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ARTICLE

Multicentric, prospective observational data show sperm capacitation predicts male fertility, and cohort comparison reveals a high prevalence of impaired capacitation in men questioning their fertility

BIOGRAPHY

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KEY MESSAGE

Sperm capacitation prospectively predicted male fertility/probability of generating pregnancy. Relative to a fertile population, reduced capacitation was highly prevalent in men questioning their fertility, even if the volume, concentration and motility of the spermatozoa were normal.

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ABSTRACT

Research questions: Can a previously defined relationship between sperm capacitation and the probability of a man generating pregnancy within three cycles, prospectively predict male fertility in diverse clinical settings? A second study asked, what is the prevalence of impaired sperm fertilizing ability in men questioning their fertility (MQF), and does this relate to traditional semen analysis metrics?

Design: In the multicentric, prospective observational study, data (n = 128; six clinics) were analysed to test a published relationship between the percentage of fertilization-competent, capacitated spermatozoa (Cap-Score) and probability of generating pregnancy (PGP) within three cycles of intrauterine insemination. Logistic regression of total pregnancy outcomes (n = 252) assessed fit. In the cohort comparison, Cap-Scores of MQF (n = 2155; 22 clinics) were compared with those of 76 fertile men.

Results: New outcomes (n = 128) were rank-ordered by Cap-Score and divided into quintiles (25–26 per group); chi-squared testing revealed no difference between predicted and observed pregnancies (P = 0.809). Total outcomes (n = 252; 128 new + 124 previous) were pooled and the model recalculated, yielding an improved fit (P < 0.001). Applying the Akaike information criterion found that the optimal model used Cap-Score alone. Cap-Scores were performed on 2155 men (with semen analysis data available for 1948). To compare fertilizing ability, men were binned by PGP (≤19%, 20–29%, 30–39%, 40–49%, 50–59%, ≥60%). Distributions of PGP and the corresponding Cap-Scores were significantly lower in MQF versus fertile men (P < 0.001). Notably, 64% of MQF with normal volume, concentration and motility (757/1183) had PGP of 39% or less (Cap-Scores ≤31), versus 25% of fertile men.

Conclusions: Sperm capacitation prospectively predicted male fertility. Impaired capacitation affects many MQF with normal semen analysis results, informing diagnosis versus idiopathic infertility.

INTRODUCTION

Infertility has often incorrectly been viewed as a 'women's health' problem, even though men contribute to 40–60% of the cases (*Agarwal et al., 2015*; *Mehta et al., 2016*; *Petok, 2015*). Despite infertility affecting 10–15% of couples globally (*Sharma et al., 2013*), the field of andrology lacks informative diagnostics (*Barratt et al., 2018*). Men are often assumed fertile if they have enough morphologically normal, motile spermatozoa to pass current World Health Organization (WHO) guidelines for lower reference values. This is despite the fact that it is well known that most male fertility problems are a result of poor sperm function/fertilizing ability and are not detected by traditional semen analysis (*Guzick et al., 2001*; *Ombelet et al., 1997*; *van der Steeg et al., 2011*). Lack of an appropriate diagnostic assessment of fertilizing ability has led to most male infertility cases being classified as 'idiopathic', or unexplained, and to repeated calls in the literature for the development of tests capable of evaluating the fertilization competency of spermatozoa (*Barratt et al., 2018*; *Lamb, 2010*; *Oehninger et al., 2014*; *Wang and Swerdloff, 2014*). New urgency is felt as it is recognized that traditional semen analysis metrics are falling precipitously in industrialized nations (*Levine et al., 2017*), and that male fertility can reflect and be prognostic for general male health (*De Jonge and Barratt, 2019*).

Clinically, this gap between need and available diagnostics has resulted in four serious negative impacts. First, it has placed the onus for extensive and often invasive diagnostic testing almost exclusively on women, with men often going undiagnosed (*Steinkeler et al., 2009*; *Stevenson et al., 2016*). Second, the failure to correctly assess male fertility has resulted in innumerable cycles of intrauterine insemination (IUI) that had low chance of success; these repeated failures are then a basis for diagnosing idiopathic infertility (*Bungum et al., 2004*; *Ruiz et al., 1997*). Conversely, efforts to avoid IUI failure due to undiagnosed defects in sperm fertilizing ability have led to a third problem, namely, that couples are sometimes advised to go straight to invasive and expensive procedures such as intracytoplasmic sperm injection (ICSI), when in fact IUI might be effective (*Bhattacharya et al., 2001*; *Evers, 2016*). Fourth, the development and use of treatments to improve male infertility has been hampered by lack of an appropriate measure of sperm fertilizing ability that could not only identify which men need treatment, but also then gauge the impact of those interventions (e.g. lifestyle changes in diet, exercise, tobacco or alcohol exposure, surgical repair of varicocele, treatment with various supplements, etc.) (*Aly and Seaman, 2018*; *Hayden et al., 2018*). In short, a test that assesses sperm fertilizing ability could provide important benefits, enabling more personalized approaches to achieve pregnancy and to improve male fertility.

