Variation in Semen Parameters Derived From Computer-Aided Semen Analysis, Within Donors and Between Donors

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ABSTRACT: The development of computer-aided semen analysis (CASA) has made it possible to study sperm motility characteristics objectively and longitudinally. In this 2-year study of 8 sperm donors, we used CASA to measure 7 semen parameters (concentration, percentage of motile spermatozoa, curvilinear velocity, average path velocity, straight-line velocity, amplitude of lateral head displacement, and beat/cross frequency). The frequency distributions of the 7 parameters in the semen samples of each donor were investigated. All parameters but one were normally distributed; concentration was distributed log-normally. Variation within individual donors and between donors was studied. Analysis of variance demonstrated that variation between donors was not explained by the longitudinal variation within individual donors. Variations in motility characteristics between donors were substantial, which may make motility characteristics of limited value as a tool for establishing fertility. Strong correlations were found between the 7 parameters, partly because by definition, motility characteristics are interdependent. Fisher's discriminant analysis demonstrated that each donor appeared to have his own set of semen characteristics and, more specifically, his own motility signature. From this data set it can be predicted that in order to find population means among sperm, it may be more efficient to measure more subjects than to increase the number of samples per subject.

Key words: Concentration, longitudinal study, motility, statistical analysis.

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In the twentieth century, scientists have begun to search for relations between semen parameters and fertility. However, the variability of semen parameters must be understood before one may gain insight into these relationships. It has been well recognized, for example, that sperm concentration values can vary considerably within individuals (World Health Organization [WHO], 1992) and that single samples may have little value in predicting fertility (MacLeod and Irvine, 1995).

The development of computer-aided semen analysis (CASA) has made it possible to measure motility characteristics objectively and precisely. Several studies have been applied to the variability of CASA-derived semen parameters, yet results have been contradictory. Some authors found that between-subject variation dominates (Mack et al, 1989; Vantman et al, 1994; Farrell et al, 1996; Tardif et al, 1997), whereas others have reported that within-subject variation dominates (Mallidis et al, 1991; Schrader et al, 1991). Whereas Katz et al (1981)

reported within-subject variation as well as between-subject variation, both Poland et al (1985) and Knuth et al (1988) reported only within-subject variations. MacLeod and Irvine (1995) stated that 1 analyzed sample is not representative of an individual, nor is an individual's semen quality static. This statement agrees with earlier studies by Irvine et al (1994) and by Irvine and Aitken (1986), but unfortunately, no data were given to support these statements. Schrader et al (1991) concluded that increasing the number of men in a study population of occupational field studies provided more useful data than increasing the number of samples per man did.

Risum et al (1984) demonstrated that donors' ejaculates could be characterized by 3 parameters. In our laboratory, long before CASA had been developed, experienced microscopists used to claim that they could recognize a donor by his semen.

Before considering within-subject variation and between-subject variation, technical and statistical aspects that may contribute to the variation should be taken into account. Numerous aspects have been discussed in studies of CASA: the type of CASA system used (Holt et al, 1994) and its settings (Davis and Boyers, 1992), the counting chamber (Ginsburg and Armant, 1990), handling of the semen sample (ESHRE Andrology Special Interest

Group, 1998), within-sample variation (Davis and Boyers, 1992), and distribution of characteristics (Gladen et al, 1991; Berman et al, 1996).

We used the Strömberg Mika cell motion analyzer (SM-CMA; Medical Technologies Montreux SA, Montreux, Switerland), which had been compared with 4 other CASA systems by Holt et al (1994), and who found that an SM-CMA gave the smallest within-sample variations for motility characteristics and concentration. Unlike other CASA systems, the SM-CMA detects immotile spermatozoa not only by size and contrast, but also through sperm tail identification. The calculated sperm tracks are overlaid onto the images of the spermatozoa on the video screen, which makes it easy to monitor the calculations of the computer program. The calculated values of each spermatozoon are shown simultaneously on the screen; thus the analysis can be checked closely.

