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Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples

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BACKGROUND: Standard semen analysis has low objectivity and reproducibility and is not closely related to fertility. We assess the prognostic value of automated measurements of sperm motility and morphology. METHODS: During 1997–1999, 1191 infertile couples with no known absolute barrier to conception were assessed by conventional semen analysis, and automated measurements of average straight-line velocity (VSL) and the percentage of sperm with characteristics that conform to those of sperm which bind to the zona pellucida of the human oocyte (%Z). During follow-up to 2001, there were 336 natural pregnancies. RESULTS: Only %Z, VSL and female age were independently significantly related to pregnancy rate by Cox regression analysis. Pregnancy rate was higher with above average %Z and VSL, indicating a continuous rather than a threshold relationship. The likelihood of pregnancy within 12 cycles can be evaluated for specific values of %Z, VSL and female age using the Cox regression model. CONCLUSIONS: The automated semen measures of sperm morphometry (%Z) and velocity (VSL) are related to pregnancy rates in subfertile couples and should assist clinicians in counselling subfertile patients about their prognosis for a natural pregnancy. Objective automated methods should replace the traditional manual assessments of semen quality.

Key words: automated semen analysis/male infertility/pregnancy rate prediction/sperm morphometry/sperm velocity

Introduction

The critical issue in the management of infertility after the detection and treatment of remediable conditions is accurate advice about the couple's prognosis for natural conception within a reasonable amount of time. If the probability is low (e.g. <20–40% in 1 year), assisted reproductive technology may be appropriate. However, prognostic interpretation of semen analysis is not straightforward, except where there is a total abnormality such as persistent azoospermia or zero sperm motility. Reference ranges derived from fertile and infertile populations are of limited clinical use because they do not separate groups of men with normal and zero fertility (World Health Organization, 1999; Guzick *et al*., 2001). Survival analysis methods can be applied to pregnancy rates. Multivariate Cox regression analysis enables identification of groups of factors that independently affect the chances of pregnancy (Baker, 2001). While studies of IVF results have indicated the importance of both sperm motility and morphology for fertilization rates *in vitro* (Liu and Baker, 1992a; Coetzee *et al*., 1998), the situation *in vivo* is more difficult to assess and the results are less clear. Prospective studies of the prognostic factors for natural pregnancy rates require extensive follow-up of large numbers (>1000) of subjects to achieve sufficient statistical power. The few such studies performed to date have not produced consistent relationships between semen analysis results and pregnancy rates, in either subfertile or general populations. For example, in a study of 1367 subfertile couples referred for male infertility, sperm concentration was a significant predictor of pregnancy rate, together with duration of infertility, previous pregnancy in the union and female age (Baker *et al*., 1985; Baker, 2001). Although sperm motility and morphology were not significant in the whole study population, they were related to pregnancy rate in the subgroup with oligozoospermia (Zaini *et al*., 1985). Other studies of large subfertile populations (>700 subjects) have found either no semen characteristic significantly related to pregnancy rate, or different combinations of female age, sperm morphology, motility and concentration, or a semen score were significant (Hargreave and Elton, 1983; Comhaire, 1987; Polansky and Lamb, 1988; Bostofte *et al*., 1990; Eimers *et al*., 1994; Wichmann *et al*., 1994; Collins *et al*., 1995; Snick *et al*., 1997; Hunault *et al*., 2002). Smaller studies of general populations (>200 subjects) are similarly inconsistent, with sperm concentration and morphology or motile sperm concentration most strongly related to pregnancy rate (Bonde *et al*., 1998; Larsen *et al*., 2000; Zinaman *et al*., 2000; Slama *et al*., 2002).

