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Analysis of the functional aspects and seminal plasma proteomic profile of sperm from smokers

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Objective

To evaluate the effect of smoking on sperm functional quality and seminal plasma proteomic profile.

Patients and Methods

Sperm functional tests were performed in 20 non-smoking men with normal semen quality, according to the World Health Organization (2010) and in 20 smoking patients. These included: evaluation of DNA fragmentation by alkaline Comet assay; analysis of mitochondrial activity using DAB staining; and acrosomal integrity evaluation by PNA binding. The remaining semen was centrifuged and seminal plasma was used for proteomic analysis (liquid chromatography-tandem mass spectrometry). The quantified proteins were used for Venn diagram construction in Cytoscape 3.2.1 software, using the PINA4MS plug-in. Then, differentially expressed proteins were used for functional enrichment analysis of Gene Ontology categories, Kyoto Encyclopedia of Genes and Genomes and Reactome, using Cytoscape software and the ClueGO 2.2.0 plug-in.

Results

Smokers had a higher percentage of sperm DNA damage (Comet classes III and IV; $P < 0.01$), partially and fully inactive mitochondria (DAB classes III and IV; $P = 0.001$ and $P = 0.006$, respectively) and non-intact acrosomes ($P < 0.01$) when compared with the control group. With respect to proteomic analysis, 422 proteins were identified and quantified, of which one protein was absent, 27 proteins were under-represented and six proteins were over-represented in smokers. Functional enrichment analysis showed the enrichment of antigen processing and presentation, positive regulation of prostaglandin secretion involved in immune response, protein kinase A signalling and arachidonic acid secretion, complement activation, regulation of the cytokine-mediated signalling pathway and regulation of acute inflammatory response in the study group (smokers).

Conclusion

In conclusion, cigarette smoking was associated with an inflammatory state in the accessory glands and in the testis, as shown by enriched proteomic pathways. This state causes an alteration in sperm functional quality, which is characterized by decreased acrosome integrity and mitochondrial activity, as well as by increased nuclear DNA fragmentation.

Keywords

acrosome, DNA damage, mitochondria, proteomics, seminal plasma

Introduction

Cigarette smoking is one of the most important lifestyle-associated factors involved in human diseases. In addition, it has been shown, in both men and women, that smoking is associated with important negative effects on reproductive health [1]. According to the WHO, one third of the world population aged >15 years smokes cigarettes [2] and there is therefore concern that smoking may have an epidemiological effect on male infertility.

Several studies have shown that tobacco is associated with a decrease in sperm concentration and in the percentage of cells with normal morphology and progressive motility [3–8]. Tobacco is also associated with increased sperm DNA fragmentation [5–8] and decreased mitochondrial activity, as well as with increased seminal plasma lipid peroxidation [5]. Smoking also leads to alterations in circulating levels of testosterone, LH and FSH, suggesting that cigarette components can act as endocrine disruptors [9–11].

Although the cellular effects of smoking on male reproductive biology have been shown, the molecular pathways by which these alterations occur are as yet unclear. The study of these pathways has led to an understanding of several mechanisms for male infertility, such as varicocele in adults and

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adolescents or in high sperm DNA fragmentation or oxidative stress [12–16]. In smokers, some changes in the proteomic profile of seminal plasma of men with varicocele were detected in relation to non-smokers with varicocele [5].

Seminal plasma is rich in proteins (~35–55 g/L), of which 70% is secreted from seminal vesicles, 20% from the prostate and 10% from the testis and epididymis. Some studies have shown that epididymal proteins have an important role in sperm quality [17], and proteins associated with testicular function can be observed in seminal plasma [18–20]. Some infertility factors have been associated with seminal plasma proteome [14,17,21–24], which reflects the activity of male sexual accessory glands and also spermatogenesis, epididymal maturation and sperm integrity [25]. The systemic effects of smoking can also alter the secretion of proteins by the male sex glands, and are reflected in the seminal plasma [26]. We hypothesized, therefore, that changes in these processes caused by smoking may alter the protein composition of the seminal plasma. The aim of the present study was to analyse the effects of smoking on sperm nuclear DNA fragmentation, acrosome integrity and mitochondrial activity and on the seminal plasma proteome.