In a previous study, SM-CMA counts were compared with hemocytometer counts separately for spermatozoa in seminal plasma and for suspensions of immotile and motile spermatozoa (Wijchman et al, 1995). In seminal plasma, linearity was obtained between hemocytometer counts and SM-CMA counts in a range of concentrations from zero to 250×10^6/mL, but the SM-CMA underestimated sperm counts. In the suspensions of immobilized spermatozoa, linearity was obtained up to 80×10^6/mL. Inspection of the video screen showed that regardless of the tail-recognition feature, the program consistently missed almost a third of the immotile spermatozoa. At the same time, the program would sometimes "invent" tails; for instance, when 2 particles touched. However, in the suspensions of motile spermatozoa, the motile sperm counts were linear up to a concentration of 160×10^6/mL, and approximately 90% of the spermatozoa was recognized by the SM-CMA. Inspection of the video screen corroborated these observations.

Neuwinger et al (1990) studied an older version of the SM-CMA, included sperm counts of up to 55×10^6/mL in seminal plasma, and found acceptable agreement. Togni et al (1995) studied SM-CMA performances by comparing the calculated values with the images on the video monitor. They found that concentrations calculated by the program were undercounted compared with concentrations that had been corrected after inspection of the video screen, a finding that agreed with ours.

Motility data were analyzed statistically by Gladen et al (1991), who advised that distributions should be normalized by a transformation, if necessary, and to consider possible interdependencies among the various parameters.

Led by the above-mentioned considerations, we decided to systematically investigate the measurements of repeated semen samples from 8 donors who had collected their samples during a 2-year period for various research projects. Because the CASA results were stored on a hard-drive, variations within and between subjects could be studied retrospectively. This longitudinal study contained 8 donors and 7 parameters.

Materials and Methods

Semen Samples

We studied the semen samples of 8 sperm donors who were selected on the basis of semen quality during their first donation. Selection criteria were $>40 \times 10^6$ spermatozoa/mL and >50% motile. The donors visited our laboratory during the period August 1993 to July 1995. The group consisted of 7 young, healthy students and 1 staff member who volunteered to donate sperm samples weekly or fortnightly for research purposes. Semen samples were usually collected at home and were brought in to the laboratory within half an hour. The students were about 20–25 years of age; their fertility status was unknown; the staff member was a 45-year-old father of 4 children. All semen samples delivered to our laboratory were analyzed with CASA. To understand the full extent of the variability, no samples were excluded from analysis.

Computer-Aided Semen Analysis

Analysis was routinely performed with an SM-CMA, which detects immotile spermatozoa by size and contrast, and by sperm tail recognition. For motility characteristics the SM-CMA provides mean values, which were used in our analyses.

At room temperature, an aliquot of 5 μL of undiluted semen was placed in a disposable, 12-μm MicroCell counting chamber (Conception Technologies, La Jolla, Calif; Ginsburg and Armant, 1990). Subsequently, the counting chambers were heated to 37°C, and within 10 minutes the SM-CMA assessed concentration; percentage motility; and the following 5 motility characteristics (WHO, 1992): curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), and beat/cross frequency (BCF, Hz). The SM-CMA operates at 50 Hz; measurements were performed at 37°C and with the 20× objective at a total magnification of 66×. The parameter settings were as described before (Wijchman et al, 1995).

A maximum of 12 fields was measured; in this way, 318 samples were evaluated. This study assesses the calculations on 76 251 spermatozoa, of which 42 712 were motile. Table 1 summarizes the number of samples and spermatozoa per donor.

Statistics

Frequency distributions of semen parameters per donor were inspected visually and tested for normality with the Kolmogorov-Smirnov test. A simple log transformation was applied on 1 parameter (concentration). For every parameter and donor, the within-subject standard deviations were calculated. Because these varied somewhat between donors, an equal number of samples (23) for each donor was used when this variation could affect the calculations by giving more weight to donors with many samples.

