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RESEARCH ARTICLE

Localization patterns of the ganglioside G_{M1} in human sperm are indicative of male fertility and independent of traditional semen measures

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Semen analysis lacks a functional component and best identifies extreme cases of infertility. The ganglioside G_{M1} is known to have functional roles during capacitation and acrosome exocytosis. Here, we assessed whether G_{M1} localization patterns (Cap-Score™) correspond with male fertility in different settings: *Study 1* involved couples pursuing assisted reproduction in a tertiary care fertility clinic, while *Study 2* involved men with known fertility versus those questioning their fertility at a local urology center. In *Study 1*, we examined various thresholds versus clinical history for 42 patients; 13 had Cap-Scores ≥39.5%, with 12 of these (92.3%) achieving clinical pregnancy by natural conception or ≤3 intrauterine insemination cycles. Of the 29 patients scoring <39.5%, only six (20.7%) attained clinical pregnancy by natural conception or ≤3 intrauterine insemination cycles. In *Study 2*, Cap-Scores were obtained from 76 fertile men (Cohort 1, pregnant partner or recent father) and compared to 122 men seeking fertility assessment (Cohort 2). Cap-Score values were normally distributed in Cohort 1, with 13.2% having Cap-Scores more than one standard deviation below the mean (35.3 ± 7.7%). Significantly, more men in Cohort 2 had Cap-Scores greater than one standard deviation below the normal mean (33.6%; p = 0.001). Minimal/no relationship was found between Cap-Score and sperm concentration, morphology, or motility. Together, these data demonstrate that Cap-Score provides novel, clinically relevant insights into sperm function and male fertility that complement traditional semen analysis. Furthermore, the data provide normal reference ranges for fertile men that can help clinicians counsel couples toward the most appropriate fertility treatment.

KEYWORDS

andrology, capacitation, Cap-Score™, infertility, sperm function

1 | INTRODUCTION

Infertility, the inability to achieve pregnancy after 12 months of unprotected intercourse, affects around 10–15% of couples around the world (Sharma, Biedenharn, Fedor, & Agarwal, 2013). Studies suggest that 50% of infertility cases are due to a female factor, 20–30% due to a male factor, and the remaining 20–30% are a combination of both (Agarwal, Mulgund, Hamada, & Chyatte, 2015).

This is a global problem, with male factor reported to contribute to between 50% and 70% of cases of infertility in several regions, and with rates of male infertility ranging from 2.5% to 12% (Agarwal et al., 2015). In the United States, 7.5% of men aged 45 years or younger (3.3–4.7 million men) reported seeing a fertility doctor (Chandra, Martinez, Mosher, Abma, & Jones, 2005). Despite the enormous and growing importance of male factor infertility worldwide, diagnostic assays for male fertility remain inadequate; the lack of sperm function tests is a particular deficit (Lamb, 2010; Oehninger, Franken, & Ombelet, 2014; Wang & Swerdloff, 2014).

The diagnosis of male infertility is currently heavily based on results of standard semen analysis, which includes the evaluation of sperm morphology, concentration, and motility. The World Health Organization establishes and updates reference values for standard semen analysis in an attempt to distinguish normal from abnormal ejaculates (World Health Organization, 2010). Normal measures are defined as sperm concentration of $\geq 15 \times 10^6$/ml, total motility of $\geq 40\%$, and morphology (Krueger strict criteria) as $\geq 4\%$ normal forms (Cooper et al., 2010; World Health Organization, 2010).

However, a methodological concern with the generation and use of these 2010 World Health Organization values exists. Although the lower reference limits (5th percentile) were obtained utilizing data generated from a population of 4,500 fertile men from 14 countries (Cooper et al., 2010), data from potential subfertile/infertile men were not taken into account. Evidence of the inadequacy of semen analysis to reflect male fertility status is raised by clinical studies highlighting the absence of correlation between semen analysis results and ability to fertilize (Guzick et al., 2001; Ombelet et al., 1997; van der Steeg et al., 2011). In fact, van der Steeg et al. (2011) showed that men with normal and abnormal semen parameters were comparable in generating spontaneous pregnancies. This group argued that semen analysis still has value, but the individual parameters must be used in aggregate with one another, as opposed to being considered in terms of simple cut-offs (van der Steeg et al., 2011).

Another factor contributing to concern about the utility of semen analysis is the high variability among different ejaculates from the same individual. An intra-individual variability of 34% in sperm concentration was observed when at least three ejaculates were examined, and the coefficient of variation remained high even when factors such as abstinence and fever were taken into account (Carlsen, Petersen, Andersson, & Skakkebaek, 2004). Another study reported very similar results, showing a variance of 54% for concentration and 74% for motility index (Mallidis, Howard, & Baker, 1991). Together, these findings on the descriptive and varying nature of the parameters of semen analysis led to an understanding that the majority of infertile men have defects in sperm function. These, unfortunately, are only diagnosed by repeated failed cycles of intrauterine insemination (Aboulghar et al., 2001; Tournaye, 2012).

One aspect of sperm function that has received much attention as the potential basis for a diagnostic assay is the process of sperm functional maturation known as "capacitation." During capacitation, sperm acquire the ability to fertilize an egg. In vivo, sperm capacitation occurs while the sperm travel through the female reproductive tract (Austin, 1951, 1952; Chang, 1951). Along this journey, sperm respond to stimuli and a series of molecular events renders them fertilization competent (Austin, 1952; Travis & Kopf, 2002). Some of these molecular events include cholesterol efflux and subsequent changes in plasma membrane composition and fluidity (Davis, Byrne, & Hungund, 1979; Visconti et al., 1999), such as altered dynamics of cholesterol and the ganglioside G_{M1} in the plasma membrane overlying the acrosome (Buttke, Nelson, Schlegel, Hunnicutt, & Travis, 2006; Selvaraj et al., 2006, 2007, 2009). Both cholesterol efflux and focal enrichment of G_{M1} were shown to trigger transient calcium influx through a voltage-gated channel in mouse sperm (Cohen et al., 2014). These transients are required for sperm to undergo acrosome exocytosis, which is a process necessary for spermatozoa to penetrate and fertilize the egg (Cohen et al., 2014).

While investigating the role that G_{M1} plays in capacitation, we noted that G_{M1} localization occurred in specific and reproducible patterns in both murine and bovine sperm that responded to stimuli for capacitation versus those that either were not incubated with the stimuli or could not respond to them (Selvaraj et al., 2007). Furthermore, we demonstrated that sperm showing the G_{M1} localization pattern associated with capacitation represented the subpopulation that could undergo acrosome exocytosis and therefore were capable of fertilization (Selvaraj et al., 2007). Based on these results, we performed studies with human sperm, finding localization patterns similar to those in the bull (Neri et al., 2013; Paniza, Neri, Rosenwaks, & Palermo, 2014; Selvaraj et al., 2007). We subsequently found that those human sperm undergoing acrosome exocytosis stemmed from the subpopulation having G_{M1} localization patterns corresponding with capacitation. We also found that those sperm having "capacitated" G_{M1} localization patterns showed evidence of communication between the plasma and outer acrosomal membranes, which was not seen in sperm having a "non-capacitated" G_{M1} localization pattern (Moody et al., 2017). These data substantiated the accuracy of the assay at the single-sperm level; namely, those sperm having a capacitated G_{M1} localization pattern were indeed capacitated (Moody et al., 2017). Based on these and other data regarding the precision and repeatability of the assay, we defined the Cap-Score™ as the percentage of sperm having G_{M1} localization patterns consistent with capacitation in relation to the total number of sperm having G_{M1} localization patterns.

