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RESEARCH ARTICLE

Cap-Score™ prospectively predicts probability of pregnancy

Jay Schinfeld[1] | Fady Sharara[2] | Randy Morris[3] | Gianpiero D. Palermo[4] | Zev Rosenwaks[4] | Eric Seaman[5] | Steve Hirshberg[6] | John Cook[7] | Cristina Cardona[8] | G. Charles Ostermeier[8] | Alexander J. Travis[8,9]

[1]Abington Reproductive Medicine, Abington, Pennsylvania

[2]Virginia Center for Reproductive Medicine, Reston, Virginia

[3]IVF1, Naperville, Illinois

[4]The Ronald O. Perelman & Claudia Cohen Center for Reproductive Medicine & Infertility, Weill Cornell Medical College, New York

[5]New Jersey Urology, Millburn, New Jersey

[6]Urology Health Specialists, Abington, Pennsylvania

[7]Singular Value Consulting, Houston, Texas

[8]Androvia LifeSciences, Mountainside, New Jersey

[9]Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York

Correspondence
Alexander J. Travis, Cornell University College of Veterinary Medicine, Baker Institute for Animal Health, Hungerford Hill Rd., Ithaca, NY 14853.
Email: ajt32@cornell.edu

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Semen analysis (SA) poorly predicts male fertility, because it does not assess sperm fertilizing ability. The percentage of capacitated sperm determined by G_{M1} localization ("Cap-Score™"), differs between cohorts of fertile and potentially infertile men, and retrospectively, between men conceiving or failing to conceive by intrauterine insemination (IUI). Here, we prospectively tested whether Cap-Score can predict male fertility with the outcome being clinical pregnancy within ≤3 IUI cycles. Cap-Score and SA were performed ($n = 208$) with outcomes initially available for 91 men. Men were predicted to have either low ($n = 47$) or high ($n = 44$) chance of generating pregnancy using previously-defined Cap-Score reference ranges. Absolute and cumulative pregnancy rates were reduced in men predicted to have low pregnancy rates versus high ([absolute: 10.6% vs. 29.5%; $p = 0.04$]; [cumulative: 4.3% vs. 18.2%, 9.9% vs. 29.1%, and 14.0% vs. 32.8% for cycles 1–3; $n = 91$, 64, and 41; $p = 0.02$]). Only Cap-Score, not male/female age or SA results, differed significantly between outcome groups. Logistic regression evaluated Cap-Score and SA results relative to the probability of generating pregnancy (PGP) for men who were successful in, or completed, three IUI cycles ($n = 57$). Cap-Score was significantly related to PGP ($p = 0.01$). The model fit was then tested with 67 additional patients ($n = 124$; five clinics); the equation changed minimally, but fit improved ($p < 0.001$; margin of error: 4%). The Akaike Information Criterion found the best model used Cap-Score as the only predictor. These data show that Cap-Score provides a practical, predictive assessment of male fertility, with applications in assisted reproduction and treatment of male infertility.

KEYWORDS
capacitation, human, infertility, male fertility, semen analysis (SA), sperm

1 | INTRODUCTION

Male fertility is a serious and growing concern globally. Yet, the field of andrology faces critical gaps in diagnostic technologies and knowledge, affecting scientific advancement as well as clinical management by both reproductive endocrinologists and urologists (Barratt, De Jonge, & Sharpe, 2018). The extent of the problem is staggering: 10–15% of

Abbreviation: AIC, Akaike Information Criterion; CAP, College of American Pathologists; Cap-Score, Cap-Score™ male fertility assay; CLEP, Clinical Laboratory Evaluation Program; CLIA, Clinical Laboratory Improvement Amendments; GM1, monosialotetrahexosylganglioside; hCG, human chorionic gonadotropin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; IVF, in-vitro fertilization; LH, luteinizing hormone; mHTF, modified Human Tubal Fluid medium; PGP, probability of generating pregnancy; SA, semen analysis.

couples have difficulty conceiving (Sharma, Biedenharn, Fedor, & Agarwal, 2013); 40–60% of these couples have a contributing male factor component (Agarwal, Mulgund, Hamada, & Chyatte, 2015); and sperm counts and concentration in Western societies have shown marked declines of over 50% since 1973 (Levine et al., 2017). Traditional semen analysis has remained the primary diagnostic tool, despite the fact that it fails to diagnose the vast majority of cases of male infertility (Guzick et al., 2001; Ombelet et al., 1997; van der Steeg et al., 2011), and has not changed appreciably in decades. Although effective at identifying descriptive parameters, traditional semen analysis fails to identify defects in sperm function. Put simply, it does not provide information about whether a man's sperm can fertilize an egg, and the probability of that man generating a pregnancy.

Being able to assess the fertilizing capacity of a man's sperm could play multiple important roles. First, particularly in couples who have delayed attempting to have children until their 30s or later, there is increasing recognition of reduction in conception rates (Dunson, Baird, & Colombo, 2004; Navot et al., 1991) and of increasing risk of genetic abnormalities (Hansen, 1986; Reichenberg et al., 2006; Wyrobek et al., 2006), associated with increasing age in both women and men. Knowledge of the male partner's fertilizing ability might help them decide whether to try at home or to pursue fertility examinations more quickly than guidelines currently suggest. Second, should an intervention or treatment be necessary, knowledge of sperm fertilizing ability could be used to personalize fertility treatment plans (Oehninger, Franken, & Ombelet, 2014; Palermo, Neri, & Rosenwaks, 2015). For example, men whose sperm have normal function and a high probability of fertilization could be guided by clinicians to approaches such as intrauterine insemination (IUI). Conversely, those with poorly functioning sperm might be able to avoid the emotional, physical, and financial costs associated with repeated IUI attempts that have little chance of success, by being guided to procedures such as intracytoplasmic sperm injection (ICSI). However, as has been pointed out (Barratt et al., 2018), approaches such as ICSI are not treatments of poor male fertility; they instead seek to overcome it.