Conventional semen analysis is subject to large methodological errors associated with counting and subjective interpretation (World Health Organization, 1999). Computer-aided semen analysis (CASA) should increase objectivity and reproducibility and allow other variables to be measured, including sperm motion parameters such as straight-line velocity (VSL). ESHRE guidelines indicate that the selection of CASA variables should be based on a relevant functional end-point (ESHRE, 1998). In a previous study of the relationship between CASA sperm motility parameters and fertilization rate *in vitro*, we found the proportion of sperm with progressive motility in the insemination medium and linearity of sperm in semen were the two sperm variables most significant in the logistic regression model (Liu *et al*., 1991). For semen analysis variables, VSL was approximately of equal significance to linearity, and as it is a machine-independent measure we chose it as the sperm kinematic parameter for this study. VSL is defined as the time-average velocity of a sperm head along the straight line between its first detected position and its last (World Health Organization, 1999).

We developed a fully automated computer image analysis method for morphology: the sperm head automated morphometric analysis system (SHAMAS), which measures 32 parameters describing dimensions, symmetry and distribution of stain density of sperm heads (Garrett and Baker, 1995). SHAMAS summary measures include %C, which is similar to conventional manual %normal morphology, and %Z, the percentage of sperm with characteristics which conform to those of sperm that bind to the zona pellucida of the human oocyte (Garrett *et al*., 1997). Binding of sperm to the zona pellucida is essential for fertilization, and the process is highly selective against sperm with abnormal morphology and for sperm with specific characteristics related to the acrosomal area (Menkveld *et al*., 1991; Liu and Baker, 1992b; Garrett *et al*., 1997). We compared morphometry measurements of sperm in insemination medium with those of sperm removed from the surface of zona after tight binding and found 12 SHAMAS parameters have 'zona pellucida preferred' values (Garrett *et al*., 1997). These are used to calculate %Z. They indicate axial symmetry, narrow neck and a large acrosomal area are important for sperm–zona binding. We hypothesized that objective assessment of the proportion of sperm with these characteristics in semen will predict pregnancy rates.

In this study, conventional semen analysis and CASA results are related to natural pregnancy rates in a sample of 1191 couples presenting with infertility, but with neither partner having a known absolute barrier to conception. This is an appropriate study group since it represents a large proportion of the patients seen for infertility in first world countries who need to decide whether to continue trying longer for a natural pregnancy or commence treatment with assisted reproductive technology.

Materials and methods

Subjects

During 1997–1999, 4582 patients had semen analysis performed in the Department of Andrology, Royal Women's Hospital (RWH). For this study, semen samples from 1780 men were selected at random for SHAMAS morphometry analysis, provided that the sperm concentration was $>2\times10^6$/ml and that the patient consented to use the semen for research. Automated morphometry and motility assessments cannot be performed with low sperm concentrations ($<2\times10^6$/ml). Some samples with sperm concentrations of $2–5\times10^6$ sperm/ml and abundant seminal debris were also excluded by the automation limitations of SHAMAS. A total of 1191 couples met the inclusion criteria of the study. Couples were excluded if there was a known complete barrier to natural pregnancy such as azoospermia, zero sperm motility, coital disorder, bilateral Fallopian tube obstruction or persistent anovulation, or if they had specific conditions such as sperm autoimmunity or necrospermia, or had received treatment involving donor gametes (Baker, 2001). Patients having treatment with clomiphene or artificial insemination for idiopathic or mild male infertility were included, since pregnancies in such couples depend upon semen quality. The means and SDs of the semen results for the 1191 patients were not significantly different from those of the total population of 4582 patients tested over the same period, indicating no major volunteer bias.

The RWH Research and Ethics Committee approved the project.

Follow-up

Follow-up of couples ceased in 2001. The duration of follow-up was from the date of the first semen analysis to the date of natural conception, the date the couple was last known to be not pregnant or when conception occurred by IVF. Couples pregnant at the time of semen analysis were excluded. Pregnancies were confirmed either by ultrasound evidence of fetal sacs or hearts (at 6–19 weeks gestation), or histological confirmation of products of conception after miscarriage or birth. Follow-up times were converted to 28-day cycles. For the 638 couples who had IVF treatment, the number of treatment cycles during the follow-up period were subtracted from the follow-up time. Analysis was also performed with censoring at the first IVF treatment.