Here, we set out to determine whether the Cap-Score could be used to indicate the fertility status of men, and therefore provide the basis for an in vitro, laboratory-developed diagnostic test of male fertility that specifically assayed sperm capacitation and functional ability to fertilize. If positive, these results would contribute to the validation of the assay (Moody et al., 2017) by providing information about the clinical accuracy, or fit-for-purpose. Historically, several assays designed to test sperm function were shown to correlate with one or more of the traditional semen analysis parameters, limiting the additional value they provided to diagnostic efforts (Aitken, 2002; Giwercman et al., 2003; Hazary, Chaudhuri, & Wishart, 2001; Zini et al., 2009). We therefore also evaluated whether G_{M1} localization patterns correlated with any of the standard semen analysis parameters or instead added distinct, complementary information.

2 | RESULTS

2.1 | Experimental design

The correspondence of G_{M1} localization patterns in sperm with capacitation status was previously identified in the Travis lab, at Cornell's College of Veterinary Medicine (Selvaraj et al., 2007). Here, we provide data from two distinct studies: *Study 1* was a post-hoc association between capacitation and fertilization, performed independently at the Ronald O. Perelman & Claudia Cohen Center for Reproductive Medicine & Infertility, Weill Cornell Medical College, New York. *Study 2* involved a cohort comparison of 76 fertile men (Cohort 1) versus 122 men questioning their fertility (Cohort 2), performed at Androvia LifeSciences' research laboratory in Mountainside, New Jersey. The involvement of different settings provided several advantages: (i) the experiments testing clinical utility were performed independently at Weill Cornell Medical College, reducing the potential for conflict of interest; (ii) the different sites had highly different patient populations in terms of age and what was known about the fertility status of the pregnant partner; and (iii) this design showed that the assay could function in both commercial and clinical settings.

For *Study 1*, Dr. Travis and his staff trained Dr. Palermo and his team at the Ronald O. Perelman & Claudia Cohen Center for Reproductive Medicine & Infertility to perform the assay. Conflict of interest was avoided and objectivity was maintained by having Dr. Palermo and his laboratory independently identify and consent all men, collect all histories, and perform all sample handling, semen analysis, incubations, and data collection without any input from Dr. Travis, his lab at Cornell, or Androvia LifeSciences, LLC. In fact, all Cap-Score data from *Study 1* were acquired before Androvia LifeSciences formed and licensed the underlying technology from Cornell.

In *Study 2*, presumed fertile men (Cohort 1) were recruited, consented, and produced samples at Androvia LifeSciences' research laboratory. Potential subfertile/infertile patients (Cohort 2) were identified and consented at the Urology Group of New Jersey, where semen analyses were also performed; these men were typically being evaluated as part of a couple experiencing fertility problems, and no attempt was made to identify or remove cases in which fertility of the

female partner might have been compromised. In this way, we could evaluate if defects in capacitation are sufficiently widespread in men questioning their fertility such that this functional test would be appropriate as part of an initial fertility screen or instead would be performed only as part of a more detailed work-up for patients in whom infertility is already strongly suspected. Aliquots of the raw ejaculates were transported to Androvia LifeSciences' research laboratory in an insulated box containing a warm pack to maintain approximate body temperature. Transportation of samples averaged 30 min, which counted towards total liquefaction time.

2.2 | Men able to conceive naturally or via intrauterine insemination produced a greater percentage of sperm capable of capacitation

Of the 63 patients for whom data were collected in *Study 1*, clinical fertility data were available for 42. Twenty-four of these 42 men either had no history of clinical pregnancy (except in vitro fertilization or intracytoplasmic sperm injection, results of which were not assessed here by design) or required greater than four cycles to achieve pregnancy through intrauterine insemination. Men with these histories were designated "subfertile/infertile." Of the 42 patients in *Study 1*, 18 required three or fewer cycles to exhibit clinical evidence of pregnancy (e.g., biochemical, ultrasonographic) or had any history of pregnancy via natural conception. Men with these histories were designated "fertile." Traditional semen parameters were measured for all men (Table 1). A difference in sperm concentration between fertile and subfertile/infertile individuals was measured ($p = 0.017$), although both groups were well above World Health Organization guidelines. No differences in ejaculate volume ($p = 0.981$), sperm motility ($p = 0.066$), or morphology ($p = 0.208$) were observed between the groups.

Cap-Scores were calculated for the 42 individuals for whom clinical fertility data were available to determine whether Cap-Score differed between fertile and subfertile/infertile individuals. The subfertile/infertile patients had mean Cap-Scores of $22.6 \pm 1.5\%$ and $28.8 \pm 1.8\%$, and the fertile population had Cap-Scores of $27.1 \pm 1.8\%$ and $38.4 \pm 2.5\%$ for Non-Cap (incubated without capacitation stimuli) and Cap (incubated with capacitation stimuli) treatments, respectively (Figure 1a). No difference was observed in mean Cap-Score between

TABLE 1 Comparison of semen quality measures between subfertile/infertile and fertile men

Measure	Subfertile/infertile[a] $n = 24$	Fertile[a] $n = 18$	*p*-value[d]
Cap-Score: Non-CAP[b]	22.6 ± 7.5	27.1 ± 7.7	0.065
Cap-Score: CAP[b]	28.8 ± 8.8	38.4 ± 10.5	0.002
Volume (ml)	2.4 ± 1.0	2.4 ± 1.3	0.981
Concentration (10^6/ ml)	49.9 ± 19.2	64.2 ± 17.5	0.017
Motility (%)	47.3 ± 6.3	50.9 ± 6.1	0.066
Morphology[c]	2.6 ± 1.0	2.9 ± 0.8	0.208

Standard deviation is given for each value.
[a]Subfertile/infertile men were defined based on a history of requiring more than three intrauterine insemination cycles to achieve conception. Fertile men achieved pregnancy with three or fewer intrauterine insemination cycles or any history of natural conception.
[b]Non-CAP samples were incubated in basal media; CAP samples were incubated with capacitating stimuli.
[c]Morphology determined using strict World Health Organization criteria.
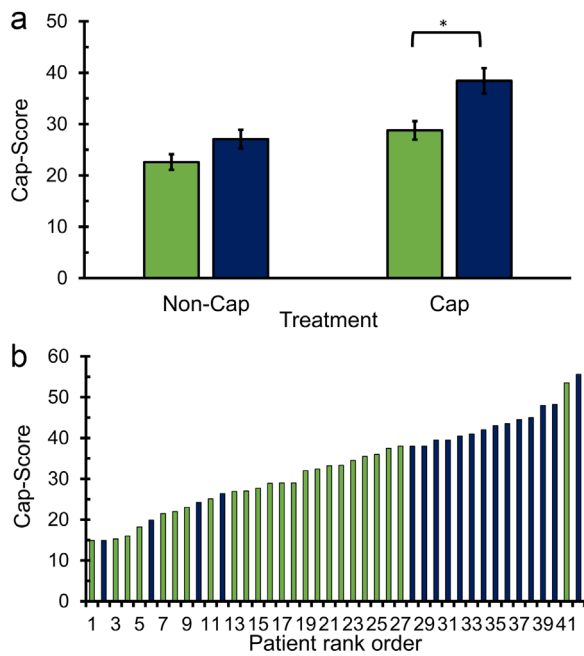[d]*p*-Value for two-tailed *t*-test for two independent samples.

