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**ORIGINAL PAPER**

# SEMEN METAL PROFILE, SPERMATOZOA MORPHOLOGY AND SEMEN BIOCHEMICAL PARAMETERS IN SUBFERTILE MEN WITH DIFFERENT LIFESTYLE HABITS*

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**Abstract**

In this study we determined the associations between lifestyle habits (alcohol intake, smoking, dietary supplements consumption) and morphological and biochemical profile of spermatozoa, as well as the metal profile of seminal plasma from subfertile patients ($n$ = 48) from northern Slovakia. Spermatozoa motility was assessed according to a four grade system, A-D, in accordance with the WHO recommendations. Commercial kits were used to carry out all biochemical analyses. Metal concentrations were measured with CVAAS (for Hg) and with FAAS (for all other metals tested). We found that alcohol consumption and smoking had no significant impact on semen quality, but subfertile men taking dietary supplements presented a slightly higher ejaculate volume, spermatozoa concentration and progressive motility, compared to those who took no supplements. With the exception of Hg and Pb, the metals tested (Ca, Cd, Cu, Fe and Zn) were found in higher concentrations in men who were taking supplements, but only in the case of Cu was the difference statistically significant ($p$ = 0.019). We also noted that numerous of the evaluated parameters were correlated. We conclude that dietary supplements may have a positive impact on semen quality. We also suggest that among people with reproductive dysfunction dietary supplements may be the first step in enhancing semen quality.

**Keywords:** human semen, lifestyle habits, metals levels, semen quality, subfertility.

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# INTRODUCTION

Infertility is an emerging problem worldwide, affecting between 60 million and 80 million people in reproductive age (Mascarenhas et al. 2012). Geographical differences (e.g. cultural, environmental, nutritional), which determine lifestyle habits are the main factors that affect rates of infertility (Ombelet 2011). Among all infertility cases, between 40% and 50% are associated with "the male factor" which is linked to a decline in the concentration of spermatozoa, motility and the percentage of spermatozoa with normal morphology (Aleisa 2013, Kumar, Singh 2015). The primary factors affecting this male dysfunction are divided into two groups: lifestyle and environmental (occupational) factors (Massanyi et al. 2011).

The above factors are responsible for oxidative stress (OS) generation, which is believed to be one of the main causes of male infertility, since high amounts of polyunsaturated fatty acids (PUFA) in the sperm plasma membranes and low concentrations of scavenging enzymes in their cytoplasm make them particularly susceptible to OS (Tvrdá et al. 2011). Fortunately, some nutritional components included in dietary supplements, such as carotenoids, trace minerals and vitamins, may help to protect against oxidative damage (Isakov et al. 2018). Taking mineral supplements is of special importance because minerals play several key roles in semen, i.e. 1) participation in spermatozoa maturation; 2) enhancement of sperm motility; and 3) a decrease in sperm vulnerability to OS (Cheah, Yang 2011).

Semen quality assessment is currently one of the most popular analyses, routinely conducted in clinical andrology and reproductive toxicology (Lukáčová et al. 2015, Slanina et al. 2016). Routine studies are, however, mainly based on morphological examination of spermatozoa. Semen evaluation thus needs to be enriched with biochemical analyses and assessments of metal concentrations in order to ascertain direct or indirect causes of infertility linked to environmental contamination, industrialization and subsequent OS. Unfortunately, such an approach is scarcely found in the literature.

The majority of the studies have investigated the impact of individual nutrients, e.g. lycopene or vitamin E on semen quality in animals and humans (Mangiagalli et al. 2012, Rengaraj, Hong 2015). The impact of commercial dietary supplements on human semen parameters is scarcely studied. Semen quality studies are, moreover, mainly based on morphological examinations of spermatozoa, which in many cases reveal nothing about biochemical and elemental aspects. The goal of this study was to assess the impact of dietary supplements on selected biochemical parameters: content of albumin (ALB), bilirubin (BLB), total protein, urea (U), uric acid (UA), and superoxide dismutase (SOD) activity, as well as morphological parameters: ejaculate volume, spermatozoa concentration, motility A-D, number of round-headed forms, percentage of spermatozoa with abnormalities, as well as concentra-

tions of chosen metals: calcium (Ca), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), lead (Pb) and zinc (Zn) in the seminal plasma of subfertile men from northern Slovakia. Additionally, the influence of lifestyle habits (alcohol consumption and smoking) on variables mentioned was also verified.