Semen analysis

Semen samples were obtained by masturbation after a requested 2–5 days (mean 3.4, SD 1.6) abstinence. Standard semen analysis was performed by World Health Organization (WHO) methods except where stated (World Health Organization, 1999). The percentage of sperm with progressive motility included WHO grades a and b, combining rapid and linear progression and other progressive motility. Manual assessment of sperm morphology (%N) was performed according to WHO 'modified strict' criteria on 200 sperm per sample by one of four technicians in the RWH Andrology laboratory.

Measurement of VSL was performed using a Hamilton–Thorne Motility Analyser (IVOS 10.8; Hamilton-Thorne Research, Danvers, MA, USA). After May 1999, manual assessment of sperm concentration and percentage progressive motility was replaced by assessment with IVOS using the 'Ident' stain, which overcame previous problems of accuracy experienced with CASA measurement of these semen variables (Farrell *et al*., 1996; Fraser *et al*., 1997; World Health Organization, 1999). If a sample was estimated during initial evaluation with the microscope to have a concentration $<50\times10^6$/ml, an aliquot of semen was mixed with equal volume of Ident stain. Otherwise, a final dilution of 1:7 was prepared by mixing 100 μl of Ident stain, 75 μl of homologous seminal plasma and 25 μl semen. The mixture was incubated at 37°C for 15 min before loading into a 20 μm Microcell (Fertility Technologies, Natik, MA, USA) chamber. A minimum of three fields containing a total of at least 200 sperm were analysed by capturing 30 frames/field at a rate of 60 frames/s. The analysis was performed at 37°C with IVOS instrument settings of 45 U

Table I. Median, mean, SD and range for semen analysis results in the study group (n = 1191)

Variable	Median	Mean	SD	Range
Volume (ml)	3.0	3.4	1.5	0.1–10.6
Concentration (10^6/ml)	71	86.7	71.8	2–618
Total sperm number (10^6)	215	270	239	0.4–2108
Progressive motility (%)	42	41.4	16.2	1–89
VSL (μm/s)	39	38.7	8.6	11–70
%N	17	19.7	13.8	0–68
%C	10	11.1	7.7	0–49
%Z	14	14.9	8.4	0–45
NCI	2.6	2.7	0.8	1.2–6.3

VSL = straight-line velocity; %N = ???; %C = percentage conformity; %Z = percentage 'zona pellucida preferred'; NCI = non-conformity index.

minimum contrast, 2 pixels minimum size, 0.6/6.5 size gates, 0.6/2.0 intensity gates, 10/100 elongation gates and Ident brightness 3000. The size default was set at 4, the default intensity at 100 and low VSL cutoff at 5.0 μm/s. Excellent precision was achieved for CASA sperm count [coefficient of variation (CV) = 5.4%], VSL (CV=4.0%) and percentage progressive sperm motility (CV = 7.4%) for six intra-sample analyses of a typical semen sample.

Throughout the study, internal quality control for the four technicians assessing the same samples (n = 8) independently, gave mean (range) CVs for sperm concentration of 7.0% (3.1–18%), for progressive motility of 10% (3.1–15%) and for morphology of 7.3% (2.4–13%). The laboratory maintains weekly means quality control charts and participates in an external quality assurance program for Australian laboratories (EQA Schemes for Reproductive Medicine), and has results consistently within 1 SD of the overall mean.

