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ORIGINAL ARTICLE

Sperm Biology

# Serum amyloid P component: a new biomarker for low sperm concentration?

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Serum amyloid P component (SAP) is present in seminal plasma, on spermatozoa, and in different tissues of the male reproductive tract, but its function is not known. The aims of this study were to determine if the concentration of SAP in seminal plasma is associated with commonly assessed semen parameters and to investigate if SAP could be a new, indirect biomarker for these parameters. In a cross-sectional study of 203 young volunteers, the concentration of SAP in seminal plasma was measured with an in-house developed enzyme-linked immunosorbent assay. Scatter plots, Pearson's correlation coefficients ( $r$ ), and linear regression models were produced, and SAP showed a statistically significant correlation with sperm concentration ( $r = 0.75$ ), sperm number ( $r = 0.68$ ), semen volume ( $r = -0.19$ ), progressive sperm motility ( $r = 0.24$ ), and sperm immotility ( $r = -0.20$ ). When the study group was dichotomized, SAP could be used to discriminate samples with a sperm concentration  $< \geq 5 \times 10^6 \text{ ml}^{-1}$ ,  $15 \times 10^6 \text{ ml}^{-1}$ , or  $40 \times 10^6 \text{ ml}^{-1}$ , and in receiver operating characteristic curves, the corresponding areas under the curves were 0.97, 0.93, and 0.82, respectively, with  $P < 0.001$  for all three cutoff values studied. The concentration of SAP in seminal plasma showed a strong, positive correlation with the concentration of spermatozoa in semen. SAP may be used as a new indirect potential biomarker for sperm concentration in fresh and in frozen, stored samples. In addition, it is envisaged that the assay could be developed into a home fertility test to differentiate between a low and a normal sperm concentration.

Asian Journal of Andrology (2021) 23, 450–455; doi: 10.4103/aja.aja\_7\_21; published online: 12 March 2021

**Keywords:** fertility; reproduction; seminal plasma; serum amyloid P component; sperm

## INTRODUCTION

In a recent study conducted in Great Britain, 1 out of 8 women and 1 out of 10 men reported that they had experienced infertility, *i.e.*, one year or more of unprotected intercourse without conceiving.<sup>1</sup> In up to 50% of cases, a male factor causing infertility can be identified.<sup>2</sup> A cornerstone in the examination of infertile men is the analysis of semen. To perform these investigations, however, several preanalytical requirements must be fulfilled. These include fresh samples, an abstinence time of 2–7 days, the sample storage temperature should be controlled, the investigation of the sample should commence within one hour, and to correct for large intraindividual variations, at least two samples should be analyzed.<sup>3</sup> According to the most recent World Health Organization (WHO) semen analysis manual,<sup>3</sup> the lower reference limits for standard semen parameters for fertile men are 1.5 ml for semen volume,  $15 \times 10^6 \text{ ml}^{-1}$  for sperm concentration,  $39 \times 10^6$  for sperm number, 4% normal forms for sperm morphology, 58% live spermatozoa for vitality, and 32% for progressive motility (PR) or 40% for total motility (PR and nonprogressive).<sup>3</sup> A large overlap in semen parameter values has been observed between fertile and infertile men.<sup>4</sup> Bonde *et al.*<sup>5</sup> showed an increased probability of conception with an increase in sperm concentration up to  $40 \times 10^6 \text{ ml}^{-1}$ . Similarly, Slama *et al.*<sup>6</sup> showed a shorter time to pregnancy at a sperm concentration above  $55 \times 10^6 \text{ ml}^{-1}$ . As a consequence of the reports that large interlaboratory discrepancies exist in semen analyses,<sup>7,8</sup> the

WHO manual<sup>3</sup> also addresses quality assurance issues. Additional biomarkers for improved diagnosis and prediction of outcome of treatment are imperative. In addition, the assays should have few preanalytical requirements and be easy to perform and standardize.

Serum amyloid P component (SAP) is a protein present in seminal plasma with a concentration of around  $1 \text{ mg l}^{-1}$ .<sup>9</sup> In serum, however, the level of SAP in men is around  $40 \text{ mg l}^{-1}$ .<sup>10</sup> SAP is also present on the sperm tail and in tissues from the male genital tract.<sup>11</sup> Together with C-reactive protein (CRP), SAP is a member of the pentraxin family and both are plasma proteins produced by the liver.<sup>10,12</sup> SAP is a 25-kDa glycoprotein with calcium-dependent ligand-binding properties; five subunits are noncovalently associated in a pentameric disc and two such discs can interact face-to-face.<sup>12,13</sup> Owing to the capacity of SAP to interact with, *e.g.*, complement (binding to C1q and to the Fcγ-receptor), apoptotic cells, certain bacteria, and nuclear structures, suggested functions for SAP include a role in innate immunity, inflammation, and autoimmune disease.<sup>12,14,15</sup>

Immunohistochemical staining of SAP present on spermatozoa in the testis, epididymis, and in the ejaculate, together with its presence in tissues from the male reproductive tract, led us to develop an enzyme-linked immunosorbent assay (ELISA) able of measuring SAP in seminal plasma.<sup>11</sup> During the development of our SAP-ELISA, we observed that some seminal plasma samples had lower SAP values and these samples also had low sperm concentrations. Therefore, the

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Received: 10 July 2020; Accepted: 22 December 2020

aims of this study were to (i) evaluate the association between seminal SAP levels and the values of established semen parameters used in the investigation of infertility, *i.e.*, sperm concentration, semen volume, sperm number, and sperm motility; and (ii) investigate if SAP could be a new, indirect biomarker for some of these parameters. To allow for comparison, three other analytes that are markers of accessory sex gland function and present in seminal plasma were also studied: fructose, total prostate-specific antigen (tPSA), and zinc.<sup>16</sup>