Therefore, the third application for a test that quantifies male fertilization competence would be as a metric for urologists attempting to treat underlying factors contributing to male infertility. For example, this test could enable an evidence-based approach to the treatment of male infertility, assessing the response of an individual man to interventions such as surgical repair of a varicocele (Seaman & Aly, 2018), or changes in medications, nutritional supplements, or lifestyle (see Hayden, Flannigan, and Schlegel (2018) for a review of the impacts of factors such as diet and exercise). Pharmaceutical companies could assess potential off-target impacts of drugs as well as on-target impacts of male contraceptives being developed. From a public health perspective, it is becoming appreciated that semen parameters can provide insights into overall male health (Glazer et al., 2017; Hanson, Eisenberg, & Hotaling, 2018), and sperm function might prove to be an even more sensitive indicator than reduction in sperm production.

Together, these pressing needs have led to numerous calls for the development of an assay of male fertility that focuses on the fertilizing ability of sperm (e.g., Lamb, 2010; Oehninger et al., 2014; Wang & Swerdloff, 2014). Much attention has focused on a process known as "capacitation," through which sperm acquire the ability to fertilize in response to stimuli within the female reproductive tract (Austin, 1952; Chang, 1951). We showed recently that localization patterns of the ganglioside monosialotetrahexosylganglioside (G_{M1}) within the plasma membrane of human sperm can identify capacitation status (Supporting Information Figure S1A; Moody et al., 2017). Importantly, the percentage of capacitated sperm determined by G_{M1} localization ("Cap-Score™"), differed between cohorts of fertile men and men questioning their fertility, and retrospectively, between men having success at, or failing, IUI (Cardona et al., 2017). Cap-Scores were highly reproducible among ejaculates within a man and had no relationship with traditional semen analysis parameters (Cardona et al., 2017). In addition, we showed at the level of single sperm, that those cells having the "capacitated" patterns were indeed those cells that could undergo acrosome exocytosis in response to ionophore (A23187; Moody et al., 2017) and progesterone (Ostermeier et al., 2018). Using repeated Cap-Score values over time, we also found that the timing of capacitation differed among men but was consistent within a man (Ostermeier et al., 2018).

Here, we build on those data and present the results of a prospective test of Cap-Score to predict a man's ability to generate a clinical pregnancy using data from IUI cycles as a well-controlled study design. We then had independent statisticians test whether a combination of Cap-Score and one or more semen analysis parameters might convey even more information, finding instead that Cap-Score alone had the best fit with clinical outcomes. The model fit on these original data was then further tested with additional data, originating from a total of five clinics, to yield a model that transformed Cap-Score results into the probability of generating a pregnancy.

2 | RESULTS

We prospectively tested Cap-Score with the outcome measure being clinical pregnancy within three or fewer cycles of IUI. We chose IUI as a rigorous study model because it offered control over the timing and number of inseminations relative to a given ovulation, as opposed to natural/spontaneous conceptions. To separate men into groups predicted to have a low versus high chance of generating pregnancy, we used a cut-off of one standard deviation below the previously identified mean of Cap-Scores in a cohort of fertile men based on comparison of their distribution with results from a cohort of men questioning their fertility (Supporting Information Figure S1B; Cardona et al., 2017). Cap-Score and semen analysis were performed ($n = 208$), with clinical outcomes available for 91 couples at the time of analysis. Survival analysis was used to compare the abilities of men with low (Table 1) and high (Table 2) predicted abilities to generate pregnancy, in relation to each IUI attempt.

The proportion of pregnancies following three rounds of IUI, in relation to the number of patients starting treatment, was 2.78-fold

TABLE 1 Clinical outcomes for men predicted to have a low chance of fertility (Cap-Score ≤27.6%)

IUI #	# Patients	# Pregnant	# Not going to next IUI	Absolute % pregnant	Proportion pregnant	Proportion not pregnant	Cumulative pregnancy	Cumulative nonpreg
1	47	2	11		4.3	95.7	4.3	95.7
2	34	2	10		5.9	94.1	9.9	90.1
3	22	1	21	10.6 (5/47)	4.5	95.5	14.0	86.0

Note. IUI: intrauterine insemination.

TABLE 2 Clinical outcomes for men predicted to have a high chance of fertility (Cap-Score >27.6%)

IUI #	# Patients	# Pregnant	# Not going to next IUI	Absolute % pregnant	Proportion pregnant	Proportion not pregnant	Cumulative pregnancy	Cumulative nonpreg
1	44	8	6		18.2	81.8	18.2	81.8
2	30	4	7		13.3	86.7	29.1	70.9
3	19	1	18	29.5 (13/44)	5.3	94.7	32.8	67.2

Note. IUI: intrauterine insemination.
IUI #: IUI attempts; # Patients: number of patients undergoing treatment; # Pregnant: number of patients generating clinical pregnancy; # Not going to next IUI: number of patients discontinuing treatment; Absolute % pregnant: # pregnant after ≤3 IUI/# patients starting treatment; Proportion Pregnant: # Pregnant/# Patients for that IUI attempt; Proportion not pregnant: 100 minus proportion pregnant for that IUI attempt; Cumulative pregnancy: 100 minus cumulative Nonpreg; Cumulative Nonpreg: Cumulative Nonpreg from previous IUI attempt * Proportion not Pregnant.

greater (29.5% vs. 10.6%; $P = 0.04$) for men prospectively predicted by Cap-Score to have a high chance of pregnancy ($n = 44$ men) versus those predicted to have a low chance ($n = 47$ men). To determine when and how these differences were established and to take into consideration that not every couple made three attempts, cumulative pregnancy rates were calculated for each round of IUI. Men predicted to have a high chance of fertility due to their Cap-Score were 4.23-, 2.94-, and 2.34-fold more likely to generate a pregnancy than men in the low group following 1, 2, and 3 IUI attempts, respectively ($p = 0.02$; cumulative predicted pregnancy for low vs. high predictions: 4.3 vs. 18.2, 9.9 vs. 29.1, and 14.0 vs. 32.8%). These results were confirmed by independent statisticians given access to all raw data.