SHAMAS analysis was performed on 200 sperm head images of Shorr-stained smears of washed semen (Garrett and Baker, 1995). The image analysis uses 32 morphometric parameters to characterize optical density contours and profiles of the sperm heads and the head–neck junction, which provide three summary measures: the percentage conformity (%C), percentage 'zona pellucida preferred' (%Z) and the non-conformity index (NCI). %C is the percentage of sperm with all 32 parameters conforming to within 2.5 SD of a 'normal' reference and is the automated equivalent of %N for sperm heads (Garrett and Baker, 1995). %Z is the percentage of sperm which conform to within 1.5 SD of the 'zona pellucida preferred' values of the 12 morphometry parameters. The selectivity of the sperm–zona pellucida binding process was determined by linear regression analysis of the differences in the means for sperm bound to the zona pellucida and in the insemination medium, against the mean value for the sperm in the insemination medium for each morphometric parameter (Garrett *et al*., 1997). The 'zona pellucida preferred' values of the morphometric parameters with statistically significant regressions were determined from the regression line, given by the value for sperm in the insemination medium at the intercept for zero difference. NCI is the average number of parameters per sperm that fall outside the 1.5 SD limits for the 12 zona-binding selected parameters. NCI quantifies the degree of non-conformity of sperm to the 'zona pellucida preferred' reference, and is analogous to the multiple anomalies index (MAI) (Jouannet *et al*., 1988) and sperm deformity index (SDI) (Aziz *et al*., 1996). The general approach of using a relevant functional endpoint, such as fertilization rate *in vitro* for VSL and sperm–zona pellucida binding for %Z, as the basis for selection of measures derived from CASA technology is consistent with ESHRE guidelines (ESHRE, 1998).

Statistical analysis

The normality of all data was examined using the Kolmogorov–Smirnov test and the cube root transformation was applied to sperm concentration and total count. Correlations between semen variables were examined by nonparametric [Spearman's rho (ρ)] tests. Pregnancy rate graphs for patients grouped by semen quality and/or age were constructed using the survival analysis method of Kaplan–Meier and compared by log rank test. Cox proportional hazards regression analysis was used to determine which groups of factors were independently significantly related to pregnancy rates. Departures from the assumption of proportional hazards were checked. Because of possible non-linearity in the regression relationships, several transformations of the covariates were examined, with untransformed age, log(%Z +1) and log(VSL) being the most significant in the Cox model.

Results

Correlation of semen variables

Table I summarizes the semen results for the 1191 subjects: 38% had normal semen analysis results using traditional limits, 13% were oligozoospermic and 49% had normal sperm concentration (>20×10^6/ml) but reduced motility and/or morphology. Results for different assessments of the same or closely related semen variables showed the expected strong correlations: %Z and %C (ρ = 0.88), %Z and NCI (ρ = –0.91), %Z and %N (ρ = 0.55) and VSL and %progressive motility (ρ = 0.55). There was a moderately strong relationship between %Z and concentration (ρ = 0.47) and also %progressive motility (ρ = 0.36). VSL was significantly but less strongly related to concentration (ρ = 0.16), %Z (ρ = 0.28) and %N (ρ = 0.36).

Factors related to natural pregnancy rate

At the time of first semen analysis for the 1191 couples, the age of the female partners ranged from 20 to 50 years [mean (SD), 33.1 (5.3)] and for male partners from 21 to 64 years [35.3 (6.0)]. Follow-up ranged from one to 50 menstrual cycles, with a median of seven cycles. The total follow-up time was 855 person years, during which there were 336 natural pregnancies. The average survival analysis pregnancy rate was 31% at 12 cycles, and 48% at 24 cycles.

The couples' ages and all semen variables except volume were significantly related to pregnancy rate by univariate Cox regression analysis (Table II). However, in multivariate Cox regression analysis only three factors were significant, of which %Z was the most important, followed by VSL and female age. None of the other variables contributed significantly to the model when considered individually as the fourth co-variate (Table II). The alternative measures of sperm morphology were less significant than %Z in combination with VSL and age. The standard variables did not add to or provide a better model than %Z, VSL and age when considered as an interaction (volume × concentration × motility × morphology), or as four sub-groups by concentration (<5×10^6/ml, n = 19; 5–20×10^6/ml, n = 129; 20–100×10^6/ml, n = 667; >100×10^6/ml, n = 376) or classification by number of defects (triple, n = 85; double, n = 276; single, n = 375; and none, n = 455, based on the criteria: sperm concentration <20×10^6/ml, %progressive motility

Table II. Summary of Cox regression analysis of factors affecting natural pregnancy rates in 1191 subfertile couples