One quantifiable measure of sperm function is capacitation status. When spermatozoa enter the female tract, they attain fertilization competence through the process of 'capacitation', in which the head acquires the ability to undergo acrosome exocytosis and the flagellum acquires hyperactivated motility (*Austin, 1952*; *Chang, 1951*; *Travis and Kopf, 2002*). Capacitation is achieved in response to stimuli including removal of membrane sterols and influx of calcium and bicarbonate (*Travis and Kopf, 2002*). Over multiple studies, the current authors identified the organization of membrane microdomains having varying compositions of sterols, the ganglioside G_{M1} and proteins involved in capacitation and acrosome exocytosis (*Asano et al., 2009*; *Asano et al., 2010*; *Asano et al., 2013*; *Buttke et al., 2006*; *Selvaraj et al., 2006*; *Selvaraj et al., 2009*; *Travis et al., 2001*). Using cell biological, pharmacological and genetic approaches, these studies identified in murine spermatozoa that G_{M1} regulates transient calcium influxes through R-type, voltage-gated channels that enable acrosome exocytosis (*Cohen et al.,*

2014). Of diagnostic relevance, it was found in murine and bovine spermatozoa that G_{M1} localization could quantify the percentage of spermatozoa capable of fertilizing (Selvaraj et al., 2007).

When tested in human spermatozoa, G_{M1} localization indicated capacitation at the level of single cells that could undergo acrosome exocytosis induced by calcium ionophores (Moody et al., 2017) and by the more physiologically relevant stimulus, progesterone (Ostermeier et al., 2018). Use of the percentage of spermatozoa in an ejaculate that capacitate (the Cap-Score Male Fertility Assay, Androvia LifeSciences, USA) was validated in terms of precision, variance within samples, and variance between readers (Moody et al., 2017). Its relationship with male fertility was initially suggested at the level of ejaculates by the finding that higher percentages of capacitated spermatozoa correlated strongly with a history of success within three or fewer cycles of IUI (Cardona et al., 2017). In repeated samples from the same individual, the percentage of capacitated spermatozoa differed by an average of 6% points of the mean for that individual, which is much lower than the variability observed with traditional semen analysis parameters (Cardona et al., 2017). The Cap-Scores of 76 men with known recent fertility (not using technologies of assisted reproduction) had a normal distribution and were significantly greater than the Cap-Scores from 122 men questioning their fertility (MQF) (Cardona et al., 2017). In the same study, minimal to no relationship was detected between traditional semen analysis parameters (morphology, motility and concentration) and Cap-Scores for those MQF (Cardona et al., 2017). Note that the cohort of MQF is heterogeneous in nature. These men are pursuing medical workup and fertility assessment at urology offices and/or fertility clinics because of difficulty conceiving as a couple. In some cases, the male partner's fertility is sound, and the challenge for conception results from female factor infertility.

A single threshold value was then tested for its ability to prospectively identify men predicted to have normal fertility (n = 44) versus men predicted to have difficulty generating pregnancy (n = 47). In that study, female partners had no factors that precluded their eligibility for IUI (Schinfeld et al., 2018). Absolute and cumulative pregnancies differed significantly, with a 4.23-fold higher first cycle pregnancy success rate in men scoring above the cut-off (P = 0.02; Schinfeld et al., 2018). There were no differences in maternal or paternal age, or semen analysis metrics, between the outcome groups (Schinfeld et al., 2018). It is increasingly recognized that male fertility does not exist as a simple binary, 'infertile' or 'fertile' state, but rather exists on a continuum (Cairo Consensus Working Group, 2019). Therefore, clinical outcomes data from a single clinic (n = 57) were used to define a continuous relationship between the percentage of spermatozoa that can capacitate and male fertility, in the form of the probability of generating pregnancy (PGP) in three cycles. The fit of this model was then tested by the addition of 67 outcomes from five clinics (total n = 124), resulting in a small average change of 4% and improved fit (Schinfeld et al., 2018). Further analysis revealed that Cap-Score alone, independent of traditional semen analysis measures, provided the optimal model (Schinfeld et al., 2018).

In the current report, a multicentric, prospective observational study was first performed to determine whether the relationship between the percentage of capacitated spermatozoa and male fertility, as defined by the published model, would match observed clinical pregnancy outcomes under actual clinical conditions with diverse patient populations and practice settings as opposed to experimental conditions. In addition, all Cap-Scores and traditional semen analysis metrics were compared between the entire MQF cohort (inclusive of men in the prospective study) against the previously characterized fertile cohort (Cardona et al., 2017). To provide context for both studies, data collected under such conditions are known in medical epidemiology as 'real world data', and interpretations from observational studies or cohort comparisons based on them are known as 'real world evidence' (USFDA, 2019).

MATERIALS AND METHODS

Study design

Methods and analyses are reported in accordance with the STROBE, 2008 (Strengthening the Reporting of Observational Studies in Epidemiology) checklist for observational studies.

Current analyses were reviewed/evaluated by the Western Institutional Review Board (November 2015 to November 2020) and Cornell University (notification September 2019). Prior collection of research samples from 76 fertile men (187 samples) was also approved by the Western Institutional Review Board. Quantification of sperm capacitation was performed by means of the Cap-Score, a laboratory-developed test approved for clinical use throughout the USA (Clinical Laboratory Improvement Amendments certified, College of American Pathologists accredited, New Jersey Department of Health licensed, and both laboratory and assay permitted by the New York State Clinical Laboratory Evaluation Program). All data included in this report were obtained from samples either produced at, or brought to, participating fertility clinics or urology offices. These samples were collected as part of regular fertility examinations of MQF (not for research purposes), although washing and preparation were specific for performance of the Cap-Score as described below.

