benefit from more direct T administration and (d) that the evaluation of androgen levels in women with DOR is beneficial.

Not shown in our result section is that we attempted by receiver operating curve (ROC) to determine whether, at least in younger women, below age 38 years, androgen conversion rates could be predictive of pregnancy outcome. Likely due to small patient sample size, we were, however, unable to demonstrate significance (area under the curve 0.694, 95%CI 0.369–1.000; $P=0.262$)

Conversion from DHEA to FT significantly differed between *FMRI* genotypes and sub-genotypes (Fig. 1b), and the interaction term between *FMRI* and age barely missed significance ($P=0.057$). These observations support that, as previously reported [15, 16], variations in pregnancy rates between *FMRI* genotypes and sub-genotypes in populations with better FOR would, be more apparent. This is also supported by the post-hoc analysis, demonstrating that, independent of age, *het-norm/high* sub-genotypes converted androgens significantly better than *het-norm/low* women, as did younger in comparison to older women.

Combined, these data point towards improved pregnancy chances for women who most efficiently convert DHEA to T. The data, however, also point towards differences in efficiency of conversion, based on *FMRI* genotypes and sub-genotypes.

FSH_{BL/CS}, AMH_{BL/CS}, FSH/androgen and AMH/androgen ratios

This study also demonstrated that FSH_{BL} was lower and AMH_{BS} higher in patients who conceived (Table 1). Lower FSH and higher AMH, of course, denote better FOR and, therefore, better pregnancy chances are expected [17, 18]. By IVF cycle start, however, only AMH was left significantly associated with pregnancy chances (Fig. 2a), which correlates well with the earlier reported observations that AMH, but not FSH improves with DHEA supplementation, and that increases in AMH levels are associated with improved pregnancy chances with IVF [21].

Comparing androgen levels at baseline and cycle start between pregnant and non-pregnant women, only FT_{CS} differed significantly, demonstrating a significant increase from FT_{BL} in pregnant women ($P=0.006$; Fig. 2b). This observation, of course, supports that efficient DHEA to FT conversion is beneficial for pregnancy chances.

Considering previously noted synergism between androgens and FSH [19, 22], and likely negative feedback of AMH on small growing follicles [23], we also attempted to investigate any possible interplay between androgens and FSH and androgens and AMH. Our hypothesis was that synergisms/antagonisms between FSH/AMH and androgens might be detectable by investigating FSH/androgen and AMH/androgen ratios. Neither had ever before been investigated, and since we presumed such interactions to occur at small growing follicle stages, we expected them to become more “visible” at baseline rather than at IVF cycle start.

Results, indeed, confirmed this by demonstrating significant differences in pregnancy chances based on the following FSH_{BL}/androgen ratios: FSH_{BL} over DHEA_{BL}, DHEAS_{BL}, DHEA_{CS}, DHEAS_{CS} and FT_{CS}. It barely missed significance with FSH_{BL}/TT_{CS} ($P=0.051$) (Fig. 2C1 and C2). In contrast, FSH_{CS} ratios did not demonstrate any significant associations. This dichotomy of results is supportive of our hypothesis that androgen/FSH ratios at baseline may accurately reflect the small growing follicle pool, representing most of FOR [1]. AMH/androgen ratios further strengthen this concept. With only one exception, all pregnancy-associated AMH/androgen ratios related to AMH_{BL}. Those included ratios with DHEA_{BL}, DHEAS_{BL}, DHEA_{CS}, DHEAS_{CS} and TT_{CS}. Only AMH_{CS}/DHEAS_{CS} related to AMH_{CS} (Fig. 2D1 and D2).

FSH/androgen and AMH/androgen ratios, thus, appear to offer new, and quite direct assessments of the small growing follicle pool, representing true FOR. Currently, AMH is considered to best reflect this stage of follicle maturation [24]. Here presented data suggest that androgen/FSH and androgen/AMH ratios at baseline may have prognostic significance that warrants further investigation. These ratios, further, raise possible therapeutic benefits: For example, adjustments in androgen supplementation, based on FSH and/or AMH levels, may make it possible to bring ratios into, yet to be determined, therapeutic ranges.