FIGURE 1 Comparison of Cap-Score between fertile and subfertile/infertile men. Patients were grouped based on clinical history. Those having no evidence of clinical pregnancy by natural conception or requiring 4 or more cycles of intrauterine insemination to achieve pregnancy are shown in green ($n = 24$); those requiring 3 or fewer cycles of intrauterine insemination or having any history of natural conception are shown in blue ($n = 18$). (a) The Cap-Score of sperm exposed to basal (Non-Cap) or capacitating (Cap) conditions. Error bars represent the standard error of the mean. The Cap-Score for samples incubated under basal, non-capacitating conditions did not differ between the two fertility groups ($p = 0.065$). In contrast, Cap-Scores for samples incubated with stimuli for capacitation were significantly lower for those men experiencing difficulties with conception (*$p = 0.002$). (b) Cap-Score was determined for 42 individuals, and then these individuals were ranked based on their Cap-Score (x-axis). The Mann–Whitney test showed that men who achieved clinical pregnancy in 3 or fewer cycles or by natural conception were more likely to have higher Cap-Scores ($p = 0.001$)

fertility groups for the Non-Cap treatment ($p = 0.065$). In contrast, men with no evidence of pregnancy or requiring greater than three cycles to achieve pregnancy had significantly lower Cap-Scores for the Cap treatment ($p = 0.002$). In addition, the fertile group had a better response to capacitation stimuli, as their percent increase in Cap-Score from Non-Cap to Cap was $45.2 \pm 8.2\%$, in comparison to $34.2 \pm 7.2\%$ for the subfertile/infertile group ($p = 0.015$; Mann–Whitney test).

A nonparametric comparison of Cap-Score was undertaken between the fertile and infertile/subfertile men to further investigate the relationship between Cap-Score and fertility status (Figure 1b). For this approach, the 42 patients with clinical fertility data were ranked based on their Cap-Score to visualize and assess the association between Cap-Score and clinical fertility. A Mann–Whitney test showed that clinically subfertile/infertile men were more likely to have lower Cap-Scores ($p = 0.001$). These results suggest that G_{M1}

localization scores tracked well with successful fertilization via intrauterine insemination and natural conception.

Several possible cut-offs were evaluated using a receiver operating characteristic curve to determine whether the Cap-Score, on its own, might be able to distinguish fertile from subfertile/infertile patients in *Study 1* (Figure 2). When a Cap-Score cut-off of 39.5% was used, 13 patients were above the cut-off and 29 were below based on the population distribution (Figure 1b). Of the 13 above the cut-off, 92.3% (12/13) were fertile. Of the 29 individuals below the cut-off, 20.7% (6/29) were fertile. When a Cap-Score of 37.5% was used as a cut-off, 17 patients were above, and 25 were below. Iteratively adjusting the cut-off revealed a tradeoff between sensitivity and specificity, which behaved as expected. A cut-off of 38.0% provided the best combination of sensitivity and specificity; however, because a cut-off of 39.5% maximized sensitivity for this population (Figure 2), this value was chosen for further analyses of *Study 1*.

The applicability of the determined cut-off was demonstrated by separating the men in *Study 1* into groups based solely on their histories of clinical pregnancy, and then evaluating for Cap-Score (Figure 3). Of the 24 patients who were designated subfertile/infertile, only one (1/24 = 4.2%) was above the cut-off (Cap-Score ≥39.5%). Of the 18 donors who were classified as fertile, 12 (12/18; 66.7%) were above the cut-off. The proportion of clinically subfertile/infertile individuals with low scores (Cap-Score <39.5%) (23/24) was larger than the proportion of clinically fertile individuals below the cut-off (6/18; $p = 0.000$). These results further suggested that the Cap-Score provided useful information that could help distinguish men who were likely to have success by natural conception or within three or fewer cycles of intrauterine insemination from those who were subfertile/infertile, and did not achieve clinical evidence of pregnancy within this limit.

2.3 | Spermatozoa from presumed-fertile men showed a robust response to capacitation stimuli

Despite their compelling nature, the above data had an important limitation; namely, they were obtained from individuals actively seeking a fertility work-up and treatment at a tertiary care clinic, often after a long history of examinations and failed cycles of intrauterine insemination at other clinics. This resulted in a highly skewed patient base, in terms of both age and need for the majority to utilize intrauterine insemination to achieve a successful fertilization. Thus, the values and cut-off in *Study 1* would likely not be applicable to a fertile population and/or a population seeking fertility treatment.

In *Study 2*, we therefore set out to determine a Cap-Score reference range for men with normal fertility in the absence of any form of assisted reproduction, and to compare that range with data from a cohort of men questioning their fertility. Cap-Scores were obtained from 187 semen samples provided by 76 men with presumed fertility (pregnant partner or fathered a child within 3 years [Cohort 1]). To determine how consistent Cap-Score readings were within a donor, multiple samples were tested from 30 of these fertile men (average four readings/donor). Collections were done at least 1-week apart, with donor assurance of two-to-five days of abstinence. A Cap-Score was obtained for each collection, and a coefficient of variation was

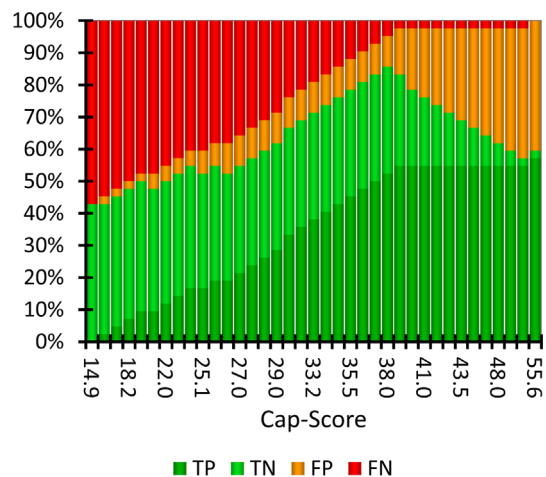| Cap-Score Assay Cut-off:	Score Group	N	Percent Fertile	Sensitivity	Specificity
39.5	Above	13	92.3 (12/13)[a]	95.8%	66.6%
	Below	29	20.7 (6/29)[b]		
38.0	Above	16	87.5 (14/16)[a]	91.7%	77.8%
	Below	26	15.4 (4/26)[b]		
37.5	Above	17	82.4 (14/17)[a]	87.5%	77.8%
	Below	25	16.0 (4/25)[b]		

FIGURE 2 Determining cut-off to optimize fertility designation in Study 1. A receiver operating characteristic curve was generated using Cap-Score and fertility history. The table shows the three Cap-Score cut-off values with best sensitivity and specificity. Score Group, "Above" denotes Cap-Scores at or above the cut-off value, and "Below" denotes Cap-Scores below the cut-off value; N, number of individuals in group; Percent fertile, proportion of men within a group that had clinical evidence of fertility, as defined by conceiving in ≤3 cycles of intrauterine insemination or by natural conception; Sensitivity, (true positives/[true positives + false negatives]); Specificity, (true negative/[true negative + false positive]). Unique superscripts denote significantly different proportions ($p < 0.05$). The graph shows the proportion of true positives (TP, subfertile/infertile individuals correctly identified as subfertile/infertile [dark green]), true negatives (TN, fertile individuals correctly identified as fertile [light green]), false positives (FP, fertile individuals incorrectly identified as subfertile/infertile [orange]), and false negatives (FN, subfertile/infertile individuals incorrectly identified as fertile [red]) along the y-axis for each potential Cap-Score cut-off on the x-axis

calculated for each donor (Figure 4). The average coefficient of variation within a donor was 12.6%, with most collections typically varying within six Cap-Score units of their average. Variation in semen assay parameters between samples collected from individuals is well documented (Carlsen et al., 2004; Mallidis et al., 1991), and these observations suggest that the Cap-Score is reasonably consistent from one collection to the next.
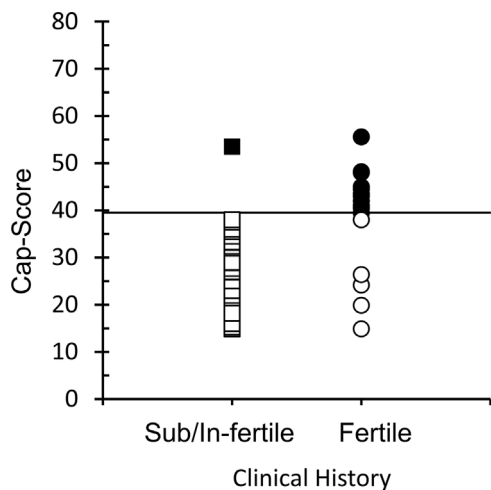
2.4 | Establishing a standard capacitation profile