Patients and Methods

Study Design

This study was approved by the Sao Paulo Federal University (UNIFESP, Brazil) institutional review board (Research Ethics Committee approval no. 05781212.6.0000.5505). Informed written consent was obtained from all the men included.

A cross-sectional study was performed in men aged 20–50 years who sought the Andrology Laboratory from the Human Reproduction Section, Division of Urology, Department of Surgery, Sao Paulo Federal University (UNIFESP). Semen samples were collected by masturbation, after 2–5 days of ejaculatory abstinence, in sterile polypropylene containers in an area attached at the Andrology Laboratory. The men included in the study were divided into two groups: those who reported that they smoked at least 10 cigarettes/day (study group) and non-smokers with normal semen analysis, according to the WHO [27] (control group). Exclusion criteria were clinical and/or surgical history that may cause testicular alterations, such as cancer, orchitis, obesity, varicocele, endocrinopathies, urogenital infection, report of fever in the 90 days preceding the clinical examination, or history of previous radio and/or chemotherapy or exposure to environmental toxicants.

After complete liquefaction of the sample, an aliquot was used for semen analysis performed according to WHO guidelines [27]. Semen volume, pH, sperm concentration, sperm motility and sperm morphology were included in the semen analysis. Motility was assessed manually using a Horwell chamber (Arnold R. Horwell Ltd, London, UK), and the sperm were classified as progressive, non-progressive and immotile. Sperm concentration was assessed by a modified Neubauer chamber (Herka, Berlin, Germany) and the result was expressed in millions per mL. For morphological evaluations, the kit Panotico (Laborclin, Santo André, Brazil) was used to stain sperm smears and Kruger's criteria were used [27]. All analyses were performed by one experienced technician, blinded to the study. Another aliquot was used for sperm functional analyses (mitochondrial activity, acrosome integrity and DNA fragmentation), as described below. The remaining semen volume was centrifuged at 800 ***g*** for 30 min to separate the supernatant seminal plasma, which was frozen without cryoprotectants and protease inhibitors and kept at −20 °C to be further used for proteomic analysis. Before these analyses, seminal plasma was thawed and centrifuged at 16 100 ***g*** for 1 h at 4 °C to remove cellular debris. All reagents used in the present study were purchased from Sigma (St Louis, MO, USA), unless otherwise stated.

Determination of Sperm Mitochondrial Activity, Acrosome Integrity and DNA Integrity

Sperm mitochondrial activity, acrosome integrity and DNA fragmentation analyses were performed as previously described [5,28,29]. For mitochondrial activity, 200 cells per sample were analysed and classified as class I (100% of the midpiece stained), class II (>50% of the midpiece stained), class III (<50% of the midpiece stained) and class IV (no staining of the midpiece). For acrosome integrity, 200 cells per sample were classified as intact (stained) or non-intact (unstained). For sperm DNA fragmentation, 100 spermatozoa were classified according to the intensity of DNA damage observed by the tail and nuclear intensity, divided into grades I (high DNA integrity: no DNA migration), II (low DNA fragmentation: little DNA migration), III (increased DNA fragmentation: an intense Comet tail and an observed nucleus), or IV (high DNA fragmentation: an intense Comet tail with no observed nucleus).

Statistical Analysis

Statistical analysis of semen and sperm function analyses were performed using SPSS 18.0 for Windows. Initially, a Kolmogorov–Smirnov test was used to verify the normality of data distribution for all variables. Groups were then compared using Student's *t*-test for unpaired samples (normally distributed variables) or a Mann–Whitney test (non-normally distributed variables). An α value of 5% was adopted for the analyses.

Proteomic Analysis of Seminal Plasma

Sample preparation

Total protein in the seminal plasma of each selected sample was analysed using a BCA protein assay (bicinchoninic acid,

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modified Lowry method) [30]. Quantification was performed by spectrophotometry in a microplate reader (EL×800 Microplate Absorbance reader; Biotek, Winooski, VT, USA) at 540 nm wavelength. Each sample was quantified in triplicate and the standard curve containing different concentrations of bovine serum albumin (0–1 000 mg/mL), in duplicate. Samples with coefficients of variation >5% were quantified again in another run.

Four different pools were prepared for each group, with different samples randomly assigned to each, in order to maintain biological variability. Each sample contributed with a volume corresponding to the same amount of total protein. The pools were the used for proteomic analysis. For each pool, 50 μg of protein was diluted in Milli-Q water (Millipore Corp., Billerica, MA, USA) to a final volume of 50 μL, and proteins were processed as previously published [16].

