RBMO

ARTICLE

Defects in sperm capacitation/fertilizing ability are equally prevalent across ages in men seeking fertility assistance

BIOGRAPHY

Fady Sharara, MD, FACOG, is a Board-Certified Reproductive Endocrinologist/Infertility Specialist (since 1996), and a Board-Certified Obstetrician/Gynecologist (since 1994). Dr Sharara has published more than 80 papers and serves on several committees to support the community in updating protocols and procedures toward the latest assisted reproductive technologies.

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KEY MESSAGE

Younger men presenting with fertility problems are more likely to have higher traditional semen analysis metrics than older men, but are equally likely to have defects in capacitation ability, making this assessment more sensitive to male infertility across age groups.

ABSTRACT

Research question: How do capacitation ability, measured by Cap-Score™, and traditional semen analysis measures (volume, concentration, motility) change with age in men questioning their fertility (MQF)?

Design: Cap-Score and semen analysis measures were obtained from MQF (n = 2652; multicentric design: 35 reproductive endocrinologist prescribers, n = 16 clinics). Morphology was not included due to differences among clinics. A Mann–Whitney test was used to compare Cap-Scores between MQF and men with known recent paternity (n = 76). The following age groups were constructed for MQF: 20–24, 25–29, 30–34, 35–39, 40–44, 45–49 and 50+. Associations between semen analysis, Cap-Score and age groups were evaluated using mixed-model analysis of variance to identify possible influence of Cap-Score collection kit type (n = 763 collected at home; n = 1889 collected at clinics).

Results: MQF had reduced capacitation ability (mean ± SE; 29.25 ± 0.15 versus 35.34 ± 0.88; $P < 0.001$). No change in Cap-Score ($P = 0.916$) or concentration ($P = 0.926$) was detected with age group. In contrast, both volume ($P = 0.008$) and % motility ($P < 0.001$) declined with age.

Conclusions: Men presenting because of difficulties in generating pregnancy showed equivalent reductions in capacitation ability regardless of age. In contrast, motility and volume declined with age. These data suggest that capacitation ability is a more sensitive indicator of male fertility across age groups than traditional semen analysis and should not be reserved for older men. Importantly, these data do not address whether sperm fertilizing ability declines in the general population as men age. Instead, they indicate that if men are having difficulty conceiving, no matter what their age, then defects in sperm fertilizing ability are equally likely to be the cause.

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FS provides clinical advice to Androvia LifeSciences. GCO is an employee of Androvia LifeSciences. AJT's 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 and holds equity interest.

KEYWORDS

Andrology
Cap-Score
Diagnostic
Infertility
Paternal age

INTRODUCTION

It is well known that increasing maternal age is negatively associated with fertility (*Schmidt et al., 2012*). This reduction results from several factors, including follicular attrition (*Wallace and Kelsey, 2010*), a reduction in oocyte number and quality (*Faddy and Gosden, 1995*) and a lack of germ cell renewal (*McGee and Hsueh, 2000*). However, the relationship between a man's age and fertility is still being debated, and whether specific causes of reduced male fertility vary with age is unknown.

Auger and his team were some of the first to investigate the impact of paternal age on semen quality (*Auger et al., 1995*). They assessed semen quality in 1351 fertile men and documented that as men aged there were declines in the percentage of spermatozoa with normal morphology, the percentage of motile spermatozoa and sperm concentration. Since then, several studies have looked at the impact of paternal age on various aspects of semen quality, including alterations in DNA fragmentation (*Brahem et al., 2011*; *Kaarouch et al., 2018*; *Sartorius and Nieschlag, 2010*). While these measures might help to assess testicular, epididymal and accessary gland function, they fail to indicate sperm fertilizing ability (*Lamb, 2010*; *Oehninger et al., 2014*) and/or male fertility, both *in vivo* and *in vitro* (*Guzick et al., 2001*; *Liu and Baker, 2002*).

Spermatozoa must go through a series of maturational events, known as capacitation, prior to gaining fertilization competency (*Austin, 1951*, *1952*; *Chang, 1951*). Capacitation involves multiple factors, including changes in the lipids that make up the sperm's plasma membrane. It has been observed, first in the mouse and bull (*Selvaraj et al., 2007*), and then in humans (*Cardona et al., 2017*), that monosialotetrahexosylganglioside (G_{M1}) localized to precise and reproducible regions of the plasma membrane in spermatozoa that responded to capacitation stimuli versus those that did not respond. Use of G_{M1} localization as a diagnostic assay was validated at the population and single cell levels (*Moody et al., 2017*), with the Cap-Score™ being defined as the percentage of spermatozoa having G_{M1} localization patterns consistent with capacitation, in relation to the total number of spermatozoa having G_{M1} localization patterns.

