Cysteine-rich secretory protein 3: inflammation role in adult varicocele

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ABSTRACT

Background: Cysteine-rich secretory protein (CRISP-3), a protein involved in inflammatory response, is highly increased in seminal plasma of adolescents with varicocele and altered semen analysis, but not in adolescents with varicocele and normal semen. It is not known, however, whether this increased seminal concentration occurs as an acute marker during the initial stages of varicocele or whether this persists as an altered protein pathway.

Objective: The purpose of this study, thus, was to test the hypothesis that this inflammatory state persists through adulthood and the correction of varicocele could correct this state, by identifying the levels of CRISP-3 in seminal plasma.

Materials and methods: This study was carried out in two substudies: (i) to verify the effect of varicocele and (ii) to verify the effect of varicocelectomy on seminal plasma CRISP-3 levels. Seminal plasma CRISP-3 levels (29 and 31 kDa isoforms) were assessed for each provided sample using standard Western blotting.

Results: The varicocele group presented higher seminal levels of CRISP-3 when compared to controls, with a 67.5-fold increase in the unglycosylated isoform (29 kDa) and a 5.2-fold increase in the glycosylated isoform (31 kDa). In contrast, CRISP-3 levels decreased following varicocelectomy, both in the unglycosylated (5.6-fold decrease) and in the glycosylated (4.3-fold decrease) isoforms.

Discussion: CRISP-3, a protein involved in inflammation, is increased in seminal plasma of men with varicocele and this is partially reversed by varicocelectomy. Monitoring its seminal levels may be useful for assessing inflammation-related alterations to fertility in men with varicocele.

Conclusion: We conclude that, in the presence of varicocele, there is a marked increase in seminal CRISP-3 levels. Surgical intervention (varicocelectomy) decreases CRISP-3 levels and improves semen quality.

INTRODUCTION

Varicocele, the main treatable cause of male infertility (Benoff & Gilbert, 2001; Hauser et al., 2001), is defined by the presence of dilated veins in the pampiniform plexus with blood reflux (Gat et al., 2004). Varicocele is detected in 35% of men with primary infertility, and up to 80% of men with secondary infertility (Pryor & Howards, 1987; Gorelick & Goldstein, 1993; Witt & Lipshultz, 1993; Smith et al., 2006), which is why it has long been suggested that varicocele causes a progressive decline in male fertility (Cозzolino & Lipshultz, 2001; Saleh et al., 2003; Zini et al., 2005). In fact, in 1967, Brown, Dubin, and Hotchkiss postulated that varicocele, when present, is likely universally detrimental and that even a fertile man with varicocele would likely present better semen if the varicocele were not present (Brown et al., 1967).

Surgical treatment of varicocele—varicocelectomy—has been associated with an increase in sperm DNA integrity and mitochondrial activity (Lacerda et al., 2011) and in testicular volume (Practice Committee of the American Society for Reproductive Medicine and Society for Male Reproduction and Urology, 2014). In adults, studies have shown improved semen quality and fertility (Madgar et al., 1995; Schlegel, 1997; Nieschlag et al., 1998), as well as differences in seminal plasma protein expression, when comparing patients before and after varicocelectomy (Camargo et al., 2013). This observed altered protein expression demonstrates that surgical repair of varicocele affects molecular pathways in the testis and that this is associated with improved semen quality.

Recently, in order to better understand how varicocele affects the seminal plasma proteome, we performed a study in...
adolescents with varicocele using a gel-free label-free proteomics approach (Del Giudice et al., 2016). In that study, different proteomic pathways were observed and a panel of potential protein biomarkers related to testicular dysfunction in varicocele was suggested. To confirm the potential function of these proteins as biomarkers, we then performed downstream Western blotting analyses (Belardin et al., 2016; Del Giudice et al., 2016) which corroborated with the mass spectrometry results.

Of special note from those studies, cysteine-rich secretory protein 3 (CRISP-3; UniProt accession—P54108) (Del Giudice et al., 2016) specifically marked adolescents with varicocele who presented altered semen—its values were greatly increased in these patients when compared to adolescents without varicocele or to adolescents with varicocele and normal semen analysis. At the time, we discussed that because CRISP-3 is associated with an inflammatory response, it may be a marker of the point in time at which the adolescent varicocele expresses itself as a de facto disease. However, we do not know whether seminal CRISP-3 values would also be increased when a varicocele is fully established (i.e., in adults).

We therefore wished to verify whether CRISP-3 levels were also increased in semen of adults with varicocele. Because of its high overexpression in adolescents, we hypothesize the adult varicocele will present altered CRISP-3 levels. Moreover, because varicocelectomy has been shown to improve semen quality (Madgar et al., 1995; Schlegel, 1997; Nieschlag et al., 1998), or sperm functional quality (Lacerda et al., 2011), we also wished to verify whether varicocelectomy would lead to alterations in CRISP-3 levels.