Variable	Univariate			Multivariate[a]		
	Coeff.	*P*	Hazard ratio (95% CI)	Coeff.	*P*	Hazard ratio (95% CI)
Female age (years)	–0.032	0.002	0.97 (0.95–1.00)	**–0.033**	**0.0015**	**0.97 (0.95–0.99)**
Male age (years)	–0.022	0.023	0.98 (0.96–1.00)	NS	0.968	
Volume (ml)	NS	0.183	NS	NS	0.775	
$^3\sqrt{}$ concentration (10^6/ml)	0.091	0.052	1.10 (1.00–1.20)	NS	0.879	
$^3\sqrt{}$ total sperm number	0.088	0.005	1.09 (1.03–1.16)	NS	0.635	
% progressive motility	0.012	0.0003	1.01 (1.01–1.02)	NS	0.827	
Log(VSL)	2.544	0.00001	12.7 (4.1–39.2)	**1.910**	**0.0013**	**6.8 (2.1–21.7)**
%N	0.010	0.011	1.01 (1.00–1.02)	NS	0.812	
%C	0.023	0.0005	1.02 (1.01–1.04)	NS	0.736	
Log(%Z +1)	0.923	0.00002	2.52 (1.65–3.84)	**0.770**	**0.0005**	**2.16 (1.4–3.3)**
NCI	–0.267	0.0007	0.77 (0.66–0.89)	NS	0.081	

NS = not significant; VSL = straight-line velocity; %N = percentage normal morphology; %C = percentage conformity; %Z = percentage 'zona pellucida preferred'; NCI = non-conformity index.
[a]Multivariate analysis results for the addition of each variable in turn as the fourth co-variate to the Cox model of female age, log (%Z +1) and log(VSL). The values in bold type correspond to the results for the multivariate model.

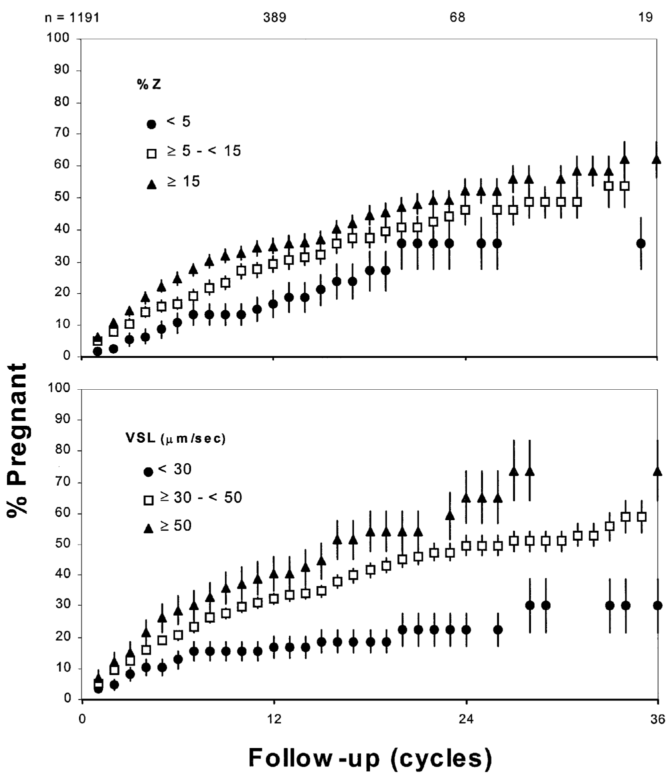

Figure 1. Effect of the percentage 'zona pellucida preferred' morphometry (%Z) and straight-line velocity (VSL) on natural pregnancy rates in 1191 subfertile couples, uncorrected for other covariates. Symbols (±SEM) indicate where pregnancy rates changed at each cycle. The number of couples (*n*) followed for 12, 24 and 36 cycles is shown at the top of the upper graph. Three curves for low, medium and high %Z and VSL are shown. The curves for the three subgroups are significantly different by log rank test: %Z high-medium (*P* = 0.033) and medium-low (*P* = 0.027); and VSL high-medium (*P* = 0.039) and medium-low (*P* < 0.0005).

