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Determination of serum amyloid P component in seminal plasma and correlations with serum hormone levels in young, healthy men.

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Determination of Serum Amyloid P Component in Seminal Plasma and Correlations with Serum Hormone Levels in Young, Healthy Men

Running title: Concentration of SAP in Seminal Plasma

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Abstract

Serum amyloid P component (SAP) belongs to the pentraxin family of proteins, and it is evolutionarily conserved, and involved in amyloidosis, innate immunity, inflammation, and apoptosis. We have previously described SAP in the male reproductive tract, where it occurs in seminal fluid, on spermatozoa, and in epididymal, seminal vesicle, and prostate tissue.

In the present investigation, our aim was to characterize SAP in male reproduction. In short, we developed and evaluated an immunoassay, analyzed the concentration of SAP in seminal plasma and serum in samples from healthy men (n = 203), and studied hormonal regulation.

SAP in seminal plasma showed a positively skewed distribution and a median concentration of 1.01 mg/L (inter quartile range [IQR] 0.56–1.65 mg/L). SAP in serum had a Gaussian distribution and a median concentration of 40.5 mg/L (IQR 34.2–49.2 mg/L). Furthermore, SAP concentrations in seminal plasma were not correlated with serum concentrations of SAP, testosterone, sex hormone-binding globulin (SHBG), the testosterone/SHBG ratio, inhibin B, or estradiol. Only a weak negative correlation was found between seminal plasma SAP and serum levels of follicle-stimulating hormone (FSH) (Spearman's rho –0.159; p = 0.023) and luteinizing hormone (LH) (Spearman's rho –0.162; p = 0.021). In conclusion, all men investigated had measurable SAP levels in seminal plasma and in serum. SAP concentrations were 40 times lower in seminal fluid than in serum, and there was no correlation between those two variables. It seems that hormonal regulation is not the major pathway regulating seminal plasma SAP, and seminal plasma SAP and serum SAP are not co-regulated.

Key words: Serum amyloid P component, ELISA, semen, fertility, reproduction

Introduction

Serum amyloid P component (SAP) is a member of the pentraxin super family, which consists of the short and the long pentraxins. The common characteristic of this group of proteins is the pentraxin domain that consists of approximately 200 amino acids and contains an eight-amino-acid pentraxin signature designated HxCxS/TWxS (x denoting any amino acid) [1]. Humans express two short pentraxins, SAP and C-reactive protein (CRP), both of which consist of five identical subunits. In the long pentraxins, for example pentraxin 3 (PTX3) and neuronal pentraxins 1 and 2 (NP1 and NP2), the pentraxin domain is located in the C-terminal region, and there is an additional N-terminal domain that is neither present in the short pentraxins nor related to other known proteins [1, 2].

The occurrence pattern of SAP in humans indicates both physiological and pathological involvement, since SAP is normally found in blood and in connective tissues throughout the body, and SAP is also detected in all forms of amyloid plaques [3]. In our earlier study [4], we found SAP in seminal plasma, in sections from testis, epididymis, seminal vesicle, and prostate tissue and, on the surface of ejaculated human spermatozoa (primarily on the tail and midpiece). Naaby-Hansen et al. also detected SAP on the surface of ejaculated spermatozoa [5].

The short pentraxins are produced mainly in the liver [3], and, by using reverse transcription PCR, we have also observed local production of SAP in seminal vesicle, testis, prostate, and epididymis [4]. The long pentraxins are expressed locally in several cell types, as exemplified by the finding that PTX3 is produced by myeloid-derived dendritic cells, endothelial cells, and fibroblasts [1].

Functional data indicate that the long and the short pentraxins have some similar characteristics related to inflammation and innate immunity. CRP is the major acute-phase reactant in humans, and concentrations of SAP and PTX3 in blood are also increased, albeit to a lesser extent, in response to inflammatory stimuli [1, 3]. SAP is also involved in clearance of apoptotic cells, and SAP is a lectin-like molecule (i.e., it can bind sugars) [1]. It has long been known that the serum concentration of SAP is higher in men than in women [6], and this gender disparity does not appear until puberty, and it is greatest during the reproductive years [7]. The SAP concentration in women is affected by hormonal changes and levels of SAP fluctuate during the menstrual cycle and are lowest in the follicular phase [7].