MATERIAL AND METHODS

Study design

Institutional review board approval was obtained from the São Paulo Federal University (Universidade Federal de São Paulo—UNIFESP; Brazil) Research Ethics Committee (CAAE: 545415156.2.0000.5505). All included subjects provided their informed, written consent. A study was carried out, recruiting men aged between 20 and 50 years old referred to the Andrology Laboratory of the Human Reproduction Section (UNIFESP; Brazil).

All participants were submitted to a clinical evaluation by a trained urologist, in which they provided their complete medical history and were submitted to physical evaluation in a temperature-controlled room with adequate illumination. Varicocele was detected by scrotal palpation, according to the method proposed by Dubin and Amelar (Dubin & Amelar, 1970). Semen samples were collected at the Andrology Laboratory by masturbation after 2–5 days of ejaculatory abstinence (WHO, 2010).

Exclusion criteria, for all patients, were as follows: fever in the 90-day period prior to semen analysis, presence of systemic diseases (such as cancer and endocrinopathies and their treatments), endocrine disorders, obesity, congenital genital malformation, genetic syndromes, history of inguinoscrotal surgery, orchitis or epididymitis, testicular torsion, or testicular dystopia, and smoking or use of other drugs.

This study was carried out as two separate substudies: a cross-sectional study and an intervention study. Both cohorts were prospectively recruited for this study. The cross-sectional study, henceforth termed ‘substudy I’, aimed at verifying the effect of the presence of a varicocele on seminal plasma CRISP-3 levels. Toward this end, two groups of patients were recruited, as follows:

- Control group (C): patients without clinical varicocele and with normal semen analysis (World Health Organization, 2010) (n = 25);
- Varicocele group (V): patients with uni- or bilateral grades II or III varicocele and normal semen analysis (World Health Organization, 2010) (n = 36).

The intervention study, henceforth termed ‘substudy II’, aimed at verifying whether treatment of the varicocele would cause a perturbation in the seminal plasma levels of CRISP-3. Toward this end, 22 patients were recruited for this branch of the study, in which they provided a sample (‘pre-varicocelectomy sample’), were then treated by a surgical approach, as stated below, and then provided another sample after 6 months of treatment (‘post-varicocelectomy sample’).

All patients participating in substudy II study had indication for intervention, as per American Urological Association (AUA) and American Society for Reproductive Medicine/Society for Male Reproduction and Urology (ASRM/SMRU) guidelines (Male Infertility Best Practice Policy Committee of the American Urological Association and Practice Committee of the American Society for Reproductive Medicine, 2004; Practice Committee of American Society for Reproductive Medicine, 2008; Practice Committee of the American Society for Reproductive Medicine and Society for Male Reproduction and Urology, 2014). These patients were referred to the Division of Urology of our University and were submitted to uni- or bilateral varicocelectomy repair by subinguinal microsurgical varicocelectomy (Marmar et al., 1985) at our University Hospital, using a Zeiss S8.8 surgical microscope. Patients returned at 3 and 6 months postsurgery for follow-up. At 3 months, patients were again examined for varicocele by scrotal palpation, as previously described, in order to verify whether the procedure was successful (no internal spermatic vein retrograde blood flow during a Valsalva maneuver). For both substudies, patients with highest grade varicocele of grade I were not included in the control group. This decision was based on conflicting reports that show that grade I varicoceles seem to affect testicular function, but its effect is not homogeneous, nor is it clearly determinant of a different seminal phenotype (Mori et al., 2008; Damsgaard et al., 2016). However, in substudy II, patients with a bilateral varicocele that presented itself as grade I in one of the sides (e.g., left grade II, right grade I) were intervened on both sides.

After semen liquefaction, an aliquot was used for semen analysis, performed according to the World Health Organization 2010 guidelines (World Health Organization, 2010). Another aliquot was used for evaluation of sperm mitochondrial activity and acrosome integrity, as described below. The remaining semen volume was centrifuged at 800 xG for 30 min to separate the supernatant seminal plasma, which was frozen without cryoprotectants and kept at −20 °C until Western blotting analysis for CRISP-3. All reagents used in this study were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Missouri, USA), unless otherwise described.