## PARTICIPANTS AND METHODS

### Participants

The study group has previously been described and included originally 305 military conscripts.<sup>17</sup> In 2000, both semen and blood samples were collected. Following an abstinence period of 48–72 h, the participants were requested to provide a semen sample. In each case, the length of the abstinence period was recorded. Approval of the study had been obtained from the Research Ethical Board of Lund University, Lund, Sweden (approval number LU 385-99), and all subjects provided signed informed consent.<sup>17</sup> Samples from 102 men were not available for the SAP analyses, 84 consecutive samples were used in other studies, and 18 random samples were excluded owing to insufficient sample volume. The 203 men studied had a mean age of 18.2 (standard deviation [s.d.]: 0.4) years, a mean body mass index (BMI) of 22.6 (s.d.: 3.4) kg m<sup>-2</sup>, and a mean abstinence time of 83.6 (s.d.: 52.4) h. No information on the fertility status of these men existed. No statistically significant differences in age, BMI, abstinence time, sperm concentration, sperm number, or sperm motility were observed between the 203 included men and the 102 excluded men. The included men, however, had statistically significant higher semen volumes (mean 3.4 ml) than the excluded men (2.9 ml).<sup>9</sup>

On the basis of three cutoff values for sperm concentration, the subjects were also divided into two subgroups: severe oligozoospermia, as defined in different studies<sup>18,19</sup> ( $<5 \times 10^6$  spermatozoa per ml [ $n = 11$ ]; and  $\geq 5 \times 10^6$  spermatozoa per ml [ $n = 192$  for SAP;  $n = 190$  for fructose, tPSA and zinc]), the WHO reference value<sup>3</sup> ( $<15 \times 10^6$  spermatozoa per ml [ $n = 27$ ]; and  $\geq 15 \times 10^6$  spermatozoa per ml [ $n = 176$  for SAP;  $n = 174$  for fructose, tPSA and zinc]) and the threshold between subfertile and fertile described by Bonde *et al.*<sup>5</sup> ( $<40 \times 10^6$  spermatozoa per ml [ $n = 79$ ]; and  $\geq 40 \times 10^6$  spermatozoa per ml [ $n = 124$  for SAP;  $n = 122$  for fructose, tPSA and zinc]).

### Semen sample preparation

The procedure for semen analysis of the samples from the study group has been previously described.<sup>17</sup> Sperm concentration was assessed with positive displacement pipettes and a modified Neubauer chamber. Seminal plasma was studied after allowing the semen samples to liquefy for at least 30 min at room temperature. The ejaculates were mixed with the protease inhibitor benzamidine, final concentration 10 mmol l<sup>-1</sup> (Merck, Darmstadt, Germany). The samples were centrifuged for 20 min at 4500g (Hettich, Tuttlingen, Germany), then they were decanted and stored at -20°C pending analyses.<sup>17</sup>

### SAP-ELISA

The concentration of SAP in the seminal plasma samples was determined with an in-house developed sandwich ELISA that has been described and validated.<sup>9</sup> Briefly, SAP purified from a pool of human serum was used as calibrator, and its concentration was determined by acid hydrolysis.<sup>9</sup> A commercially available polyclonal anti-SAP antibody (A0302, Dako, Santa Clara, CA, USA) was used as both primary and secondary antibody. For detection, the secondary

antibody was biotinylated, and a streptavidin-ABCComplex/Horse Radish Peroxidase solution (Dako) was added followed by a peroxidase substrate (o-phenylenediamine dihydrochloride [Dako]). The reaction was quenched with 0.5 mol l<sup>-1</sup> sulfuric acid (Merck) and the absorbance was determined at 490 nm on an Emax precision microplate reader (Molecular devices, San Jose, CA, USA). The intra-assay coefficient of variation (CV) was 7.4% (at 5.0 mg l<sup>-1</sup>) and 4.4% (at 17.2 mg l<sup>-1</sup>) and the inter-assay CV was 22.6% (at 3.1 mg l<sup>-1</sup>) and 12.6% (at 19.4 mg l<sup>-1</sup>).<sup>9</sup>

### Analyses of fructose, tPSA, and zinc

Analyses of fructose, tPSA, and zinc have been previously performed ( $n = 201$ , missing = 2) and described.<sup>20</sup> Briefly, fructose was measured with a spectrophotometric method (Beckman Synchron LX20, Brea, CA, USA) and the inter-assay CV was 5% (at 12.7 mmol l<sup>-1</sup>); tPSA was measured with the Delfia™ method (Wallac Oy, Turku, Finland) and the inter-assay CV was 12% (at 660 mg l<sup>-1</sup>); and zinc was measured with a colorimetric method (Beckman Synchron LX20) and the inter-assay CV was 7% (at 2.0 mmol l<sup>-1</sup>).<sup>20</sup>

### Statistical analyses

Initially, scatter plots were performed to determine visually whether the assumption of linearity was reasonable for the associations between the concentration of SAP in seminal plasma and sperm concentration, sperm number, semen volume, and sperm motility (progressive sperm motility, nonprogressive sperm motility, and immotile spermatozoa). As linear associations were observed, Pearson's correlation coefficients ( $r$ ) for pairwise comparisons were then used. In addition, linear regression analyses were performed to generate  $\beta$ -coefficients (corresponding to the units of increase or decrease per 1 mg l<sup>-1</sup> increase in SAP) with 95% confidence intervals (CI), and  $P$  values and the fraction of explained variance (adjusted  $r^2$ ) were also presented. Moreover, in the multivariate models, the following potential confounders were simultaneously included: BMI, abstinence time, and smoking. Model assumption was assessed by residual analyses. To ensure the robustness of the results, the five individuals (arbitrary number) with the highest SAP concentrations were excluded and the analyses were repeated.

Box plots were used to illustrate the distributions among the subgroups and the Mann-Whitney U test was performed to determine statistically significant differences between the subgroups.  $P < 0.05$  was defined as statistically significant.

All samples were dichotomized on the basis of sperm concentration at three cutoff values:  $5 \times 10^6$  cells per ml,  $15 \times 10^6$  cells per ml, and  $40 \times 10^6$  cells per ml, and the concentration of SAP was evaluated as a discriminator to identify samples as  $<$  or  $\geq$  the respective cutoff value, using receiver operating characteristic (ROC) curves with a corresponding area under the curve (AUC), 95% CI, and  $P$  values. In addition, sensitivity and specificity were calculated. To enable comparison with SAP, corresponding analyses were also performed with the fructose, tPSA, and zinc data. IBM SPSS Statistics, version 24 (IBM Corporation, New York, NY, USA) was used for statistical analyses.