The MEANS procedure in the Statistical Package for the So-

Table 1. *Number of samples and of spermatozoa*

Donor	B	E	F	H	L	P	R	V
No. of samples/donor	61	28	23	33	64	36	36	37
Median no. of measured spermatozoa/sample	235	177	132	325	248	186	215	288
Median no. of motile spermatozoa/sample	152	55	74	170	131	101	75	195
Total no. of measured spermatozoa/donor	14 928	4752	3050	11 156	16 782	6872	8019	10 692
Total no. of motile spermatozoa/donor	9510	2328	1717	5593	9562	3859	2833	7310

cial Sciences (SPSS) produced one-way analysis of variance (ANOVA) and several useful figures. Three sums of squares (SSQs) are given; the combined within-subject SSQ, the between-subject SSQ, and the sum of these. First, the average variance within subjects was calculated from the within-SSQ group and the variance between subject means was calculated from the between-SSQ group. Standard deviations (SDs) were computed as the square roots of these variances. We also used the coefficient of variation (CV), which is the SD expressed as a per-

centage of its corresponding mean, because it represents the SD in a normalized way. Second, an intraclass correlation was computed as the proportion of between-SSQs to the total SSQ. It can also be seen as a degree of the repeatability of measurements within a subject, relative to the variation found within the population. Its square root is given as Eta by the SPSS MEANS procedure.

The correlation between the parameters was measured with Pearson's *r*. The proportion of the total variance explained by the association of 2 parameters is represented by r^2. Scatter plots and regression lines were visually inspected.

Fisher's discriminant analysis was applied to identify the donors from their peers, based on their semen parameters. To this end, first, 10 samples from each donor were set apart; the samples were sorted chronologically and per donor, and every n/10th sample was chosen. Second, the remaining 238 samples of the data set of 318 were used to define the discriminant functions. In this way, classification parameters for each donor were calculated. Third, the classification parameters were applied to the 10 samples per donor, which had initially been set apart.
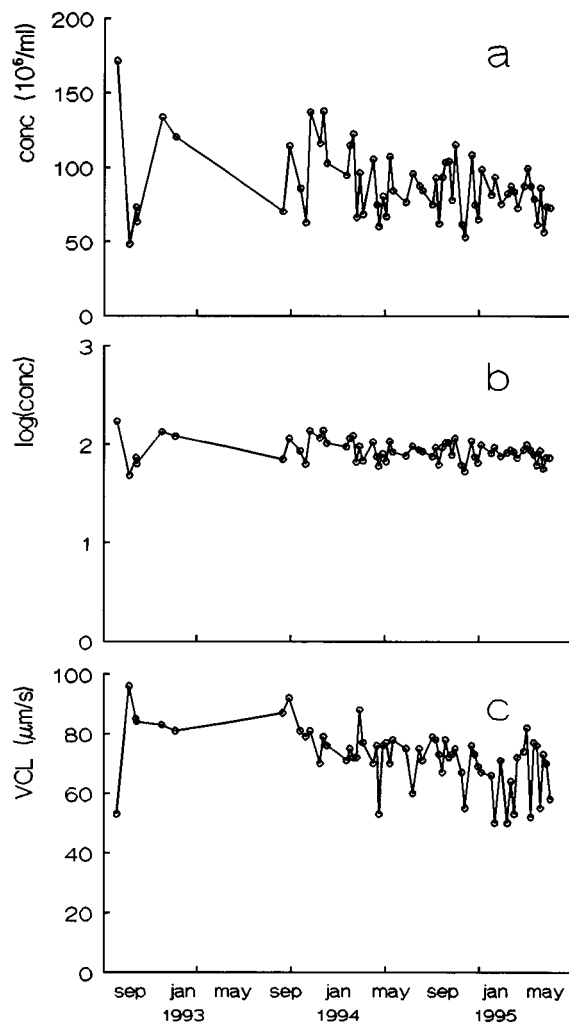
Results

Distribution

Within a subject the frequency distributions of motility characteristics (VSL, VAP, VCL, ALH, and BCF) and percentage motility did not differ from a normal (Gaussian) distribution. Concentration values were distributed log-normally. The averages and medians were quite close, which indicates symmetric distributions (data not shown).