Mass spectrometry

Liquid chromatography (LC)-tandem mass spectrometry (MS) was conducted using a nanoACQUITY UPLC (Waters, Milford, MA, USA) system coupled to a quadrupole mass spectrometer hybrid-OrbitrapThermo Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) for high resolution. MS conditions were as previously published [16].

The MS raw files were analysed using MaxQuant software (version 1.4.1.2) and Andromeda search engine for protein identification and quantification [31]. The processing conditions were: maximum of two missed trypsin cleavages; carbamidomethylation as a fixed modification; protein N-terminal acetylation and methionine oxidation as variable modifications; and up to 2 min retention time distortion (in each direction) for chromatographic alignment. The SwissProt UniProt database was used for protein assignment (version 2014_07_09, downloaded on 9 July 2014). The false discovery rate was set to 1% and was determined by searching in a reverse database. Quantification was performed label-free using intensity-based absolute quantification (iBAQ), in which the protein content is normalized to the total number of potential peptides [32].

Data analysis

For each group, four biological replicates were formed, which were acquired in triplicate (technical replicates) during LC-MS/MS experiments. To maximize the number of observations in each study and because MS data are variable, each technical replicate of the pools was considered as a different observation. Each experimental group, therefore, presented a total of 12 observations. Contaminant proteins and those identified only by site and/or reverse sequence database were excluded. The iBAQ data, generated by the MaxQuant software for each protein, were normalized to the total iBAQ value of each observation (%iBAQ). Proteins were only considered when quantified in at least three of the 12 observations.

For univariate statistical analyses, PASW (SPSS) 18.0 software for Windows was used. A descriptive analysis was performed to calculate the fold-change of %iBAQ (ratio of the study group to the control group means). For comparison between groups, an unpaired Mann–Whitney test was carried out, and an α value of 5% was used. Three different protein lists were generated: a list of proteins exclusive to or in higher concentration in the seminal plasma of the men in the smoker group; a list of proteins exclusive to or in higher concentration in the men in the control group; and a list of conserved proteins (common to both groups).

To validate our findings, multivariate statistical analyses were performed, using the online platform Metaboanalyst [33] and the PASW (SPSS) 18.0 software for Windows. Data were transformed to their logarithmic values, and a partial least-squares discriminant analysis (PLS-DA) was performed. The components (groups of correlated variables) were extracted, and in each component the complete data matrix received a Variable Importance in the Projection (VIP) score. For PLS-DA results, the tridimensional graph showing the separation of the groups was reported. Thereafter, proteins with a VIP score of at least 2.0 were used for logistic regression and discriminant analyses in order to identify the most important proteins to differentiate the groups. For these analyses, non-transformed %iBAQ data for each protein were used as an independent variable, and group was used as a dependent variable. For logistic regression analysis, independent variables were inserted in the model using the enter method. Results were presented as the model's predictive values and the receiver-operating characteristic (ROC) curve. For discriminant analysis, a linear model was constructed.

Functional enrichment analysis

Quantified proteins were used for Venn diagram construction with Cytoscape 3.2.1 software [34], using the PINA4MS plug-in [35]. Because many different functions may be assigned to individual proteins, functional enrichment studies have been used, in which shared functions among different proteins are statistically more frequent (enriched), thus offering a functional aspect to proteomic analysis. Differentially expressed proteins were also used for functional enrichment analysis of Gene Ontology categories, Kyoto Encyclopedia of Genes and Genomes and Reactome, using the Cytoscape software and the ClueGO 2.2.0 plug-in [36]. Only enriched functions with a P value <0.05 were considered.

Results

The clinical data (age and semen analysis) and sperm functional analyses results are shown in Tables 1 and 2,

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Table 1 Clinical data from the control (non-smokers) and study (smokers) groups.