Sperm mitochondrial activity

Sperm mitochondrial activity was evaluated by midpiece sperm staining with 3,3′-diaminobenzidine (DAB) (Hrudka,
with 60 for 15 min and air-dried again. Spermatozoa were stained methanol (Merck Millipore, Burlington, Massachusetts, USA) microscope slides and air-dried. The slides were fixed in a modified Lowry et al.

were then collected and used for proteomics experiments.

for CRISP-3 levels analysis by Western blotting

Sperm acrosome integrity

Acrosome integrity was verified by staining with peanut agglutinin, a lectin that binds to the outer sperm acrosome membrane (Roth et al., 1998; Intasqui et al., 2015; Antoniassi et al., 2016). Briefly, two 15 µL smears were prepared on microscope slides and air-dried. The slides were fixed in methanol (Merck Millipore, Burlington, Massachusetts, USA) for 15 min and air-dried again. Spermatozoa were stained with 60 µg/mL fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (Sigma Aldrich) in phosphate-buffered saline for 30 min in the dark and subsequently washed with milli-Q water to remove background staining.

A total of 200 spermatozoa were analyzed using an Olympus BX-51 phase contrast upright microscope (Olympus Corporation, Tokyo, Japan) under 1000× magnification. Cells were classified as follows: class I = 100% of the midpiece stained; class II = more than 50% of the midpiece stained; class III = less than 50% of the midpiece stained; and class IV = absence of staining in the midpiece (Blumer et al., 2008; Fariello et al., 2012a,b; Intasqui et al., 2015; Antoniassi et al., 2016).

CRISP-3 levels analysis by Western blotting

In order to assess seminal plasma CRISP-3 levels, frozen seminal plasma samples, that had been stored at −20 °C, were thawed at room temperature and centrifuged at 15 100 xG for 30 min at 4 °C in order to remove cellular debris. Supernatants were then collected and used for proteomics experiments.

Total protein concentration of each sample was measured using a modified Lowry–bicinchoninic acid (BCA) assay, according to the manufacturer’s recommendation (Smith et al., 1985). Samples were diluted (1:80) in milli-Q water and measured in triplicate, while standard curve points (0, 200, 400, 600, 800, and 1000 mg/mL bovine serum albumin in water) were measured in duplicate, in a 96-well plate. Absorbance was measured using a microplate reader at 540 nm, and concentrations were calculated from the curve. Only standard curves with over 0.99 r² values were accepted, and samples with a coefficient of variation during quantification of over 5% were quantified, to ensure accuracy.

A volume of seminal plasma containing 50 µg from each patient was then suspended in milli-Q water to a final volume of 7.5 µL. This was then diluted 1:1 (vol:vol) in sample buffer (0.125 mol/L Tris-HCl, pH 6.8, 4% [w:vol] sodium dodecyl sulfate [SDS], 20% [vol:vol] glycerol, 5% [vol:vol] β-mercaptoethanol, 0.01% [w:vol] bromophenol blue) and heated to 100 °C in a dry bath for 5 min. Samples were then loaded onto 10% polyacrylamide gels (8.5 × 8.5 cm) under denaturing conditions, ensuring samples from different groups/substudies were run in a same gel (a full-range rainbow marker was used as a molecular mass marker (GE Healthcare, Amersham Place, UK)). Gels were submitted to electrophoresis at 50 V for 30 min, followed by 150 V until the end of the gel. After one-dimensional electrophoresis, proteins were transferred from the gels to 0.45-µm nitrocellulose membranes by using a wet transfer system (MiniVE, GE Healthcare, Amersham Place, UK).

Nitrocellulose membranes were then incubated in blocking buffer (3% [w:vol]) bovine serum albumin [BSA] in Tris-buffered saline (20 mmol/L Tris, 150 mmol/L NaCl [w:vol] pH 7.6) with 0.1% Tween 20 (TTBS) for 1 h at room temperature, washed in TTBS, and incubated with primary antibodies against the protein of interest (CRISP-3—anti-human CRISP-3 polyclonal antibody produced in rabbit [C9996, Sigma-Aldrich, Saint Louis, Missouri, USA]), diluted in blocking buffer (1:1000 [vol:vol]) for 2 h at room temperature under constant agitation. Membranes were then washed (three times, 10 min) with TTBS under constant agitation, followed by incubation with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (A0545, Sigma-Aldrich, Saint Louis, Missouri, USA) secondary antibody (1:1000 dilution in TTBS) for 1 h at room temperature under constant agitation. Membranes were again washed for 10 min, three times with TTBS under constant agitation.

Following antibody binding, membranes were incubated with a chemiluminescence (ECL) reagent (Amersham ECL Western Blotting Detection Reagent [25006262], GE Healthcare, Amersham Place, UK), and images were acquired using a LAS-4000 system. Stored images were finally processed using ImageQuant TL 7.0 Software (GE Healthcare, Amersham Place, UK). Both CRISP-3 unglycosylated [29 kDa] and glycosylated isoforms [31 kDa]) were quantified.