Within-Subject Variation

The values for 1 donor were used to illustrate within-subject variations in the course of time. Figure 1a shows the typical variation in concentration, such as it was seen in all donors. The variation in log(concentration) (Figure 1b) was much smaller than the variation in concentration, as was expected. Variation in VCL as an example of variations in motility characteristics is shown in Figure 1c. For this donor, the CV of concentration was 26.8%; the CV of log(concentration) was 5.8%, and the CV of VCL was 14.1%.

Between-Subject Variation

Average values (± SD) for the 7 parameters appear for each donor in Figure 2. For concentration, the logarithm

Figure 1. Within-donor variation in the course of almost 3 years for donor B (n = 61). **(a)** Variation between the measured values of concentration (10⁶/mL). **(b)** Variation between the logarithms of concentration. **(c)** Variation between the measured values of VCL (μm/s).
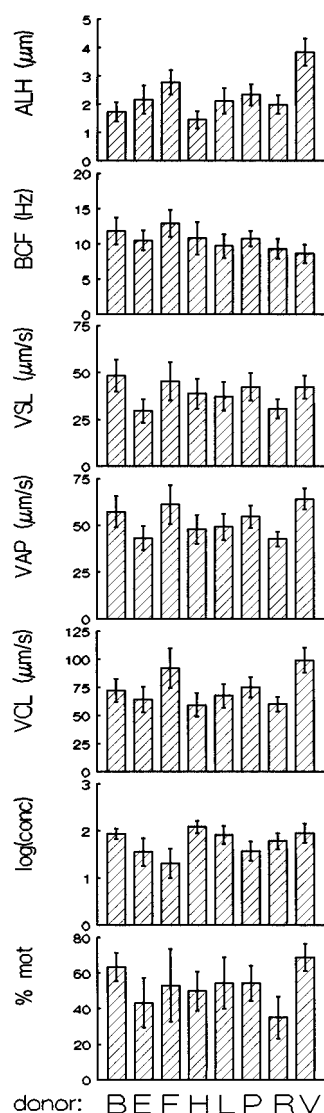
Figure 2. Representation of the 7 parameters of 8 donors (B, E, F, H, L, P, R, and V) by mean values ± SD. Logarithms are given for concentration. Means and SDs were calculated for 61, 28, 23, 33, 64, 36, 36, and 37 samples, respectively, over the course of 3 years.

is shown (see above). Semen parameters varied within subjects as well as between subjects. Between-subject variation is depicted by the heights of the hatched bars, and within-subject variation is indicated by the lengths of the error bars. For all parameters, ANOVA showed that the differences between donors could not be explained by variations between the samples of a donor ($P < .001$).

Correlations Among Semen Parameters

Table 2 shows Pearson's *r* values calculated across all donors. Correlation coefficients differed per donor; therefore, for each donor, an equal number of samples was used. Motility characteristics are interdependent by definition. The correlations of these characteristics with log(concentration) showed low r^2 values, but among the correlations with percentage motility, some showed rather high r^2 values.

Discriminant Analysis

The dissimilitude between the 8 donors (Figure 2) was illustrated with discriminant analysis, which is a way of distinguishing donors from each other, based on a combination of their semen parameters. Ten samples were tested per donor. A selection of combinations is given with the results of the discriminant analyses in Table 3. Per combination, the number of correctly attributed samples is shown per donor and overall. By mere chance, the overall result would have been 10 out of 80 correctly classified samples.