The Cap-Score has been shown to predict male fertility in two prospective studies. In the first, a threshold value was tested, with men having normal Cap-Scores showing 423% higher success in the first round of intrauterine insemination (IUI) than men with low Cap-Scores, and 278% higher over three cycles (*Schinfeld et al., 2018*). Data from five clinics (n = 124 pregnancy outcomes) were used to create a model showing the relationship between a man's Cap-Score and his fertility, assessed as the 'probability of generating pregnancy' within three cycles (*Schinfeld et al., 2018*). This relationship was then itself tested prospectively, with data from six clinics (n = 128 new pregnancy outcomes) showing that the number of pregnancies predicted by Cap-Score matched those observed (*Sharara et al., 2020*). In a cohort comparison, almost half of men questioning their fertility (MQF; n = 2155) had Cap-Scores considered to be low, indicative of subfertility with poor probability of conception within three cycles (*Sharara et al., 2020*). Although these data match clinical knowledge that semen analysis fails to identify half the cases of male infertility, in which men have defects in fertilizing ability (*Guzick et al., 2001*; *Oehninger et al., 2014*), no one has previously examined whether Cap-Score varies with age in MQF. This study builds on previous work (*Sharara and Ostermeier, 2021*), investigating the relationship between capacitation ability, as measured by Cap-Score, and age in MQF. For comparison, associations between age and traditional semen analysis measures (volume, concentration, motility) were also investigated.

MATERIALS AND METHODS

Study design

Methods and analyses are reported in accordance with the STROBE checklist for observational studies (*Adams et al., 2018*). The current analyses were assessed and approved by the Western Institutional Review Board (20152233; 11 January 2021). The ability of spermatozoa to capacitate was determined by means of the Cap-Score test, a laboratory-developed test that is CLIA compliant (Clinical Laboratory Improvement Amendments, 31D2115698), accredited by CAP (College of American Pathologists, 9443749), licensed by New Jersey CLIS (Clinical Laboratory Improvement Services, 0010296) and New York CLEP (Clinical Laboratory Evaluation Program, 9256), and has received a PLA code (Proprietary Laboratory Analyses, 0255U) from the American Medical Association.

A retrospective cohort study design was used to investigate the association of capacitation and traditional semen analysis measures (volume, concentration, motility) among age groups in MQF. The age groups were selected both to distribute the number of observations among the bins, while having enough bins to provide detailed understanding of any age-related changes.

Settings

Semen samples from men seeking fertility assessment at reproductive endocrinology/fertility clinics across the USA were sent to Androvia, where Cap-Score was determined (n = 2652; multicentric design, 35 reproductive endocrinologist prescribers, n = 16 clinics). Traditional semen analysis values were either reported to Androvia by clinics performing their own semen analysis or measured in Androvia's laboratory for samples obtained using home collection. Following routine procedures, test results were provided to physicians, who then used the information to help in decision-making, patient counselling, and design and implementation of treatment.

Participants

De-identified data were obtained from Androvia's database and represented all clinical samples obtained from reproductive endocrinology clinics between December 2016 and August 2021. Male age ranged from 22 to 69 years. Men with moderate to severe oligozoospermia or azoospermia were excluded if they had insufficient numbers of spermatozoa post-washing to perform the Cap-Score.

To ensure that men in the youngest age group (20–24 years of age) were presenting for difficulty conceiving, as opposed to efforts to preserve fertility prior to some sort of procedure or treatment, an examination of medical records was performed by the submitting clinic. This examination by the submitting physicians confirmed that at least 20/21

of MQF in this age group presented because of struggles conceiving, with one being non-responsive.

Variables and outcomes

Semen analysis was performed according to WHO guidelines (*World Health Organization, 2010*). Because morphology assessment varied among clinics, it was not included in this analysis. In a previous study, however, no relationship was observed between Cap-Score and morphology (*Cardona et al., 2017*).

Cap-Scores were determined by trained personnel at Androvia's laboratory. Sample processing and scoring were done as previously described, with slight modifications (*Moody et al., 2017*). Briefly, semen samples were collected by masturbation and then processed for delivery using two different kit types that were provided by Androvia. For 763 samples, the semen was diluted in TEST-Yolk Buffer (FUJIFILM; catalogue# 90129), packaged to ensure transit at 4°C, and shipped overnight to Androvia, where staff performed washing and incubation prior to Cap-Score determination (home collection kits). For 1889 samples, the ejaculates were collected, washed and incubated at clinics before being fixed and sent to Androvia for Cap-Score determination (clinical pack kits). All samples were processed after liquefaction by washing through a density gradient (S Plus 90%; VitroLife; ref# 1523). With both kit types, spermatozoa were then incubated under capacitating conditions, fixed and imaged as described previously (*Cardona et al., 2017*; *Moody et al., 2017*). Trained individuals, passing proficiency and daily quality assurance testing, identified G_{M1} localization patterns of both non-capacitated and capacitated spermatozoa (*Moody et al., 2017*). Previous studies have shown no difference in Cap-Score when using these two processing kits, although as expected, reductions in motility were observed with home collection and transport (*Ostermeier et al., 2019*, *2022*).

Bias

Participants were men seeking fertility assistance. It is anticipated that in approximately 50% of cases, the couple's infertility would have originated from a female factor (*Agarwal et al., 2015*) and so not all men were of equal fertility status. This heterogeneity could obscure differences across the age groups, if there were not equal distribution across those groups.