An average Cap-Score was determined for each donor in Cohort 1 of *Study 2* to ensure that each individual had similar weight in the cohort comparison. The average Cap-Score for Cohort 1's sperm incubated with stimuli for capacitation was 35.3 ± 0.9% (76 unique donors, 187 observations) and 23.7 ± 1.0% for the basal, non-capacitating treatment (76 unique donors, 177 observations). The mean percent increase in Cap-Score from basal to capacitating conditions for Cohort 1 was 66.6 ± 4.9% (n = 76), suggesting that sperm from fertile individuals had a robust ability to respond to capacitation stimuli.

2.5 | Comparison of men questioning their fertility against the standard capacitation profile

The major goal in clinical settings is to diagnose the fertility status of a specific individual. Therefore, an understanding of the distribution of the individual scores is necessary to establish a useful test. For this purpose, we compared Cap-Scores obtained from 122 individuals seeking semen analysis because of questions regarding their fertility (potential subfertile/infertile men [Cohort 2]) against the Cap-Scores obtained from the 76 individuals in Cohort 1. No patients in *Study 2* were removed from the population of men questioning their fertility as a result of female factor infertility, making it likely that a number of these men were actually fertile.

FIGURE 3 Variation in Cap-Score based on fertility status. Individuals in Study 1 were grouped on the x-axis based on their history of clinical pregnancy. Twenty-four patients were designated as subfertile/infertile (no history of natural conception or by intrauterine insemination in three or fewer cycles [squares]). Of these, only one scored above the threshold of having a Cap-Score ≥ 39.5 (1/24 = 4.2% [closed square]). Eighteen patients were classified as fertile (successful conception in three or fewer cycles of intrauterine insemination or natural conception [circles]). Of these, 12 scored above the threshold of having a Cap-Score ≥ 39.5 (12/18 = 66.7% [closed circles])
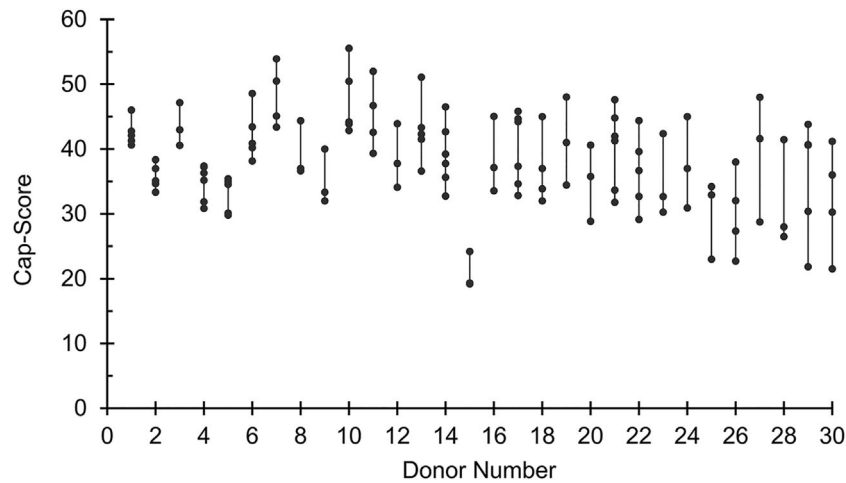
FIGURE 4 Cap-Score reproducibility within individuals. Multiple ejaculates were tested from 30 individual men with known fertility (Study 2, Cohort 1; average four ejaculates per donor). The x-axis shows donor number and the y-axis shows Cap-Score. Each point aligned vertically along the bar above a single donor number represents a different collection. Average coefficient of variation (standard deviation/mean [σ/μ]) within each donor was 12.6%. On average, repeated collections from an individual varied within 6% points of the average for that individual

To compare the distributions of these two populations, we first converted the data from Cohorts 1 and 2 to z-scores using the mean (μ) and standard deviation (SD; σ) for Cohort 1 ($z\text{-score}_i = (X_i - \mu)/\sigma$; X_i = observation (i), $\mu = 35.3$; $\sigma = 7.7$). This transformed the mean of Cohort 1 to 0, and each standard deviation from the mean became equivalent to 1 unit on the x-axis. This approach enables one to visualize the mean and distribution of samples in the known-fertile Cohort 1 with the remainder of the converted values simply representing the distance of any observation from the mean in units of σ. The Lilliefors test for distribution normality showed that Cohort 1 followed a normal distribution ($p = 0.24$). In a population with a perfectly normal distribution, 68% of the values should be within one standard deviation of the mean, and 95% of the values should be within two standard

deviations. Here, 72.4% were within one standard deviation of the mean and 94.7% were within two standard deviations of the mean.

Clinical attention is focused on the lower end of the male fertility spectrum, so we next compared Cap-Scores from Cohort 2 against the data from Cohort 1. In Cohort 1, 13.2% of the observations had z-scores at or below −1 (Figure 5). A greater proportion of individuals in the potential subfertile/infertile Cohort 2 had z-scores at or below −1 (33.6%; $p = 0.001$) (Figure 5). These data show that, in comparison to fertile men, many men questioning their fertility had sperm that responded relatively poorly to stimuli for capacitation. Cap-Score population means were also compared between Cohorts 1 and 2; however, since the vast majority of Cohort 2 was not vetted for female fertility factor, this population likely represents a rather
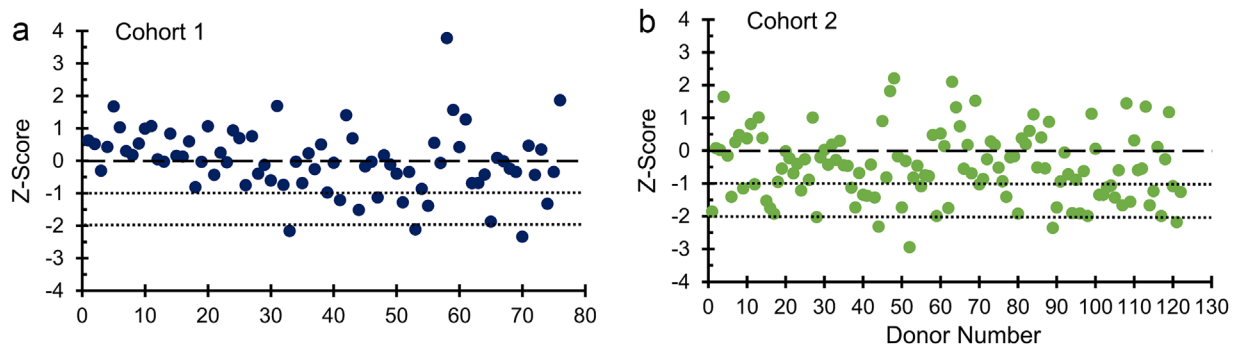

FIGURE 5 Comparison of Cap-Scores between sperm of fertile men and men questioning their fertility. (a) Scatter plot of Cap-Scores obtained from men with known fertility (Study 2, Cohort 1). Blue dots represent average Cap-Scores for 76 unique fertile donors (total 187 observations), plotted as z-scores. (b) Scatter plot of Cap-Scores obtained from a potential subfertile/infertile cohort (Study 2, Cohort 2). Green dots represent the Cap-Score from each patient ($n = 122$). Cap-Scores from both populations were converted to z-scores ([$X-\mu$]/σ; X = observation, $\mu = 35.3$; $\sigma = 7.7$), and are shown on the y-axis and the donor number is on the x-axis. The dashed horizontal line represents the mean and the dotted lines represent 1 and 2 standard deviations below the mean. In the fertile population of Cohort 1, 13.2% (10/76) of the observations had z-scores ≤ -1, which is consistent with a normal distribution. In contrast, 33.6% (41/122) of the individuals in the potential subfertile/infertile cohort had z-scores ≤ -1 ($p = 0.001$)

heterogeneous distribution that includes a number of fertile men. Nonetheless, the average Cap-Score for sperm incubated with capacitation stimuli for Cohort 2 was 31.6 ± 0.73% ($n = 122$), which is significantly less than the value obtained for Cohort 1 ($p = 0.001$). Altogether, these data strongly suggest that the population of men questioning their fertility produced fewer sperm that could respond to capacitation stimuli.