Table 3 shows that to distinguish a donor from his peers, the combination of ALH and VCL gave comparable results with the combinations of log(concentration) and VCL. The 2 motility characteristics, VCL and ALH, illustrate the differences and similarities between donors in Figure 3. Three characteristics were used to draw individual motility patterns of each donor's sperm (Figure 4). These patterns consisted of the average values of VSL, ALH, and BCF per donor. Although VCL was not used to compose the patterns, it is visible as the length of the curve. Only straightness is not visible. Spermatozoa of different donors seemed to have more or less different

Table 2. *Correlations between the seven parameters, calculated over 23 samples per donor**

	Log(concentration)	Percentage Motility	VCL	VAP	VSL	ALH
Percentage motility	0.314					
VCL	−0.180	0.545				
VAP	−0.079	0.607	0.920			
VSL	0.23	0.543	0.664	0.876		
ALH	−0.112	0.371	0.825	0.588	0.186	
BCF	−0.366	0.078	0.241	0.380	0.578	−0.210

* Independent parameters are shown from right to left, dependent parameters are shown from top to bottom. Pearson's correlation coefficients are given.

Table 3. *Discriminant analysis results per donor*

	B	E	F	H	L	P	R	V	Total
All 7 parameters	8	3	5	8	5	5	7	10	51
Log(concentration) + VCL + ALH	9	3	8	9	2	3	7	9	50
Log(concentration) + VCL	7	3	8	9	2	3	7	9	48
ALH + VCL	9	2	8	10	1	3	4	9	46
ALH alone	6	1	6	10	1	1	2	9	36

Ten samples per donor were evaluated with discriminant analysis, a total of 80. The second row gives the results if all parameters are being used, the third row gives the best combination of 3 parameters, the fourth row gives the best combination of 2 parameters, the fifth row gives the best combination of 2 motility parameters, and the last row shows the best single discriminating parameter (ALH). The columns show the number of samples that were attributed to the correct donor per combination of parameters. The last column shows the total number of correctly attributed samples. By mere chance alone, this would have been 10.

patterns of motion. We propose to refer to these patterns as "motility signatures."

Intraclass Correlations and Reliability

Table 4 shows the within-subject CV for each parameter, averaged for 23 samples per donor, the population mean, the between-subject CV, the within-subject SSQ, the between-subject SSQ, and Eta squared. ALH had the highest intraclass correlation, percentage motility had the lowest. For these parameters, Figure 5 shows what the standard error of the population mean would be using either more samples per subject or more subjects.

Discussion

In this longitudinal study, the variability among 7 semen parameters was evaluated. We aimed to find a way to differentiate between 8 donors using the data of 318 semen samples, analyzed with SM-CMA.
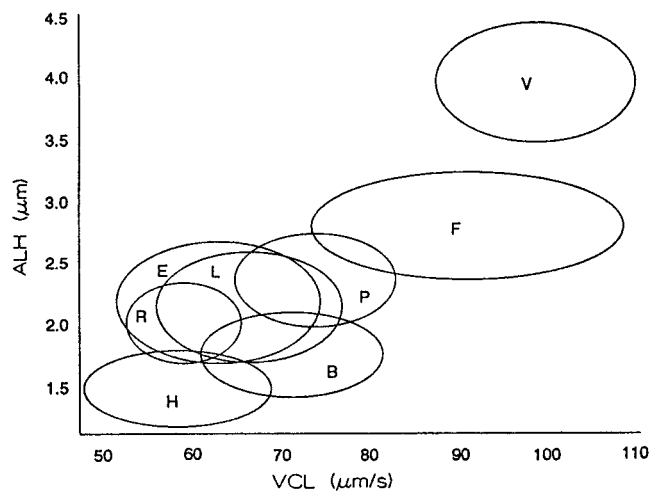
Concentration values were distributed log-normally