Remarkably, only AMH_{CS}/DHEAS_{CS} maintained significance after adjustment for patient age. This confirms the importance of age in predicting IVF success but also points to the possibility that the AMH_{CS}/DHEAS_{CS} ratio may, independent of age, predict IVF pregnancy chances. Like all FOR assessment tools, AMH levels, in principle, are age-specific [18]. At 1.05 ng/mL, AMH, however, independent of age, has been reported in DOR to differentiate between better and poorer IVF pregnancy chances [24], and, in comparison to FSH, may, therefore, be more reflective of age-independent characteristics of FOR, required for pregnancy success. These data, ultimately suggest that, aside from the AMH_{CS}/DHEAS_{CS} ratio, all other here reported desirable ratios, likely, will have to be established in age-specific ways.

DHEA and other androgens binned for low, normal and high levels

DHEA_{BL}, and other androgens at baseline did not affect overall pregnancy chances or in association with individual *FMRI* genotypes or sub-genotypes. In contrast, DHEA_{CS} did: If DHEA_{CS} was low, increasing FT_{CS} improved odds of pregnancy (Fig. 3a). As DHEA_{CS} improved towards normal, FT_{CS} slowly lost significance ($P=0.074$), with high DHEA_{CS} levels no longer showing any association with FT_{CS}, and odds of achieving pregnancy only affected by rising DHEAS_{CS}.

This again points towards the importance of effective DHEA conversion to T but, likely, only if androgens are initially low.

As baseline androgens increase, importance of further androgen conversion declines, suggesting the possibility of a therapeutic androgen range for maximal pregnancy chances. That at normal DHEA_{CS} levels changes of FT_{BL} to FT_{CS} only almost reached significance ($P=0.053$) and that at high levels of DHEA_{CS}, once again, only the DHEA_{CS}/DHEA_{CS} ratio significantly affected pregnancy chances (Fig. 3b) also support such an interpretation. It is further supported by the observation that after adjustments for length of DHEA supplementation, female age and oocyte yields, in presence of low DHEA_{CS}, FT_{CS}, still, was significantly associated with increasing pregnancy odds, and in presence of high levels of DHEA_{CS}, only increasing DHEA_{CS} and the DHEA_{CS}/DHEA_{CS} ratio were positively affecting pregnancy chances (Fig. 3c).

The data, therefore, also suggest that in women who poorly convert DHEA to T, the bottleneck in sequential conversions appears to lie at the conversion of [DHEA ↔ DHEAS] to androstenedione. So identified patients may, therefore, benefit from direct administration of T rather than DHEA.

FMRI genotypes and sub-genotypes

FMRI genotypes and sub-genotypes appear to reflect different degrees of androgen effectiveness, as TT_{CS} in *het-norm/high* women was significantly associated with greater odds of pregnancy ($P=0.043$), and in *norm* and *het-norm/low* patients only suggested a trend towards significance (Fig. 4a). Similarly, FT_{CS} almost reached significance for *het-norm/low* and demonstrated trends for *norm* and *het-norm/high*.

Interaction between DHEA_{CS} and FT_{CS} in *het-norm/low* patients decreased odds of pregnancy ($P=0.056$). However, most importantly, after adjustment for length of DHEA supplementation, female age and oocyte yields, the interaction between DHEA_{CS} and FT_{CS} still significantly affected pregnancy chances for women with *het-norm/low* sub-genotype; but only trends were observed with *het-norm/high* and *norm* (Fig. 4b). Least affected by conversion from DHEA_{CS} to FT_{CS} appeared to be women with *norm* genotypes but they, interestingly, demonstrated almost statistically significant odds of increased pregnancy rates ($P=0.062$) based on changes in the DHEA_{BL} to DHEA_{CS} ratio, suggesting that in these patients androgen conversion rates may differ from other *FMRI* genotypes (Fig. 4b), and higher T levels may be less important.

FMRI genotypes and sub-genotypes and age

Stratification by age is important because it allows insights into two distinct forms of DOR, so-called premature ovarian aging (POA) in younger women, and physiologic aging with advanced female ages [17, 19]. Such stratification, once more, demonstrates differences in androgen conversion: young women (≤ 38 years) demonstrated significant differences in

progression from TT_{BL} to TT_{CS}. Specifically, women with *het-norm/low* sub-genotypes demonstrated significantly slower increases in TT between these two time points than *het-norm/high* and *norm* patients (Fig. 5a). These data, therefore, suggest distinct differences in how *FMRI* genotypes and sub-genotypes process androgens at different ages.