## RESULTS

### SAP levels and semen parameters

There were statistically significant associations between the concentration of SAP in seminal plasma and sperm concentration ( $r = 0.75$ ;  $P < 0.001$ ) and between the concentration of SAP and sperm number ( $r = 0.68$ ;  $P < 0.001$ ) as shown in **Figure 1** and **Table 1**. In the regression models, an increase of 1 mg l<sup>-1</sup> in SAP corresponded to an increase in sperm concentration of  $51.8 \times 10^6$  per ml (explained variance 0.56) and an increase in sperm number of  $143 \times 10^6$  (explained variance 0.45). Although statistically

significant, the correlations were lower between the concentration of SAP and semen volume ( $r = -0.19$ ;  $P = 0.007$ ) and sperm motility (PR:  $r = 0.24$ ,  $P = 0.001$ ; nonprogressive motility:  $r = -0.15$ ,  $P = 0.03$ ; and immotility:  $r = -0.20$ ,  $P = 0.004$ ). In the adjusted models, the effect estimates ( $\beta$ -values) between SAP concentration and semen volume were changed by 13.1%, whereas for the other semen parameters (sperm concentration, sperm number and sperm motility), the estimates were changed by <6.5%.

#### SAP levels and correlations with markers of accessory sex gland function

The correlations between the concentration of SAP and the concentrations of markers of accessory sex gland function were all statistically significant (tPSA:  $r = 0.27$ ,  $P < 0.001$ ; fructose:  $r = -0.16$ ,  $P = 0.024$ ; and zinc:  $r = 0.28$ ,  $P < 0.001$ ).

#### Fructose, tPSA, and zinc concentrations and semen parameter

Statistically significant correlations were observed between sperm concentration and the concentration of fructose ( $r = -0.22$ ,  $P = 0.001$ ), tPSA ( $r = 0.35$ ,  $P < 0.001$ ), and zinc ( $r = 0.36$ ,  $P < 0.001$ ), as shown in **Supplementary Figure 1a–1c**. When simultaneously adjusted for smoking, BMI, and abstinence time, an increase in fructose of 1 mmol l<sup>-1</sup> corresponded to a decrease in sperm concentration of  $2.1 \times 10^6$  (95% CI:  $-3.4 \times 10^6$  to  $-0.8 \times 10^6$ ) ml<sup>-1</sup>. Correspondingly, an increase in tPSA of 1 mg l<sup>-1</sup> led to an increase in sperm concentration of  $0.05 \times 10^6$  (95% CI:  $0.03 \times 10^6$  to  $0.08 \times 10^6$ ) ml<sup>-1</sup>; and an increase in zinc of 1 mmol l<sup>-1</sup> also resulted in an increase in sperm concentration of  $17 \times 10^6$  (95% CI:  $8.2 \times 10^6$  to  $26.2 \times 10^6$ ) ml<sup>-1</sup>. In addition, tPSA and zinc concentrations were associated with sperm number (tPSA:  $r = 0.26$ ,  $P < 0.001$ ; and zinc:  $r = 0.30$ ,  $P < 0.001$ ) and fructose and tPSA were associated with semen volume (fructose:  $r = 0.23$ ,  $P = 0.001$ ; and tPSA:  $r = -0.23$ ,  $P = 0.001$ ). None of the three analytes showed statistically significant correlations with the sperm motility parameter values. However, none of the regression models including fructose, tPSA, and zinc explained more than 16% of the variance in the semen parameters.

#### Associations when the study group was dichotomized on the basis of sperm concentration

Irrespective of which cutoff value of sperm concentration that was studied, men with a sperm concentration lower than the cutoff level had statistically significant lower SAP concentrations than men with a sperm concentration higher than or equal to the cutoff value,  $P$  values for all three cutoff levels were <0.001 (**Figure 2a–2c**). With respect to fructose, tPSA, and zinc concentrations, the association varied with the sperm concentration cutoff value studied (**Supplementary Figure 2a–2c, 3a–3c, and 4a–4c**). No statistically significant differences were apparent when the lowest cutoff value ( $5 \times 10^6$  spermatozoa per ml) was investigated.

For the cutoff value of  $15 \times 10^6$  spermatozoa per ml, men with a sperm concentration below this cutoff value had a higher fructose concentration as compared to men with a sperm concentration above this cutoff value ( $18.2$  mmol l<sup>-1</sup> vs  $14.3$  mmol l<sup>-1</sup>;  $P = 0.007$ ), and this was also seen for the cutoff value of  $40 \times 10^6$  spermatozoa per ml ( $16.5$  mmol l<sup>-1</sup> vs  $13.8$  mmol l<sup>-1</sup>;  $P = 0.025$ ).

tPSA and zinc showed statistically significant differences only for the highest cutoff value studied ( $40 \times 10^6$  spermatozoa per ml), and men with a sperm concentration below this cutoff value had both a lower tPSA concentration ( $561$  mg l<sup>-1</sup> vs  $677$  mg l<sup>-1</sup>;  $P < 0.001$ ) and a lower zinc concentration ( $1.0$  mmol l<sup>-1</sup> vs  $1.7$  mmol l<sup>-1</sup>;  $P < 0.001$ ) than men with  $\geq 40 \times 10^6$  spermatozoa per ml.

#### AUCs from ROC curves, sensitivity, and specificity of SAP

ROC curves for the concentration of SAP as a discriminator of a sperm concentration below the three cutoff values studied are presented in **Figure 2d–2f**. At all cutoff values, the AUCs were >0.82 and all were statistically significant (all  $P < 0.001$ ). The highest AUC was 0.97 and this was obtained from the lowest sperm concentration cutoff value studied ( $5 \times 10^6$  spermatozoa per ml), and at a cutoff value for SAP concentration of  $0.48$  mg l<sup>-1</sup>, the sensitivity was 100% (*i.e.*, the probability that an individual with a SAP concentration  $\leq 0.48$  mg l<sup>-1</sup> had a sperm concentration  $< 5 \times 10^6$  spermatozoa per ml) and the specificity was 84% (*i.e.*, the probability that an individual with a SAP concentration  $> 0.48$  mg l<sup>-1</sup> had a sperm concentration  $\geq 5 \times 10^6$  spermatozoa per ml). AUCs and corresponding 95% CIs and  $P$  values, along with the combination of highest sensitivity and specificity for all three cutoff values in sperm concentration, are presented in **Table 2**.