Cap-Scores and semen analysis measures were obtained from samples that were processed using two different kit types. As described above, almost three-quarters of the samples had semen analysis data obtained at the clinic. The remaining samples were obtained at home and shipped directly to the Androvia laboratory. Delays in assessment of motility, even in the presence of refrigeration medium and under controlled temperature conditions, are well known to have negative effects on sperm motility (*Baek et al., 2006*; *Jaskey and Cohen, 1981*; *Johnson et al., 1984*; *Morris et al., 2021*).

For those kits processed and prepared at clinics, the instructions required 10 million or more total cells, and 3 million spermatozoa post-wash. Because of clinical interest, 8 samples falling below these cut-offs were scored out of 1889 (0.4%). For those samples being diluted in TEST-Yolk buffer, there was no pre-set limit on required cell number for submission, as many of these samples were collected by patients at home. Because these samples were processed at Androvia, sample volumes could be modified to accommodate as few as 1.2×10^6 initial cells in the assay. Using modified approaches, Cap-Scores were determined for 16 samples with fewer than 10 million total spermatozoa, out of 763 (2%). If men with moderate to severe oligozoospermia were not evenly distributed across age groups, this could confound interpretation of data. As presented below, this distribution and its potential impact was examined.

To account for the possibility of kit type impacting the investigation, mixed-model analyses of variance (ANOVA) were used where kit type was crossed with the age groups. This is an extension of ANOVA, where groups are divided into random subgroups. With this approach it was possible to simultaneously account for variation between kit type and age group, as well as the interaction of kit type by age group.

Study size

Two populations were used, the first of which was a group of men with proven paternity (n = 76). This population was collected previously, and the sample size was based on a power analysis done to determine a robust Cap-Score reference range (*Cardona et al., 2017*). The second sample was a population of men questioning their fertility and visiting a reproductive endocrinology clinic (MQF; n = 2652; multicentric design, 35 reproductive endocrinologist prescribers, n = 16 clinics). The analysis was restricted to those men visiting a reproductive endocrinologist, as these men were less likely to have a previously identified male factor than men referred to a reproductive urologist, for example (*Najari, 2019*).

Quantitative variables

Cap-Score reports the proportion of spermatozoa having G_{M1} localization patterns that are consistent with capacitation, out of all spermatozoa having G_{M1} localization patterns (*Moody et al., 2017*). Methodologies for traditional semen analysis have been established by the WHO (*World Health Organization, 2010*).

Statistical methods

Statistical analyses (Mann–Whitney, ANOVA) were carried out in XLSTAT Version 2021.2.2 (Addinsoft, New York, USA, https://www.xlstat.com).

Data were assessed for normality using the formal Jarque–Bera test, P–P plots, Q–Q plots and by comparing the data histogram to a normal distribution (Supplementary Figures 1–4) (*Ghasemi and Zahediasl, 2012*). Data are represented by means ± SE for the Mann–Whitney result and as least square means (LSM) ± SE for the ANOVA results. LSM are most appropriate in multi-way designs and are probably closer to reality than arithmetic means, as they control for potential differences in the covariates. Multiple comparisons were done using both Fisher's least significant difference (LSD) and Tukey's honest significant difference (HSD). Looking at both helps to balance type II (false-negative) and I (false-positive) errors.

Following best practices, Fios Genomics (Edinburgh, Scotland) was contracted and given all data related to this study. They assessed both the appropriateness of the analyses and determined their accuracy.

RESULTS

The objective of the current study was to evaluate the association of age with the percentage of spermatozoa deemed fertilization competent (Cap-Score), and