In addition to this prospective test of Cap-Score, traditional semen analysis measures were compared retrospectively between those men who were and were not successful in generating a pregnancy (Table 3). Only Cap-Score differed significantly between the pregnant and not-pregnant groups ($p < 0.01$). These observations support our previous findings that Cap-Score differs between fertile and subfertile men and does not correlate with traditional semen analysis parameters (Cardona et al., 2017). In addition, these results are consistent with the observation of others, that traditional semen analysis measures have significant overlap between fertile and infertile men (Guzick et al., 2001; van der Steeg et al., 2011).

The relationship between Cap-Score and the probability of generating a pregnancy was defined by the independent statisticians using logistic regression analysis for those men generating a pregnancy within or completing 3 IUI attempts (Figure 1a; $n = 57$ men). A significant association was observed (probability of generating pregnancy [PGP] = 1/[1 + exp[-[−3.810 + 0.102*Cap-Score]]]; $p = 0.01$), which supported the finding above that those men having higher Cap-Scores were more likely to generate a pregnancy. To test the fit of this model, an additional 67 data points, now representing a total of five clinics, were added to the analysis (Figure 1b). A small change (approximately 4% for a given Cap-Score) was observed in the model, but the fit improved (PGP = 1/[1 + exp[-[− 2.863 + 0.0776*Cap-Score]]]; $n = 124$; $p < 0.01$). These results further substantiate the strong association between Cap-Score, sperm function/fertilizing ability, and ability to generate a clinical pregnancy. As discussed further below, the equation itself could have important clinical applications because it can transform Cap-Score values into the probability of a man being able to generate a pregnancy.

To determine if inclusion of one or more traditional semen analysis parameters could improve fit even further, logistic regression models were calculated using Cap-Score and semen analysis measures alone and in combination for both the single- (one clinic; $n = 57$) and multiclinic (five clinics; $n = 124$) data sets (Table 4). The deviance and Akaike Information Criterion (AIC; Akaike, 1974) were then used to test the relative quality of the models. In general, the smaller the deviance, the better the model fits. However, as more parameters are added, the fit will appear to improve in large part because of increased complexity. To guard against the model being tailored to the random noise of the sample, rather than representing the overall population, the AIC was used. The AIC penalizes increasing model complexity without a reciprocal increase in fit. Those models with smaller AIC are considered most appropriate. For both the single- and multiclinic data sets, Cap-Score alone was deemed most appropriate. These observations are consistent with Cap-Score being the best predictor of male fertility.

TABLE 3 Semen analysis measures for men who were and were not successful in generating pregnancy

	N	Volume (ml)	Conc (M/ml)	Motility (%)	Male age (years)	Female age (years)	Cap-Score (%)
Preg	18	2.3 ± 0.2	85.3 ± 15.3	66.4 ± 4.3	33.9 ± 1.2	33.5 ± 0.9	32.5 ± 1.7
NP	73	2.6 ± 0.2	60.9 ± 5.4	60.4 ± 2.8	34.5 ± 0.5	32.9 ± 0.4	26.7 ± 0.8
Preg versus NP (*p* value)		0.45	0.07	0.32	0.62	0.52	<0.01

Note. Individuals completed at least one round of IUI. Men were placed in the pregnant (Preg) category if their partner conceived in ≤3 rounds of IUI. Otherwise, they were placed in the not pregnant (NP) group. *p*-value (Preg vs. NP): *p*-value from two-tailed *t* test. *N*: number of observations. Conc: sperm concentration. Cap-Score™: proportion of sperm having G_{M1} localization patterns consistent with capacitation.

3 | DISCUSSION

It is widely appreciated that our reliance on semen analysis—either to diagnose male infertility or to assess a functional response to treatment of male infertility—is inadequate (Barratt et al., 2018). Assays proposed over the years to fill these needs have not demonstrated efficacy at prospectively predicting the clinical outcome of generating a pregnancy (Barratt et al., 2018), and are often found to correlate with the traditional semen analysis parameters (Aitken, 2002; Giwercman et al., 2003; Hazary, Chaudhuri, & Wishart, 2001; Zini et al., 2009). Prospective data presented here strongly demonstrate that the Cap-Score, a measure quantifying the percentage of sperm that can capacitate and undergo acrosome exocytosis (Cardona et al., 2017; Moody et al., 2017; Ostermeier et al., 2018), successfully predicted low or high success at achieving pregnancy in three or fewer attempts at IUI. Yet from a clinical perspective, we know that male fertility does not simply switch between a binary "infertile" to "fertile" categorization at a single cut-off; rather, it is complex, multifactorial, and exists along a continuum with men having different probabilities of fertilizing. Importantly, logistic regression analysis of the raw data performed by independent statisticians showed that Cap-Score provided a straightforward means of identifying the probability of a man to generate a pregnancy, given a female partner who was eligible for IUI.

These data support the findings of prior retrospective and cohort comparisons, that Cap-Score can be used as a measure of male fertility (Cardona et al., 2017). In the current investigation, a previously determined Cap-Score reference range (Cardona et al., 2017) was used to separate individuals prospectively into groups having either a low or high chance of generating a pregnancy. A survival analysis then prospectively showed that the absolute pregnancy rate of couples with men in the high Cap-Score group was 278% that of men predicted to have difficulty conceiving due to low Cap-Score. In contrast to the predictive power of Cap-Score, traditional semen analysis parameters and male and female age were not related to clinical outcome, which also supported a previous finding that Cap-Score does not correlate with traditional semen analysis parameters (Cardona et al., 2017).