The participating physicians and clinics then shipped samples to Androvia's laboratory, where the test was performed. Results were generated and reported to the physicians to inform their medical practice and decision-making, including patient counselling, and design and implementation of treatment pathways (e.g. whether to pursue natural conception, IUI, IVF or ICSI; or whether to use various treatments aimed at improving male fertility such as varicocele repair or nutritional supplementation). Clinics performing IUI tracked pregnancy outcomes, which were later reported to Androvia. All data were de-identified for analysis. All methods were performed as described previously (Schinfeld et al., 2018) and are presented briefly below.

Settings and IUI methods

Multiple reproductive endocrinology/fertility clinics and reproductive urologists generated data. Clinics providing pregnancy outcomes included Abington Reproductive Medicine, IVF1, New Jersey Urology, Piedmont Reproductive Endocrinology Group, Virginia Center for Reproductive Medicine and Weill Cornell Medicine. Methods of IUI for each clinic were as described (Schinfeld et al. 2018)). Piedmont Reproductive Endocrinology Group was not involved in that prior study, and their methods are

reported here. Briefly, IUI was performed in stimulated cycles using 5 mg/day letrozole or clomiphene citrate 100 mg/day on days 3–8 of the menstrual cycle. Ultrasound monitoring was performed on cycle day 12, and 250 U of human chorionic gonadotrophin (HCG) was administered when developing follicles reached 18–22 mm in diameter. IUI was performed 24–36 h later. Semen samples produced by masturbation after 2–3 days of sexual abstinence were analysed for sperm counts and motility, and then processed for IUI. All IUI samples were processed through a gradient wash using ISolate Sperm Separation Medium (catalogue #99275, Irvine Scientific, Fujifilm, USA) according to the manufacturer's protocol for discontinuous gradients. Post-wash assessment of motility and concentration was performed to calculate the total motile sperm count for insemination.

Participants

All clinical samples on which Cap-Scores were generated, and all corresponding clinical IUI outcomes and semen analysis metrics, are included. These were collected over a 2.7-year period from November 2016 to July 2019. The only pregnancy outcomes excluded were those using donor spermatozoa, on which Cap-Scores were not performed. Inclusion criteria were based on kit instructions, which stipulate 10×10^6 spermatozoa on initial count prior to density gradient centrifugation and incubation. However, 14 samples from men with fewer spermatozoa were submitted. The Cap-Scores generated were included in the overall analysis and were also analysed separately. Selection criteria varied among physicians, taking into consideration the details of the specific patient/couple. No information on possible comorbidities in the MQF was collected for the current analyses.

Variables and outcomes

Semen analyses were performed at each participating clinic according to WHO guidelines (*WHO, 2010*). However, morphology assessment varied among clinics, precluding its inclusion in overall analysis. Prior investigation of Cap-Score and morphology in 122 MQF showed no relationship (*Cardona et al., 2017*). For Abington Reproductive Medicine, IVF1, Weill Cornell Medicine and the Virginia Center for Reproductive Medicine, clinical pregnancies were identified and confirmed as described previously using blood HCG followed by ultrasonography to confirm fetal heart activity (typically performed at or around gestational week 5.5; *Schinfeld et al., 2018*). At Piedmont Reproductive Endocrinology Group, pregnancy outcomes were first determined by a urine pregnancy test performed 2 weeks after insemination. Blood HCG concentrations were obtained if a home pregnancy test was positive, and were then was followed to ensure an appropriate rise. A transvaginal ultrasound scan was performed at 6–7 weeks gestational age to confirm embryonic cardiac activity.

Measurement of the Cap-Score

Cap-Scores were all assessed by trained personnel at Androvia's laboratory (*Moody et al., 2017*). Sample processing and scoring were performed as previously described (*Schinfeld et al., 2018*). Briefly, semen samples were collected by masturbation and processed at the various clinics using kits provided by Androvia. After liquefaction and washing by density gradient centrifugation, spermatozoa were incubated in modified HTF (catalogue # 90126, Irvine Scientific, Fujifilm), with or without 2-hydroxypropyl-β-cyclodextrin (catalogue # C0926 Sigma, USA), a stimulus for capacitation. Following incubation, the samples were fixed and shipped overnight from the clinics to Androvia's laboratory, where the Cap-Score test was performed. Upon receipt, samples were labelled with Alexa Fluor 488-conjugated CTB = cholera toxin B subunit (catalogue # C34775, Thermo Fisher, USA), placed on a slide and moved to a fluorescence microscope where images were collected.

Readers were trained to identify G_{M1} localization patterns associated with both non-capacitated and capacitated human spermatozoa (*Moody et al., 2017*). All readers passed proficiency testing and daily quality assurance testing as previously described (*Moody et al., 2017*). All samples were prepared and scored using these methods except an initial 37 samples provided by Weill Cornell, which were processed and scored prior to the formation of Androvia (*Cardona et al., 2017*). Those data were included in the generation of the relationship between Cap-Score and PGP that was previously published (*Schinfeld et al., 2018*) and is now tested here.

Bias

Bias could result from inclusion of women with reduced fertility. In a prior study (*Schinfeld et al., 2018*), a minimum suite of tests for female factor infertility was defined. The published relationship between Cap-Score and male fertility in the form of PGP within three cycles was therefore based on data from women without most severe identifiable forms of female factor infertility (e.g. tubal occlusion or hydrosalpinges; *Schinfeld et al., 2018*). Although there is general agreement among clinics regarding tests that would be performed on women before pursuing IUI, in this report data were not excluded based on the female partner's fertility diagnosis; grounds for inclusion were only that IUI was attempted. Inclusion of infertile/subfertile women would make the number of observed pregnancies fall below those predicted based solely on the male partner's fertility.