	Control group ($n = 20$)	Study group ($n = 20$)	P
Age, years			
Mean (SD)	35.4 (3.69)	35.4 (5.42)	1.00
95% CI	33.7–37.1	32.9–37.9	
Volume, mL			
Mean (SD)	4.0 (1.30)	3.5 (1.54)	0.23
95% CI	3.4–4.6	2.7–4.2	
Progressive, %			
Mean (SD)	55.2 (10.12)	46.1 (15.64)	0.08
95% CI	49.4–61.1	37.1–55.2	
Non-progressive, %			
Mean (SD)	4.4 (2.30)	5.6 (3.48)	0.37
95% CI	3.3–5.5	3.6–6.9	
Immotile, %			
Mean (SD)	41.9 (10.00)	47.1 (12.94)	0.16
95% CI	37.2–46.5	41.0–53.1	
Concentration, $\times 10^6$/mL			
Mean (SD)	91.2 (96.70)	69.9 (56.70)	0.40
95% CI	46.0–136.5	43.3–96.4	
Totalcount, $\times 10^6$			
Mean (SD)	330.8 (291.59)	195.6 (138.72)	0.07
95% CI	194.3–467.3	130.6–260.5	
Morphology, %			
Mean (SD)	7.0 (2.62)	6.6 (3.80)	0.50
95% CI	5.8–8.2	4.5–8.0	
Round cell, %			
Mean (SD)	1.1 (0.87)	1.3 (1.75)	0.77
95% CI	0.7–1.5	0.4–2.1	
Neutrophils, $\times 10^6$/mL			
Mean (SD)	0.1 (0.11)	0.2 (0.45)	0.52
95% CI	0.01–0.1	0.03–0.4	

Groups were compared using Student's t-test, except for neutrophils, which were compared using a Mann–Whitney test ($P < 0.05$).

Table 2 Sperm functional analysis of the study group (smokers) and the control group (non-smokers).

	Control group ($n = 20$)	Study group ($n = 20$)	P
Comet class I (%)			
Mean (SD)	27.7 (19.02)	5.8 (7.97)	<0.001*
95% CI	18.8–36.6	2.1–9.5	
Comet class II (%)			
Mean (SD)	64.5 (15.56)	57.6 (17.99)	0.212
95% CI	57.2–71.8	49.4–66.3	
Comet class III (%)			
Mean (SD)	5.6 (5.02)	23.6 (15.20)	<0.001*
95% CI	3.2–7.9	16.3–30.6	
Comet class IV (%)			
Mean (SD)	2.6 (3.57	12.9 (12.59)	<0.001*
95% CI	0.6–3.9	7.0–18.8	
Intactact acrosome (%)			
Mean (SD)	77.6 (7.18)	68.6 (8.59)	<0.001*
95% CI	74.2–81.0	64.7–72.3	
DAB class I (%)			
Mean (SD)	14.3 (12.84)	7.5 (13.51)	0.111
95% CI	8.3–20.3	1.2–13.9	
DAB class II (%)			
Mean (SD)	60.6 (11.50)	47.9 (15.64)	0.008*
95% CI	54.8–65.5	40.6–55.2	
DAB class III (%)			
Mean (SD)	20.8 (10.06)	34.6 (13.95)	0.001*
95% CI	16.1–25.5	28.1–41.1	
DAB class IV (%)			
Mean (SD)	4.7 (3.97)	10.0 (6.93)	0.006*
95% CI	2.9–6.6	6.7–13.2	

*Groups were compared using the Student's t-test, except 'Comet class IV', which was compared using a Mann–Whitney test ($P < 0.05$). *Significant difference.*

respectively. Groups were similar in terms of age and conventional semen analysis. The study group (smokers) had higher sperm DNA fragmentation values and lower sperm acrosome integrity and mitochondrial activity values when compared with the control group.

A total of 422 proteins were identified and quantified, of which one protein was absent, six were over-represented and 27 were under-represented in smokers (Table 3, Fig. 1). A total of 388 proteins were conserved between the groups (Table S1).

In the study group, functional enrichment analysis showed the enrichment of antigen processing and presentation, positive regulation of prostaglandin secretion involved in immune response, protein kinase A signalling and arachidonic acid secretion, complement activation, regulation of cytokine-mediated signalling pathway and regulation of acute inflammatory response (Fig. 2). In the control group, glycosaminoglycan degradation, keratan sulphate catabolic process, substrate adhesion-dependent cell spreading, long-chain fatty acid transport and extracellular matrix receptor interaction were enriched (Fig. 2).