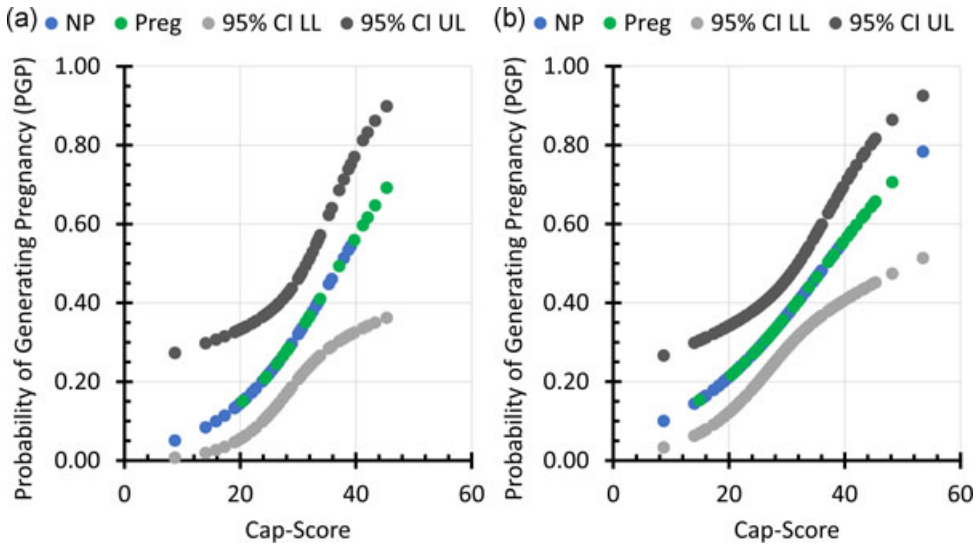

FIGURE 1 Cap-Score™ and its association with the probability to generate pregnancy. Logistic regression defined the relationship between Cap-Score and a man's PGP. Panel A shows data from a single clinic ($n = 57$; $p = .01$) with the PGP ranging from 5% to 69%. In Panel B, the single-clinic model was tested by adding 67 additional data points obtained from this and four other clinics ($n = 124$; $p < 0.001$; PGP range: 5–78%). The green and blue points respectively show those men generating (Preg) and not generating (NP) a pregnancy. The light and dark grey points respectively show the lower (LL) and upper (UL) limits for the 95%CI. CI: confidence intervals; NP: not pregnant; PGP: probability of generating a pregnancy

TABLE 4 Model evaluation and selection using Akaike Information Criterion (AIC)

	Single-clinic		Multi-clinic	
Model	Deviance	AIC	Deviance	AIC
Cap-Score	61.7	65.7	148.6	152.6
Motility	63.5	67.5	157.7	161.7
Concentration	64.5	68.5	158.5	162.5
Volume	65.2	69.2	159.4	163.4
Cap-Score+motility	60.6	66.6	147.5	153.5
Cap-Score+concentration	60.5	66.5	147.7	153.7
Cap-Score+volume	60.1	66.1	148.6	154.6
Motility+concentration	63.2	69.2	157.5	163.5
Motility+volume	62.8	68.8	157.6	163.6
Concentration+volume	64.3	70.3	158.2	164.2
Cap-Score+motility +concentration	60.1	68.1	147.2	155.2
Cap-Score+motility +volume	58.8	66.8	147.5	155.5
Cap-Score+concentration +volume	59.5	67.5	147.7	155.7
Concentration+motility +volume	62.7	70.7	157.2	165.2
Cap-Score+concentration +motility+volume	58.8	68.8	147.2	157.2

Note. AIC: Akaike Information Criterion. Model: shows the measures considered. Single-clinic: dataset obtained from one site, $n = 57$. Multi-Clinic: dataset obtained from five sites, $n = 124$. Deviance: analogous to sum of squares of residuals in ordinary least squares.

Of interest, the proportion of pregnancies generated for each of three IUI attempts for men in the low group was consistent (4.3%, 5.9%, and 4.5%), with an average of 4.9%. In contrast, success in the first IUI attempt for men in the high group was 18.2% or 423% that of the low group. Success for the high group declined to 13.3% in the second attempt, and 5.3% in the third, still yielding cumulative pregnancy rates that were 294% and 234% those of the men predicted to have a low chance of conceiving. Although requiring further investigation beyond the scope of this report, the decline in success in the third cycle of men with normal-range Cap-Scores is suggestive that the IUI protocol being used was not in some way optimized for the sperm function of this subpopulation. That is, their success was no better than that of men whose sperm had low capacitation ability. One possible explanation is offered by our recent finding that the timing of capacitation differs among men, but is reproducible within repeated ejaculates of a given man (Ostermeier et al., 2018). In that study, 44% of men were shown to have sperm that took significantly longer to capacitate than those of their peers (Ostermeier et al., 2018). Currently, insemination is typically performed 24–36 hr after human chorionic gonadotropin (hCG) injection. Ovulation has been reported to occur over a range of 26–46 hr after the hCG injection with an average of 38 hr (Andersen, Als-Nielsen, Hornnes, & Franch Andersen, 1995; Testart, Thebault, Souderes, & Frydman, 1982). Because oocytes are believed to be fertilization competent for less than 24 hr (Lash & Whittaker, 1974; Miao, Kikuchi, Sun, & Schatten, 2009), inseminations occurring around or after the time of ovulation might not allow enough time for sufficient numbers of sperm to capacitate, systemically and specifically disadvantaging those men whose sperm take longer to capacitate. For men with high Cap-Scores but no success over the first two IUI attempts, a potentially simple point of study could be to evaluate the impacts of an earlier insemination time (i.e., sooner after the hCG), which might allow more sperm to capacitate and improve pregnancy rates.