<40% and %N <15%). When only the standard semen analysis variables and patient ages were considered in the multivariate model, female age and %progressive motility were the only independently significant factors (*P* = 0.003 and 0.0004 respectively). No improvement over linearity was found in the decline of pregnancy rate with female age by applying various transformations and testing a range of thresholds aged between 25–40 years. When the data were analysed with cessation of follow-up at the time of first IVF treatment cycle, female age was not significant (*P* = 0.12) in the univariate Cox regression analysis, but %Z, %C, %N, %progressive motility and VSL remained individually significant (*P* < 0.01). Only VSL and %Z were significant in the multivariate model.

The survival curves in Figure 1 show the effect of low, medium and high values of %Z and VSL on pregnancy rates, uncorrected for the other co-variates. Men with higher than average %Z or VSL produced higher pregnancy rates.

Figure 2 shows curves for a 20 and 40% chance of pregnancy within 12 cycles for female ages between 20 and 40 years, calculated as a function of %Z and VSL using the Cox regression model. Alternatively, the likelihood of natural conception within 1, 12 and 24 cycles can be calculated using the formula in the Appendix. The calculated rates are generally within 1 SE of the Kaplan–Meier estimates for groups of ~100 patients with specified ranges of %Z, VSL and female age. For example, for the 188 patients with %Z ⩽ 10 and VSL ⩽ 35 μm/s, the pregnancy rate at 12 cycles was 20 ± 4% and the Cox regression estimate for %Z, VSL and female age equal to the mean values for the subset is 19%.

Discussion

The aim of this study was to ascertain the value of standard semen variables and additional parameters specific to automated methods of semen analysis in the prognosis of natural pregnancy rates in subfertile couples who have no obvious absolute barriers to fertility. These couples have usually been trying to conceive naturally for more than 12 months, on average about 2 years, and need assistance to decide whether to continue trying for longer or to commence assisted reproductive technology such as IVF. When all available factors were considered, the uniquely automated measures %Z and VSL were the only semen variables independently significantly