Sample preparation kits included instructions that the current version of the test is designed for men with 10 million or more total cells, ideally yielding 3 million or more spermatozoa after washing. Because of clinical interest, some samples from men with lower numbers (n = 14 men) were prepared and submitted. These results were included in the overall count and were also broken out and analysed separately. Men with moderate to severe oligozoospermia or azoospermia who were not considered eligible for IUI were typically not selected by their physicians to have their spermatozoa's ability to capacitate quantified. Another potential source of bias would include physicians preferentially selecting men for the assay because of reproductive or other medical history or disclosed behaviour/lifestyle (e.g. smoking or alcohol consumption). To assess selection bias, data were evaluated from the one practice performing the test as an initial screen on every man (n = 423) in comparison to the rest of the clinics, which did not use it in their initial fertility examinations for every patient.

Study size

The decision of when to analyse/report data was determined by the desired patient numbers for the prospective, observational study, in which the previously published model was tested (*Schinfeld et al., 2018*). That original relationship between Cap-Score and

TABLE 1 PROSPECTIVE TEST OF PREDICTED PROBABILITY OF GENERATING PREGNANCY BASED ON CAP-SCORE VERSUS PREGNANCIES OBSERVED WITHIN THREE IUI CYCLES

Cap-Score quintile	*n*	Mean Cap-Score ± SD	Observed pregnancies	Predicted pregnancies ± σ
1st	26	19.7 ± 2.0	8	5.46 ± 2.07
2nd	25	24.7 ± 1.1	7	6.98 ± 2.24
3rd	26	28.2 ± 1.5	11	8.84 ± 2.41
4th	25	32.3 ± 1.3	8	10.40 ± 2.46
5th	26	40.2 ± 5.0	15	14.58 ± 2.49

There were no statistically significant differences between predicted and observed pregnancies (χ^2 = 2.28, with five degrees of freedom; P = 0.809).

male fertility was based on 124 outcomes. Pregnancy data were collected monthly until at least that same number of new outcomes had been reached (i.e. the dataset had doubled). Because outcomes were reported in batches, in practice 128 new outcomes of patients who completed treatment (achieved pregnancy or completed three cycles of IUI) were collected and all were included in the analyses.

The second observational study evaluating how the ability to capacitate is distributed in MQF versus fertile men, and how it compares with traditional semen analysis metrics, was included at this time to provide more in-depth understanding of the prevalence of impaired capacitation in MQF. In this cohort comparison, *all* Cap-Score data (n = 2155) collected over the study period of about 2.7 years were included in the comparison of distributions. No exclusion criteria were applied to clinical samples for MQF. Samples from fertile men collected by Androvia for research purposes were not included in the clinical population of MQF. Androvia did not receive semen analysis data for all men; therefore, those results were not included in comparisons of Cap-Scores and semen analysis parameters (n = 1948 men for whom both Cap-Score and semen analysis data were available).

Quantitative variables

Cap-Score reflects the percentage of spermatozoa having G_{M1} localization patterns consistent with capacitation, out of all spermatozoa having G_{M1} localization patterns (*Moody et al., 2017*). Methodologies for traditional semen analysis were established by the WHO (*WHO, 2010*) and were adhered to by participating clinics; however, differences in scoring/reporting of morphology prevented that metric from being included.

Statistical methods

Statistical analyses (logistic regression, Akaike information criterion [AIC], analysis of variance [ANOVA] and and chi-squared and two-tailed t-tests) were carried out in XLSTAT (Version 2019.2.2.59398, Addinsoft, Inc., New York, New York, USA). For prospective comparison of the predicted PGP versus observed pregnancies, the results were rank-ordered by Cap-Score, and the data were then divided into quintiles. The expected number of pregnancies was calculated by summing the PGP values in each quintile

$$(\textit{expected \# preg} = \textit{average PGP} * n = \frac{\sum_{i=1}^{n} x_i * n)}{n}$$

with PGP being predicted by the previously published logistic regression model (*Schinfeld et al., 2018*). A goodness of fit chi-squared statistic was generated to determine whether predicted and observed outcomes differed. The AIC (*Akaike, 1974*) penalizes increasing model complexity without a reciprocal increase in fit.

Following best practice of having analyses confirmed/performed by independent statisticians, Singular Value Consulting (USA) was contracted and given Androvia's complete raw dataset related to this study, to both assess the appropriateness of the analyses and determine their accuracy. Statistics and logistic regression analysis were carried out in R (*Team, 2014*) and SciPy (*Jones et al., 2001*).

RESULTS

The percentage of capacitated, fertilization-competent spermatozoa and traditional semen analysis results were measured for men from six clinics (n = 292), with pregnancy outcomes subsequently collected. Of these patients, 128 finished treatment (i.e. the couple became pregnant within, or completed, three cycles of IUI) when data were analysed. Three tests were employed to assess the predictive relationship between sperm capacitation and male fertility as defined previously for Cap-Score and PGP.