There are very few reports concerning the short pentraxins and reproduction, whereas many papers describing the long pentraxins have been published in the last years. For example, it has been found that female PTX3 knockout mice are sub-fertile due to defects in the cumulus surrounding the oocyte [8], and studies of humans have shown that cumulus cells express PTX3 mRNA [9], and PTX3 is present in the follicular fluid [10]. Furthermore, in another recent investigation [11], PTX3 was detected in seminal plasma in concentrations similar to those observed in blood, and it was also found attached to the neck and subacrosomal region of ejaculated spermatozoa, and in epithelial cells in the male reproductive tract. In the guinea pig, the long pentraxin apexin is present in the sperm acrosome, and, due to this localization, it has thus been suggested that apexin participates in the fertilization process before and/or after the acrosome reaction takes place [12, 13].

Inasmuch as SAP is found in the male reproductive tract and is associated with functions that play a role in male reproduction, and it also seems that this protein is influenced by hormonal changes, we wanted to investigate and elucidate SAP in the context of reproduction. To achieve

that goal we developed and characterized an ELISA to quantify SAP in seminal plasma and serum, and we also studied the distribution of SAP in a group of healthy men. The previous finding of local SAP mRNA prompted us to examine whether a correlation exists between SAP in seminal plasma and serum, and whether hormones regulate the levels of this protein in seminal plasma.

Methods

Study subjects

Blood and semen samples were collected from 305 Swedish military conscripts. The participants completed a questionnaire, underwent a physical examination, and delivered a semen sample. Approval had been obtained from the Research Ethics Board of Lund University. The material has previously been described by Richthoff et al. [14]. A total of 102 subjects were excluded in this study (84 consecutive samples that were used in other studies and therefore not accessible and 18 random samples, due to insufficient remaining semen sample volume), leaving 203 subjects to be analyzed. Those 203 males had a mean age of 18.2 years (SD 0.4), a mean BMI of 22.6 kg/m^2 (SD 3.4), a mean abstinence time of 83.6 h (SD 52.4), a mean sperm concentration of 69.2 x 10^6/mL (SD 62.2), and a mean semen volume of 3.4 mL (SD 1.3). To investigate the risk of selection bias, we used the Mann-Whitney test to compare the study group (n = 203) with the excluded subjects (n = 102) regarding the above-mentioned parameters. No statistically significant differences were observed, with the exception of a larger semen volume for the members of the study group (3.4 mL (SD 1.3)) than for the 102 males who where excluded (2.9 mL (SD 1.4)).

Sample preparation

Seminal plasma samples were prepared according to the guidelines of the World Health Organization [15]. In short, after liquefaction for 30 min, 450 µL of an ejaculate was mixed with 50 µL of benzamidine (100 mM). Thereafter the sample was centrifuged for 20 min at 4500 g, and the seminal plasma was subsequently decanted and stored at −20°C pending analysis [16].

Characterization of the standard and the antibody used in the SAP-ELISA

SAP purified from serum was used as a standard. The purification was performed as previously described [17], and the concentration was determined after acid hydrolysis (24 h in 6 M HCl, 110 °C, *in vacuo*) on a Beckman 6300 amino acid analyzer (Beckman Coulter Inc., Fullerton, CA, USA). The purity of the standard was assessed after performing a 10 % Coomassie-stained sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) [4]. The standard curve was obtained using the purified protein diluted to 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 µg/L and buffer as a blank. Pooled seminal plasma (diluted 1:50 and 1:300) and pooled serum (diluted 1:1000 and 1:6000) were used as controls. In general, seminal plasma samples were diluted 1:100 and serum samples 1:2000. To address the issue regarding antibody specificity, an absorption experiment was performed. Purified SAP was coupled to Affi 10 gel (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. The polyclonal anti-human-SAP antibody, used in the ELISA, (A0302, Dako, Glostrup, Denmark) was incubated with the gel (2 x 1 h). An SDS-PAGE followed by Western blot of seminal plasma and purified SAP in duplicates was performed [4]. Half the membrane was incubated with the polyclonal anti-SAP antibody and the other half with the antibody that had been subjected to absorption with purified SAP coupled to Affi 10 gel.