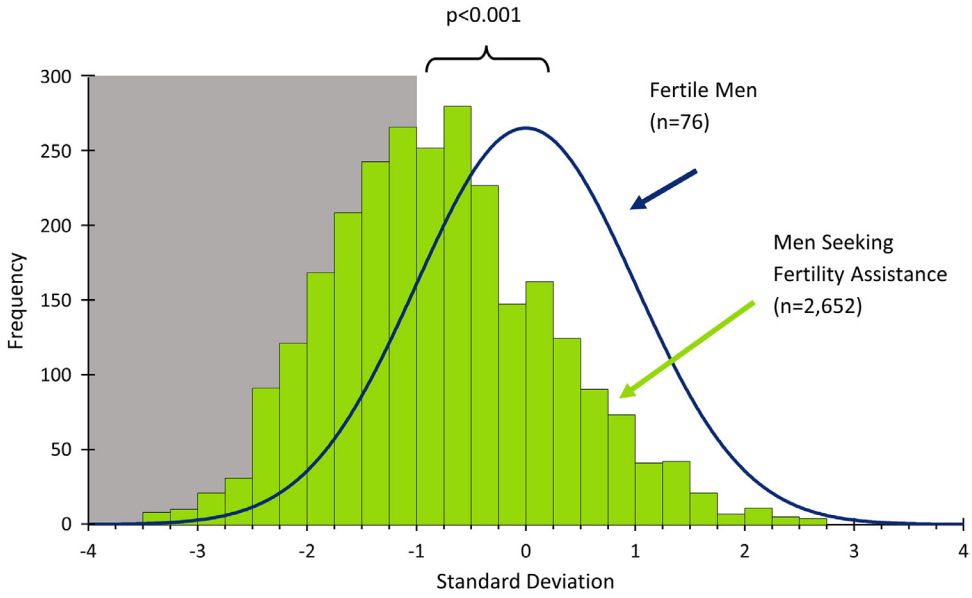

FIGURE 1 Comparison of Cap-Scores between men of known fertility and men questioning their fertility (MQF) and seeking assistance from reproductive endocrinologists. Cap-Score results were collected from MQF (n = 2652; green histogram) and a previously acquired population of known fertile men (n = 76; blue bell curve approximates distribution; *Cardona et al., 2017*). Both distributions were standardized so that the Cap-Score mean (35.3) and SD (7.7) of the fertile population was set to 0 and 1 unit, respectively. There was a significant reduction in Cap-Scores in MQF (0.01 ± 0.11 versus −0.79 ± 0.02; P < 0.001). The grey box illustrates a previously determined reference range (*Cardona et al., 2017*), with a threshold at 1 SD below the mean of the fertile population. Multiple prospective tests have shown that men falling below this cut-off are significantly less likely to generate a pregnancy (*Schinfeld et al., 2018*; *Sharara et al., 2020*; *Kloos et al., 2021*).

with traditional semen analysis measures, in MQF and visiting reproductive endocrinologists. To determine whether this population had reduced capacitation ability, the MQF were compared with a previously measured cohort of men with known fertility (n = 76). Both distributions were standardized so that the mean (μ = 35.3) and SD (σ = 7.7) of the fertile population was set to 0 and 1 unit, respectively ($z_i = (x_i - \mu)/\sigma$). A significant reduction in Cap-Score was detected in those men who were seeking fertility assistance (FIGURE 1; 0.01 ± 0.11 versus −0.79 ± 0.02, P < 0.001, Mann–Whitney).

To assess the relationship between age, Cap-Score and traditional semen analysis measures, the data were binned into the following age groups: 20–24, 25–29, 30–34, 35–39, 40–44, 45–49 and 50+ years of age (n = 21, 280, 926, 844, 374, 143 and 64, respectively). The data were then assessed using mixed-model ANOVA, and multiple comparisons done using Fisher's LSD and Tukey's HSD. No association was detected between Cap-Score and the age groups (P = 0.916; FIGURE 2). Further, kit type (P = 0.481) and kit type by age group (P = 0.386) had no impact on Cap-Score.

The distribution of sperm concentration was skewed (Supplementary Figure 2) and was normalized using a log transformation. No associations were detected between the log of sperm concentration and the age groups (P = 0.926; FIGURE 3) or the kit type by age group term (P = 0.939). However, a reduction in log concentration was detected in association with the home collection kit (1.72 ± 0.02 versus 1.65 ± 0.03, P = 0.049). This result was predictable, as noted above, because home collection enables the assay to be performed on samples having lower concentrations and absolute numbers of spermatozoa.

Significant differences in the % motile spermatozoa were detected among the age groups (P < 0.001; FIGURE 4). Multiple comparisons using Fisher's LSD and Tukey's HSD indicated that motility declined with age, with reductions first detectable in the 40–44 years age group, as they had fewer motile spermatozoa than the 20–24 years age group (55.31 ± 4.89 versus 43.38 ± 1.21%, P = 0.018, using Fisher's LSD). If one applies a correction for multiple tests, then the 50+ age group has significantly lower % motility (36.54 ± 3.25) than all age groups below 40: 20–24 (55.31 ± 4.89, P < 0.001), 25–29 (48.91 ± 1.31, P = 0.001), 30–34 (46.60 ± 0.72, P = 0.017) and 35–39 (46.75 ± 0.77, P = 0.014). Also, as predicted, there was a reduction in the % motile spermatozoa detected in association with the home collection kit (55.9 ± 0.95 versus 35.5 ± 1.61%, P < 0.001). There was no association between the % motile spermatozoa and the term kit type by age group (P = 0.691).

The ejaculate volume distribution was skewed (Supplementary Figure 4) and was normalized using a square root (SQRT) transformation. Significant differences in SQRT (ejaculate volume) were detected among the age groups (P = 0.008; FIGURE 5). Multiple comparisons, using Fisher's LSD, showed that reductions were first apparent in the 40–44 years age group, as they had smaller SQRT (ejaculate volumes) than the 20–24-year-olds (1.73 ± 0.10 versus 1.58 ± 0.03 ml, P = 0.015). Further, there was a gradual reduction in SQRT (ejaculate volume) that was associated with increasing age, which started with the 25–29 years age group and that reached significance by the 45–49 years age group (P = 0.028). Although men in the 50+ age group had reduced SQRT (ejaculate volume) when compared with those men in the 20–24 (P < 0.001) and the 25–29 (P = 0.010) years age groups, their mean ejaculate volume