In multivariate analysis, a complete separation between the groups was obtained with PLS-DA using 15 proteins (VIP score >2; Fig. 3), which were used for logistic regression and discriminant analyses. In the logistic regression analysis, prolargin was included in the final model as the most important protein to differentiate the groups ($P < 0.047$). The model presented positive, negative and total predictive values of 91.7, 83.3 and 87.5%, respectively, and an area under the ROC curve of 90.4% (Fig. 3). In the discriminant analysis, the protein S100A9 was capable of predicting those who were smokers (study group), and prolargin and mammaglobin B were capable of predicting the control group.

Discussion

Male reproductive potential is sensitive to environmental factors, such as obesity, smoking, alcoholism and pollution [37]. Conventional semen quality is usually decreased [8,11,38–40], although there are conflicting results [9]. Although we did not observe differences in semen quality between smokers and non-smokers in the present study, our experiment was not set up to test that hypothesis. The results, in terms of sperm functional testing and seminal plasma proteomics, should therefore be interpreted in light of the fact that semen quality was similar to normozoospermic samples. It is interesting to note, however, that in the present study, differences were observed with regard to sperm functional tests and semen proteomics, showing that these tests have a greater sensitivity than conventional semen analysis [41].

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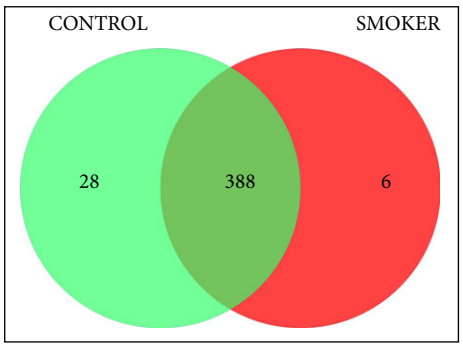
Table 3 Absent, under-represented and over-represented proteins in smokers.

UniProt accession no.	Gene symbol	Protein name	*P*	$\frac{iBAQ_{(smokers)}}{iBAQ_{(control)}}$	
O75556	SCGB2A1	*Mammaglobin-B*	–	Absent	
P51888	PRELP	*Prolargin*	0.011	0.206	Under-represented
Q15365	PCBP1	*Poly(rC)-binding protein 1*	0.044	0.318	
P19835	CEL	*Bile salt-activated lipase*	0.015	0.338	
P16152	CBR1	*Carbonyl reductase [NADPH] 1*	0.025	0.393	
P14555	PLA2G2A	*Phospholipase A2, membrane associated*	0.020	0.416	
P16070	CD44	*CD44 antigen*	0.017	0.496	
Q7Z304	MAMDC2	*MAM domain-containing protein 2*	0.006	0.513	
Q14507	EDDM3A	*Epididymal secretory protein E3-alpha*	0.036	0.522	
Q8IZP9	GPR64	*G-protein coupled receptor 64*	0.010	0.561	
Q9H3G5	CPVL	*Probable serine carboxy peptidase CPVL*	0.026	0.562	
P16278	GLB1	*β-galactosidase*	0.038	0.564	
P39060	COL18A1	*Collagen α-1(XVIII) chain*	0.019	0.595	
P49327	FASN	*Fatty acid synthase*	0.034	0.612	
P17931	LGALS3	*Galectin-3*	0.033	0.624	
P14625	HSP90B1	*Endoplasmin*	0.032	0.633	
P63231	M2	*Matrix protein 2*	0.045	0.665	
Q9H173	SIL1	*Nucleotide Exchange factor SIL1*	0.043	0.689	
P10909	CLU	*Clusterin*	0.038	0.797	
P0CG06	IGLC3	*Ig lambda-3 chain C regions*	0.047	1.541	Over-represented
P01876	IGHA1	*Ig alpha-1 chain C region*	0.025	1.666	
P02647	APOA1	*Apolipoprotein A-*	0.029	1.764	
P06702	S100A9	*Protein S100-A9*	0.009	5.313	
P02763	ORM1	*Alpha-1-acid glycoprotein 1*	0.032	17.771	
P80188	LCN2	*Neutrophil gelatinase-associated lipocalin*	0.010	36.165	

iBAQ, intensity-based absolute quantification. Proteins were identified by Mann–Whitney test for unpaired samples ($P < 0.05$).

Fig. 1 Venn diagram of proteins in the study quantified by mass spectrometry. Proteins assigned to the control group (non-smokers) represent those absent or under-represented in the study group (smokers), whereas proteins exclusively or over-represented in the study group are assigned to this group. The intersection represents proteins conserved to both groups.