ELISA

Microtitre plates (Maxisorp 473709, Thermo Fisher Scientific, Waltham, MA, USA) were coated with a polyclonal rabbit anti-human-SAP antibody (A0302, Dako), 0.5 µg/well diluted in 0.05 M carbonate buffer (pH 9.6) and then incubated overnight at 5 °C. The plates were then washed three times in wash buffer (0.05 M Tris, 0.15 M NaCl, and 0.1 % w/v Tween 20; pH 7.5) on a Wallac Delfia Plate washer (model no. 1296-024, PerkinElmer, Waltham, MA, USA). Thereafter, 100 µL of incubation buffer (0.05 M Tris, 0.15 M NaCl, 1 mM EDTA, and 1 % w/v BSA; pH 7.5) was applied to each well to block unspecific binding; this incubation and all subsequent incubations were performed at room temperature on a Wallac Delfia Plate shaker (model no. 1296-001). After a wash step, the standard, controls, and samples were diluted in the incubation buffer and duplicates were applied; the controls were applied both in the beginning and end of each plate. Incubation was performed for 2 h, followed by a wash step, after which biotinylated anti-SAP antibody was applied (A0302; 11.8 ng per well). The antibody had been biotinylated according to standard procedures [18] using biotinamidohexanoic acid N-hydroxysuccinimide (B2643, Sigma-Aldrich St Louis, MO, USA). The plates were subsequently incubated for 1 h and then subjected to another wash step. Next, 50 µL of streptavidin ABComplex/HRP solution (Dako; used according to the manufacturer's instructions) was applied to each well followed by incubation for 1 hour. After the last wash step, 100 µL of a mixture containing; an OPD tablet (2 mg, Dako), and 1.25 µL of hydrogen peroxide in 3 mL deionized water, was applied to each well. The subsequent incubation step (5 min at room temperature and shielded from light) was interrupted by adding 100 µL of 0.5 M H_2SO_4, to each well, and the absorbance was read at 490 nm on an Emax^{TM} Precision microplate reader (MDS Analytical technologies, Toronto, Canada). The data were processed by Multicalc 2000 v 2.7 (Perkin Elmer), and the standard curve fitting algorithm used was spline smoothed 1.0.

ELISA characterization

To determine the *intra-assay coefficient of variation (CV),* 20 replicates of the seminal plasma and serum controls were applied in the same analysis run. *Inter-assay CV* was determined using controls from five consecutive runs (11 microtitre plates). *Linearity* was assessed by diluting three seminal plasma samples and three serum samples 1:2 in two parallel dilution series, for seminal plasma starting at 1:10 and 1:15, and for serum starting at 1:100 and 1:150. A concentration obtained from the straight part of the standard curve (conc. 6.25–12.5 µg/L) was set as 100% and the deviations from that point were calculated. *Analytical sensitivity* was calculated from 20 replicates of buffer (mean + 3 SD). *Recovery* was evaluated by adding purified SAP (0.75 µg) to three seminal plasma (20 µL) and three serum (10 µL) samples with known concentrations of SAP. *Stability* of SAP after repeated thaw and refreeze cycles was analyzed in five seminal plasma and five serum samples. The samples were aliquoted in four portions (designated A–D), which were placed at –20 °C directly after preparation: aliquot A was kept at –20 °C and was thawed immediately prior to analysis; aliquot B was thawed once for 4 h; aliquot C was thawed twice for 4 h; aliquot D was thawed three times for 4 h and then refrozen pending analysis.

Analysis of hormones

SHBG, testosterone, LH, FSH, and estradiol were measured on an Autodelfia® system (PerkinElmer), and inhibin B was determined using a specific immunometric assay [19] These analyses were performed in a routine clinical chemistry laboratory (Uppsala University Hospital), and they have previously been described [16].