## MATERIAL AND METHODS

### Subjects

The study comprised ejaculate from 48 men (between 21 and 35 years of age), who had failed to procreate over one year of unprotected sexual intercourses and had therefore undergone treatment in the Center for Assisted Reproduction in Košice (Slovakia). All subjects claimed to have had no infections or diseases in the preceding 3 months and never suffered serious injuries to the sex organs. Informed consent was obtained from all individual participants in the study. They answered a questionnaire which included information on lifestyle habits (yes, $n = 21$ vs no, $n = 27$ for active smoking of at least 10 cigarettes per day; yes, $n = 24$ vs no, $n = 24$ for alcohol consumption). In the case of alcohol, an affirmative answer meant that the subject consumed more than 14 units (112 g) of alcohol weekly. A number of the subjects ($n = 18$) admitted to taking dietary supplements twice a day (typically commercially available containing vitamins A, C, D, E, K and vitamin B complex; minerals such as Ca, Cr, Cu, Fe, I, Mg, Mn, Mo, P, Se and Zn; lutein and folate).

### Sample collection and semen quality assessment

Semen samples were obtained by masturbation after between 2 and 3 days of sexual abstinence. The motility was evaluated according to a four grade system at 37°C: grade A (progressive motility, movement fast in a straight line – $\geq 25$ μm $s^{-1}$); grade B (progressive motility, but tendency to move in a curve – $< 25$ μm $s^{-1}$; $\geq 5$ μm $s^{-1}$); grade C (non-progressive motility, movement only in their tails – $< 5$ μm/s); and grade D (immotile spermatozoa) – WHO (2010).

### Spermatozoa morphological analysis

A drop of a semen sample was smeared on a slide and air-dried; fixed with Hancock's solution and stained with Giemsa. All slides were analyzed at 1000x using an Olympus AX 70 Provis (Japan) light microscope. For each slide, at least 500 spermatozoa were evaluated and the percentage of pathological spermatozoa (classified as round forms, separated flagellum, broken flagellum, flagellum torso, twisted flagellum, flagellum ball, retention of cytoplasmic drop) was recorded.

**Biochemical analyses**

The samples were centrifuged (10 min, 300x g, 4°C) to obtain the cell sediment and seminal plasma fraction. The fractions were separated, seminal plasma was transferred into 1.5 mL tubes and kept frozen (-80°C) until further analysis.

SOD activity was assessed using the Randox RANSOD commercial kit (Randox Laboratories, Crumlin, Great Britain) at 505 nm using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.). A uric acid (UA) commercial kit (PLIVA-Lachema, Brno, Czech Republic) was used for the assay and the measurement was made at 550 nm. Urea (U) concentration was assessed at 340 nm, according to a Urea DiaSys (DiaSys, Holzheim, Germany) commercial kit. ALB concentration was measured using an ALB BioLa Test (PLIVA-Lachema, Brno, Czech Republic) commercial kit and measured photometrically at 578 nm. Protein concentration was assessed using a DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and a semi-automated clinical chemistry photometric analyzer Microlab 300 (Merck, Darmstadt, Germany) at 540 nm. The determination of total bilirubin (BLB; free and direct) was performed photometrically with the help of a Bilirubin DiaSys commercial kit (DiaSys, Holzheim, Germany) at 440 nm.

**The assessment of metal levels**

The concentrations of metals (Ca, Cd, Cu, Fe, Hg, Pb and Zn) were measured in 1 mL of semen samples, which were mineralized with hot nitric acid (65%, Baker Analyzed, JT Baker, Phillipsburg, NJ, USA) in an open mineralization system (DK20, Velp Scientifica, Usmate, Italy). Mineralized solutions were diluted with ultrapure water (18.2 MΩ cm at 25°C, Direct-Q 3, Merck-Millipore, Germany) up to 10 mL and analyzed with a flame atomic absorption spectrometer (AAnalyst 200, PerkinElmer, Waltham, MA, USA).