Women with *het-norm/low* sub-genotypes have been reported to demonstrate significantly reduced pregnancy chances with IVF [15, 16]. Considering the poor androgen conversion rates observed in this study in *het-norm/low* women, lower pregnancy chances with this *FMRI* sub-genotypes should, therefore, not surprise.

While young women demonstrated no differences in mean androgen levels between *FMRI* genotypes and sub-genotypes, older women demonstrated distinct differences in DHEA_{CS}. Specifically, collapsed *hom* patients demonstrated significantly elevated DHEA_{CS} in comparison to both *het* sub-genotypes (Fig. 5b) and higher DHEA_{CS}/DHEA_{CS} ratios than *het* sub-genotypes and *norm* patients (Fig. 5c). Moreover, *hom* patients increased mean levels of DHEA_{BL} to DHEA_{CS} to a significantly larger degree than *het-norm/high* and *norm* women (Fig. 5d).

Based on their *FMRI* genotype and sub-genotype, younger and older women with DOR, thus, appear to convert androgens at different rates. FOR differs in young oocyte donors, depending on race. At somewhat older ages the differences have, however, already significantly diminished in women with infertility [25]. At least partially, FOR differences observed between races are due to varying *FMRI* genotype distributions, with African women demonstrating disproportionally more *het-norm/low* sub-genotypes and Asian women more *het-norm/high* [16]. IVF outcome differences between races, partially attributed to such variations in *FMRI* genotype distributions [25], may, therefore, ultimately, reflect differences in androgen processing.

The increased importance of DHEA_{CS}/DHEA_{CS} ratio in older women suggests that, lacking ability to convert [DHEA ↔ DHEAS] to T as well as younger women, they accumulate DHEAS. High DHEA_{CS}/DHEA_{CS} ratios, independent of age, therefore, likely, suggest a poorer pregnancy prognosis. Younger women with poor androgen conversion, and older women in general may, therefore, benefit from direct T supplementation in place of or in addition to DHEA.

Here presented data offer also a first glimpse at the possible function of the *hom* genotype of *FMRI*. While functions of *norm* and *het* genotypes have previously been well defined [14–16], *hom*, because it is comparatively rare, is not well understood. Based on higher DHEA_{CS}, this study suggests that *hom* women convert androgens poorer than either *norm* or *het* females. *Hom* patients, therefore, can be expected to demonstrate poorer responses to DHEA supplementation, and poorer clinical outcomes with IVF, though that remains to be confirmed by clinical studies.

Summary and conclusions

The evolving picture, therefore, suggests that younger women convert androgens better than older women, though how well androgens are converted appears to differ between *FMRI* genotypes and sub-genotypes at younger as well as older ages, though with greater impact at younger ages. Better IVF pregnancy rates after DHEA supplementation in younger than older women support this observation [8].

Whether androgens are beneficial for normal follicle maturation and female fertility has in humans remained controversial [1]. Indeed, only recently, did two commentaries suggest that DHEA supplementation in women with DOR should not be considered [26, 27]. So far only one, underpowered clinical trial of DHEA supplementation has been reported [28], and most reported studies utilized lower power of evidence [6]. Lack of recruitment resulted in the abandonment of at least two prospectively randomized studies of DHEA in the U.S. and Europe, in which our center was involved (Government ID# NCT00419913). Two trials are still underway but recruitment is excruciatingly slow (Government ID# NCT00948857 and #NCY00650754), as, perceiving themselves “out of time,” DOR patients are difficult to recruit into randomizations to placebo.