Statistical analysis

Microsoft Excel (Office 2003, Microsoft Corporation, Redmond, WA, USA) was used for all calculations performed to evaluate the ELISA. The SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA) was employed to determine the presence of Gaussian distribution, and to achieve logarithmic transformations, descriptive statistics, histograms, dot plots, correlations (Spearman's rank correlation), and Mann-Whitney tests. Non-parametric statistics were used in this paper since SAP in seminal plasma did not show a normal distribution.

Results

ELISA characterization

The *purity of the standard* was considered to be > 95 % (3 µg applied), since no additional protein bands could be detected on the Coomassie-stained gel of the standard. The *specificity of the polyclonal anti-SAP antibody* used in the ELISA, was high since the prominent band corresponding to SAP seen on the Western blot could not be seen when the antibody was pre-incubated with purified SAP coupled to Affi 10 gel prior to Western blot. The *analytical sensitivity* of the ELISA was 0.003 µg/L and the *inter- and intra-assay* results are presented in Table I. The method was considered to be linear if the deviation from the expected concentration in a sample was no more than +/− 20%. *Linearity* was observed at 2.5–108 µg/L for seminal plasma and at 1–100 µg/L for serum. Figure 1 shows absorbance plotted against serial dilutions of SAP standard, seminal plasma samples and serum samples. One standard curve, two seminal plasma samples and two serum samples are presented. *Recovery* was assessed as the ratio of the expected concentration of SAP to the concentration measured, after addition of purified SAP. Three independent samples were analyzed, which resulted in recoveries of 75%, 115% and 100%

from seminal plasma and 87%, 117% and 121% from serum. *Stability of samples* subjected to repeated thaw and refreeze cycles did not show a systematic decrease in SAP concentrations.

SAP in seminal plasma and serum

The distributions and descriptive statistics of SAP concentrations in the studied group of 203 men are shown in Figure 2. Considering seminal plasma, the distribution of SAP was positively skewed, (skewness 1.849 and kurtosis 6.353) with a median concentration of 1.01 mg/L and an interquartile range (IQR) of 0.56–1.65 mg/L and the reference interval (mean ± 1.96 SD, determined after logarithmic transformation of the data), was 0.23–4.05 mg/L. Since semen volume varied in this material (median 3.2 mL; IQR 2.6–4.2 mL; range 0.9–8.4 mL), the median amount of SAP was determined (concentration of SAP x semen volume). The amount of SAP in seminal plasma showed a distribution similar to that seen for the concentration of SAP in seminal plasma with a median amount of 3.12 µg (IQR 1.81–5.45 µg, range 0.33–21.9 µg, skewness 1.832 and kurtosis 6.134). For SAP in serum, the distribution was normal with a median concentration of 40.5 mg/L (IQR 34.2–49.2 mg/L, skewness 0.515 and kurtosis 0.649).

We found no correlation between SAP concentrations in serum and seminal plasma, Spearman's rho correlation coefficient was 0.058 (p-value 0.415) (Figure 3). Furthermore, the amount of SAP in seminal plasma was not correlated with the SAP concentration in serum (Spearman's rho 0.029; p-value 0.683). The concentration of SAP in seminal plasma did not correlate with serum levels of SHBG, testosterone, the testosterone/SHBG ratio, inhibin B, or estradiol, whereas it showed a negative correlation with FSH and LH in serum. The amount of SAP in seminal plasma did not correlate with any of the mentioned hormones. Spearman's correlation coefficients and p-values are presented in Table II.

Discussion

Several investigations have suggested that SAP has a number of different functions, some of which are of interest in relation to fertility. Examples of these are inflammation, innate immunity, and apoptosis, and even potential carbohydrate interactions that might be involved in actions such as the binding of spermatozoa in the female reproductive tract before and during fertilization [1, 20]. Nevertheless, to our knowledge no functional studies have considered SAP in the context of reproduction. The aim of the present investigation was to further examine the role of SAP in male reproduction, and therefore we designed and evaluated an ELISA for determination of SAP in seminal plasma and serum.