#### AUCs from ROC curves of fructose, tPSA, and zinc

ROC curves for the concentration of fructose, total PSA, and zinc as discriminators of a sperm concentration  $<$  or  $\geq$  the three different cutoff values studied are presented in **Supplementary Figure 2d–2f, 3d–3f, and 4d–4f**. None of the three analytes showed statistically significant AUCs at the sperm concentration cutoff value of  $5 \times 10^6$  ml<sup>-1</sup>. At the sperm concentration cutoff value of  $15 \times 10^6$  ml<sup>-1</sup>, only fructose showed a statistically significant AUC of 0.66 (95% CI: 0.57 to 0.76,  $P = 0.007$ ). All three analytes, however, did show statistically significant AUCs at the sperm concentration cutoff value of  $40 \times 10^6$  ml<sup>-1</sup>, and the corresponding AUCs were 0.59 for fructose (95% CI: 0.51 to 0.67,  $P = 0.025$ ), 0.65 for tPSA (95% CI: 0.57 to 0.73,  $P < 0.001$ ), and 0.65 for zinc (95% CI: 0.58 to 0.73,  $P < 0.001$ ).

## DISCUSSION

In this study, we investigated the association between the concentration of SAP in seminal plasma and values of semen parameters that are commonly used in an investigation of infertility. The strong correlation observed for sperm concentration was not surprising since SAP has been detected on spermatozoa present in the testis, epididymis, and in the ejaculate.<sup>11</sup> There was also a strong, positive

**Table 1: Linear regression analyses of semen parameter values and the concentration of SAP (mg l<sup>-1</sup>;  $n = 203$ )**

Parameter	$\beta$	95% CI	$P$	$r^2$
Sperm concentration ( $\times 10^6$ ml <sup>-1</sup> )	51.8	45.4 to 58.2	<0.001	0.56
Sperm number ( $\times 10^6$ )	143	121 to 165	<0.001	0.45
Semen volume (ml)	-0.27	-0.46 to -0.07	0.007	0.03
Motility, PR (%)	3.90	1.67 to 6.13	0.001	0.05
Motility, NP (%)	-1.37	-2.60 to -0.13	0.03	0.02
Motility, IM (%)	-2.65	-4.43 to -0.86	0.004	0.04

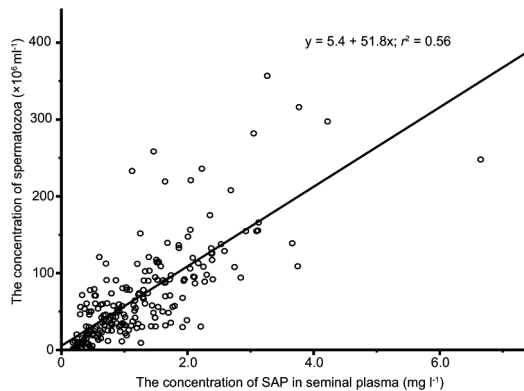
SAP: serum amyloid P component;  $\beta$ : estimated effects on semen parameters by 1 mg l<sup>-1</sup> increase in SAP; CI: confidence interval; PR: progressive motility; NP: nonprogressive motility; IM: immotile

**Table 2: Data from the receiver operating characteristic curves at the three cutoff values for sperm concentrations ( $n = 203$ )**

Cutoff values <sup>a</sup>	AUC	95% CI	$P$	Sensitivity (%)	Specificity (%)
< or $\geq 5$	0.97	0.94 to 1.00	<0.001	100 <sup>b</sup>	84 <sup>b</sup>
< or $\geq 15$	0.93	0.87 to 0.98	<0.001	89 <sup>c</sup>	83 <sup>c</sup>
< or $\geq 40$	0.82	0.76 to 0.88	<0.001	71 <sup>d</sup>	73 <sup>d</sup>

<sup>a</sup>Sperm concentration ( $\times 10^6$  ml<sup>-1</sup>); <sup>b</sup>cutoff for SAP concentration was 0.48 mg l<sup>-1</sup>; <sup>c</sup>cutoff for SAP concentration was 0.59 mg l<sup>-1</sup>; <sup>d</sup>cutoff for SAP concentration was 0.92 mg l<sup>-1</sup>. AUC: area under the curve; CI: confidence interval; SAP: serum amyloid P component



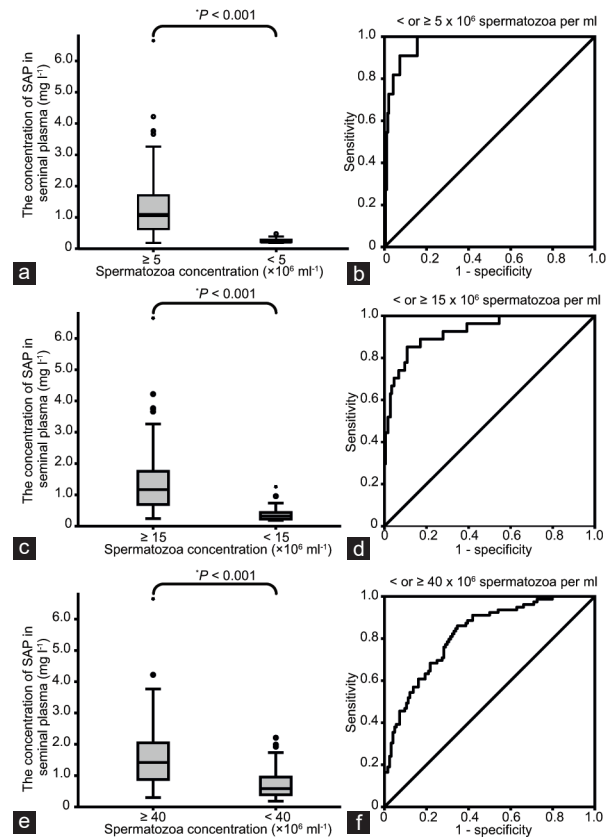


**Figure 1:** Scatter plot and linear regression analysis for the concentration of SAP in seminal plasma and sperm concentration ( $n = 203$ ). SAP: serum amyloid P component.