2.6 | Minimal-to-no relationship was found between Cap-Score and standard semen analysis parameters

Several assays designed to test sperm function were previously found to correlate with one or more of the traditional semen analysis parameters, greatly reducing their diagnostic value (Aitken, 2002; Giwercman et al., 2003; Hazary et al., 2001; Zini et al., 2009). Therefore, we evaluated whether Cap-Score provided novel functional data or merely tracked with standard semen analysis parameters by comparing sperm morphology, concentration, and motility metrics to the Cap-Scores for each man in Cohort 2.

First, morphology scores were obtained applying the World Health Organization criterion (2010): 78.7% (96/122) of the population was teratozoospermic, with less than 4% normal forms; 21.3% (26/122) passed the World Health Organization criteria for normal morphology, and of these men, 19.2% (5/26) had a Cap-Score more than one standard deviation below the mean of the presumed fertile population (e.g., Cohort 1). Samples were classified as having 0, 1, 2, 3, or ≥4% normal forms, and the grouped Cap-Scores were compared using analysis of variance. No relationship was found between Cap-Score and morphology ($p = 0.28$) (Figure 6).

Regarding motility, the World Health Organization (2010), classifies a sample as asthenozoospermic when total motility is less than or equal to 40%. Motility is traditionally presented as a univariate graph (range 15–80%) (Figure 7a) that can be expanded to include Cap-Score (Figure 7b). Within Cohort 2, 93.4% (114/122) exhibited normal motility; of these men, 30.7% (35/114) had Cap-Scores more than one standard deviation below the mean of the presumed fertile population. Linear

regression suggested a minor relationship between total motility and Cap-Score ($r = 0.22$, $p = 0.02$; $r^2 = 0.05$), with total motility accounting for only 5% of the variability in Cap-Score, leaving 95% of the variability unrelated to motility. As shown, total motility data were collected in an ordinal fashion, rather than as a continuous measure, so total motility data were also analyzed in bins of 5% to reflect the manner in which the data were collected. Bins having less than three observations were removed, followed by analysis of variance. No difference in Cap-Score was detected across the six bins ($p = 0.14$), highlighting the minor nature of any potential relationship.

Finally, the relationship between sperm concentration and Cap-Score was evaluated. Again, traditional semen analysis plots concentration as a univariate distribution (Figure 8a). The World Health Organization (2010) identifies a concentration of sperm less than or equal to 15×10^6/ml as abnormal and oligozoospermic; 17.2% (21/122) of men in Cohort 2 exhibited oligozoospermia. Incorporation of Cap-Score data versus sperm concentration (Figure 8b) revealed that of the 82.8% (101/122) of the men exhibiting normal sperm concentration, 32.7% (33/101) had Cap-Scores more than one standard deviation below the mean of the presumed fertile population. Linear regression analysis revealed no correlation between concentration and Cap-Score ($r = 0.04$, $p = 0.67$).

3 | DISCUSSION

Here, we report that changes in patterns of G_{M1} localization in human sperm correspond with male fertility. This finding was consistent in different settings, with different study designs, different patient populations, and when performed by different operators. When combined with the absence of relationship between the Cap-Score and conventional semen analysis parameters, these clinical findings suggest that G_{M1} localization patterns, the basis of the Cap-Score, can provide important information on sperm function that will complement traditional semen analysis.
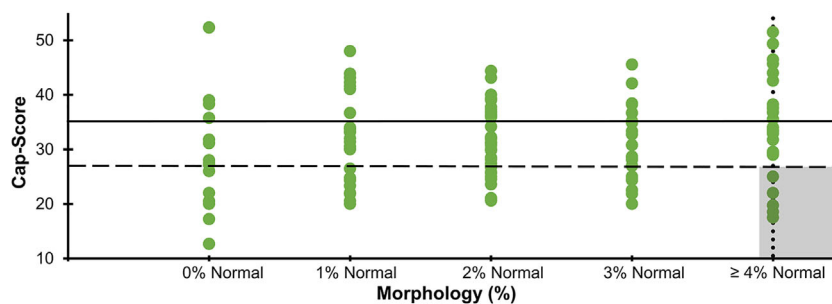

FIGURE 6 Association of sperm morphology and Cap-Score. Scatter plot of morphology (% normal forms; x-axis) versus Cap-Score (y-axis) obtained for 122 samples from men questioning their fertility (Study 2, Cohort 2). 78.7% (96/122) of the population had abnormal morphology (<4% normal forms; cut-off shown by vertical dotted line). 21.3% (26/122) of the population had normal morphology (≥4% normal forms). The solid horizontal line marks the mean and dotted horizontal line denotes one standard deviation below the mean for a population of presumed fertile men (Study 2, Cohort 1). 19.2% (5/26, gray shaded area) had a Cap-Score more than one standard deviation below the mean of the presumed fertile population. Analysis of variance revealed no relationship between morphology and Cap-Score ($p = 0.28$)
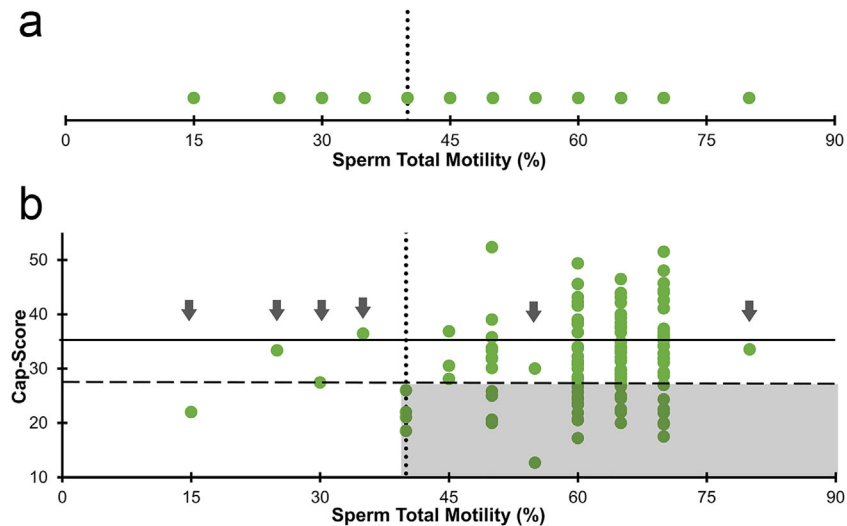
FIGURE 7 Association of sperm motility and Cap-Score in men questioning their fertility. (a) Traditional view of motility (Study 2, Cohort 2, n = 122). (b) Motility plotted against Cap-Score (Study 2, Cohort 2, n = 122). Total motility was collected in an ordinal fashion and data are presented in bins, or increments of 5% (x-axis). Bins with less than three observations were removed from the analysis, and are indicated by gray arrows. The corresponding Cap-Score for each observation is shown on the y-axis. The solid horizontal line marks the mean while the dotted horizontal line denotes one standard deviation below the mean for a population of fertile men (Cohort 1). No difference in Cap-Score was detected across the six bins (n = 115; p = 0.144 by analysis of variance) with sufficient numbers to be assessed. 6.6% (8/122) of men were asthenozoospermic by World Health Organization criteria (≤40% total motility; cut-off represented by vertical dotted line). 93.4% (114/122) of men had normal motility. 30.7% (35/114) had normal motility, but exhibited Cap-Scores ≤1 standard deviation below the mean (gray shaded area)