within the subjects; the other semen parameters were distributed normally. Risum et al (1984), in a longitudinal study, also found that concentration values per donor were distributed log-normally. Mallidis et al (1991) proposed cube root transformations as the best fit for concentration values. In our data set, both transformations were suitable but we applied the log transformation, which is more straightforward.
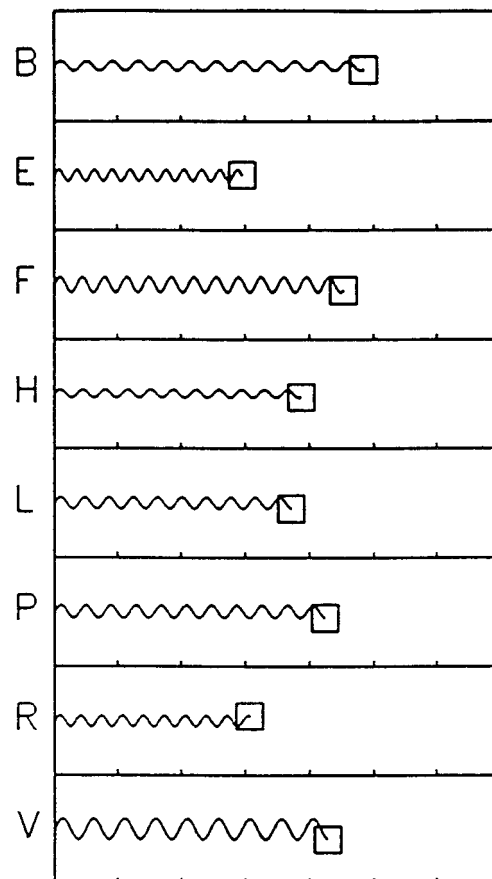

Figure 3. Classification of 8 donors by VCL (μm/s) and ALH (μm). The centers of the ovals are determined by the averages, the perimeters by 1 SD. The total number of samples is 318. Fisher's discriminant analysis attributed 46 of 80 samples in the test set to the correct donor.

Figure 4. "Motility signatures" of 8 donors. Idealized sperm tracks were calculated from the combination of VSL, ALH, and BCF. These idealized tracks are shown as sinusoids. VSL is presented as the distance from the beginning to the end of the curve, ALH as the amplitude, and BCF as the number of waves. As a result, VCL is the total length of the curve.

Table 4. *Statistical data, calculated over 23 samples per donor*

	Average Within-Subject CV	Population Means	Between-Subject CV	Within-Subject SSQ	Between-Subject SSQ	Eta Squared
Log(concentration)*	12.5	57.5	13.9	8.39	9.61	0.53
Percentage motility	24.1	52.6	18.4	29 493	15 418	0.34
VCL (μm/s)	14.5	73.7	20.1	21 787	35 683	0.62
VAP (μm/s)	13.0	52.6	15.6	8842	10 931	0.55
VSL (μm/s)	18.4	39.4	17.8	9714	7998	0.45
ALH (μm)	16.4	23.0	31.7	2389	8588	0.78
BCF (Hz)	15.2	10.6	12.6	437	293	0.40

* Back-transformed mean.

Davis and Boyers (1992) mentioned a number of difficulties that may render CASA motility results invalid, such as the ability of the technician, the number of spermatozoa or microscopic fields analyzed, the time between semen collection and measurement, and the period of abstinence. Factors such as counting chamber and machine settings were constant. The factors that varied in our study are discussed below.

The numbers of spermatozoa and samples per donor are summarized in Table 1. Although we did not always measure >200 spermatozoa (WHO, 1992), the median number of spermatozoa per sample was >200 for 4 donors and <200 for 3 donors (Table 1). Also, the median number of motile spermatozoa was >100 for 4 donors, and <100 for 3 donors (WHO, 1992; Table 1). No samples were excluded from analysis because of a low number of counted spermatozoa. Indeed, donor F had higher within-subject CVs than the other donors did (Figure 2).