In the present study, we showed that smoking has a detrimental effect on functional aspects of sperm. Smokers had a lower percentage of sperm with high DNA integrity (Comet class I) as well as higher percentages of sperm with mid- to high sperm DNA fragmentation, when compared with non-smokers. Sperm DNA fragmentation can occur by several mechanisms [42], but the most important involves oxidative stress [43]. Cigarettes contain a complex mixture of components, of which many present oxidative activity, such as cadmium and nicotine, generating increased levels of superoxide anion and hydroxyl radical [44], leading to oxidative stress [45], which in turn leads to several alterations, among which is sperm DNA fragmentation [46].

Moreover, from the time when the input of Cd^{2+} in sperm occurs, the mechanisms of DNA damage are potentiated by topoisomerase IIB activity, which degrades DNA into ~50-Kb fragments. This mechanism may be reversible, but becomes decisive with other nucleases that completely degrade sperm DNA [47]. This type of mechanism has been shown in some animals, such as hamsters and mice, as well as in men [48]. Nicotine, the main alkaloid present in tobacco [44], is an important oxidant and reacts with the sperm membrane [49], which, in turn, affects sperm chromatin integrity, leading to a high frequency of single and double DNA strand-breaks [46].

Another finding with regard to sperm functional analysis in the present study was that smokers had less acrosome integrity. The sperm membrane is rich in polyunsaturated fatty acids [50] and their cytoplasm contains low concentrations of antioxidant enzymes. Because of a limited ability to repair damaged membranes, the sperm acrosome and mitochondrial membranes are thus highly susceptible to reactive oxygen species (ROS)-induced damages [51]. In the acrosome, ROS are capable of causing a transient permeability of the acrosome membrane to Ca^{2+}, which in turn may prematurely trigger exocytosis. This is probably the mechanism through which lower rates of intact acrosome occurred in the present study cohort; oxidative stress induced premature acrosome exocytosis [52].

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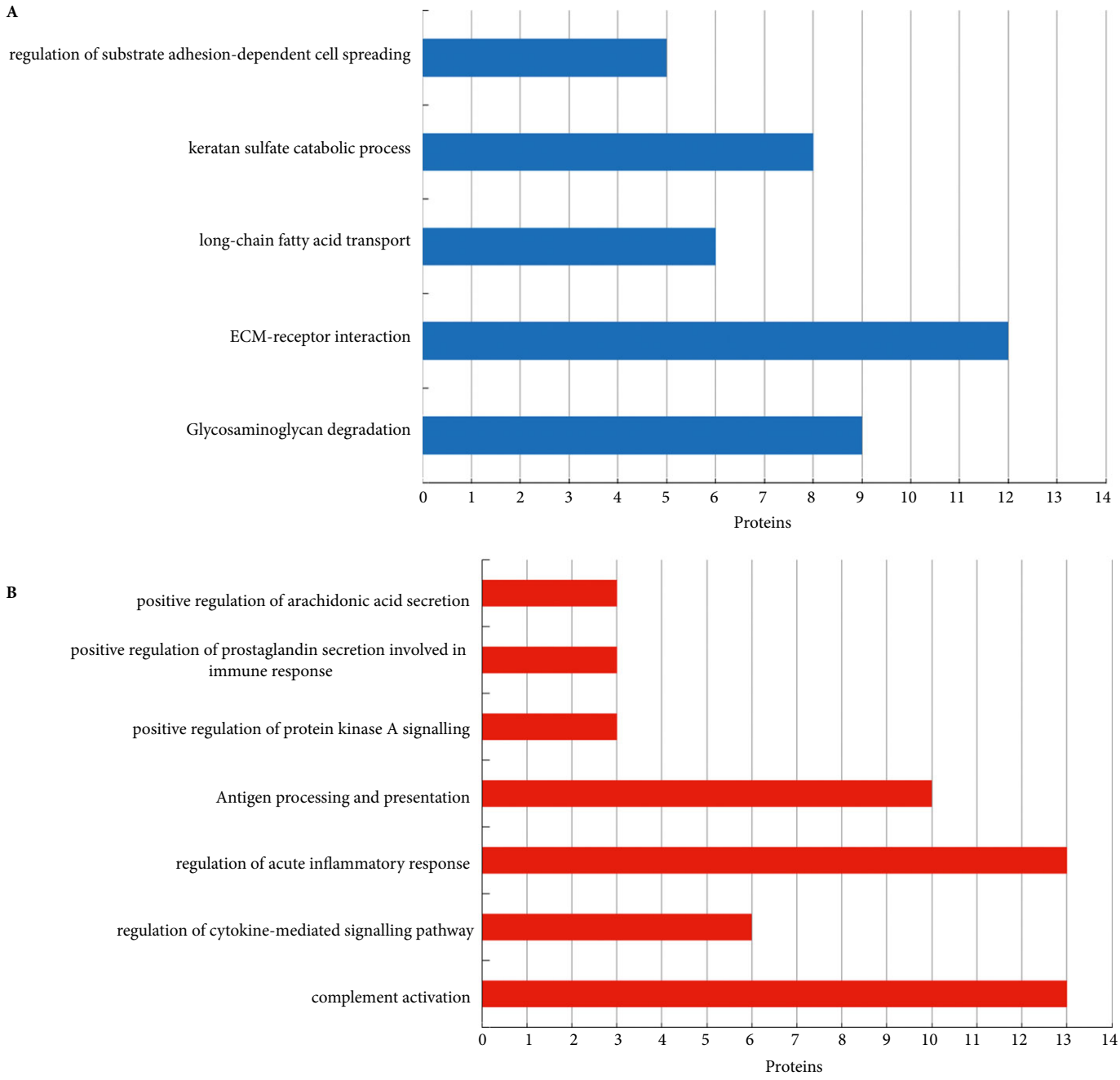
Fig. 2 Main statistically enriched functions in the control group (panel A) and in the smokers group (panel B) and number of proteins identified in this study related to each of these functions.