Prospective test of the predictive relationship between capacitation and male fertility

First, to test whether the new data on Cap-Scores and pregnancy outcomes were consistent with the previously published model (*Schinfeld et al., 2018*), the results were rank-ordered by Cap-Score and divided into quintiles (n = 25 or 26 per group). The expected number of pregnancies for each quintile was calculated using PGPs that were predicted by that logistic regression model (*Schinfeld et al., 2018*). The number of pregnancies observed and those predicted are presented in TABLE 1. In each quintile, the differences between observed and expected numbers of pregnancies are as expected due to the uncertainty in the model. To quantify this statement, a chi-squared statistic was computed (χ^2 = 2.28). This value was compared to a chi-squared random variable with five degrees of freedom. Such a random variable would have a mean of 5 and a 95% confidence interval (CI) of 0.83–12.83. The observed value of 2.28 is well within the 95% CI, indicating that the results are typical of what one would expect based on the logistic model. In short, the pregnancies prospectively predicted by the model are consistent with those observed (P = 0.809, showing no statistically significant difference between predicted and observed pregnancies).

Evaluation of fit of the logistic model

Second, the new outcomes were added to the previous 124. Logistic regression

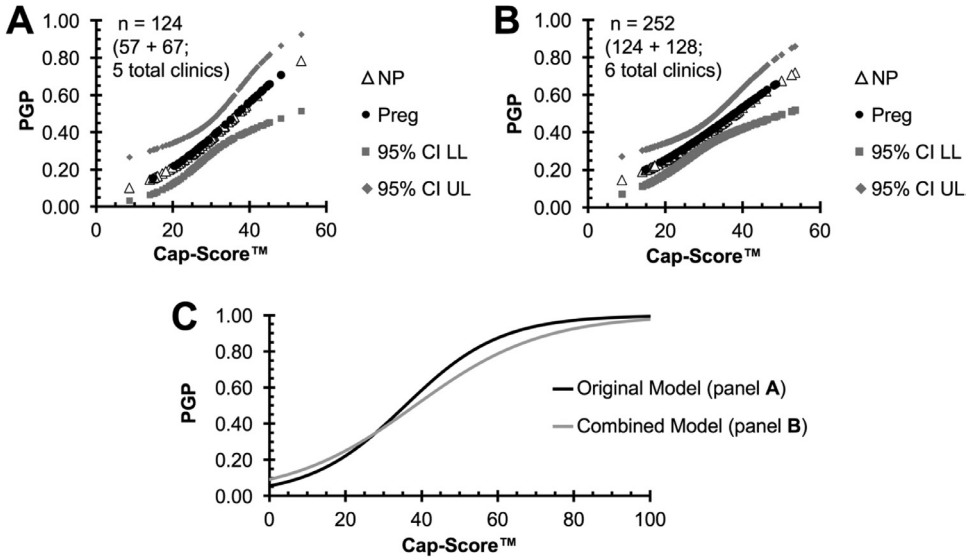

FIGURE 1 Doubling of dataset size with prospective observational clinical data had only minor impact on the relationship between Cap-Score and male fertility. Original (A; *Schinfeld et al., 2018*) and combined (B) logistic regression models defining the relationship between Cap-Score and probability of generating pregnancy (PGP) within three cycles. Overlay of the original and combined models (C). Coefficients for the two models were not significantly different ($P > 0.05$). CI LL, lower limit of confidence interval; CI UL, upper limit if confidence interval; NP, non-pregnant cycles; Preg, cycles resulting in pregnancy.

models PGP as a function of Cap-Score as

$$PGP = 1/(1 + \exp(-(a + b*\text{Cap-Score})))$$

where the coefficients a and b are determined from data. Using the full dataset ($n = 252$) the estimates $a = -2.301$ and $b = 0.061$ were obtained. The fact that b is positive shows that PGP increases with increasing Cap-Score. The P-values associated with both coefficients were <0.001.

The new logistic regression model was consistent with the previous model, which was demonstrated by overlapping CI for the logistic regression coefficients and by how similar the predictions were. The previous intercept term a was –2.863 with a 95% CI of –4.555 to –1.331. The new estimate for a is –2.301 with a 95% CI of –3.316 to –0.330. The previous linear term b was 0.078 with a 95% CI of 0.029 to 0.131. The new estimate for b is 0.061 with a 95% CI of –0.004 to 0.095. In each case, the new coefficient estimates are within the CI of the previous model and vice versa. Overlapping 95% CI show that there is no significant change in the logistic regression coefficients when the number of observations in the dataset was doubled ($P > 0.05$; FIGURE 1).

The third test of the relationship between capacitation and male fertility involved discerning whether the inclusion of one or more traditional semen analysis parameters would improve fit. To test this, logistic regression models were fitted to the combined dataset using Cap-Score and semen analysis measures alone and in every possible combination (Supplemental Table 1). The AIC (*Akaike, 1974*) was performed to test the relative quality of the models. Cap-Score alone was found to provide the optimal model, underscoring that capacitation served as the primary metric of male fertility.

Impact of maternal age

Use of IUI data enabled the study to focus to some degree on male fertility, in that clinicians did not feel IUI was contraindicated. That being said, for many of these patients, findings of female factor including polycystic ovary syndrome, diminished ovarian reserve, repeated pregnancy loss, amenorrhoea, myoma, endometriosis, and so on were made but did not preclude performance of IUI. These patients are included in the dataset so that the results best reflect the performance/predictive ability of the assay under actual clinical conditions, with the diverse patient base and medical histories that are represented in couples pursuing IUI. In addition to these variables, the impacts of advanced maternal age on multiple aspects of female fertility are well documented (*Wyndham et al., 2012*). To test whether maternal age impacted the relationship defined for male fertility, the outcomes for which maternal age was available were combined. When maternal age was added as a term in the logistic regression, the coefficient of age was not significant ($P = 0.42$).