correlation between the concentration of SAP and total sperm number. This was not an unexpected finding, as total sperm number is determined by sperm concentration and semen volume. When smoking, BMI, and abstinence time were taken into consideration, these correlations were only marginally altered. On the basis of the sperm concentration, the study group was dichotomized in three ways related to increased probability of conception,<sup>5</sup> the reference range for sperm concentration,<sup>3</sup> and severe oligozoospermia.<sup>18,19</sup> The SAP concentration was low in samples with low sperm concentration and only a minor overlap was seen in the two lower cutoff values, but the overlap was more pronounced in the highest cutoff value. The ROC curves showed that SAP was a good discriminator of sperm concentration at all three cutoff values. SAP was a better predictor of sperm concentration at the two lower cutoff values and these AUCs were above 0.92.

None of the three markers for accessory sex gland function was correlated as highly with semen parameters as was SAP. The highest explained variance was determined for sperm concentration with the concentrations of tPSA and zinc. Once the confounders were added to the model, however, less than 16% of the variance could be explained. The box plots for fructose, tPSA, and zinc at the three sperm concentration cutoff values studied showed a much greater overlap than that for SAP at all the three cutoff levels. With respect to the AUCs that were determined from the ROC curves, the tendency was that the three analytes discriminated slightly better at the highest cutoff level for sperm concentration. Here, all three analytes showed statistically significant differences, but the AUCs were  $<0.66$ . None of these three markers were as predictive as SAP.

In another study, local production of SAP in the male reproductive tract was suggested because SAP mRNA was observed in tissue from testis, seminal vesicle, epididymis, and prostate. In addition, SAP protein was located in epithelial cells in the epididymis, prostate and seminal vesicle, and also on the tail of spermatozoa in the testis and epididymis<sup>11</sup> and also on ejaculated spermatozoa.<sup>11,21,22</sup> In the present study, the correlations between SAP and the markers of accessory sex gland function were all statistically significant, but the correlation coefficients were low. This could indicate that the majority of SAP in semen is neither coproduced with tPSA and zinc in the prostate nor with fructose from the seminal vesicles.<sup>16</sup> SAP production in testis and/or epididymis needs to be further studied. One possibility is that an



**Figure 2:** Box plots of the concentration of SAP in seminal plasma ( $\text{mg l}^{-1}$ ),  $P$  value from Mann–Whitney U test and ROC curves for the different cutoff values for sperm concentrations studied. (a) Box plot of the subgroups with  $< \text{or } \geq 5 \times 10^6$  spermatozoa per ml. (b) ROC curve of the concentration of SAP in  $< \text{or } \geq 5 \times 10^6$  spermatozoa per ml (AUC: 0.97;  $P < 0.001$ ). (c) Box plot of the subgroups with  $< \text{or } \geq 15 \times 10^6$  spermatozoa per ml. (d) ROC curve of the concentration of SAP in  $< \text{or } \geq 15 \times 10^6$  spermatozoa per ml (AUC: 0.93;  $P < 0.001$ ). (e) Box plot of the subgroups with  $< \text{or } \geq 40 \times 10^6$  spermatozoa per ml. (f) ROC curve of the concentration of SAP in  $< \text{or } \geq 40 \times 10^6$  spermatozoa per ml (AUC: 0.82;  $P < 0.001$ ). In a, c, and e, the boxes represent the 1<sup>st</sup> and 3<sup>rd</sup> quartile, the band inside the box is the median, the whiskers represent 1.5 times the box or the minimum or maximum values; if no outliers are present, the circles and stars are outliers. \* $P < 0.05$ . ROC: receiver operating characteristic; AUC: area under the curve; SAP: serum amyloid P component.

equilibrium is formed between SAP levels in seminal plasma and SAP present on spermatozoa either rapidly at ejaculation, or in the epididymis during maturation of spermatozoa or during the liquefaction of semen *in vitro* before analyses of seminal plasma.

The strengths of this study are that a large group of well-characterized, healthy, young men were studied. At the same time, this is also somewhat of a limitation because men of varying age and genetic and environmental background were not included in the cohort. Although approximately 13% of the participants had a sperm concentration lower than the WHO fertile reference range, the dichotomized group with the lowest sperm number was small (11 subjects). A further weakness was that the intraindividual variation in SAP levels could not be assessed. However, if a high level of intraindividual variation in SAP exists, this would rather reduce the predictive power of SAP analysis and cannot explain the significant AUCs reported by us. Furthermore, the samples had been stored from 2000 to 2007 at  $-20^\circ\text{C}$ . A previous study has shown that repeated freezing and thawing of samples does not lead

to a systematic decline in SAP concentration.<sup>9</sup> Nevertheless, whether storage time influences the SAP concentration has not been studied. The storage time, however, was identical for all samples and thus the assumption was made that this parameter would not influence the overall conclusions of the study.

To compare the data obtained from this study with previous work is difficult because only few such reports have been published. Nevertheless, one investigation of the proteome of a few seminal plasma samples showed that SAP levels in semen from azoospermic and postvasectomized men were lower than controls.<sup>23</sup> This is indicative of a relationship between SAP and sperm concentration and our findings are in accordance with this.

Counting spermatozoa is a laborious process that requires fresh samples and skilled technical staff to maintain a high level of quality, and the process is difficult to standardize.<sup>3</sup> Thus, the suggestion from this study is that the SAP-ELISA could be used as a complementary approach. The SAP-ELISA is based on commercially available reagents and can be easily implemented in other laboratories. In general, immunoassays are easier to standardize and automate than cell counting assays. SAP can also be measured in thawed seminal plasma samples, a major advantage in cases where there is a long distance between a patient and the fertility clinic, for example. Another possible advantage is in research studies involving multiple centers where SAP analyses can be performed in one single laboratory to minimize this variability.