Hg concentrations were measured with a cold vapour atomic absorption spectrometer (MA-2, Nippon Instruments Corporation) in 100 μL of each semen sample (with two repetitions).

The validity of the whole procedure was checked against the certified reference materials (CRM). Spikes and control samples were run every 10 analyses. The final results were presented as μg of metal per 1 mL of the sample (Table 1).

**Statistical analysis**

The data fulfilled the assumptions of parametric analyses (based on the results of the Levene and Shapiro-Wilk tests). Factorial ANOVA was then used to evaluate the influence of smoking, alcohol intake and dietary supplementation on the above-mentioned variables. The results were presented as mean ± standard deviation (SD). The relationships between variables were evaluated with *r* Pearson correlation coefficients. The significance level in all the analyses was set as 0.05. All the calculations were done on Microsoft Excel 2016 for Mac (Microsoft) and Statistica 12 (StatSoft).

Table 1

Characteristics of the analytical method used: limits of quantification (LoQ) established for semen samples; recoveries for certified reference materials (CRM)* with relative standard deviation (RSD) of 10 replicates

| Metal | Wavelength $\lambda$ (nm) | LoQ (mg $L^{-1}$) | CRM | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| Ca | 422.7 | 0.514 | SRM1577b | 108.2 | 6.6 |
| Cd | 228.8 | 0.010 | ERMCE195 | 91.6 | 4.3 |
| Cu | 324.8 | 0.035 | SRM1577b | 99.6 | 2.7 |
| Fe | 248.3 | 0.415 | SRM1577b | 101.6 | 6.2 |
| Hg | 253.7 | 0.208** | BCR463 | 97.1 | 2.6 |
| Pb | 217.0 | 0.107 | ERMCE195 | 93.7 | 6.2 |
| Zn | 213.9 | 0.024 | SRM1577b | 106.2 | 5.3 |

* no human semen CRM was found on the market, so other biological CRMs were used
** LoQ value for Hg expressed as a ng per sample

# RESULTS

Three factor ANOVA revealed that contrarily to supplementation, smoking and alcohol intake have an insignificant impact on most variables tested. We thus focused on the influence of dietary supplements on semen parameters.

### Metal concentrations

The general ascending order of metal concentrations was as follows Pb<Hg<Cd<Cu<Fe<Zn<Ca for subjects who took supplements and Hg<Pb<<Cd<Cu<Fe<Zn<Ca for subjects who did not. Taking supplements slightly increased levels of most elements in the seminal plasma (Figure 1). At the same time, concentrations of Hg and Pb seemed to decrease. Nevertheless, a significant impact of taking supplements was observed solely in the case of Cu concentration in the seminal plasma (0.076 vs. 0.127 μg $mL^{-1}$, $p = 0.019$). A positive relationship between the concentrations of Fe and Ca ($r = 0.568$), and between the Hg concentrations and the percentage of spermatozoa with the retention of cytoplasmic droplets ($r = 0.421$) was noted. A negative relationship between spermatozoa motility D and Cd concentrations ($r = -0.441$) in the seminal plasma was also observed.

### Morphological parameters

Taking supplements has a statistically significant impact only on total morphological changes (12.32 vs. 15.48%, $p = 0.027$). In the case of other parameters, only trends (not statistically significant) were noticed (Table 2).