Type III Sum of Squares analysis (Cap-Score):					
Source	DF	Sum of squares	Mean squares	F	Pr > F
Kit type	1.0	30.699	30.699	0.496	0.481
Age group	6.0	126.435	21.073	0.341	0.916
Kit type*Age group	6.0	392.854	65.476	1.058	0.386

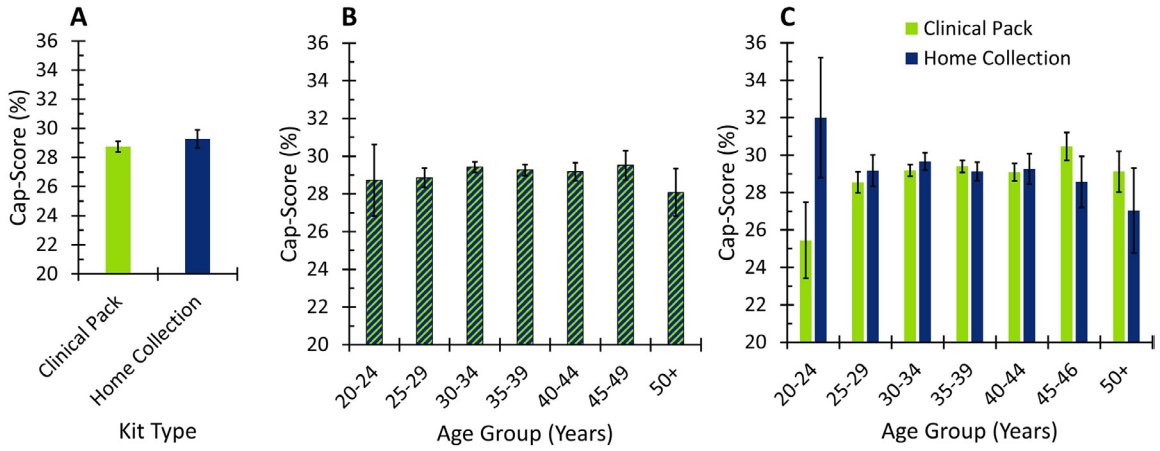

FIGURE 2 The relationship between age and Cap-Score in men questioning their fertility (MQF). The table shows the type III sum of squares analysis for the analysis of variance (ANOVA). The bar charts show the Cap-Score least square means (LSM) and their SE. Panel A illustrates that kit type (P = 0.481) did not have a significant impact on Cap-Score. No association was detected between the age groups and Cap-Score (P = 0.916; Panel B). The data shown in Panel C demonstrate that there was no significant effect of kit type by age group (P = 0.386). Although it appears that there is a difference in Cap-Score between kit type in the 20–24 years age group, this group had the smallest sample size (n = 21 total; 6 from home collection). This apparent difference was entirely due to a single outlier in the home collection group that was left in the analysis, as there was no justification for removal (i.e. the value was within normal limits and may have been from a fertile individual). Source = independent variable; DF = degrees of freedom; F = the value obtained when running an ANOVA and informs if a group of variables are jointly significant; Pr>F = the probability of getting the calculated F statistic and is obtained from an F distribution with DF calculated from the number of groups and the total number of subjects in the experiment.

was still within the normal WHO range (2.46 ± 0.23 ml). In contrast, Tukey's HSD was unable to discern differences in SQRT (ejaculate volumes) among the groups. No significant differences were detected between kit type (P = 0.664) or among the kit type by age groups (P = 0.274).

DISCUSSION

Studies have repeatedly shown that Cap-Score is reduced in MQF (*Cardona et al., 2017*; *Schinfeld et al., 2018*; *Sharara et al., 2020*). This finding was substantiated in the current study (FIGURE 1), which further demonstrated that reductions in Cap-Score were equally common across the age groups in MQF (FIGURE 2). In contrast, reductions in % motility (FIGURE 4) and SQRT (ejaculate volume) (FIGURE 5) were observed across the age groups. Because even the youngest men (20–24 years age group) were experiencing similar difficulties in generating a pregnancy as were men in the older groups, these data show that defects in sperm fertilizing ability are equally prevalent across ages in MQF. Because half of all men with fertility problems are not diagnosed with traditional semen analysis (*Guzick et al., 2001*), and because semen analysis parameters are more subject to the influence of age, these data suggest that Cap-Score is a more sensitive indicator of male fertility regardless of age, and should not be reserved for older men.