It recently occurred to us that the investigation of androgen conversion rates after DHEA supplementation might improve our understanding of DHEA effects on ovaries. Like in the mouse, in humans granulosa cells of small growing follicles demonstrate AR, FSHR and AMHR [1]. Synergistic activity on granulosa cells between androgens and FSH at these stages appears likely [20, 22]. Effects of AMH are less well understood. Kristensen et al. recently reported that peripheral AMH correlates with peripheral androgens [11]. Androgens, FSH and AMH, through their interaction, likely, affect follicle recruitment and small follicle growth, androgens and FSH synergistically, and AMH inhibitory. In high recruitment situations, like polycystic ovarian syndrome (PCOS), AMH, therefore, is high, and in low recruitment environments, like hormonal contraception, pregnancy and DOR, AMH is low [25]. Genotypes and sub-genotypes of the *FMRI* gene also appear to influence follicle recruitment, and are closely associated with ovarian aging patterns [14, 15]. That they vary in androgen conversion rates may offer a potential physiological link between these observations.

This study investigated a unique patient population of 213 DHEA-supplemented infertile women with DOR. All, because of a DOR diagnosis, had undergone *FMRI* evaluations and had a complete set of baseline androgen data at DHEA supplementation start and, after at least 6 weeks of DHEA supplementation, at IVF cycle start. Finally, all patients completed a first IVF cycle, allowing for detailed outcomes assessments.

In such an unfavorable population the observed clinical pregnancy rate (positive fetal heart on ultrasound) of 22.1% is noteworthy, with women under age 38 actually conceiving in 34.1% of cycles. That pregnancy rates did not differ based on *FMRI*, may on first glance surprise since we previously reported that *het-norm/low* women experience significantly lower pregnancy rates than women with *norm FMRI* genotype [15]. Those data were, however, generated in women with better FOR. Here presented data suggest that, once ovarian reserve is significantly compromised, ovarian *FMRI* genotypes and sub-genotypes no longer matter to the same degree. This interpretation of results is also supported by the observation that FORs in young donors vary significantly based on *FMRI*; but in older infertility patients differences have largely disappeared [29].

DOR appears to represent an androgen deficient state, whether in younger or older women. To what degree, is currently under investigation at our center. Since androgens mostly derive from adrenal precursors, DOR, at least in part, has to reflect a clinical insufficiency state of the adrenal zona reticularis. DOR, therefore, mirrors in opposing features the hyperandrogenism of PCOS, for decades recognized as adrenal and ovarian in etiology.

FSH and AMH/androgen ratios are, to a rather surprising degree, predictive of pregnancy success. One can hypothesize that these ratios may better represent the small growing follicle pool than either FSH or AMH alone. They also offer potential therapeutic benefits by allowing for the individualization of androgen dosages, based on FSH and AMH levels, and, therefore, for diagnostic and therapeutic reasons deserve further investigations.

Financial disclosure NG, AW and DHB have received research and grant support, travel funds and speaker honoraria from various pharmaceutical and medical device companies, none, however, related to here presented topics. NG and DHB are listed as inventors on two already awarded and other still pending United States patents, claiming beneficial effects on diminished ovarian reserve (DOR) and embryo ploidy from dehydroepiandrosterone (DHEA) or from other androgen supplementations. NG is owner of CHR, where this research was conducted. NG and DHB are also listed as co-inventors on a number of pending United States patents, claiming diagnostic relevance for the assessment of triple CGG repeats on the *FMRI* gene in determining risk towards DOR and related issues. At time of this submission only two United States user patents with relevance to this manuscript, both DHEA-related, have been awarded (November 10, 2009; #7615544 and November 29, 2011; #8067400) The first claims benefits from supplementation of DHEA/androgens on ovarian reserve and IVF pregnancy rates in women with DOR; and the second claims decreased aneuploidy (and, therefore, miscarriage rates) and improved pregnancy rates. Among *FMRI*-related pending patent applications, one describes ovarian genotypes and sub-genotypes, as used in this manuscript, and claims that these genotypes and sub-genotypes reflect different ovarian aging patterns. All patent applications filed by researchers at CHR are 50 % owned by CHR and 50 % by the investigators who did the research that led to the application. A full list of all patent information can be provided on request.

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Contributions to authorship

N.G. and D.H.B. contributed equally to the manuscript. N.G. contributed to study design and data analysis, and wrote the manuscript, A.K. and D.H.B. performed data analyses and statistical analyses, A.W. contributed to study design. All authors approved the final manuscript