In addition to the regulation of the mechanisms mentioned above, Ca^{2+} also acts on mitochondrial activity [52]. The men in our study group (smokers) had lower sperm mitochondrial activity when compared with control group. Mitochondria captures cytosolic Ca^{2+} through uniporter channels present in the inner mitochondrial membrane and uses it for energy production in the form of ATP, and also in other cellular functions [52,53]. Moreover, oxidation of mitochondrial polyunsaturated fatty acids may also destabilize the membrane, leading to decreased selectivity in its permeability, which compromises maintenance of the mitochondrial membrane potential, ultimately compromising mitochondrial energy generation and further potentiating oxidative stress through the release of mitochondrial oxidants [54–56]. These mechanisms may be responsible for the increase in the percentage of mostly and completely inactive mitochondria

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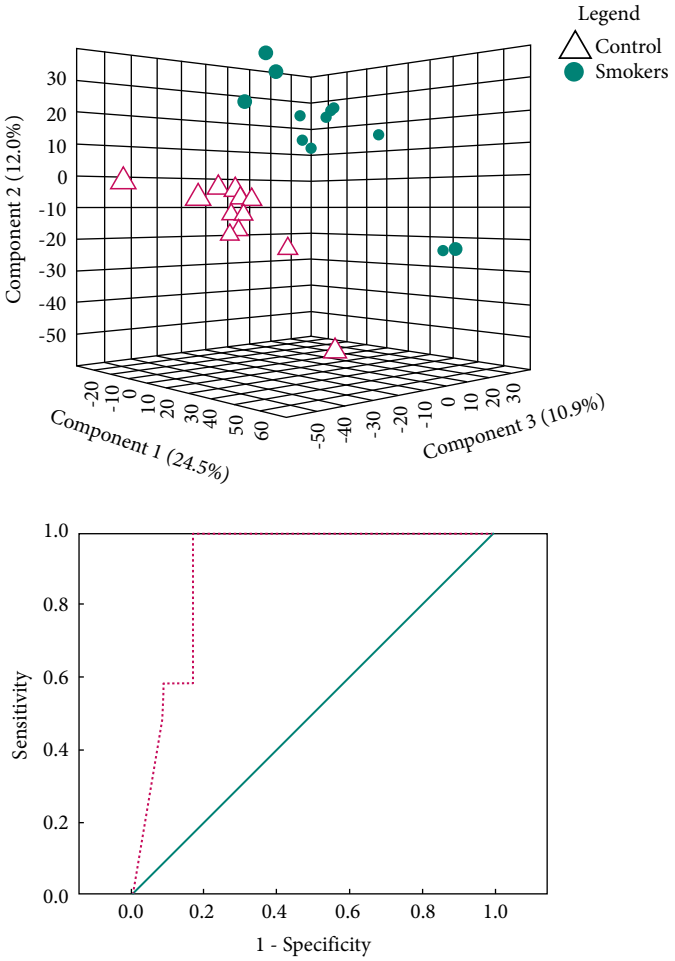
Fig. 3 Multivariate statistical analyses. Top: tridimensional graphic obtained in partial least-squares discriminant analysis, showing the groups separation. Bottom: receiver-operating characteristic curve obtained in the logistic regression analysis, with an area under the curve of 90.4%.