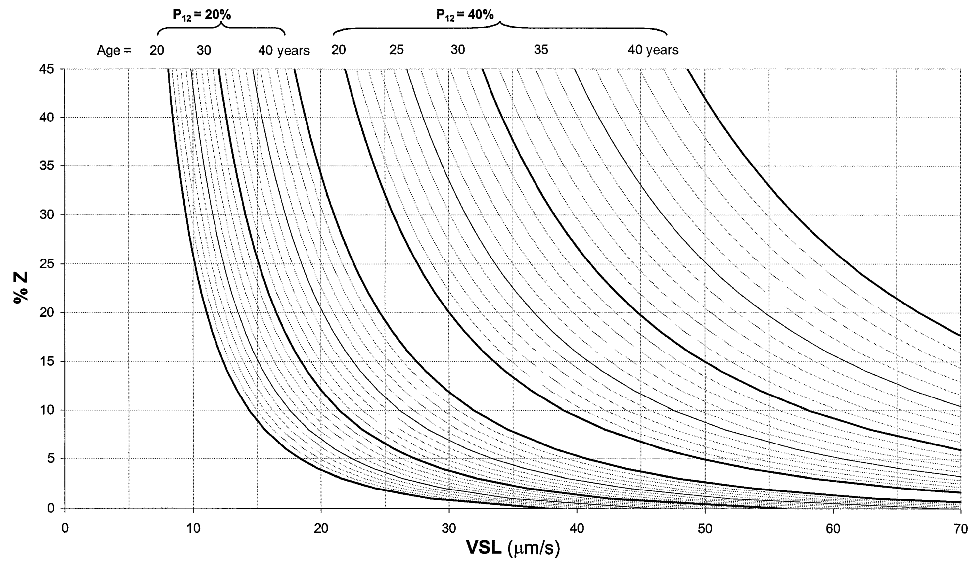

Figure 2. Combined effects calculated from the Cox regression model of the percentage of 'zona pellucida preferred' morphometry (%Z), straight-line velocity (VSL) and female age on natural pregnancy rates in subfertile couples. The curves show 20% (P_{12} = 20%) and 40% (P_{12} = 40%) chances of pregnancy in 12 cycles for different female ages as a function of %Z on the *y*-axis and VSL on the *x*-axis. For example, a woman aged 30 years and a partner with %Z = 20, the chance of pregnancy in 12 cycles would be <20% if VSL = 15 μm/s, 20–40% if VSL = 30 μm/s or >40% if VSL = 50 μm/s, and for a woman aged 30 years and a partner with VSL = 35 μm/s, the probability of pregnancy in 12 cycles would be <20% if %Z = 2, 20–40% if %Z = 10 or >40% if %Z = 40.

related to pregnancy rate in the multivariate regression analysis. While most semen results and indices were significantly related to pregnancy rate individually, they did not add significantly to the multivariate model including %Z, VSL and female age. Our hypothesis of the importance of sperm capable of binding to the zona pellucida for fertility is confirmed by the finding that the most significant factor in the regression analysis was the morphometry summary measure %Z, the percentage of sperm conforming to characteristics of zona-bound sperm.

The significant correlations between semen variables reflect the fact that all aspects of semen analysis (sperm concentration, motility and morphology) are related and tend to change together. However, %Z was only moderately, and VSL only weakly, related to other semen variables and thus these two measures jointly reflect a wide spectrum of semen quality, which may explain why they alone remain statistically significant in the multivariate model. The identification of %Z and VSL as the only semen variables significantly related to the natural pregnancy rates is impressive considering the omission from the analysis of other explanatory variables such as duration of infertility, previous pregnancies in the relationship, undiagnosed adverse female factors, smoking, coital frequency and timing to ovulation (Hargreave and Elton, 1983; Baker *et al*., 1985; Howe *et al*., 1985; Comhaire, 1987; Bostofte *et al*., 1990; Eimers *et al*., 1994; Wichmann *et al*., 1994; Collins *et al*., 1995; Snick *et al*., 1997; Zinaman *et al*., 2000; Hunault *et al*., 2002). While couples known to be sterile were excluded, only age and semen variables were analysed. Non-inclusion of potentially influential data, such as these prognostic factors and information on recent illness that might transiently impair semen quality, would generally tend to obscure rather than promote semen variable–pregnancy rate relationships. Duration of infertility and previous conceptions in the relationship are strong predictors of subsequent pregnancies in subfertile couples (Baker *et al*., 1985; Comhaire, 1987; Eimers *et al*., 1994; Wichmann *et al*., 1994; Collins *et al*., 1995; Snick *et al*., 1997). However, they reflect the severity of the infertility, and while useful clinically in advising couples about their chances of conception, they are not directly related to the causes of infertility. Some researchers have chosen to use the sum of the duration of infertility plus the follow-up time (i.e. overall time to achieve a pregnancy) as the dependent variable to examine the value of different semen measures (Irvine *et al*., 1994). We deliberately omitted duration of infertility and previous conceptions from the analysis as we wanted to study the effect of semen factors on pregnancy rate. More prospective studies are needed to confirm and refine the clinical value of %Z and VSL in relation to other prognostic factors.

Values of VSL, %Z and female age can be used to calculate (see formula in Appendix) or estimate from a graph (Figure 2) the likelihood of a pregnancy occurring with time. Pregnancy rates of 20 and 40% in 12 cycles have been chosen for illustration, since patients with a <20% chance in 1 year would benefit from assisted reproductive technology, while those with >40% chance might wish to try longer for a natural conception. Patients with severe oligospermia ($<2\times10^6$/ml) were excluded, and those with moderate oligozoospermia ($2–5\times10^6$/ml) were under-represented in this study, thus possibly reducing the significance of sperm concentration as a predictor of pregnancy rate in this population. However, from a previous study including patients with severe and moderate oligospermia, the average pregnancy rate was similar to this study and the chance of pregnancy in 1 year for couples with severe oligospermia was found to be low, at 12%, and 18% for moderate oligospermia (Baker, 2001). Since a poor prognosis for this subset of men is known, their omission from the present study group does not detract from the clinical importance of the findings.