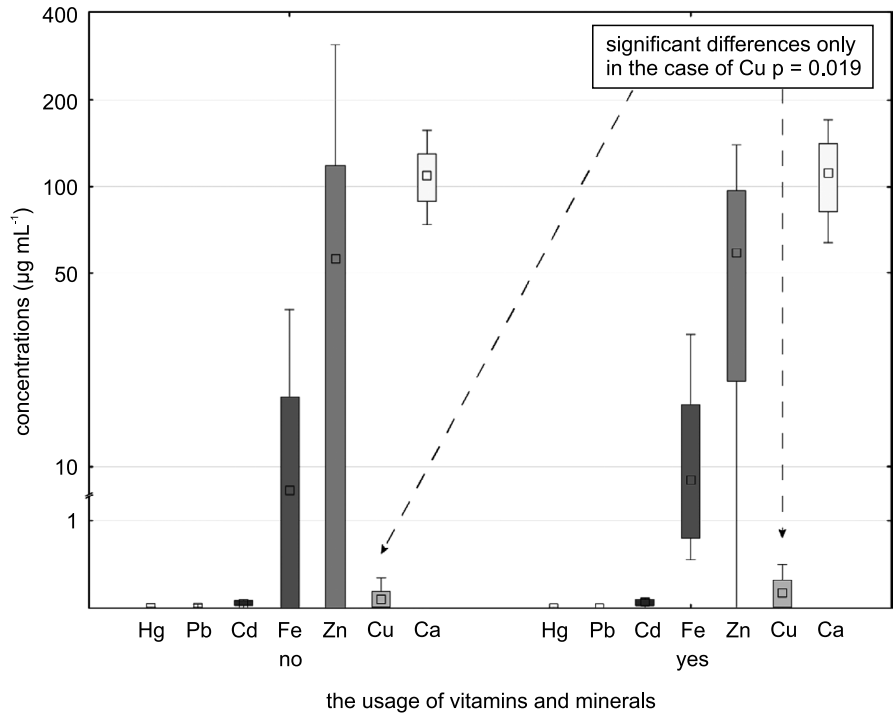


Fig. 1. Comparison of metal concentrations (expressed on a logarithmic scale) in the seminal plasma of men who took vitamin supplements ($n$ = 18) and those who did not ($n$ = 30)

Positive relationships between the percentage of total morphological changes and several other parameters, such as the percentage of spermatozoa with broken flagellum ($r$ = 0.715), the percentage of spermatozoa with twisted flagellum ($r$ = 0.705) and the percentage of spermatozoa presented with a flagellum ball ($r$ = 0.545) were observed. Negative relationships between spermatozoa motility A and D ($r$ = -0.737), between spermatozoa motility B and D ($r$ = -0.535), and between spermatozoa motility B and number of round forms ($r$ = -0.447) were also found.

## Biochemical parameters

A statistically significant impact of taking supplements was observed for SOD activity (5.44 vs. 4.22 U $mL^{-1}$, $p$ = 0.02), total protein content (29.29 vs. 37.14 g $L^{-1}$, $p$ < 0.001) and U content (4.45 vs. 3.7 mmol $L^{-1}$, $p$ = 0.023). For other parameters only trends (not statistically significant) were noticed (Table 3). SOD activity in the seminal plasma was lower in men taking supplements. A similar trend was observed in the case of ALB content and U content in the seminal plasma. Contrarily, a higher content of BLB, UA and total protein in the seminal plasma was observed in cases where supple-

Table 2

Characteristics of morphological parameters of semen samples. Data for patients who took vitamin supplements over data for patients who did not, followed by a statistical comparison (three-way ANOVA)