(DAB classes III and IV, respectively) observed in smokers compared with non-smokers. The control group, by contrast, had a higher percentage of predominantly active mitochondria (DAB class II), which was also consistent with previous results from our group [5].

Although the cellular mechanisms are well established [7,57], little is known regarding the molecular pathways which determine these effects [58]. Several studies have shown a relationship between infertility and alterations in the seminal plasma proteome [12–14,28,59,60] as well as an association with sperm functional quality [15,19]. In the present study, 422 proteins were observed, of which one was absent, six over-represented and 27 under-represented in smokers.

In the study group, most of the enriched functions were related to immune response. Cigarette smoking seems to promote an inflammatory response in the male reproductive tract, as a result of excessive ROS levels caused by its pro-oxidative components. This condition is further confirmed by the identification of the S100A9 protein in the multivariate statistical analysis in the study group. This protein belongs to the group of Ca^{2+}-binding proteins that have the EF-hand domain [61], and binds to pro-inflammatory receptors in order to initiate the inflammatory cascade [62]. Extracellular overexpression of S100A9, as we observed in the present study has been associated with chronic inflammation [63]. It is important to note that smokers commonly have leukocytospermia [9,64], which may have a prostatic origin as a result of chronic exposure to Cd^{2+} present in cigarettes, generating intense inflammation [65]. Moretti et al. [66] found increased levels of inflammatory markers such as resistin, interleukin-6 and TNF-α in the seminal plasma of smokers, suggesting that this panel may reduce sperm quality, which was demonstrated in our study group (increased DNA fragmentation and decreased mitochondrial activity and intact acrosomes) and may also decrease fertilization potential [66].

In addition, regulation of protein kinase A signalling, which is involved in sperm motility, acrosome reaction and capacitation [67–69], was hyper-regulated. This hyper-regulated function may trigger premature exocytosis, leading to early acrosome reaction. In this context, we can correlate this information with our findings regarding decreased acrosome integrity in smokers.

Furthermore, the protein Mammaglobin B, a marker in the control group, is present in several types of tissues including the prostate and epididymis, and its main function is associated with binding to steroid hormones [70] and chemotherapeutic agents (http://www.uniprot.org/uniprot/O75556). Its absence in the smokers group may suggest a pathway through which these men commonly experience changes in hormone profile, although this was not evaluated in the present study [9–11].

Additionally, the Prolargin protein also predicted the control group. This protein binds to groups of heparan sulphate, proteoglycans that constitute the basement membrane of various cells, including those of the male genitourinary system [71]. Its main function is to anchor the basement membrane to the underlying connective tissue, and binding to collagen type I [72]. This action of cell support is essential for cellular function, because the basement membrane interacts with cell receptors, controlling various mechanisms of cell survival, such as migration, proliferation, gene expression and apoptosis [73]. Thus, the expression of prolargin suggests that smokers can experience a damage to the basement membrane of the male urogenital tract, epididymis or other male sexual glands.

In conclusion, cigarette smoking is associated with an inflammatory state in the accessory glands and in the testis, as shown by enriched proteomic pathways. This state effects an alteration in sperm functional quality, which is characterized by decreased acrosome integrity and mitochondrial activity as well as by increased nuclear DNA fragmentation.

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Conflict of Interest

None declared.

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Abbreviations: LC, liquid chromatography; MS, mass spectrometry; iBAQ, intensity-based absolute quantification; PLS-DA, partial least-squares discriminant analysis; VIP, Variable Importance in the Projection; ROC, receiver-operating characteristic; ROS, reactive oxygen species.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Complete list of conserved proteins, equally represented both in control and smoker group.

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