Alternatively, the reduced success in the third cycle of men within the normal Cap-Score range might support limiting attempts to only two IUI cycles if the lack of success simply reflects other determinants, such as subtle female factors or potentially other male components that are left undetected by current diagnostics. For example, male fertility is multifaceted (Amann, 1989; Amann & Hammerstedt, 1993). Men producing sperm within the normal Cap-Score range and failing to generate pregnancy within two IUI cycles might be producing sperm that lack the ability to initiate embryo development (Swann, Saunders, Rogers, & Lai, 2006; Yoon et al., 2008). As yet another alternative, recent evidence suggests that although there is little association between the presence of seminal human papillomavirus and semen analysis parameters (Luttmer et al., 2016), men with this infection have reduced ability to generate pregnancy (Garolla et al., 2016). The infected sperm are still able to gain access to oocytes and fertilize (Foresta et al., 2011), but appear to have an early reduction in embryo viability beyond that event. If either of these alternatives is correct as opposed to the issue of capacitation timing, then the data would support attempting only two IUI cycles even in couples in which men are producing sperm within the normal Cap-Score range. Of course, it is possible that all three alternatives contribute to the reduced success in the third IUI attempt.

There are several reasons to evaluate male fertility. These range from counseling couples on the best technology of assisted reproduction to generate pregnancy (Palermo et al., 2015), to determining the effects of interventions designed to improve male fertility, such as changes in diet and exercise (Hayden et al., 2018), nutritional supplements (Walczak-Jedrzejowska, Wolski, & Slowikowska-Hilczer, 2013), administering or withdrawing various medications (Brezina, Yunus, & Zhao, 2012), surgical repair of varicoceles (Comhaire & Kunnen, 1985), vasectomy reversal, and so forth. One could also evaluate the efficacy of sperm cryopreservation (Bailey, Blodeau, & Cormier, 2000) or other protocols to extend or preserve male fertility. This latter application could have use beyond human medicine in various animal agriculture industries such as dairy or turkey production, in which artificial insemination is almost universally practiced. Indeed, before our tests of human fertility, the relationship of G_{M1} localization and capacitation status was first identified in nonhuman animals such as mice and bulls (Selvaraj et al., 2007).

Whether using Cap-Score results to personalize the technology chosen to try to conceive, or to measure the impact of a treatment for male infertility or protocol for sperm handling, it is important to circle back to the fact that male fertility represents a continuum rather than a binary "fertile versus infertile" categorization defined by a specific cut-off. The equation generated by logistic regression of results from the controlled study design of IUI cycles is critical in enabling doctors and their patients to conceptualize how changes in Cap-Score might influence a man's ability to generate a pregnancy. For example, if a man's Cap-Score goes up by 10 points in response to a treatment, we can now calculate how much more likely he is to generate a pregnancy (e.g., a change in Cap-Score from 6% to 16% is modeled to increase the PGP by 8%, whereas a change in Cap-Score from 25% to 35% would increase the probability by 18%). To date, we have performed 1,126 clinical Cap-Scores with the lowest and highest values being 7.6% and 54.7%. The logistic function converts these into a probability of generating a pregnancy, with the equation beginning and ending with asymptotes just over 0% and approaching 100%. In practice, however, 95% of the observations gathered thus far have probabilities of generating pregnancies between 14% and 60% (data not shown). To overcome the initial limitation of generating the logistic equation with data from a single clinic, additional data were added to the analysis representing a total of five clinics, and more than doubling the sample size from 57 to 124 men. Despite the potential for the introduction of noise from variation in IUI techniques and patient characteristics from multiple sites, there was no appreciable change in the prediction model. That finding and the use of the AIC to evaluate model quality together confirmed that the optimum model for establishing the probability of fertilization was based on Cap-Score alone.

Although only Cap-Score reflected male fertility, it is important to identify limitations in our study design that argue that traditional semen analysis should still be performed. For Cap-Score evaluation, there must be at least six million sperm following removal of the seminal plasma. Thus, men experiencing severe oligozoospermia and (or) azoospermia were excluded from the analysis. Similarly, couples in this study were all eligible for IUI based on the female partner passing several assays diagnostic of causes of female factor infertility (as described in the Methods). No matter how good a man's sperm function might be, there will not be fertilization if sperm cannot reach the oocyte. In this regard, our prior assessment of Cap-Scores of 122 men questioning their fertility was performed without any screening for female factor infertility (Cardona et al., 2017). In that cohort, a full third of the men had Cap-Scores that were more than one standard deviation below the mean of a cohort of 76 fertile men (Cardona et al., 2017), which would have placed them in the group predicted to have a low chance of generating pregnancy in the current study. That finding, coupled with the current prospective results, demonstrate that the Cap-Score is effective at identifying the majority of cases of what had previously been considered idiopathic male infertility—encouraging its use as a first-line, primary screen of male fertility along with traditional semen analysis.

One might question why this test of capacitation should prove to be such a strong indicator of male fertility. Unlike genetic analyses that look for specific mutations, or screens that cover a panel of genes that have unclear contributions to male fertility (and do not preclude the possibility that other gene products might compensate for the reduction in function of any given gene), Cap-Score is a test that reflects the end product of the interactions of hundreds or possibly thousands of proteins and lipids in multiple pathways and developmental processes. For example, to capacitate and contribute toward a normal-range Cap-Score, a sperm must successfully complete germ cell development and differentiation within the testis, mature within the epididymis to achieve both normal membrane architecture and be able to capacitate, and interact first with seminal plasma and then respond appropriately to stimuli for capacitation. The complexity of spermatogenesis and the signaling pathways involved in sperm functional maturation offer a vast number of potential opportunities for sperm function to be deranged. Although traditional semen analysis can correctly identify profound problems that would result in reduced or abnormal sperm production or impaired motility, the literature repeatedly finds that more subtle defects in function are responsible for the majority of male factor infertility. By providing a downstream assessment of the final product—the percentage of sperm that can fertilize—the single measure of the Cap-Score has proven capable of identifying a wide range of these problems.