| Specification | Mean | Min | Max | SD | Suppl. *p* | Alcohol *p* | Smoking *p* |
|---|---|---|---|---|---|---|---|
| Ejaculate volume (mL) | 4.04 | 1.50 | 14.50 | 2.47 | 0.454 | 0.736 | 0.188 |
| | 3.75 | 1.80 | 7.50 | 1.88 | | | |
| Round form (mln $mL^{-1}$) | 1.43 | 0.20 | 3.60 | 0.84 | 0.216 | 0.337 | 0.339 |
| | 1.18 | 0.00 | 4.00 | 0.86 | | | |
| Spermatozoa concentration (mln $mL^{-1}$) | 76.58 | 6.50 | 244.00 | 67.99 | 0.246 | 0.083 | 0.463 |
| | 64.19 | 3.20 | 216.00 | 53.15 | | | |
| Spermatozoa motility A (%) | 22.50 | 4.00 | 46.00 | 11.14 | 0.615 | 0.778 | 0.708 |
| | 20.43 | 3.00 | 36.00 | 8.95 | | | |
| Spermatozoa motility B (%) | 16.42 | 9.00 | 24.00 | 4.36 | 0.485 | 0.49 | 0.283 |
| | 17.92 | 3.00 | 36.00 | 7.12 | | | |
| Spermatozoa motility C (%) | 9.03 | 4.00 | 16.00 | 3.18 | 0.376 | 0.077 | 0.439 |
| | 11.08 | 0.00 | 32.00 | 6.08 | | | |
| Spermatozoa motility D (%) | 50.39 | 16.00 | 78.00 | 16.17 | 0.836 | 0.358 | 0.428 |
| | 50.50 | 25.00 | 78.00 | 11.27 | | | |
| Separated flagellum (%) | 1.42 | 0.06 | 2.25 | 0.57 | 0.565 | 0.906 | 0.29 |
| | 1.21 | 0.00 | 2.05 | 0.62 | | | |
| Broken flagellum (%) | 5.69 | 2.65 | 8.36 | 1.65 | 0.148 | 0.136 | 0.174 |
| | 4.91 | 0.92 | 8.01 | 2.10 | | | |
| Torso (%) | 0.86 | 0.14 | 1.99 | 0.64 | 0.358 | 0.63 | 0.322 |
| | 0.70 | 0.13 | 1.87 | 0.47 | | | |
| Twisted flagellum (%) | 4.45 | 1.23 | 8.45 | 1.94 | 0.106 | 0.198 | 0.642 |
| | 3.12 | 0.00 | 8.21 | 2.43 | | | |
| Flagellum ball (%) | 2.91 | 1.03 | 7.89 | 1.65 | 0.578 | 0.455 | 0.531 |
| | 2.48 | 0.00 | 7.22 | 1.55 | | | |
| Retention of cytoplasmatic drop (%) | 0.15 | 0.00 | 1.02 | 0.22 | 0.515 | 0.371 | 0.138 |
| | 0.18 | 0.00 | 0.78 | 0.21 | | | |
| Total morphological changes (%) | 15.48 | 8.01 | 25.87 | 4.20 | <u>0.027</u> | 0.054 | 0.796 |
| | 12.32 | 4.94 | 21.07 | 4.03 | | | |

Underlining indicates statistically significant differences.

ments were taken. Correlations were observed between the content of ALB and BLB ($r$ = 0.638), as well as between ALB and total protein content ($r$ = 0.517) in the seminal plasma.

Table 3

Characteristics of biochemical parameters of semen samples. Data for patients who took vitamin supplements over data for patients who did not, followed by a statistical comparison (three-way ANOVA)

| Parameters | Mean | Min | Max | SD | Suppl. *p* | Alcohol *p* | Smoking *p* |
|---|---|---|---|---|---|---|---|
| SOD (U $mL^{-1}$) | 4.22 | 2.25 | 7.25 | 1.32 | <u>0.020</u> | 0.419 | 0.847 |
| | 5.44 | 2.50 | 10.50 | 1.86 | | | |
| Uric acid (μmol $L^{-1}$) | 118.73 | 46.73 | 221.19 | 50.55 | 0.534 | 0.467 | 0.574 |
| | 112.26 | 31.15 | 320.88 | 56.87 | | | |
| Albumin (g $L^{-1}$) | 5.00 | 3.50 | 7.63 | 1.08 | 0.972 | 0.373 | 0.328 |
| | 5.14 | 3.45 | 7.21 | 1.01 | | | |
| Total protein (g $L^{-1}$) | 37.14 | 18.70 | 60.54 | 10.39 | <u>≤0.001</u> | 0.091 | 0.1 |
| | 29.29 | 18.34 | 37.68 | 5.82 | | | |
| Urea (mmol $L^{-1}$) | 3.70 | 2.60 | 5.50 | 0.85 | <u>0.023</u> | 0.943 | 0.333 |
| | 4.45 | 2.30 | 6.30 | 0.97 | | | |
| Bilirubin (μmol $L^{-1}$) | 3.87 | 1.60 | 9.48 | 1.78 | 0.521 | 0.471 | 0.109 |
| | 3.72 | 1.90 | 7.99 | 1.34 | | | |

Underlining indicates statistically significant differences.

# DISCUSSION

In our study, we found that alcohol consumption and smoking had no significant impact on semen quality, while dietary supplements had significant effects on the semen parameters measured. Mean values of most morphological semen parameters were higher in men who took supplements. The mean concentrations of micro- and macronutrients were also higher in these men. We observed no such trends in the case of biochemical parameters.