Second, Mortimer et al (1982) found that delays of up to 3 hours between the time of semen collection and measurement did not seriously affect mean semen parameters, which had been established in a group of patients. In our study group, samples were usually measured within 1.5 hours.

Third, the period of abstinence may influence CASA results. Indeed, some studies have shown that a period of abstinence influenced sperm concentration (eg, Schwartz et al, 1979; Heuchel et al, 1981; Levin et al, 1986), but not percentage motility (Heuchel et al, 1981; Mortimer et al, 1982). Both Knuth et al (1988) and Cooper et al (1993) found that a period of abstinence did not influence motility characteristics. Moreover, a study by Farrell et al (1996) reported significant between-subject differences in motility characteristics for human, rabbit, and bull in a study with controlled periods of abstinence, suggesting that the effect of a period of abstinence is very small at most. We have no information on the length of abstinence of donors, but, as was argued above, our results on motility characteristics were unlikely to be affected.

Because of the ability of the SM-CMA to measure high concentrations of spermatozoa (Wijchman et al, 1995), we did not dilute the semen samples. Dilution with an artificial medium may change the motility characteristics (Farrell et al, 1996; Tardif et al, 1997) and dilution with homologous seminal plasma may introduce errors (Comhaire et al, 1992; Mortimer et al, 1989), which would make concentration assessments inaccurate. We also did not use a fluorescent staining for the concentration assessments (ESHRE Andrology Special Interest Group, 1998) because we found that even though parts of immotile spermatozoa were consistently missed by the SM-CMA, motile sperm counts were linear up to a concentration of 160×10^6 (Wijchman et al, 1995).
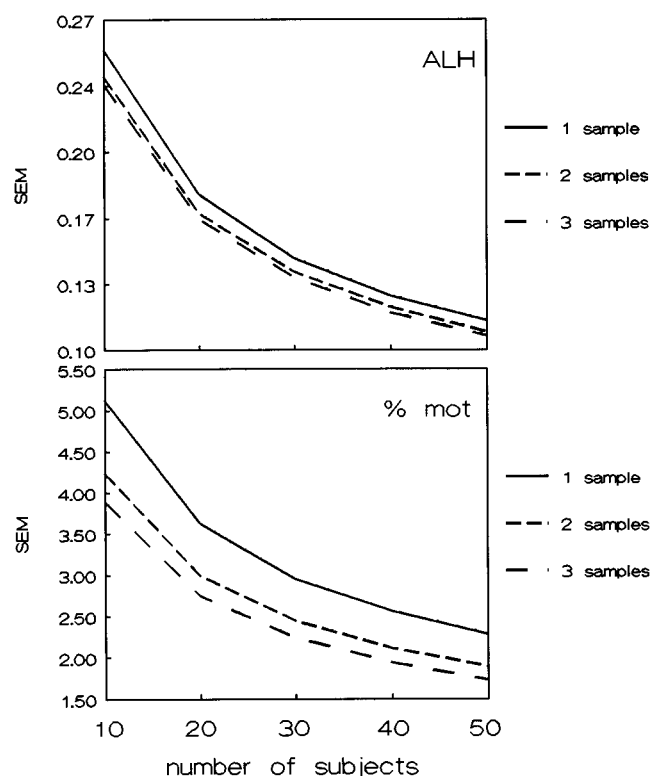

Figure 5. These 2 graphs show how the standard error of a population mean (SEM) (y-axis) is influenced by using either more subjects (x-axis), more samples per subject (3 lines), or both. The top graph shows this for ALH, which had the highest Eta squared; the bottom graph shows this for percentage motility, which had the lowest Eta squared. The mean of ALH was 2.3 μm; the mean percentage motility was 52.6%.