It should be reiterated that the data presented do not suggest a steady state of male fertility as men age. Instead, it is proposed that if a man is having difficulty in generating a pregnancy, no matter his age, then defects in sperm capacitation are equally likely to be the cause. Additional studies, focusing on men representing the general population, rather than men seeking fertility assistance, could show a decline in Cap-Score with age, as it is known that advancing paternal age can influence several reproductive functions that could impact sperm capacitation. For example, it is known that regulation of reproductive hormones is altered as men age (*Juul and Skakkebæk, 2002*; *Kaufman and Vermeulen, 2005*). Specifically, it has been documented that in healthy men, testosterone concentrations decline with age (*Snyder, 2001*), while little to no decline is observed in patients (*Gray et al., 1991*). This would suggest that as healthy men age, there is a disruption in the hypothalamic–pituitary–gonadal axis. Interestingly, the ability of spermatozoa to capacitate is first established in the epididymis (*Orgebin-Crist, 1967*), whose function is largely dependent on 5α-reductase activity and the presence of high concentrations of androgens from the testes (*Robaire and Viger, 1995*; *Sullivan and Saez, 2013*). Thus, one could envisage that as healthy men age, there could be a disruption in sperm capacitation ability and/or male fertility due to alterations in epididymal function, caused by reduced testosterone.

Similarly, changes in a man's lifestyle as he ages might also impact his ability to produce capacitation-competent spermatozoa. For example, as men age there tends to be an increase in body mass index (*Cohen, 2008*; *Vermeulen et al., 1999*). Interestingly, paternal obesity has been linked to decreased

Type III Sum of Squares analysis (LOG (Concentration)):					
Source	DF	Sum of squares	Mean squares	F	Pr > F
Kit type	1.0	0.474	0.474	3.867	0.049
Age group	6.0	0.237	0.039	0.322	0.926
Kit type*Age group	6.0	0.218	0.036	0.296	0.939

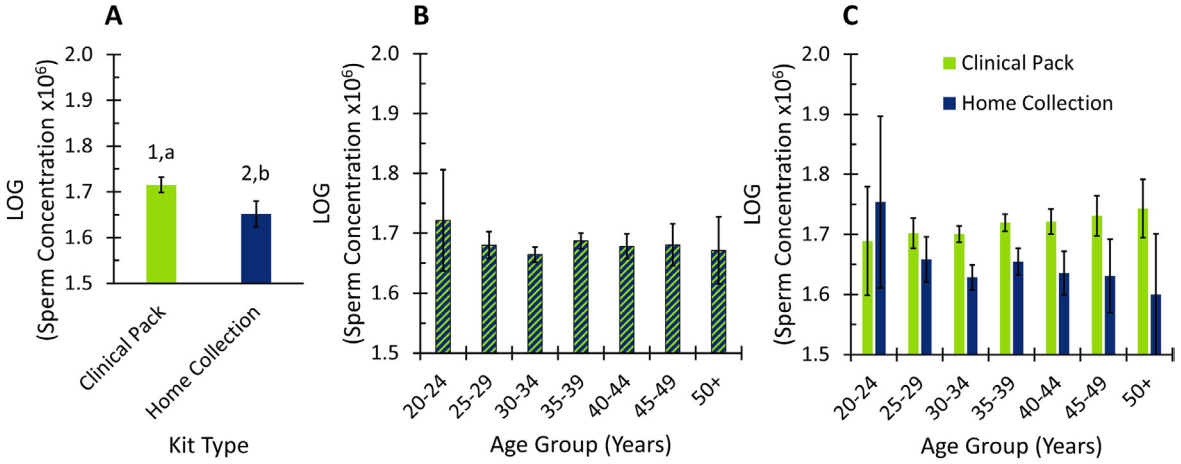

FIGURE 3 The relationship between age and concentration in men questioning their fertility (MQF). To better normalize the measures of sperm concentration, they were log transformed prior to analysis. The table shows the type III sum of squares analysis for the analysis of variance (ANOVA). The bar charts show the Cap-Score least square means (LSM) and their SE. A significant relationship was found between log concentration and kit type ($P = 0.049$; Panel A). No significant differences were detected in log(concentration) among the age groups ($P = 0.926$; Panel B) or kit type by age groups ($P = 0.939$; Panel C). Those groups with different letter superscripts were deemed unique by Fisher's LSD. Those with different number superscripts were different, as assessed by Tukey's HSD. Source = independent variable; DF = degrees of freedom; F = the value obtained when running an ANOVA and informs if a group of variables are jointly significant; Pr>F = the probability of getting the calculated F statistic and is obtained from an F distribution with DF calculated from the number of groups and the total number of subjects in the experiment.

Type III Sum of Squares analysis (Percent Motile Sperm):					
Source	DF	Sum of squares	Mean squares	F	Pr > F
Kit type	1.0	49021.407	49021.407	119.372	<0.001
Age group	6.0	10810.067	1801.678	4.387	<0.001
Kit type*Age group	6.0	1600.095	266.682	0.649	0.691