The lower limit of detection of the ELISA was satisfactory, since all samples had to be diluted prior to analysis and no concentration was close to the detection limit. Indeed, this novel ELISA measures SAP at concentrations of the same magnitude as previously reported assays. This is demonstrated by the observation that the median concentration of SAP in serum samples from our study population was 40.5 mg/L (range 13.1–80.9 mg/L, Figure 2), which agrees with the findings of two earlier studies showing corresponding concentrations of 43 and 32 mg/L, respectively [6, 21].

Considering SAP in seminal plasma, our earlier investigation indicated a concentration of 2 mg/L [4], whereas the median concentration in the present study was 1.01 mg/L, and the levels varied from one fifth to six times that concentration (range 0.18–6.65 mg/L; Figure 2). The discrepancy in these results can be explained by differences between the two studies with regard to the number of participants (11 men in the first study, compared to 203 here) and the analytical method used (electroimmunoassay and a novel ELISA, respectively). Since semen volume varies

considerably between men [22], we also investigated the amounts of SAP in our study population and found a median amount of 3.12 µg SAP (range 0.33–21.9 µg). The distributions of the amounts and concentrations of SAP in seminal plasma were similar, and the positive skewness observed implies that the majority of the men had SAP concentrations of 0.2–2 mg/L (Figure 2), whereas around 10% had higher levels. In serum, however, the concentrations of SAP were normally distributed. This difference in distribution between levels of SAP in seminal plasma compared to serum might be explained by a greater inter-individual variation in levels of this protein in seminal plasma, which suggests that control mechanisms differ for production of SAP occurring locally in the reproductive tract compared to that derived from the liver. The assumption that production of SAP is controlled differently in serum and seminal plasma is further supported by the observed lack of correlation between SAP concentrations found in seminal plasma and those detected in serum (Figure 3), which of course might also be explained by the different roles that this protein might play in the male reproductive tract and the blood. The lack of correlation between SAP concentrations in seminal plasma and serum also supports our previous reverse transcription PCR results demonstrating that SAP is produced in testis and seminal vesicle, as well as in prostate and epididymis [4].

As mentioned in the introduction, several studies have provided evidence of hormonal regulation of SAP levels in serum, and thus we investigated possible correlations between levels of various hormones in serum and the amount and concentration of SAP in seminal plasma. These analyses indicated that, in our samples, neither the concentration nor the amount of SAP in seminal plasma was correlated with serum levels of testosterone, SHBG, estradiol, or inhibin B, or the serum testosterone/SHBG ratio. However, for serum FSH and LH we did observe a weak negative correlation ($p < 0.05$) with the concentration, but not the amount of SAP in seminal plasma

(Table II). Thus our results suggest that SAP in seminal plasma is not extensively regulated by the blood concentrations of the examined hormones. The mentioned findings were somewhat surprising because it seems that concentrations of SAP in serum are influenced by hormones. However, it should be kept in mind that the hormonal processes in the male sexual organs are complex and involve endocrine, paracrine, and autocrine modes of action, along with hormone modifications and receptor interactions [23] most of which were not addressed in our study. Therefore, further research is needed to investigate the possible impact of local hormones on production of SAP in the male reproductive tract.

To our knowledge, the literature contains no studies of SAP levels in seminal plasma that we could refer to for comparison with our data, although there is one article concerning PTX3 [11]. Both SAP and PTX3 are found in tissues of the male reproductive tract (e.g., testis and prostate), attached to spermatozoa, and in seminal plasma, and concentrations of both these proteins exhibit a positively skewed distribution [4, 11]. However, there are also several differences between the two proteins. Doni and colleagues found the following with regard to PTX3: concentrations were similar in serum and seminal plasma; levels were not measurable in seminal plasma from some of the investigated men; some of the men had higher concentrations in seminal plasma compared to levels normally observed in serum. By comparison, we made the following observations concerning SAP: the concentration was 40 times higher in serum compared to seminal plasma; all the investigated men had a detectable concentration; and none of the subjects had a higher concentration in seminal plasma than in serum. The two studies are not completely comparable, since we investigated SAP in a normal population, whereas Doni et al. analyzed PTX3 in men who had been referred to a fertility clinic. Furthermore, there are biological differences between

SAP and PTX3 with respect to expression patterns and functions as reviewed by Mantovani et al. [1].