There are home fertility tests available on the market to evaluate semen samples for sperm concentration, motility, and viability.<sup>19,24</sup> One test is based on an antibody against an acrosome protein called SP-10. For 96% of the samples, this test correctly identified the sperm concentration as  $\geq 20 \times 10^6$  spermatozoa per ml,  $5 \times 10^6$  to  $20 \times 10^6$  spermatozoa per ml, or  $< 5 \times 10^6$  spermatozoa per ml.<sup>19</sup> In this study, sensitivity and specificity of 83%–100% were obtained for SAP to identify a sample as  $\geq 5 \times 10^6$  ml<sup>-1</sup> and/or  $\geq 15 \times 10^6$  ml<sup>-1</sup> in sperm concentration. Our data are promising and a home test with high performance could be developed.

Our results give rise to several questions concerning possible functions of SAP in male reproduction. Is SAP of importance for sperm production in the testes, sperm maturation in the epididymis, or protection of sperm integrity after ejaculation?

In recent years, amyloids, *i.e.*, proteins that self-assemble into cross-beta-sheet rich structures, have also been suggested to perform normal biological functions, including reproduction.<sup>25</sup> Studies in mice have shown presence of functional amyloid in the acrosome and in the lumen of the epididymis, suggesting a role in the acrosome reaction and in sperm maturation.<sup>26,27</sup> SAP has a strong connection to amyloid since SAP is bound to all amyloid involved in pathological processes.<sup>28</sup> Further studies are required to address a possible connection between SAP, functional amyloid, and reproductive functions.

In summary, this study showed that the concentration of SAP in seminal plasma was correlated positively with sperm concentration and this can enable distinction between samples with a sperm concentration of  $<$  or  $\geq 5 \times 10^6$  ml<sup>-1</sup> and  $<$  or  $\geq 15 \times 10^6$  ml<sup>-1</sup> with an AUC of 0.97 and 0.93, respectively. Thus, SAP in seminal plasma is potentially a new, indirect biomarker for sperm concentration that can be easily performed and stored samples can be used which is applicable in a research setting.

#### AUTHOR CONTRIBUTIONS

AG was involved in enrolling the participants, and was responsible for the funding of the semen analyses and other initially performed analyses. AS performed the ELISA and together with LR performed

the statistical analysis. AH developed the SAP-ELISA and together with JM, LR, and AG designed the research study. AS prepared the draft of the manuscript except the result section which was done together with LR. All authors contributed in revising the paper critically and read and approved the final manuscript.

#### COMPETING INTERESTS

AS, JM, LR, and AH declare no competing interests. AG has received lecturing fee from Sandoz, IBSA, and Finox.

#### ACKNOWLEDGMENT

Financial support was received from Southern Health Care Region, Skane County Council's Research and Development Foundation – PhD Study Grant. Grants were also received from ReproUnion (InterregV EU Regional Fund).

Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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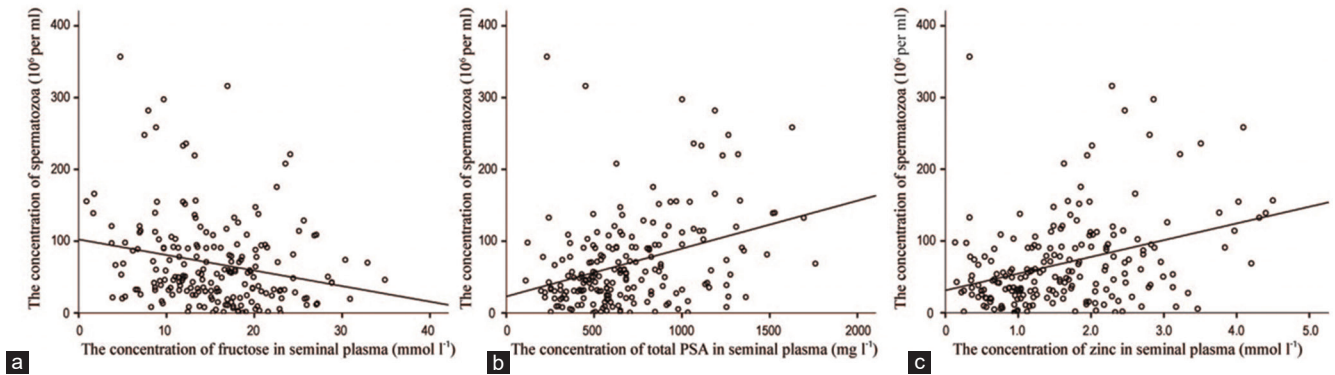
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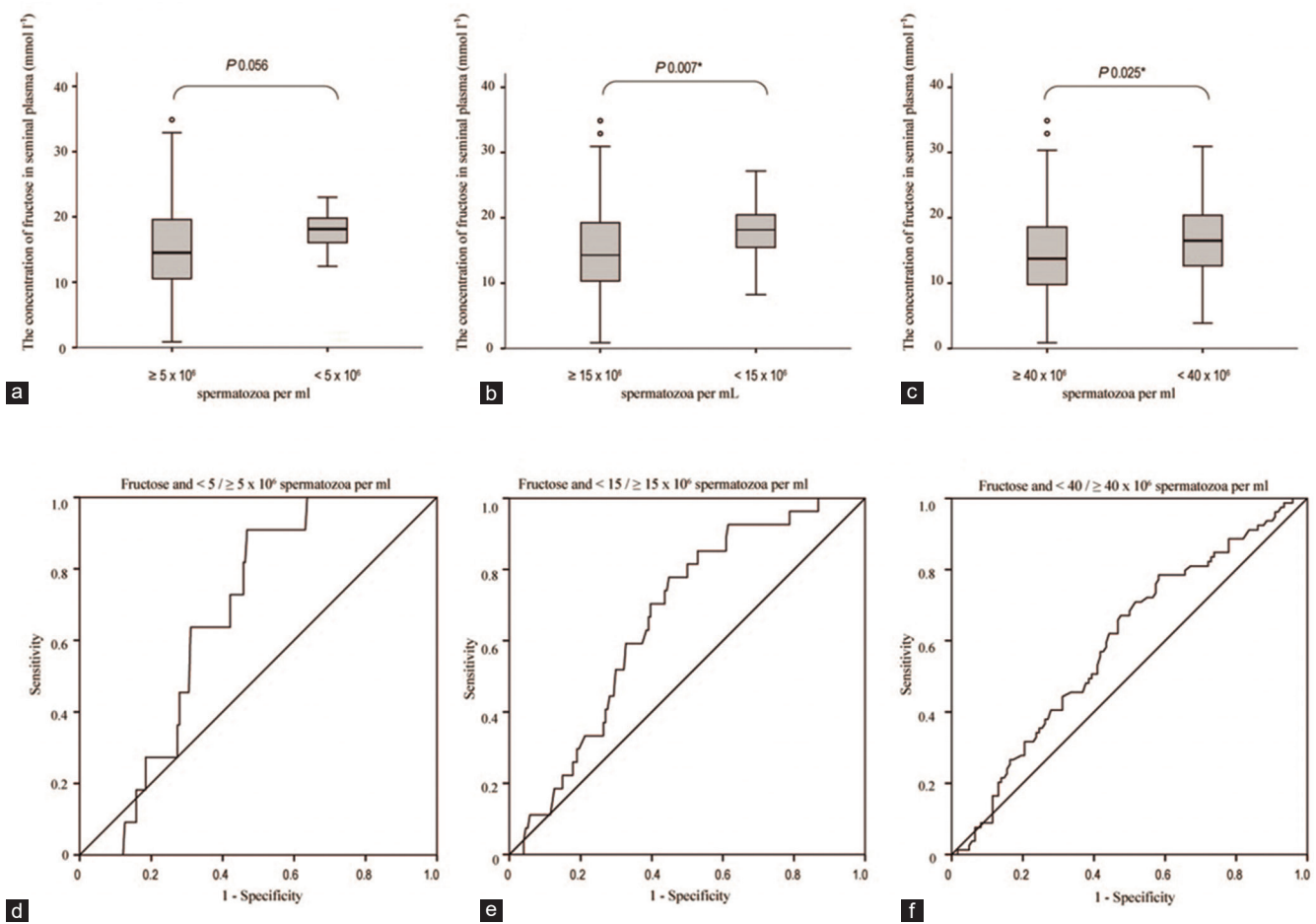
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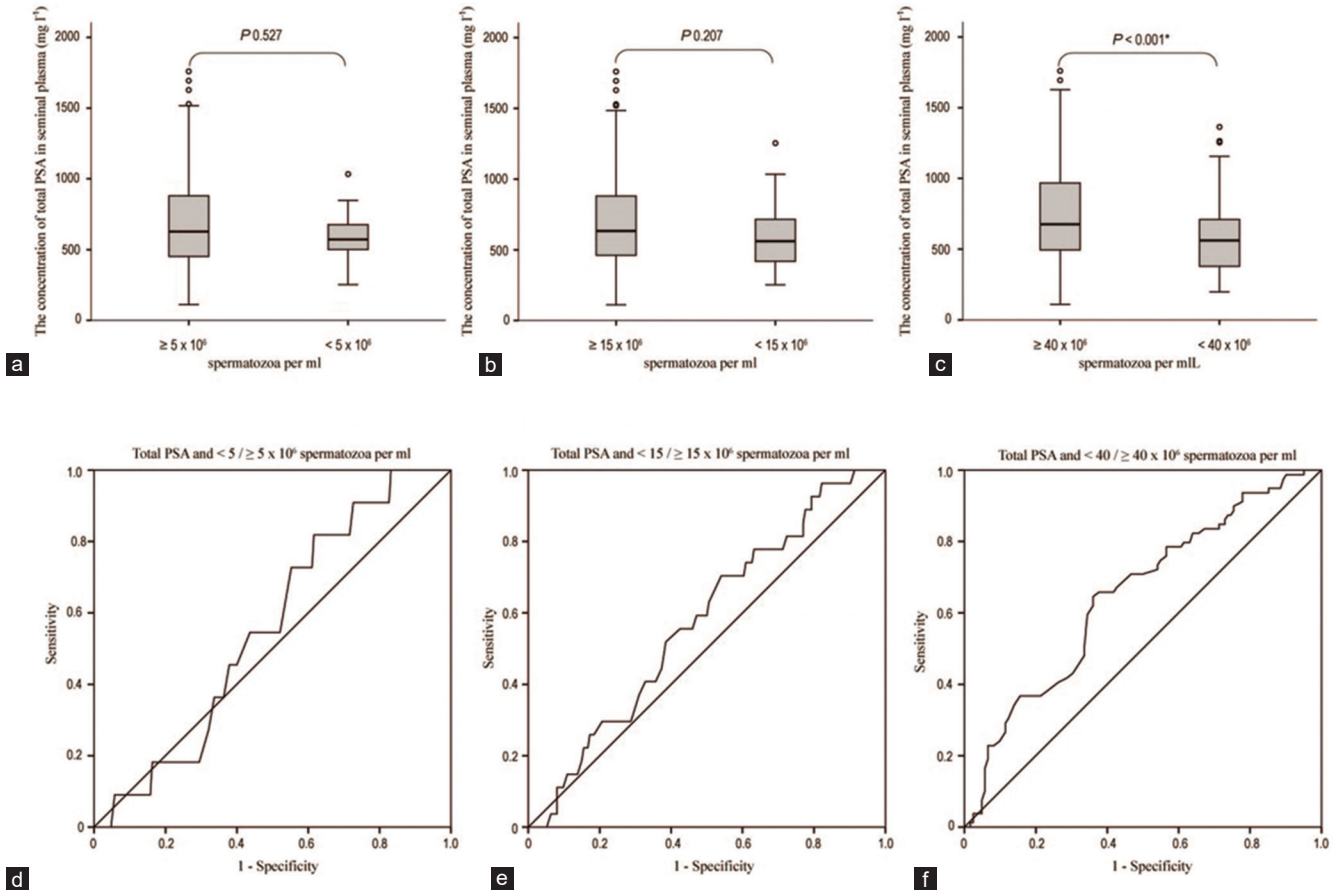




**Supplementary Figure 1:** Scatter plot and linear regression analyses for respectively the concentration of fructose, total PSA and zinc in seminal plasma and the sperm concentration ( $n = 201$ ). (a) Concentration of fructose. (b) Concentration of total PSA. (c) Concentration of zinc. PSA: prostate-specific antigen.