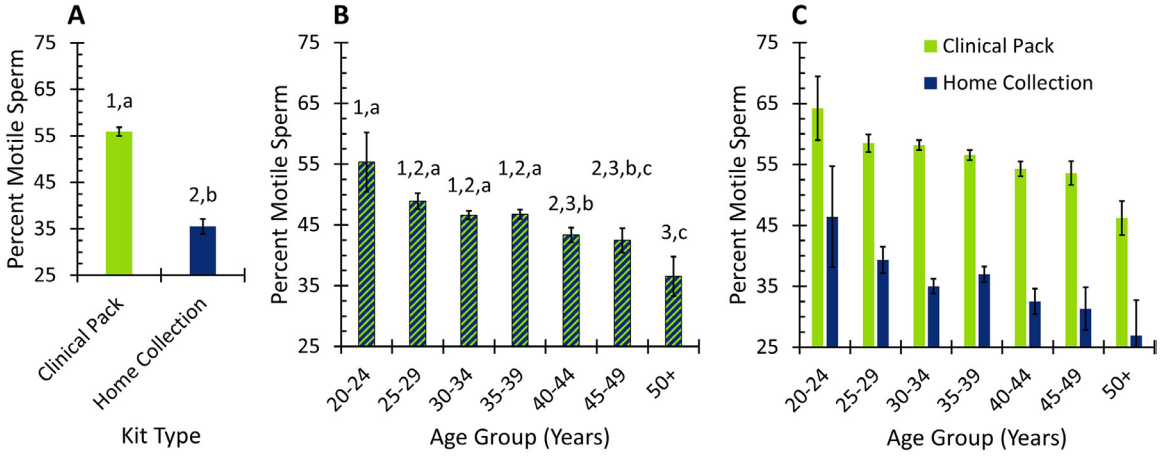

FIGURE 4 The relationship between age and motility in men questioning their fertility (MQF). The table shows the type III sum of squares analysis for the analysis of variance (ANOVA). The bar charts show the Cap-Score least square means (LSM) and their SE. Significant relationships were detected between % motile spermatozoa and kit type ($P < 0.001$; Panel A) and among the age groups ($P < 0.001$; Panel B). In contrast, no relationship was found between % sperm motility and kit type by age group ($P = 0.691$; Panel C). Those groups with different letter superscripts were deemed unique by Fisher's LSD. Those with different number superscripts were different, as assessed by Tukey's HSD. Source = independent variable; DF = degrees of freedom; F = the value obtained when running an ANOVA and informs if a group of variables are jointly significant; Pr>F = the probability of getting the calculated F statistic and is obtained from an F distribution with DF calculated from the number of groups and the total number of subjects in the experiment.

Type III Sum of Squares analysis (SQRT (Volume)):					
Source	DF	Sum of squares	Mean squares	F	Pr > F
Kit type	1.0	0.035	0.035	0.189	0.664
Age group	6.0	3.200	0.533	2.904	0.008
Kit type*Age group	6.0	1.386	0.231	1.258	0.274

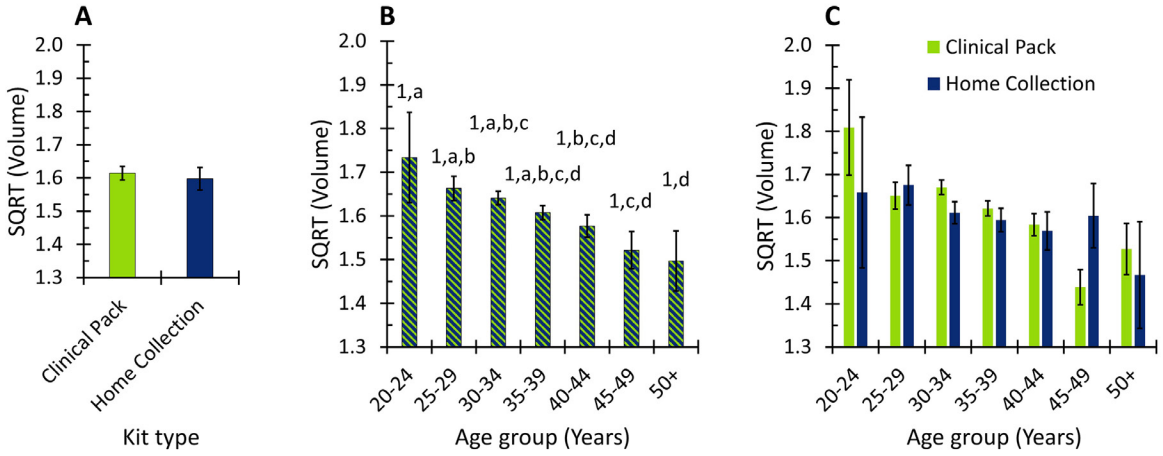

FIGURE 5 The relationship between age and ejaculate volume in men questioning their fertility (MQF). The table shows the type III sum of squares analysis for the analysis of variance (ANOVA). The bar charts show the Cap-Score least square means (LSM) and their SE. There was no significant difference in SQRT (ejaculate volume) between kit type (P = 0.664; Panel A) and among the kit type by age group (P = 0.274; Panel C). In contrast, SQRT (ejaculate volume) declined with age (P = 0.008; Panel B). Those groups with different letter superscripts were deemed unique by Fisher's LSD. Those with different number superscripts were different, as assessed by Tukey's HSD. Source = independent variable; DF = degrees of freedom; F = the value obtained when running an ANOVA and informs if a group of variables are jointly significant; Pr>F = the probability of getting the calculated F statistic and is obtained from an F distribution with DF calculated from the number of groups and the total number of subjects in the experiment.