In conclusion, our observations in healthy young men showed that the median concentration of SAP was 40 times lower in seminal plasma than in serum, and the distribution of this protein in seminal plasma was positively skewed, which implies a greater inter-individual variation in SAP levels in seminal plasma compared to serum. Moreover, it seemed that SAP levels in seminal plasma and serum were not co-regulated, and the concentration of SAP in seminal plasma was not regulated by hormones. The literature in this area is still too scarce to reveal the functions of SAP in male reproduction, and hence it is too early to ascertain whether the concentration of SAP can serve as a clinical biomarker. The current study has provided baseline data for comparison with the results of future investigations, and, in continued research, we will also examine infertile populations to determine whether SAP can be used as a prognostic or a diagnostic marker in clinical reproductive medicine.

Acknowledgements

Legends to figures

Figure 1

A standard curve and serial dilutions of two seminal plasma and two serum samples are shown. The dilution factor is 1:2, starting at 1:3000 for the standard, 1:10 for seminal plasma and 1:100 for serum samples.

Figure 2

The distribution of SAP is positively skewed in seminal plasma and is Gaussian-like in serum. Descriptive statistics and reference intervals are presented in the inserts.

Figure 3

No correlation detected between SAP in serum and seminal plasma. Spearman's rho correlation coefficient was 0.058 (p-value 0.415).

Figure 1

Descriptive statistics	(mg/L)
Min	0.18
25th percentile	0.56
Median	1.01
75th percentile	1.65
Max	6.65
Reference interval	0.23 – 4.05

Descriptive statistics	(mg/L)
Min	13.1
25th percentile	34.2
Median	40.5
75th percentile	49.2
Max	80.9
Reference interval	21.2 – 62.5

SAP in seminal plasma (mg/L)

SAP in serum (mg/L)