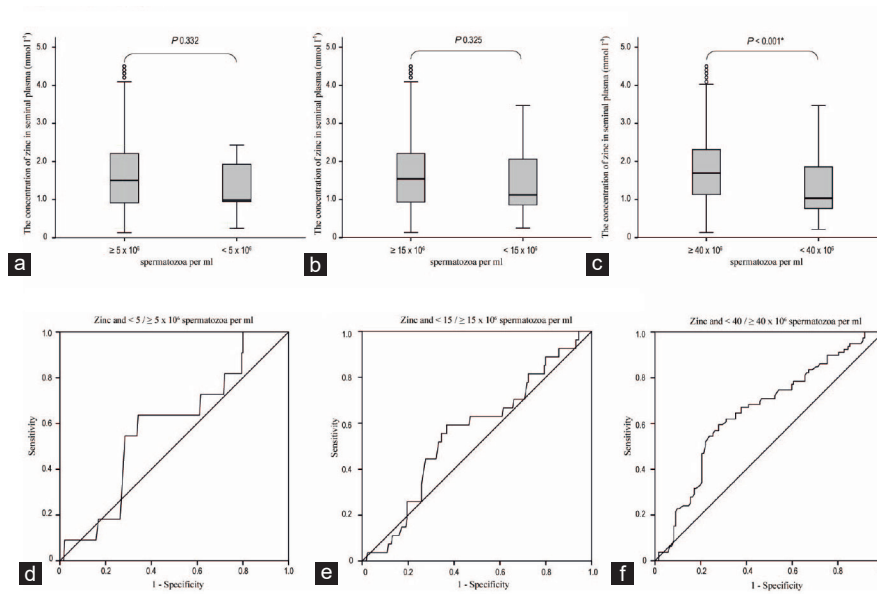


**Supplementary Figure 2:** Box plots of the concentration of fructose in seminal plasma ( $\text{mmol l}^{-1}$ ),  $P$  value from Mann–Whitney  $U$  test and ROC curves for the different cutoff values for sperm concentrations studied. (a) Box plot of the subgroups with  $<$  or  $\geq 5 \times 10^6$  spermatozoa per ml. (b) Box plot of the subgroups with  $<$  or  $\geq 15 \times 10^6$  spermatozoa per ml. (c) Box plot of the subgroups with  $<$  or  $\geq 40 \times 10^6$  spermatozoa per ml. (d) ROC curve of the concentration of fructose in  $<$  or  $\geq 5 \times 10^6$  spermatozoa per ml (AUC 0.67;  $P = 0.056$ ). (e) ROC curve of the concentration of fructose in  $<$  or  $\geq 15 \times 10^6$  spermatozoa per ml (AUC 0.66;  $P = 0.007$ ). (f) ROC curve of the concentration of fructose in  $<$  or  $\geq 40 \times 10^6$  spermatozoa per ml (AUC 0.59;  $P = 0.025$ ). In a–c, the boxes represent the 1<sup>st</sup> and 3<sup>rd</sup> quartile, the band inside the box is the median, the whiskers represent 1.5 times the box or the min or max values, if no outliers are present, the circles and stars are outliers. \* $P < 0.05$ . ROC: receiver operating characteristic; AUC: area under the curve.



**Supplementary Figure 3:** Box plots of the concentration of total PSA in seminal plasma ( $\text{mg l}^{-1}$ ),  $P$  value from Mann–Whitney U test and ROC curves for the different cutoff values for sperm concentrations studied. (a) Box plot of the subgroups with  $<$  or  $\geq 5 \times 10^6$  spermatozoa per ml. (b) Box plot of the subgroups with  $<$  or  $\geq 15 \times 10^6$  spermatozoa per ml. (c) Box plot of the subgroups with  $<$  or  $\geq 40 \times 10^6$  spermatozoa per ml. (d) ROC curve of the concentration of total PSA in  $<$  or  $\geq 5 \times 10^6$  spermatozoa per ml (AUC 0.56;  $P = 0.528$ ). (e) ROC curve of the concentration of total PSA in  $<$  or  $\geq 15 \times 10^6$  spermatozoa per ml (AUC 0.58;  $P = 0.207$ ). (f) ROC curve of the concentration of total PSA in  $<$  or  $\geq 40 \times 10^6$  spermatozoa per ml (AUC 0.65;  $P < 0.001$ ). In a–c, the boxes represent the 1<sup>st</sup> and 3<sup>rd</sup> quartile, the band inside the box is the median, the whiskers represent 1.5 times the box or the min or max values, if no outliers are present, the circles and stars are outliers. \* $P < 0.05$ . PSA: prostate-specific antigen; ROC: receiver operating characteristic; AUC: area under the curve.





**Supplementary Figure 4:** Box plots of the concentration of zinc in seminal plasma ( $\text{mmol l}^{-1}$ ),  $P$  value from Mann–Whitney U test and ROC curves in the different cutoffs for sperm concentration studied. **(a)** Box plot of the subgroups with  $<$  or  $\geq 5 \times 10^6$  spermatozoa per ml. **(b)** Box plot of the subgroups with  $<$  or  $\geq 15 \times 10^6$  spermatozoa per ml. **(c)** Box plot of the subgroups with  $<$  or  $\geq 40 \times 10^6$  spermatozoa per ml. **(d)** ROC curve of the concentration of zinc in  $<$  or  $\geq 5 \times 10^6$  spermatozoa per ml (AUC 0.59;  $P = 0.332$ ). **(e)** ROC curve of the concentration of zinc in  $<$  or  $\geq 15 \times 10^6$  spermatozoa per ml (AUC 0.56;  $P = 0.325$ ). **(f)** ROC curve of the concentration of zinc in  $<$  or  $\geq 40 \times 10^6$  spermatozoa per ml (AUC 0.65;  $P < 0.001$ ). In **a–c**, the boxes represent the 1<sup>st</sup> and 3<sup>rd</sup> quartile, the band inside the box is the median, the whiskers represent 1.5 times the box or the min or max values, if no outliers are present, the circles and stars are outliers. \* $P < 0.05$ . ROC: receiver operating characteristic; AUC: area under the curve.