pregnancy rates and increased pregnancy losses in couples undergoing fertility treatments (*Bakos et al., 2011*; *Hinz et al., 2010*; *Keltz et al., 2010*). In part, these characteristics are a result of reduced blastocyst formation, as well as limited sperm binding and fertilization during IVF (*Bakos et al., 2011*; *Hwang et al., 2011*). With capacitation being necessary for spermatozoa to bind and fertilize (*Bailey, 2010*), it is tempting to postulate that age-associated male obesity may have a negative impact on sperm capacitation. However, there was no reduction in capacitation ability observed in the current population (FIGURE 2), suggesting that capacitation ability was equally reduced across the ages in men actively seeking fertility assistance. Regardless of potential cause, a finding of reduced male fertility with age would be consistent with more men entering the pool of MQF with age. Current results suggest that the proportion of men with defects in capacitation ability would remain relatively unchanged across the groups.

Home collection systems for semen evaluation and cryopreservation are becoming more popular (*Morris et al., 2021*; *Samplaski et al., 2021*; *Yu et al., 2018*). With these systems, semen is collected at home and then either evaluated by the patient using an instrument that is provided or they are extended with a refrigeration medium and sent to a clinic for processing and/or evaluation. This study used data obtained using two different approaches. In the first approach samples were collected at a clinic, where they were processed, fixed and then sent to Androvia for evaluation. In the second approach, samples were collected at home by the patient, extended, cooled and then sent to Androvia for processing and evaluation. No differences in Cap-Score (FIGURE 2) or SQRT (ejaculate volume) (FIGURE 5) were detected between the two collection types. This is consistent with observations that fertility can be maintained in samples that have been extended and cooled (*Zavos et al., 2006*). In contrast, it has been well documented in many species that sperm motility declines with cooling and maintenance (*Ahmad et al., 2021*; *Baek et al., 2006*; *Fernandez-Novo et al., 2021*; *Jaskey and Cohen, 1981*; *Johnson et al., 1984*; *Morris et al., 2021*; *Samplaski et al., 2021*). The current data support these observations (FIGURE 4).

It may seem counterintuitive that a reduction in sperm concentration was observed with home collection (FIGURE 3). However, this was anticipated, as a minimum of 10×10^6 cells was required when samples were processed at clinics, whereas no minimum was set for home collection. When samples were processed at clinics, 3×10^6 post-wash spermatozoa were placed into a fixed sample volume of 300 µl. This resulted in a concentration of 10^6 spermatozoa/ml (*Cardona et al., 2017*). With home collection, trained individuals prepared the samples and could modify the sample volume for ejaculates with reduced sperm numbers. The main requirement was to keep the sperm concentration consistent. Using this approach, it was possible to assess semen samples with as few as 1.2×10^6 total spermatozoa. While this flexibility made the assay available to more individuals, caution must be used when interpreting the results in two ways. First, when samples were split and processed fresh versus being extended and shipped, no differences in concentration were observed (data not shown). Thus, the difference in concentration in the current study arose from expansion of the patient base, as

opposed to an inherent difference in the clinical pack versus home collection. Second, when interpreting the Cap-Scores of men with moderate to severe oligozoospermia for clinical purposes (e.g. trying to decide between IUI versus intracytoplasmic sperm injection), the modelled and validated associations of Cap-Score with male fertility might not hold, considering that they were established using samples with at least 10×10^6 spermatozoa (*Cardona et al., 2017*; *Schinfeld et al., 2018*; *Sharara et al., 2020*). Significant numbers of additional patient samples should be tested, and pregnancy outcomes obtained, before sufficient sample size is obtained that would allow one to model the relationships between Cap-Score, sperm concentration and absolute number, and male fertility in men with severe oligozoospermia.

In this study, 'real world' observations were obtained from multiple clinics. This approach has the benefit of constructing varied patient and clinical profiles and therefore more robust data sets. For example, in this study data were obtained from 2652 men seeking fertility treatment with 35 reproductive endocrinologists at 16 clinics. That said, one limitation of the current data set was the limited number of observations on either end of the age distribution when compared with the central part of the distribution. In addition, the current study does not indicate whether Cap-Score values decline with age in the general population. To address that, one would need to study large numbers of men in each age group, with diversity representative of the population as a whole.

In men seeking fertility assistance, motility and volume decreased with age, while Cap-Score was equally reduced across the age groups. These data show that defects in the sperm's ability to fertilize, which would have previously been diagnosed as idiopathic infertility, are equally prevalent across age groups in MQF. These observations indicate that Cap-Score was a more sensitive indicator of male fertility than traditional semen analysis. Further, they support the use of Cap-Score as an initial screen when evaluating male fertility, no matter the patient's age. Together, Cap-Score and traditional semen analysis can provide clinicians with information needed to make decisions regarding a couple's fertility treatment, as well as the impact of protocols designed to enhance male fertility.

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SUPPLEMENTARY MATERIALS

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