Figure 2

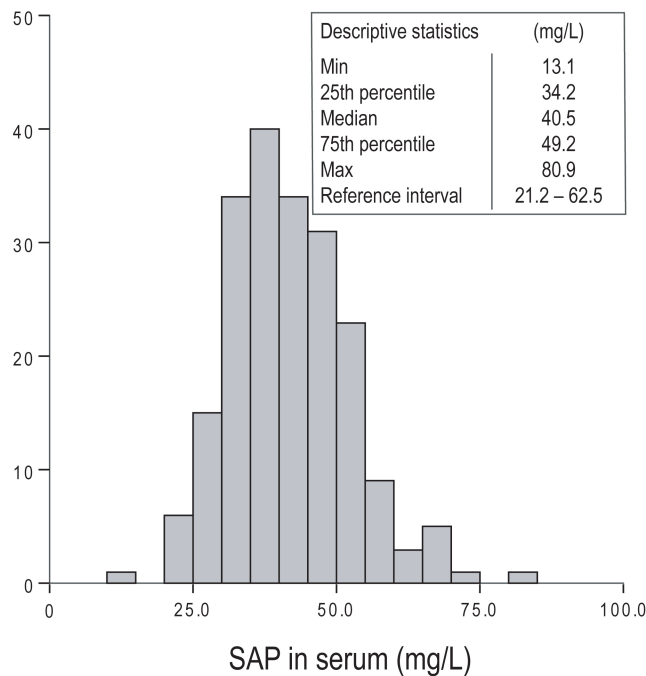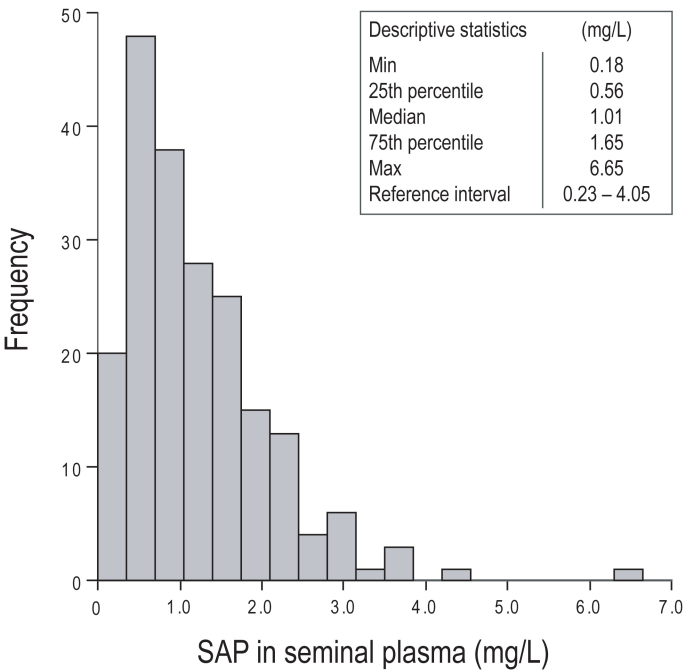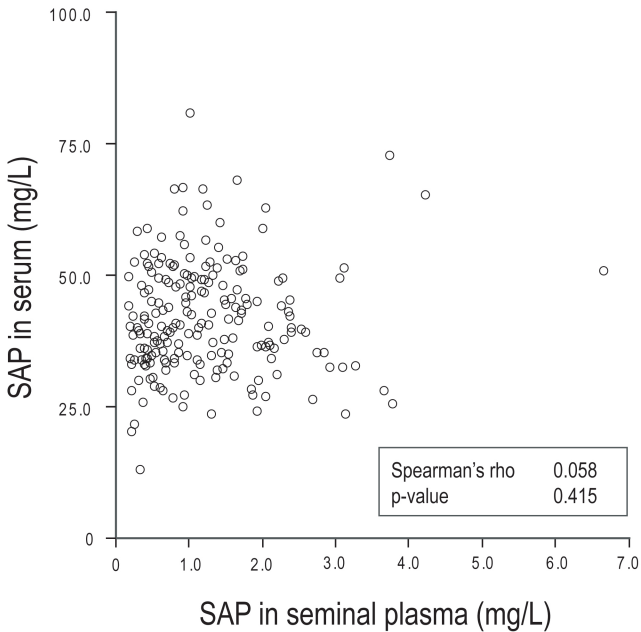|		
Spearman's rho	0.058	
p-value	0.415	

SAP in seminal plasma (mg/L)

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TABLE I.

Intra- and inter-assay CV of the SAP ELISA

	Seminal plasma		Serum	
	low	*high*	*low*	*high*
Concentration (µg/L)	5.0	17.2	4.3	36.0
Intra-assay CV (%)	7.4	4.4	4.6	6.4
Replicates (n)	20	20	20	20
Concentration (µg/L)	3.1	19.4	6.4	36.9
Inter-assay CV (%)	22.6	12.6	7.7	11.6
Microtitre plates (n)	11	11	11	11

TABLE II. Correlations between the concentration and the amount of SAP in seminal plasma (SemP) and different serum (S) markers

	SemP-SAP (mg/L)		SemP-SAP (μg)	
	Spearman's rho	*p-value*	*Spearman's rho*	*p-value*
S-SAP (mg/L)	0.058	0.415	0.029	0.683
S-SHBG (nmol/L)	–0.099	0.159	–0.064	0.363
S-testosterone (nmol/L)	–0.103	0.145	–0.034	0.634
S-testosterone/S-SHBG	0.069	0.331	0.076	0.280
S-FSH (IU/L)	–0.159	**0.023**	–0.124	0.077
S-LH (IU/L)	–0.162	**0.021**	–0.118	0.095
S-inhibin B (ng/L)	0.082	0.244	0.085	0.230
S-oestradiol (pmol/L)	–0.013	0.856	0.041	0.559

Bold text indicates a p-value < 0.05.