### Metal concentrations

Due to the fact that human semen is noninvasive in collection, it has become a popular matrix in which biomarkers of exposure and subsequent infertility are measured (Thornton et al. 2002). Mean concentrations of most of the metals tested, with the exception of Fe, were lower in both groups studied when compared to our previous study (Slivkova et al. 2009). The administration of minerals may lead to an increase of these elements in the body fluids, which is probably the reason we observed slightly higher levels of micro- and macronutrients in the seminal plasma when supple-

ments were taken. More importantly, lower concentrations of Hg and Pb when supplements were taken was observed, which is consistent with studies on substances with known antioxidant properties (Al-Attar 2012, Garcia, Pedraza-Chaverri 2014). Their action against xenobiotic metal accumulation and toxicity is connected with their ability to scavenge free radicals, to reduce lipid peroxidation and to chelate metals (Sharma et al. 2014). As such, the slightly higher Cd concentration in men taking extra supplements we recorded was surprising.

In our study, a strong positive correlation between Ca and Fe concentrations in the seminal plasma was observed, which contradicts other studies that have shown that Ca can inhibit Fe absorption independently of sources. The Ca effects on Fe nevertheless rely on the duration of the taking of mineral supplements (Lönnerdal 2015). Retention of cytoplasmic droplets is associated with disturbances in spermatozoa maturation, which may be evoked by Hg-catalyzed OS (Ibrahim 2015). This may provide an explanation to the medium strong correlation between Hg level in the seminal plasma and the percentage of spermatozoa with cytoplasmic droplet retention observed in this study. The action of Cd on spermatozoa is connected with the inhibition of microtubules sliding, which play a key role in the flagellar motion. A medium strong and negative relationship between the number of immotile spermatozoa and Cd concentrations in the seminal plasma were unexpected (Oliveira et al. 2009).

**Morphological parameters**

Since vitamins act as important elements in the sperm antioxidant mechanisms, membrane protectants against ROS, and maintaining proper spermatogenesis, the taking of vitamin supplements may improve semen parameters (Ogli et al. 2009, Cheah, Yang 2011). Our results agree with studies reporting an increase in semen quality after treatment with vitamins (Ayinde et al. 2012). Furthermore, minerals such as Cu, Fe, Mg, Mn and Zn which act as cofactors for mitochondrial enzymes are believed to enhance spermatozoa motility (Singh et al. 2010). Interestingly, we observed a higher percentage of total morphological changes in men taking supplements, which is inconsistent with the literature available (Young et al. 2008). To take vitamin supplements to excess may adversely affect the spermatogenesis rate, thus contributing to the occurrence of morphological changes (Ogli et al. 2009). Positive correlations between several of the morphological parameters of spermatozoa that indicate their abnormalities is in accordance with other reports investigating the effect of trace elements on rabbit semen (Lukáč et al. 2009).

**Biochemical parameters**

We expected that dietary supplementation would increase the SOD activity (Zeitoun, Al-Damegh 2015). We observed a decreased SOD activity,

however, which may be explained by the assumption that antioxidant supplements may take over the function of antioxidant enzymes to oppose ROS. Their activity is thus lower than expected. This hypothesis, however, needs further examination.

The lower ALB content in men who take additional supplements may be a result of a low protein and amino acid diet, as well as the activity of hormones that reduce the ALB synthesis. Since we know that the total protein content depends on the albumin/globulin ratio, a higher content of total proteins in men taking supplements should correlate with a higher concentration of globulin in their seminal plasma (Attia et al. 2017). UA and BLB represent a part of non-enzymatic antioxidant defense mechanisms and play physiological roles in stifling free radicals (Aliahmat et al. 2012). Higher concentrations of UA and BLB in the seminal plasma of men who take dietary supplements may be due to improvements in their antioxidant properties through the addition of natural antioxidants to the diet (Pushpakiran et al. 2004). Last but not least, a positive relationship between ALB and BLB content may be a derivative of their bonding properties in blood fractions (Singh et al. 2011).

## CONCLUSIONS

Administration of vitamins and minerals may positively affect semen quality in men with fertility problems. Nevertheless, this impact was statistically significant only in the case of a few parameters. We suggest that among people with reproductive dysfunction taking supplements should be the first step in enhancing semen quality. Moreover, the monitoring of daily habits, which may have an association with semen quality seems to be essential for clinical practice.

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