Correlations between the parameters are expressed in Table 2 by Pearson's *r* values, calculated across 23 samples from all donors. Because VCL, VAP, and VSL all describe the velocity of a spermatozoon in different ways, their strong correlation is understandable. VAP is even directly calculated from VCL, and ALH is defined as such, so that an increasing ALH would result in an increasing VCL. For other CASA systems in different setups, motility characteristics influenced by concentration have been described (Vantman et al, 1988; Wetzels et al, 1993). However, in our group of donors, motility characteristics measured with the SM-CMA were not strongly correlated with log(concentration), but they were correlated with percentage motility. High concentrations of spermatozoa in a counting chamber probably did not cause artifacts with the SM-CMA. If this is also true for high-percentage motility, we have to consider a physiological relation. This is supported by the positive correlation (ie, high percentage motility was associated with high velocities). It could be that both simply indicate a "healthy" semen sample.

For all parameters, highly significant variations between subjects were found with ANOVA. With discriminant analysis it was illustrated that each donor had his own set of semen parameters. Some donors (H, V, B, and F) were better distinguishable than others (donors E and L) were (Table 4; Figure 3). Donor F had the highest within-subject CVs. Overall, the best combination of 2 parameters to identify a donor between the others was the combination of log(concentration) with VCL. Table 4 also shows that adding more parameters to the discriminant analysis increased the total number of correctly attributed samples perhaps only slightly, but that it did not simultaneously lead to more correctly attributed samples to the individual donors. This phenomenon can be explained by the interdependence of the parameters.

When discriminant analysis was applied to the motility characteristics alone, the best combination was VCL with ALH. Forty-six out of 80 samples were attributed to the correct donor. By mere chance alone this would have been 10 out of 80. In Figure 3, VCL and ALH illustrate the differences and similarities between donors. The spermatozoa of different donors had more or less different patterns of motion or "motility signatures" (Figure 4). A few recent studies on CASA-derived motility characteristics also pointed to motility signatures (Farrell et al, 1996; Tardif et al, 1997). Risum et al (1984) demonstrated that donors' ejaculates could be characterized by a set of 3 parameters, among which is log(concentration).

The intraclass correlations in Table 2 ranged from 0.34 for percentage motility to 0.78 for ALH. Figure 5 shows that even for percentage motility it would be more useful in future comparative studies to have more subjects than more samples per subject (see also Schrader et al, 1991).

The average within-subject CVs are relatively low. MacLeod and Irvine (1995) stated that 1 sample is not representative of an individual, but they were referring to patients who were subfertile.

The values provided by the SM-CMA are the averages of all spermatozoa measured in a sample, and they ignore variations between spermatozoa. Still, we could show differences between the donors. Meanwhile, the data of all 76 251 single spermatozoa were stored. Variations between single spermatozoa within samples is the subject of a current study. In a future study we will also examine data from patients' samples.

The movement of the head of a spermatozoon, which is measured by CASA, may very well be influenced by its morphological characteristics (Katz et al, 1982), the implantation of the tail, the size of the midpiece, or the force of flagellar movement. Motility signatures are probably influenced by such intrinsic sperm features. On the other hand, differences in the composition of seminal plasma may also be responsible for differences between donors. It will be interesting to investigate variations in swimming patterns by comparing semen plasma and culture medium.

The inducement for the present study was to test whether the claim by our microscopists that they could recognize a donor by his semen could be confirmed by CASA. We demonstrated a substantial variety of semen parameters among 8 "normal" men; young, healthy volunteers who were selected on the basis of their semen quality. They may or may not be fertile. Each of them appeared to have his own set of semen characteristics and his own motility signature in particular. Differences between subjects such as we found need not be related to fertility. As discussed by Morris and Morrissey (1989), semen parameters that exhibit limited between-subject variation will be more valuable, so the parameters in our study may not be of much use for differentiating between fertile and nonfertile subjects. In a recent CASA study, Larsen et al (2000) found that 1n(concentration) and percentage of motile spermatozoa were the only parameters for predictive value of a man's fertility, and that motility characteristics were not. However, despite the variability of our data, the range of our donor data was such that subfertility data may well fall below that range.

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