Manual semen analysis has limited objectivity and reproducibility. Despite attempts to standardize techniques, differences of opinion on methodology and interpretation have paralysed progress (World Health Organization, 1999). Results of external quality assurance programmes indicate that agreement between laboratories is not achievable for sperm motility and morphology, and adds impetus to automate semen analysis (Jorgensen *et al.*, 1997; Franken *et al.*, 2000). The results of this study indicate that the objective semen variables available from the automated image analysis of semen are of greater clinical predictive value than the standard semen variables of concentration, motility and morphology. The %Z assessment used in this study could be incorporated into any CASA system with a morphometry module that analyses stain density (Garrett and Baker, 1995). However, as all semen analysis will not be automated in the immediate future, modification of manual morphology assessment may be possible to obtain a reasonable estimate of %Z. The morphology assessment should emphasize large relative size of the acrosome area, longitudinal head symmetry and optimal neck width, all important characteristics of sperm capable of binding to human zona pellucida. Sperm head dimensions, eccentricity and ellipticity, which form part of the standard assessment of morphology, are less important (Garrett *et al.*, 1997). Tail defects are not included in this morphometry assessment but are taken into account by the motility measure.

Historically, threshold values of semen variables have been sought to define fertility potential (MacLeod and Gold, 1953; World Health Organization, 1999). However, the interacting combination of male and female factors affect pregnancy rate and, except with extremes such as azoospermia, zero motility or uniform total teratozoospermia, the relationship between semen variables and pregnancy rate is not a step function. Figure 1 shows that subfertile couples with higher or lower than average values of %Z or VSL have corresponding significantly higher or lower pregnancy rates, indicating no evidence of threshold effects. The better fit with logarithmic transformation in the Cox model is consistent with the relationships between %Z and VSL with pregnancy rate being continuous, although not linear. This has also been shown in the results of donor insemination, which indicate that the selection of donors with above average semen will increase pregnancy rates, particularly for morphology and post-thaw motility (McGowan *et al.*, 1983; CECOS, 1993; Clarke *et al.*, 1997).

It is well known that fertility declines with female age for natural pregnancies, donor insemination and IVF (Howe *et al.*, 1985; CECOS, 1993; Speirs *et al.*, 1996; Baker *et al.*, 2000). In this study, several transformations of female age involving a constant value below a threshold age were tested in the model, but gave no significant improvement on linearity in the prediction of natural pregnancy rate. In IVF data, the effect of female age on fertilization rates appears after the mid-30s, whereas a more continuous effect is seen with natural conceptions. It is possible that this apparent difference is due to different definitions of female age in the two types of studies: age at the time of IVF treatment and age at the start of follow-up in the natural pregnancy rate studies. The increase in female age during the follow-up period could obscure a threshold effect of age in the analysis.

In summary, the specific automated morphology and motility measures of %Z and VSL are strongly related to pregnancy rates in subfertile couples. These results confirm that aspects of sperm morphology and motility known to affect human fertilization *in vitro* are important for natural fertility, and demonstrate that automation of semen analysis has come of age.

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Appendix

The formula for calculating the probability of pregnancy after m cycles P_m is given by the Cox regression model as:

$P_m = 1 - \exp[\alpha_m \exp \Sigma_{i\ =\ 1,2,3} (\beta_i\ x_i)]$, where

α_m describes the underlying (general) probability of pregnancy after m cycles

$\alpha_1 = -0.0030$; $\alpha_{12} = -0.0213$; $\alpha_{24} = -0.0375$

x_1 = female age (years); $x_2 = \log(\%Z+1)$; $x_3 = \log(VSL)$ are the co-variates and

$\beta_1 = -0.0332$; $\beta_2 = 0.7704$; $\beta_3 = 1.910$ are the corresponding regression coefficients.

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