Additionally, these data were disaggregated into the following maternal age groups: ≤29, 30–34, 35–39 and ≥40 years (Supplemental Table 2). No difference was observed between predicted and observed pregnancy outcomes across maternal age groups ($\chi^2 = 0.585$; $P = 0.965$; four degrees of freedom). ANOVA showed that Cap-Scores did not vary across maternal age stratifications ($P = 0.266$). Although female age and fertility are indisputably linked, sperm capacitation accurately predicted pregnancy outcomes across maternal age in women pursuing IUI. Limitations in interpretation are discussed further below.

Although not necessarily related to age, other maternal effects might manifest themselves in failure to carry to term. As a preliminary investigation of whether pregnancies from IUI might be more likely to result in miscarriage, data were assessed from one clinic of 38 couples pregnant by IUI and 23 by natural conception. There were no differences ($P > 0.05$) in couples that miscarried (34% and 35% with IUI and natural

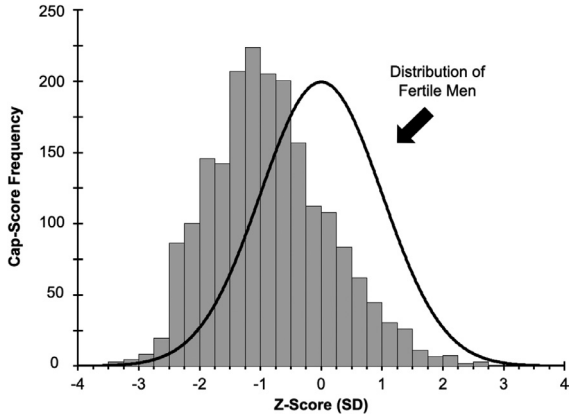

FIGURE 2 Impaired capacitation ability is highly prevalent in men questioning their fertility. Cap-Scores from 2155 men questioning their fertility (histogram) were significantly lower than the distribution of Cap-Scores previously defined for a cohort of fertile men (*Cardona et al., 2017*) (the black curve approximates the normal distribution of a fertile cohort; $P < 0.001$). The *x*-axis shows *Z*-scores, with the mean of 35.3 (the 'fertile mean' determined by Cardona et al.) set to 0, and every unit equal to 1 SD of 7.7 (*Cardona et al., 2017*).

conception, respectively) or delivered (66% and 65% with IUI and natural conception, respectively).

Cohort comparison of MQF versus fertile men

To evaluate whether the percentage of capacitated spermatozoa in a man's ejaculate differed between MQF and fertile men, all Cap-Score data generated from the clinics (n = 2155 men, 22 clinics;) were compared against those from a cohort of men with known fertility (n = 76 men, 187 samples,) (*Cardona et al., 2017*). No exclusion criteria beyond kit criteria were applied to the MQF population, although physicians used their own judgement in selecting the patients for whom they prescribed the assay. The distribution of Cap-Scores in MQF was significantly different from that in fertile men (FIGURE 2; $P < 0.001$), with 81% (1741/2155) falling below the fertile mean of 35.3% (*Cardona et al., 2017*).

Of these 2155 men, accompanying semen analysis data were available for 1948. TABLE 2 shows the distribution of data relating Cap-Scores, PGP and traditional semen analysis metrics. Because the relationship between Cap-Score and PGP is not linear, data are presented in bins by PGP (≤19%, 20–29%, 30–39%, 40–49%, 50–59%, ≥60%). The lower distribution of Cap-Scores and associated PGPs is revealed in this presentation through several comparisons, although it bears repeating that because male fertility is best viewed as a continuum, there is no single value that should be interpreted as a definitive 'cut-off'. For the purposes of comparison of result ranges only, 67% of MQF (1313/1948) had PGPs ≤39%, in comparison to 25% of fertile men (19/76)

Consistent with multiple prior reports (*Guzick et al., 2001*; *Ombelet et al., 1997*; *van der Steeg et al., 2011*), traditional semen analysis results did not correlate

TABLE 2 DISTRIBUTION OF DATA RELATING CAP-SCORES, PGP AND TRADITIONAL SEMEN ANALYSIS METRICS

Cap-Score (%)	PGP (%)	% of all men having fertility exams	% men with normal concentration, motility and volume, having fertility exams	% men having fertility exams >10 million TMC	% fertile men [a]
≤18	≤19	8 (151/1948)	6 (69/1183)	7 (128/1809)	1 (1/76)
19–25	20–29	28 (551/1948)	27 (322/1183)	28 (499/1809)	9 (7/76)
26–31	30–39	31 (611/1948)	31 (366/1183)	32 (573/1809)	14 (11/76)
32–36	40–49	17 (330/1948)	19 (224/1183)	18 (320/1809)	36 (27/76)
37–42	50–59	10 (186/1948)	10 (124/1183)	10 (176/1809)	24 (18/76)
>42	≥60	6 (119/1948)	7 (78/1183)	6 (113/1809)	16 (12/76)

A non-linear relationship exists between Cap-Score and PGP. Thus, the data bins presented as rows were established using PGP.