In the absence of a test such as Cap-Score, these defects in sperm function are identified only after repeated failure with natural conception and IUI (Aboulghar et al., 2001; Tournaye, 2012). Accordingly, many couples being treated for infertility are faced with substantial emotional, physical and monetary burdens. When these burdens become too great, it is not uncommon for couples to drop out of treatment before attaining pregnancy (Domar, Smith, Conboy, Iannone, & Alper, 2010). Our data suggest that Cap-Score results could be used by clinicians to inform their patients of that man's ability to generate pregnancy and help guide a treatment pathway. The impact provided by such an approach was previously modeled and shown to not only increase clinical pregnancy rates but also to greatly reduce medical costs, in an age-dependent fashion (Babigumira, Sharara, & Garrison, 2018).

As noted above, Cap-Score results should be interpreted in the context of both a semen analysis and a complete medical work-up inclusive of both the male and female partner. The Cap-Score should never be used as the sole criterion in the determination of male fertility. A low Cap-Score could be transient in nature, influenced by factors occurring before, during and(or) after sample collection and preparation. If the treatment plan allows, it is recommended to repeat the Cap-Score test in three months. Yet, other factors notwithstanding, the data reported here clearly demonstrated that Cap-Score was predictive of an individual man's chance of generating a pregnancy. Importantly, the equation translating the Cap-Score results into probabilities is based on actual clinical outcomes and settings, and not with an artificial system designed to mimic an idealized population of eggs with maximum fertility. The "real world"

nature of these probabilities is an important consideration for clinicians who wish to use the results to counsel their patients.

For example, for patients with very low probabilities of generating a pregnancy, the clinician might counsel that couple toward classical in-vitro fertilization (IVF) or ICSI. For patients with borderline low-normal scores, but with advanced age, those couples might also be counseled toward a more aggressive treatment, whereas a couple in which the man has an identical Cap-Score and semen analysis results but both he and his partner are much younger, might be counseled toward a less aggressive initial approach. These examples highlight just a few of the many scenarios in which knowledge of sperm fertilizing ability would enable a more personalized approach to the pursuit of parenthood. Such personalization could potentially encourage more couples to seek examination and possible treatment and enable an evidence-based approach to the treatment of underlying male factor infertility.

4 | MATERIALS AND METHODS

This study was designed as an observational, prospective feasibility trial of utilization of the Cap-Score™ Male Fertility Assay at a small number of fertility clinics and urology practices. Cap-Score is a Laboratory Developed Test, performed at Androvia LifeSciences' laboratory facility (Mountainside, NJ), which is Clinical Laboratory Improvement Amendments (CLIA) compliant and College of American Pathologists (CAP) certified. Cap-Score is commercially available and based on prior published data falls under standard of care at multiple clinics. For the current research, clinics provided Androvia with de-identified data that were collected during an individual's normal medical work-up. Samples were read and the Cap-Scores were reported back to the clinicians before receipt of any clinical outcomes. Inclusion/exclusion criteria, components of medical work-up, and IUI procedures are described below, broken out for each clinic to reflect differences. All work and methods were reviewed and approved by the Western Institutional Review Board (https://www.wirb.com/Pages/Default.aspx; Protocol #20152233).

4.1 | Specimen collection and processing

Semen samples were collected by manual masturbation as part of a standard male fertility evaluation exam. Any sample having fewer than 10×10^6 motile sperm on initial count was excluded. The samples were collected and processed using Cap-Score™ Male Fertility Assay kits obtained from Androvia LifeSciences LLC. Briefly, ejaculates were liquefied for up to 2 hr (Moody et al., 2017). Following liquefaction, the sperm were removed from the seminal plasma by centrifugation through Enhance S-Plus Cell Isolation Media (Vitrolife; Göteborg, Sweden; catalogue #15232 ESP-100-90%) and washed with modified Human Tubal Fluid medium (mHTF; Irvine Scientific, Santa Ana, CA; catalogue #90126 [97.8 mM NaCl; 4.69 mM KCl; 0.20 mM $MgSO_4$; 0.37 mM KH_2PO_4; 2.04 mM $CaCl_2$; 4 mM $NaHCO_3$; 21 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]; 2.78 mM $C_6H_{12}O_6$; 0.33 mM sodium pyruvate; 21.4 mM sodium lactate; 10 μg/ml gentamicin; 5 mg/L phenol red]). The sperm were resuspended in mHTF with (CAP) and without (NonCap) 2-hydroxypropyl-β-cyclodextrin (Sigma; St. Louis, MO; catalog #C0926) to promote capacitation. Following incubation, the samples were fixed, packaged and shipped overnight to Androvia for the test to be performed and Cap-Score™ determined.

All samples were sent in this way to Androvia LifeSciences, with the exception of 37 samples provided by Weill Cornell Medical Center, which were processed and scored at Weill Medical Center before the formation of Androvia LifeSciences. The processing and scoring of these samples have been detailed previously (Cardona et al., 2017); these samples were not part of the prospective study but contributed to the additional 67 data points that were used to test the model generated by the logistic regression analysis.

For all 124 patients considered in this study, semen analysis was performed in conjunction with the Cap-Score. All semen analyses were performed by licensed technicians following WHO guidelines (WHO, 2010).

4.2 | Sample labeling

Following incubation, fixation, and shipping, samples were labeled with 2 μg/ml of Alexa Fluor 488-conjugated cholera toxin beta subunit (Thermo Fisher Scientific; Waltham, MA; catalog #C34775). After ten minutes, 5 μl of the labeled sperm were placed on a microscope slide, overlaid with a coverslip (50 mm no. 1), and moved to an imaging station.

4.3 | Image acquisition

Imaging was performed on Nikon Eclipse NI-E microscopes equipped with CFI60 Plan Apochromat Lambda 20× Objectives; C-FL AT GFP/FITC Long-Pass Filter Sets; Hamamatsu ORCA-Flash 4.0 cameras; H101F – ProScan III Open Frame Upright Motorized H101F Flat Top Microscope Stages; and 64-bit imaging workstations running NIS Elements software (Nikon; Melville NY).