Supporting this assertion, data collected independently in *Study 1* showed a marked difference in Cap-Score between fertile men versus subfertile/infertile men. When viewed from the opposite perspective, men who scored above a threshold Cap-Score were very likely to be fertile (92.3%), whereas men who scored below that cut-off were unlikely to be fertile (20.7%). Another profound difference was revealed by the cohort comparison (*Study 2*), in which 33.6% of men questioning their fertility had Cap-Scores lower-than or equal-to one
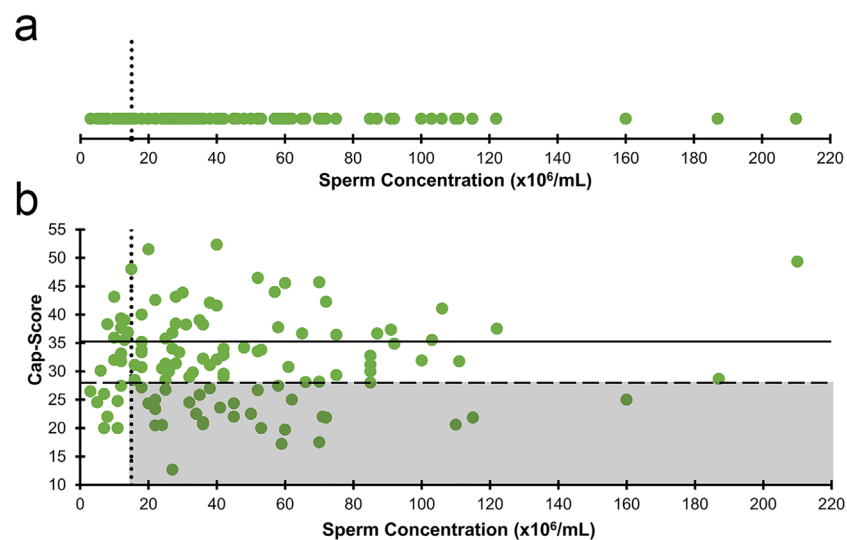

FIGURE 8 Comparison of sperm concentration and Cap-Score in men questioning their fertility. (a) Traditional view of concentration (Study 2, Cohort 2, n = 122). (b) Concentration (x-axis) plotted against Cap-Score (y-axis) (Study 2, Cohort 2, n = 122). Horizontal lines denote the mean Cap-Score and one standard deviation below the mean for fertile men (Study 2, Cohort 1). 17.2% (21/122) of men demonstrated oligozoospermia (≤15 × 10⁶/ml; cut-off shown by vertical dotted line). 82.8% (101/122) of men had normal sperm concentration. 32.7% (33/101) of men with normal sperm concentration had Cap-Scores more than one standard deviation below the mean of the presumed fertile population (gray shaded area). 33.6% (41/122) of men exhibited Cap-Scores ≤1 standard deviation below the mean; of these, 80.5% (33/41) had normal sperm concentrations

standard deviation below the mean, as compared to 13.2% for the fertile cohort. These data are especially remarkable in that none of the men questioning their fertility (Cohort 2) were excluded because of infertility in their female partners. Thus, one would expect a number of the men in Cohort 2 to be fertile, with a purely female factor fertility issue driving that couple to be examined (Agarwal et al., 2015). If those fertile men had been removed from Cohort 2, then the assay might have revealed an even higher percentage of cases of men whose sperm responded poorly to stimuli for capacitation.

As noted, several assays initially thought to provide information on sperm fertilizing ability were later shown to track with one of the existing parameters of semen analysis. We found that traditional semen analysis parameters, including sperm morphology, total motility, and concentration, had little to no correlation with the Cap-Score (at most, motility accounted for 5% of the Cap-Score); thus, the Cap-Score provides novel information.

Over the past decades, several assays were shown to measure or assess sperm capacitation. For example, capacitation can be detected by performing immunoblots for phosphotyrosine residues (Osheroff et al., 1999; Visconti et al., 1995). This is an excellent laboratory tool, though it is not by nature quantitative, and the technical effort required has precluded this approach from being developed for commercial use. Other assays of capacitation also involve incubating sperm under capacitating conditions, and then treating them with a stimulus to induce acrosome exocytosis. For similar reasons of technical effort, as well as the subjective nature of interpreting the signal in the sperm head (e.g., as when using chlortetracycline as a calcium indicator [Saling & Storey, 1979]), these assays have also not been used extensively in clinical practice.

Additional data on the inter- and intra-operator reliability of Cap-Score measurements (Moody et al., 2017), along with observations on

how semen must be handled in order to perform this assay and optimize function (Moody et al., 2017), support the technical feasibility of this assay and how it might fit into the work flow of a traditional semen analysis. The current demonstration of clinical "fit for purpose" combine with those data to provide a validation of the assay.

Our results strongly suggest that the Cap-Score assay addresses recent calls for the development of practical tests of sperm function to act as a complement to semen analysis (Lamb, 2010; Oehninger et al., 2014; Wang & Swerdloff, 2014). Clearly, a large percentage of men questioning their fertility and passing World Health Organization semen analysis cut-offs had sperm that were poorly responsive to stimuli for capacitation. The Cap-Score Sperm Function Test should not, however, be the singular test administered and/or considered when evaluating the fertility status of the male partner. This caveat is applicable to all tests of sperm function, as these tests are not meant to detract from or replace, but instead build upon and strengthen, the information provided in the traditional semen analysis (Sakkas, Ramalingam, Garrido, & Barratt, 2015).

When used in conjunction with traditional, descriptive measures of semen quality, knowledge of the Cap-Score should help clinicians counsel couples to the most appropriate fertility treatment (Palermo, Neri, & Rosenwaks, 2015). Currently, defects in sperm function go undiagnosed by the traditional semen analysis. This results in half of all cases of male infertility being idiopathic, identified only by repeated failure at natural conception and intrauterine insemination (Aboulghar et al., 2001; Tournaye, 2012). The current diagnostic algorithm imparts enormous emotional, physical, and financial costs on couples trying to conceive. Integration of the Cap-Score Sperm Function Test into the contemporary diagnostic/treatment algorithm is relatively simple (Figure 9), and would allow couples identified with reduced sperm function to be spared cycles of intrauterine insemination that are
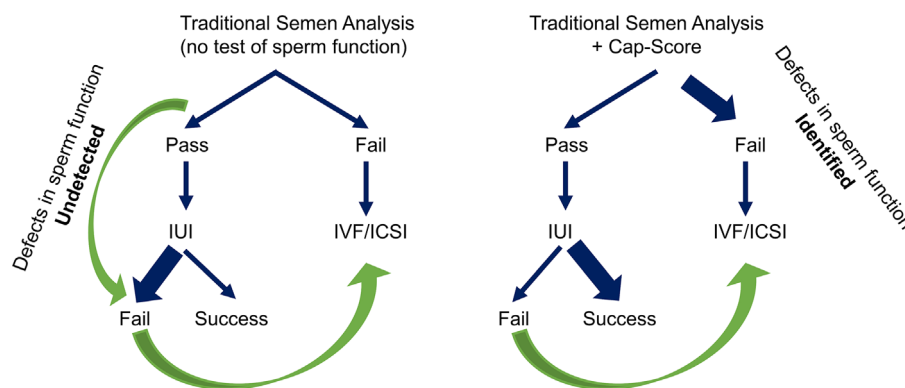