[a] This column is not part of the MQF population. Rather, it represents the distribution of Cap-Scores in a group of 76 men with known fertility (conceptions achieved without assisted reproduction; previously published in *Cardona et al., 2017*).

MQF, men questioning their fertility; PGP, probability of generating pregnancy; TMC, total motile cells.

with sperm fertilizing ability or male fertility. A total of 61% (1183/1948) of all MQF met or exceeded WHO lower reference value criteria for volume, concentration and motility. Of these men, 64% (757/1183) had PGPs ≤39%. Failure to generate pregnancy in men passing traditional semen analysis metrics would typically result in a diagnosis of idiopathic infertility; these data revealed that impaired sperm capacitation (relative to fertile men) was highly prevalent in MQF. Finally, impaired sperm capacitation was equally prevalent regardless of an individual man's passing any single or multiple semen analysis metric(s), or having >10 million total motile cells (TMC), which is sometimes thought of as an indicator of minimally acceptable overall semen quality (*Leushuis et al., 2014*) (TMC $P = 0.987$). The majority of MQF had >10 million TMC (93%, 1809/1948), but 66% of them had PGPs ≤39% (1200/1809).

One potential limitation or source of bias in interpreting these data would be if clinicians were successful at identifying men who would have 'idiopathic infertility' based on habitus or history, and preferentially ordered Cap-Scores on these men. To evaluate the existence or impact of this potential confounder, the 423 items of Cap-Score data from the Virginia Center for Reproductive Medicine, the only clinic to perform the assay on all eligible men, were disaggregated and compared against the remaining data from the other clinics. No difference was found when using the Mann–Whitney comparison of two samples ($P = 0.107$), indicating that the trends in the MQF population were not the result of selection bias.

Relationship of Cap-Score/PGP and traditional semen analysis metrics

Previously, minimal to no relationship was found between Cap-Score and semen analysis metrics (*Cardona et al., 2017*; no relationship was identified for any metric via ANOVA, $P > 0.05$; linear regression analysis suggested a small but statistically significant relationship with motility, in which motility accounted for 5% of the Cap-Score, $r^2 = 0.05$). Here, it was re-evaluated whether relationships might be revealed based on the considerably larger sample size (1948 versus 122). Supplemental Figure 1 shows scatterplots and associated regressions exploring potential relationships between volume, motility and concentration with Cap-Score. No relationship was found between volume and Cap-Score ($r^2 < 0.001$, $P = 0.65$). Small, but statistically significant, relationships were found for motility and concentration ($P < 0.001$ for each). Motility was found to contribute approximately 2% to the Cap-Score ($r^2 = 0.018$), and concentration was found to contribute around 1% to the Cap-Score ($r^2 = 0.013$). These data support prior reports that traditional semen analysis parameters have little relationship with the fertilizing ability of spermatozoa, or with male fertility.

For the sake of additional comparisons, Supplemental Table 3 summarizes the proportion of men having normal and abnormal semen volume, sperm concentration and percentage of motile spermatozoa and provides the respective average Cap-Scores for each subgroup. Supplemental Table 4 compares Cap-Score and semen analysis measures based on having fewer or more than 10 million TMC. Although the average Cap-Scores were statistically lower for the men having low concentration, low motility or <10 million TMC (each $P < 0.001$), it is well accepted that as sample size increases, even small differences that are not clinically informative will reach statistical significance. The size of the relationship between Cap-Score and each semen analysis metric is conveyed by the regressions in Supplemental Figure 1 and described in the preceding paragraph. As a final comparison, although kit criteria specified 10 million spermatozoa prior to washing, 14 samples were submitted with numbers below this threshold. Although this was a very low population size, Cap-Scores for these specimens averaged 28.5 ± 7.0, which is contained within a 95% CI of the MQF population mean (28.77 ± 7.53 (±SD)).

DISCUSSION

These studies yielded several findings. First, a measure of sperm capacitation, the Cap-Score, prospectively predicted male fertility across diverse clinical settings. Second, the previously defined mathematical relationship between Cap-Score and a metric of male fertility – PGP within three cycles – changed minimally with a doubling of the outcomes dataset. Third, impaired or reduced capacitation ability was highly prevalent in MQF, and finally, there was minimal to no relationship between sperm capacitation and traditional semen analysis metrics.

In terms of interpretation of the data and comparison with other studies, these data confirm that traditional semen analysis metrics fail to identify impairments in fertilizing ability, which typically lead to a diagnosis of idiopathic infertility (*Guzick et al., 2001*; *Ombelet et al., 1997*; *van der Steeg et al., 2011*). The predictive power of measuring capacitation confirms the important contribution of male factor in determining successful generation of pregnancy, and validates prior calls for development of tests of sperm function/fertilizing ability (*Barratt et al., 2018*; *Oehninger et al., 2014*; *Wang and Swerdloff, 2014*). Sperm capacitation involves a number of intracellular signalling and metabolic responses, presenting multiple alternative metrics such as protein tyrosine phosphorylation events, phospholipid scramblase activity, membrane potential, intracellular pH and so on (*Puga Molina et al., 2018*). Despite capacitation having first been identified close to 70 years ago (*Austin, 1952*; *Chang, 1951*), clinical measurement of this essential component of male fertility is not commonly performed because its predictive relationship with fertility is only now being described, and a practical means of measurement has been lacking.