4.4 | Cap-Score determination

Readers were trained to identify G_{M1} localization patterns associated with both non-capacitated and capacitated human sperm (Moody et al., 2017). All readers passed proficiency testing and daily quality assurance testing as described (Moody et al., 2017). The proportion of sperm within a sample having undergone capacitation was determined and reported as the Cap-Score (# of sperm with patterns associated with capacitation/(# of sperm with patterns associated with capacitation + number of sperm with other patterns)). All readings were performed according to validated methods (Moody et al., 2017), and consistent with CLIA-, CAP- and Clinical Laboratory Evaluation Program (CLEP)-approved best practices for quality control and assurance. Briefly, if sufficient sperm were present in the sample, at least 150 total patterns were determined for each condition. If insufficient cells were available, a

minimum of 100 patterns was necessary to compute Cap-Score. Otherwise, the samples were rejected.

4.5 | Patients

Cap-Scores were performed on men whose fertility was being assessed for purpose of trying to conceive. The initial exclusion criterion for men was having fewer than 10×10^6 motile sperm on initial count. The fertility of female partners was examined, but findings of female factor (e.g., polycystic ovary syndrome, diminished ovarian reserve, repeated pregnancy loss, amenorrhea, myoma, anovulation, endometriosis, etc.) that did not preclude attempts at IUI were not considered grounds for exclusion so that the test population could most accurately reflect the patient population pursuing IUI. Only couples that pursued IUI were included in the study because that approach afforded the most rigorous control of the number and timing of insemination(s) relative to a given ovulation. Therefore, couples that were advised to pursue a course of expectant management and/or achieved natural/spontaneous conception, were excluded, as were couples who pursued classical IVF or ICSI. Differences among clinics reflecting their patient base, practices, and the components of the study to which they contributed are described below.

4.5.1 | Abington Reproductive Medicine

All patients were offered the Cap-Score™ Male Fertility Assay (n = 208 performed). Patients were included in the survival analysis if outcomes were available from at least one IUI attempt at the time of initial data analysis (n = 91). The data set used for this initial logistic regression analysis was from those couples who generated a pregnancy within, and (or) completed, at least three rounds of IUI at the time of data analysis (n = 57). Data were collected between 11/2016 and 1/2018.

4.5.2 | IVF1

Data from 13 consecutive couples looking for initial fertility treatment who generated a pregnancy within and (or) completed at least three rounds of IUI were included in the test of the original model generated by logistic regression. Data were collected between 3/2017 and 2/2018.

4.5.3 | Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine and Infertility, Weill Cornell Medicine

Men scheduled for semen analysis of any age that had normal semen parameters (WHO, 2010) and a female partner equal to or less than 35 years old were considered eligible. Data from four consecutive couples who generated a pregnancy within and (or) completed at least three rounds of IUI were included in the test of the original model generated by logistic regression. Data were collected between 3/2017 and 2/2018. As described above, data previously collected and described (Cardona et al., 2017) from 37 couples that fell under the current criteria for participation were also included in the test of the original model generated by logistic regression.

4.5.4 | Virginia Center for Reproductive Medicine

Data from nine consecutive couples looking for initial fertility treatment who generated a pregnancy within and (or) completed at least three rounds of IUI were included in the test of the original model generated by logistic regression. Data were collected between 12/2016 and 12/2017.

4.5.5 | New Jersey Urology

No data related to female factor were obtained. Data from four patients who had a Cap-Score™ performed and responded to a followup questionnaire to self-report clinical outcomes were included in the test of the original model generated by logistic regression. Data were collected between 12/2016 and 7/2017.

4.6 | Intrauterine insemination

4.6.1 | Abington Reproductive Medicine

IUI was performed in stimulated cycles. Patients were stimulated either with clomiphene citrate (CC), letrozole (Let), or gonadotropins. All patients were inseminated 24–36 hr after hCG injection. In rare cases, patients had a second insemination the following day, primarily due to low sperm numbers in the initial sample. Semen samples were produced by masturbation and allowed to liquefy. Semen analysis was performed to assess volume and concentration. Samples were washed as follows: First, 1 ml of warmed lower medium was pipetted into the bottom of 15 ml conical tube, then 1 ml of warmed upper medium was slowly layered on top. The semen sample was carefully layered on top. After centrifugation at 400g for 20 min, the supernatant was removed and the pellet was resuspended in 0.25–2.0 ml of wash medium with protein (volume dependent on the size of the sperm pellet). Post-wash count and motility were assessed. The sample was then centrifuged for 10 min at 400g, and the supernatant was carefully removed. The pellet was resuspended in 0.5 ml wash media with protein and used for insemination.

4.6.2 | IVF1

IUI was performed in stimulated cycles. Patients were stimulated either with CC, Let, or gonadotropins. For some of the patients stimulated with CC or Let, ovulation detection was performed by urine luteinizing hormone (LH) test. Patients tested their urine sample once or twice a day and were inseminated 20–24 hr after LH surge. Other patients stimulated with CC or Let were monitored at the fertility center and inseminated 30–36 hr after hCG injection. Ovulation was

triggered with hCG when a patient's follicles reached 20 mm or more in diameter. Patients stimulated with gonadotropins were inseminated 30–36 hr after hCG trigger. Ovulation was triggered with hCG when a patient's follicles reached at least 17 mm in diameter. Semen samples were produced by masturbation either at the fertility center or home. Samples underwent a simple wash and the pellet was resuspended in 0.3 ml of medium and used for insemination.