FIGURE 9 Comparison of clinical algorithms between historical reliance on semen analysis versus semen analysis performed in conjunction with Cap-Score. The inclusion of Cap-Score to the clinical algorithm will provide a test of sperm function, offering a valuable complement to the descriptive parameters in the traditional semen analysis. Currently, semen analysis fails to diagnose or identify defects in sperm function. More than 50% of male infertility cases are idiopathic, identified only by repeated failure at natural conception and intrauterine insemination (IUI) (large arrow in flow chart on left). In a new algorithm, inclusive of a traditional semen analysis and the Cap-Score Sperm Function Test, couples with low sperm function would be identified earlier (large arrow near the top of the flow chart on right), and immediately directed to a more appropriate form of assisted reproduction, such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Removal of these couples with low sperm function from those pursuing intrauterine insemination would be predicted to result in a higher relative success rate of intrauterine insemination for couples with appropriately high Cap-Scores (large arrow near bottom of flow chart on right)

doomed to fail. Instead, these couples could immediately be directed to a more appropriate form of assisted reproductive technology, such as in vitro fertilization or intracytoplasmic sperm injection (Forti & Krausz, 1998).

Why should an assay of the sperm's ability to capacitate in vitro provide clinically relevant information on sperm function for a large percentage of men? Unlike tests that look for mutations in single genes, a cell-biology approach that evaluates the entire pathway of capacitation enables one to assess the functions of, and relationships among, hundreds or possibly thousands of gene products. Male germ cell development and differentiation in the testis, maturation during epididymal transit, and interaction with seminal plasma and stimuli for capacitation must all be normal for sperm to capacitate and yield a Cap-Score within the reference range.

G_{M1} localization is highly conserved in the spermatozoa of diverse mammalian species, including mouse, bull, and human (Buttke et al., 2006). This ganglioside is enriched in the plasma membrane overlying the acrosome, matching the localization of sterols and Caveolin-1, a protein associated with membrane rafts and involved in the regulation of acrosome reaction and capacitation (Selvaraj et al., 2006; Travis et al., 2001). G_{M1} is bound by substances in seminal plasma that help keep the sperm functionally quiescent; as G_{M1}-binding proteins such as SVS2 (Seminal vesicle secretory protein 2) are lost from the sperm, these gametes can then capacitate and acquire the ability to fertilize an egg (Kawano & Yoshida, 2007; Kawano, Yoshida, Iwamoto, & Yoshida, 2008). G_{M1} acts as an important control point for capacitation because it regulates transient calcium flux required for acrosome exocytosis in response to sterol efflux (Cohen et al., 2014).

We previously showed that specific patterns of G_{M1} localization are found in mouse and bull sperm that are responsive to stimuli for capacitation (Selvaraj et al., 2007). In the cohort of fertile men, our present finding of a mean Cap-Score of 35.3% is roughly consistent with these previous studies in murine and bovine spermatozoa, in which approximately 40% of the spermatozoa showed specific changes in G_{M1} localization upon exposure to capacitation stimuli (Selvaraj et al., 2007). These results are also consistent with other studies in which capacitation in murine sperm was measured by protein tyrosine phosphorylation events (Urner, Leppens-Luisier, & Sakkas, 2001). Together, these data support the view that in diverse mammalian species, including humans, not all sperm in an ejaculate are functionally equivalent and that G_{M1} localization is a reasonable readout that is informative of these functional differences.

Of interest, the mean Cap-Score for fertile men in *Study* 1 (38.4), and the cut-off value that had the most sensitivity (39.5), were both slightly higher than the mean Cap-Score for fertile men in Cohort 1 of *Study* 2. While the lower sample size of fertile men in *Study 1* (18 out of the 42) could well contribute to this difference, it should be recalled that the *Study 1* patient base was skewed toward older individuals with more complicated reproductive histories who were largely reliant on intrauterine insemination for conception. These differences in the size and nature of *Study 1*'s patient base also likely account for the slight, but statistically significant, difference in concentration between the fertile and subfertile/infertile individuals. To reduce variability within

and between studies, we could have introduced age-related exclusion criteria; we decided against this because the age range of patients in *Study 1* reflected those individuals actually being treated for infertility. In both *Study 1* and Cohort 2 of *Study 2*, we felt it would be practically important to account for the variation being observed in real life situations.

The combination of study settings and design enabled us first to determine that the Cap-Score tracks with clinical evidence of pregnancy (*Study 1*), and then to establish that a population of fertile men had Cap-Scores with a normal distribution (*Study 2*). These data herein provide a reference against which the Cap-Score of any individual might be compared. Despite the reasonably consistent Cap-Scores obtained from repeated ejaculates from the same individuals, we expect that changes in the ability to capacitate could be transient in nature, influenced by other components of that man's health. Nevertheless, Cap-Score was somewhat more consistent than the reported variance of other semen analysis measures: An intra-individual variability of 34% in sperm concentration was observed when at least three ejaculates were examined (Carlsen et al., 2004), while a variance of 54% for concentration and 74% for motility index was also reported (Mallidis et al., 1991). Despite the consistency in Cap-Score results within an individual, we suggest that, as with the traditional semen analysis parameters, clinicians utilizing the Cap-Score exercise restraint in conveying to a patient a status of "fertile" or "subfertile/infertile" on the basis of a single Cap-Score measurement.

The Cap-Score can be a powerful tool to evaluate male fertility, when used to complement standard semen analysis parameters. This assay provides unique, quantitative insight into sperm capacitation, the process by which sperm become functionally able to fertilize an egg. The ability to identify defects in sperm function would allow clinicians to personalize reproductive therapies, more quickly directing patients towards an appropriate technology of assisted reproduction. For example, assuming no contra-indicating female factor, men with semen analysis parameters around the World Health Organization thresholds but with high Cap-Scores might be encouraged to try intrauterine insemination. In contrast, men with those same semen analysis results but a low Cap-Score might be encouraged to pursue in vitro fertilization or intracytoplasmic sperm injection, sparing them from cycles of intrauterine insemination that would be unlikely to succeed.

4 | MATERIALS AND METHODS

4.1 | Participant identification and specimen collection

In *Study 1*, consenting subjects were identified from men actively undergoing fertility examinations. Ages for 63 consenting individuals ranged from 27 to 53. All infertile couples included in the current study underwent comprehensive infertility screening. A detailed history of any gynecologic, menstrual, medical, or surgical problems was elicited from all female partners. An in-office sonogram was performed,

including measurement of antral follicle counts. Hysterosalpinography and saline infusion sonography was performed to confirm patency of the Fallopian tubes and to rule out any uterine cavity lesions, respectively. Blood measurements of the following hormones were also performed: cycle Day-2/3 follicle stimulating hormone, cycle Day-2/3 estradiol, anti-Müllerian hormone, and luteal-phase progesterone levels. No known female-factor infertility was present in these couples, other than the risk factor associated with age of some female partners, which ranged from 24 to 46, with 20 being ≥40 years old. All male partners also provided a detailed history and underwent a physical examination. A semen analysis was performed in all men, which was repeated at least once. Semen samples were collected from consenting men after a minimum of 2 days and a maximum of 5 days of sexual abstinence by manual masturbation. Of these 63 initial couples, sufficient reproductive medical history was available for 42. Criteria for sufficient history included knowledge of any natural conception or the results from a total of at least three cycles of intrauterine insemination, combining history from before and after the assay. The assay was performed at the time of semen sample collection, without consideration of history. Outcomes of cycles of classical in vitro fertilization or intra-cytoplasmic sperm injection were not considered.