The studies presented here had several strengths. To test the relationship between sperm capacitation and male fertility, an outcome measure of pregnancy within three cycles of IUI was used. This design enabled more rigorous and focused evaluation of male fertility by providing some control regarding timing of inseminations relative to ovulation and a basic level of female fertility. Although they also control timing, classical IVF and ICSI bypass important physiological aspects of male fertility.

Multicentric observational data have the advantage of being generated under actual clinical conditions reflecting diverse patient bases and clinical practices, and avoid potential unconscious bias with non-randomized, directed assignment to interventions. The prospective nature of testing the predicted PGP and inclusion of *all* non-donor pregnancy outcomes later observed were primary strengths of the first study. The primary strengths of the

cohort comparison were the size of the pool of MQF, the inclusion of *all* clinical data and the diversity of the participating clinics.

However, these studies investigating the relationship of sperm capacitation and male fertility do have several limitations worth noting. Of greatest importance, the logistic relationship between Cap-Score and male fertility in the form of PGP is predicated upon a fertile female partner. Inclusion of some women having female factor infertility would cause a systematic bias of lowering the number of observed pregnancies relative to predicted. However, there was no statistically significant evidence for that here.

Another bias might have had the opposite effect and increased observed pregnancies; namely, several participating physicians reported modifying their clinical practices when receiving the result of a low Cap-Score. For example, several recommended to their patients with impaired capacitation ability that they make changes in lifestyle, take nutritional supplements, undergo varicocele repair and/or have two inseminations performed in a single IUI cycle. The effects of these changes in practice might be reflected in the new outcomes, which were slightly elevated relative to those predicted for men with low Cap-Scores. Although the two logistic regression equations did not differ statistically, the potential impacts of inclusion of patients with female factor infertility, changes in IUI practice and/or possible treatment of men argues for the continued use of the original equation (*Schinfeld et al., 2018*) in reporting Cap-Scores and PGP.

Interpretation of outcomes data stratified by maternal age must be viewed with caution. The lack of difference across age ranges may result, in part, from the original relationship between Cap-Score and PGP being defined using clinical pregnancy outcomes generated from a variety of maternal ages (*Schinfeld et al., 2018*). Although there was no difference between predicted and observed pregnancies for women aged 40 years or over, it must be noted that the sample size of that group was the smallest of any age group tested.

A potential source of 'noise' in the cohort comparison is the fact that although the study used WHO guidelines, the current semen analysis data were generated by multiple andrologists at different clinics. While providing the advantage of a more diverse patient base, this approach undoubtedly introduced variations in technique and practice, such as those leading to the inability to compare morphology data across clinics. There were also no data regarding comorbidities in the MQF; further research would be needed to evaluate whether conditions such as varicocele might have a particular relationship with impaired capacitation.

The current results have a number of implications for clinical practice. These results demonstrate that the percentage of capacitated spermatozoa can provide important predictive information about male fertility, directly impacting a couple's chances of conception. Tests of capacitation, such as the Cap-Score, can provide a functional complement to the traditional semen analysis. These can aid in identifying impairments in fertilizing ability that might otherwise be found only through repeated failed attempts at conceiving, resulting in diagnoses of 'idiopathic infertility' and their associated physical, emotional and financial costs. Indeed, a successful measure of capacitation has been modelled to not only improve outcomes, but also reduce cost per couple (*Babigumira et al., 2018*). Of course, if men produce so few spermatozoa that a Cap-Score cannot reasonably be performed (i.e. they exhibit severe oligozoospermia), these men would probably be advised to move toward ICSI.

A straightforward application for predictive information on male fertility is the personalized counselling and treatment of couples seeking assistance with fertility. When considered as part of the couple's medical findings and personal context, this information will help clinicians and couples identify an approach that is optimal for them at that point, whether it be tailored expectant management, IUI, IVF or ICSI. Of course, the man is only part of the fertility equation. Various factors related to the female partner's health and fertility will be critical elements in that decision-making. Data presented here show that information on sperm capacitation and male fertility provide critical, previously missing input, and highlight that knowledge of both partners' fertility is essential for the practice of reproductive medicine.

A finding of impaired capacitation could also identify those men who stand to benefit from seeing reproductive specialists and undergoing various treatments to improve male fertility, including change in lifestyle, taking nutritional supplements or undergoing varicocele repair as appropriate (*Aly and Seaman, 2018*). A quantifiable metric of male fertility would also provide a way to assess response to such treatment. Measurement of impact on capacitation might also enable optimization of cryopreservation or semen-handling practices (*Moody et al., 2017*).

Other applications with clinical relevance might include the testing of various drugs or nutritional supplements designed to promote male fertility or act as male contraceptives (whether intended or off-target). Whether sperm fertilizing ability can provide a window into the overall future health of a man, as is being discussed for other semen analysis metrics (*De Jonge and Barratt, 2019*), is an intriguing possibility that will require new research. This line of investigation could also be facilitated by collection of semen samples at home, since that would broaden geographical availability and overcome social and/or economic barriers such as concerns of privacy or conflicts with employment.

The present findings prospectively show a clear relationship between capacitation and male fertility, and reveal a very high prevalence of impaired capacitation in men questioning their fertility, typically because of difficulty conceiving. Together, these findings demonstrate that capacitation is a highly sensitive indicator of male fertility, and show both the need and ability to bring men back into the fertility equation, complementing the multiple assays performed on their female partners.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.105939.

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