4.6.3 | Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine and Infertility, Weill Cornell Medicine

Ovarian stimulation was performed with CC at a dose of 50 or 100 mg daily for five days. The response to stimulation and endometrial thickness were monitored by serial transvaginal ultrasounds. Serum hormone assays were also used to measure estradiol and LH levels. In the absence of LH surge, ovulation was triggered with 10,000 IU hCG when the dominant follicle(s) reached 20 mm. IUI was performed within 24 hr after hCG injection. Semen samples were collected at the laboratory after 2–5 days of abstinence. Semen analysis was performed after 30 min of liquefaction. The samples were first diluted in HEPES-buffered human tubal fluid supplemented with human serum albumin for centrifugation at 600*g* for 10 min. For each sample, the pellet was then resuspended and layered on a density gradient. It was then centrifuged for 10 min at 300*g*. The bottom layer containing motile spermatozoa was collected by aspiration with a glass Pasteur pipette and resuspended for a final 10 min centrifugation at 600*g* to remove silica gel particles. The final pellet was resuspended in 0.5 ml of medium and used for insemination after reassessing concentration and motility.

4.6.4 | Virginia Center for Reproductive Medicine

IUI was performed in stimulated cycles. Depending on the patient's medical history, simulations were done either with Let, Tamoxifen, gonadotropins or a combination of medications. Ovulation was triggered with hCG when a patient's follicles reached 18–20 mm in diameter. Time from hCG trigger and insemination depended on whether there was single or double insemination. If single insemination, IUI was performed 36 hr after the trigger. If double insemination, the first IUI was done 24 hr after the trigger and the second IUI was done 48 hr thereafter. Cycles were supplemented with progesterone, starting the night after the insemination. Patients stayed on progesterone until nine weeks of pregnancy.

Semen samples were kept in a 36°C warmer for 30 min for liquefaction. Semen analysis was then performed to assess volume, concentration, motility, and morphology. The semen sample was then divided into two equal volumes between two 14-ml conical tubes. Two ml of prewarmed Quinn's Sperm Wash was added to each tube and mixed by pipetting. After centrifuging at 1,500 rpm for 5 min, the supernatant was removed from each tube, and both pellets were combined into one tube. Another 2 ml of warm Quinn's Sperm Wash was added to the combined pellet and mixed. After centrifuging at 1,500 rpm for 5 min, the supernatant was removed until 0.3–0.5 ml media was left covering the pellet. The medium-covered pellet was then kept in the warmer to allow the sperm to swim up for 2–4 hr. About 30 min before the scheduled IUI time, the medium containing motile sperm was removed from the pellet and placed in a new, pre-warmed tube ready for IUI. The volume, concentration, and motility of the final media were assessed to calculate the percentage of motile sperm recovered before IUI.

4.7 | Pregnancy outcome

4.7.1 | Abington Reproductive Medicine

Pregnancies were confirmed by beta hCG blood tests starting 14 days after ovulation (confirmed by LH and progesterone levels). All blood tests were repeated every 48–72 hr and ultrasound typically scheduled at 5.5 weeks of gestational age.

4.7.2 | IVF1

Clinical evidence of pregnancy was determined by beta hCG blood levels. If positive, the test was repeated two days later. If the hCG rise was deemed to be appropriate, then the patient was brought back to the office for a transvaginal ultrasound when it was predicted that the hCG level would be at least 2,000 IU/ml. If the hCG level did not rise appropriately, then the patient would return for additional hCG levels. A clinical pregnancy was determined to be present if a fetal pole with evidence of heart motion was seen.

4.7.3 | Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine and Infertility, Weill Cornell Medicine

Clinical pregnancies were identified by the presence of at least one fetal heartbeat using ultrasound.

4.7.4 | Virginia Center for Reproductive Medicine

Two weeks after insemination a urine test was performed; if positive, hCG and progesterone blood levels were determined. Blood tests were repeated every two days, to make sure that hCG was doubling every 48 hr. Once hCG blood levels of 1,000 U/ml were detected, ultrasonography was performed.

4.8 | Statistical analyses

Initial statistical analyses (Kaplan–Meier survival analysis and two sample *t* test) were carried out in XLSTAT Version 19.03.45087. Following the best practice of having analyses performed by independent statisticians, Singular Value Consulting (Houston, TX) was contracted and given Androvia's complete raw data set related to this study including updated clinical outcomes as they came in. First, the survival analysis and two sample *t* test results were confirmed. Second, logistic regression was used to evaluate Cap-Score and traditional semen analysis results alone and in combination in relation to the PGP for men who were successful in, or completed, three

rounds of IUI ($n = 57$). The model fit on these data was then tested using data from 67 additional patients (five total clinics). The AIC was used to assess model quality when the Cap-Score was combined with traditional semen analysis parameters. Confirmational statistics and logistic regression analysis were carried out in R (Team, 2013) and SciPy (Jones, Oliphant, & Peterson, 2001).

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DISCLOSURES

C. Cardona and G. C. Ostermeier are employees of Androvia LifeSciences, LLC. We know of nothing to disclose for Z. Rosenwaks and S. Hirshberg. J. Schinfeld, F. Sharara, R. Morris, G. D. Palermo, and E. Seaman have provided clinical advice to Androvia LifeSciences. G.D. Palermo is involved in intellectual property with Androvia that extends beyond the current publication. A. J. Travis' laboratory at Cornell identified the underlying technology, which was licensed by, and has been developed by, Androvia LifeSciences. He serves as a consultant to Androvia LifeSciences with duties of a Chief Scientific Officer.

AUTHOR CONTRIBUTIONS

J. Schinfeld, F. Sharara, R. Morris, G. Palermo, Z. Rosenwaks, E. Seaman, and S. Hirshberg identified patients, obtained histories, performed medical workups and procedures, and wrote the manuscript. C. Cardona and G. C. Ostermeier performed the Cap-Score Male Fertility Assay, maintained all datasets, analyzed data, and wrote the manuscript. J. Cook and A. J. Travis analyzed data and wrote the manuscript.

ORCID

G. Charles Ostermeier http://orcid.org/0000-0002-3951-512X
Alexander J. Travis http://orcid.org/0000-0001-5010-7568

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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