In *Study 2*, fertile men (Cohort 1) were defined as having a pregnant partner or having fathered a child within the last 3 years without the use of assisted reproduction. This cut-off was used to facilitate sample acquisition from a sufficiently large population. If men in this group developed secondary (acquired) infertility in this time span, their presence would have resulted in a conservative error that lowered the normal mean and reduced the difference from the subfertile/infertile men (Cohort 2). Fertility in Cohort 1 was documented prior to specimen collection either by a birth certificate of their child, a note from an obstetrician or gynecologist, or an annotated ultrasound image. Ages in this group ranged from 24 to 49 years. Fertile donors were recruited through local advertising; men with a history of infertility were not considered. We performed a power analysis (Faul, Erdfelder, Lang, & Buchner, 2007) to ensure that our sample size was adequate to establish a normal reference range; this was done using preliminary data from 34 fertile men (mean Cap-Score ± SD; 40 ± 7.1%). An acceptable range about the mean was set at 3%, and a two-tailed *t*-test at $p < 0.01$, with a probability of detecting a difference this large of 90%, was applied. Results showed that a valid standard range could be established with a minimum of 61 individuals, below our cohort of 76 unique individuals. For Cohort 2, semen samples from 122 consenting men, who had been referred to the Urology Group of New Jersey for fertility evaluation, were assessed over a 5-month period. None of the men in this Cohort were excluded because of confounding infertility in their partner, and it is highly likely that a number of these men were actually fertile. In fact, within an unscreened population of couples questioning their fertility, 30–50% of the men would be expected to be fertile since female-factor infertility is expected in 50–70% of infertile couples (Agarwal et al., 2015). In this Cohort, ages ranged from 22 to 56 years. A single semen sample was obtained from each of the 122 patients. The referring urologist

included a semen evaluation with each sample inclusive of morphology, concentration, and total motility. Samples from Cohort 2 were also used to investigate potential relationships between the Cap-Score and traditional semen analysis measures.

4.2 | Sample processing

Samples having fewer than 10 million sperm cells were not included in any of the *Studies*. Samples were liquefied at 37°C for at least 15 min but for no more than 2 h (Moody et al., 2017). Subsequent to liquefaction, sperm were removed from the seminal plasma by centrifugation through Enhance S-Plus Cell Isolation Media (Vitrolife, Englewood, CO, reference: 15232 ESP-100-90%) at 300g for 10 min. The cells were collected, resuspended with approximately 4 ml of Human Tubal Fluid (HTF) (Irvine Scientific, Santa Ana, CA, reference 90125) (*Study 1*) or modified Human Tubal Fluid medium (mHTF) (Irvine Scientific, reference 90126) (*Study 2*), and centrifuged at 600g for 10 min. The resultant pellet was re-suspended in HTF (*Study 1*) or mHTF (*Study 2*) and divided into two separate aliquots incubated with (Cap) and without (Non-Cap) capacitation stimuli. Sperm concentration was adjusted to 10 million/ml per tube, and then incubated for 3 h at 37°C. For *Study 1*, cells were incubated in an incubator with 5% CO_2, therefore, HTF containing a HCO_3^- buffer was used. For *Study 2*, an air incubator and mHTF, containing a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, was used. Preliminary studies observed no difference in Cap-Score, viability, or sperm recovery whether HCO_3^- or HEPES-buffered medium was used. The capacitation stimuli consisted of 2-hydroxypropyl- β-cyclodextrin (Sigma, St. Louis, MO; reference C0926) (Osheroff et al., 1999; Parinaud, Vieitez, Vieu, Collet, & Perret, 2000). Pilot studies showed that this stimulus was as effective in promoting capacitation in human sperm, as measured with the Cap-Score, at 3 h of incubation as albumin was at 6 h (Vairo et al., 2013). Following incubation, the samples were fixed with paraformaldehyde (Electron Microscopy Services: Hatfield, PA reference 15712) as described (Selvaraj et al., 2006).

4.3 | Sample labeling

Samples were labeled with 2 µg/ml of Cholera Toxin B (Buttke et al., 2006), conjugated with Alexa Fluor 488 (Thermo Fisher, C34775, USA). After 10 min, 5 µl of the labeled sperm were placed on a microscope slide, overlaid with a cover slip, and moved to an imaging station to be scored.

4.4 | Image acquisition

Study 1: a Nikon NIU microscope equipped with CFI60 Plan Apochromat Lambda 10,40, 60, and 100× Objectives, an Andor Clara Digital Camera, and a 64-bit imaging workstation running NIS Elements software (Nikon, U.S.A). *Study 2*: Nikon Eclipse NI-E microscopes equipped with CFI60 Plan Apochromat Lambda 40× Objectives, C-FL AT GFP/FITC Long Pass Filter Sets, Hamamatsu ORCA-Flash 4.0 cameras, and 64-bit imaging workstations running NIS Elements software.

4.5 | Determination of patterns of G_{M1} localization

In all *Studies*, approximately 150–200 sperm with no gross morphological abnormalities were analyzed per sample, and G_{M1} localization patterns were determined. Readers were trained to identify G_{M1} localization patterns as per Moody et al. (2017). Studies demonstrated that the same and independent readers reproducibly replicate Cap-Score values when evaluating distinct subsamples of the same ejaculate (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 capacitation patterns/[# of sperm with capacitation patterns + number of sperm with other patterns]).

4.6 | Statistical analyses

Analysis of variance, coefficient of variation, Mann–Whitney test, Lilliefors test, and Chi-square test were performed using XLSTAT (2015). Linear regression analysis and Student's *t*-test were carried out using Microsoft Excel (2013). Standard error is reported unless otherwise noted.

4.7 | Ethical approval

All research and study protocols were approved by either Weill Cornell's IRB (Protocol# 1210013187; *Study* 1) or WIRB (https://www.wirb.com/Pages/Default.aspx; Protocol# 20152233; *Study* 2).

AUTHORS' CONTRIBUTION

C. Cardona, Q. V. Neri, G. C. Ostermeier, M. A. Moody, A. J. Simpson, E. K. Seaman, and T. Paniza performed experiments. C. Cardona, Q. V. Neri, A. J. Simpson, G. C. Ostermeier, T. Paniza, G. D. Palermo, and A. J. Travis analyzed data. E. K. Seaman, G. D. Palermo, C. Cardona, Q. V. Neri, G. C. Ostermeier, M. A. Moody, and A. J. Simpson identified and consented subjects. C. Cardona, A. J. Simpson, G. C. Ostermeier, Z. Rosenwaks, G. D. Palermo, and A. J. Travis wrote the manuscript.

DISCLOSURES

G. C. Ostermeier, C. Cardona, M. A. Moody, and A. J. Simpson are employees of Androvia LifeSciences, LLC. We know of nothing to disclose for Q. Neri. T. Paniza, and Z. Rosenwaks. After the completion of both data collection and analysis in *Study 1*, G.D. Palermo became a clinical advisor to Androvia LifeSciences and is involved in intellectual property with Androvia that extends beyond the current publication. E. K. Seaman is a urologist specializing in male fertility at the Urology Group of New Jersey and his participation is in compliance with guidelines established by the American Medical Association. A. J. Travis' laboratory at Cornell identified the underlying technology, which was licensed by, and is being developed by, Androvia LifeSciences. He serves as a consultant to Androvia LifeSciences with duties of a Chief Scientific Officer.

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