Following protein detection, membranes were incubated in mild stripping buffer (0.2 mol/L glycine, 0.1% [w:vol] SDS, 1% [vol:vol] Tween 20, pH 2.2) and probed with a primary anti-human DJ-1 (Protein deglycase DJ-1, gene name: PARK7; UniProt accession Q99497) goat polyclonal antibody as loading control protein (SAB2500750, Sigma-Aldrich, Saint Louis, Missouri, USA) (Belardin et al., 2016; Del Giudice et al., 2016; Wiśniewski & Mann, 2016), diluted in blocking buffer (1:1000 [vol:vol]) for 2 h at room temperature under constant agitation. Membranes were washed, incubated with an HRP-conjugated secondary antibody, and submitted to ECL, as described above.

Statistical analysis

For statistical analysis, the SPSS (PASW) 18.0 software was utilized. Semen analysis, sperm functional analysis, and Western blotting data were tested for normality of distribution using a Kolmogorov–Smirnov test, and for homogeneity of variance using a Levene test. For substudy I (effect of varicocele), normally distributed and homoscedastic variables were compared between control and varicocele groups using an unpaired
RESULTS
Clinical and seminal characteristics of the study populations for both cohorts are presented in Tables 1 and 2. In this study, samples from patients with varicocele presented lower semen quality, observed by lower ejaculate volume, sperm concentration, total sperm count, and sperm morphology (Table 1). Sperm functional quality was also decreased in men with varicocele in this study, observed by a lower percentage of acrosome intact spermatozoa and a higher percentage of spermatozoa with inactive mitochondria (DAB IV) when compared to controls without varicocele (Table 1).

For our prospective intervention cohort, at 6 months post-varicocelectomy, patients generally presented improved semen quality, as observed by an increase in ejaculate volume and in the percentage of spermatozoa with normal morphology (Table 2). Moreover, sperm function was also somewhat improved, as observed by an increase in the percentage of spermatozoa with all active mitochondria (DAB I) (Table 2).

Protein expression levels are all presented in Figure 1, for both cohorts. Figure 1e presents typical Western blotting membranes submitted to enhanced chemiluminescence captured by the LAS 4000 imaging equipment, cropped to the area of interest, without any adjustment for contrast. An uncropped original image is supplied as a supplementary figure as well (Figure S1).

Seminal plasma CRISP-3 levels were higher in men with varicocele when compared to controls. This was observed for both the unglycosylated and the glycosylated isoforms. The 29 kDa unglycosylated isoform was 68-fold higher in patients with varicocele (mean; standard deviation = 26.6; 23.1 in varicocele, 0.25; 0.25 in controls. Data are arbitrary units from the imaging equipment). The 31 kDa glycosylated isoform was fivefold higher in patients with varicocele (mean; standard deviation = 30.1; 27.5 in varicocele, 4.1; 4.2 in controls). Effect sizes for these results were also high (d = 1.58 and 1.42, respectively) as was achieved statistical power (0.99 for both). These data are summarized in Figure 1a,b.

DISCUSSION AND CONCLUSION
Varicocele is the most prevalent clinical finding in infertile men. Diagnosis, as recommended by the AUA and the ASRM/SMRU, is quite straightforward—a physical examination of a patient in a room with adequate illumination and temperature suffices for determination of the presence of the pathognomonic venous dilation and blood reflux in the pampiniform plexus (Male Infertility Best Practice Policy Committee of the American Urological Association and Practice Committee of the American Society for Reproductive Medicine, 2004; Practice Committee of American Society for Reproductive Medicine, 2008; Practice Committee of the American Society for Reproductive Medicine and Society for Male Reproduction and Urology, 2014). However, defining a varicocele that is determining, or at least

Table 1 Seminal and functional analysis (substudy I)

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 25)</th>
<th>Varicocele (n = 36)</th>
<th>p</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>33.0; 10.00</td>
<td>34.0; 9.00</td>
<td>0.547</td>
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<tr>
<td>Volume (mL)</td>
<td>3.4; 1.80</td>
<td>2.3; 1.70</td>
<td>0.011*</td>
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<tr>
<td>Sperm concentration</td>
<td>89.0; 138.0</td>
<td>38.6; 97.00</td>
<td>0.001*</td>
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<tr>
<td>Total count (×10⁶/mL)</td>
<td>299.5; 243.00</td>
<td>70.2; 289.00</td>
<td>&lt;0.00001*</td>
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<tr>
<td>Progressive motility (%)</td>
<td>51.3; 8.88</td>
<td>49.9; 13.05</td>
<td>0.140</td>
</tr>
<tr>
<td>Non-progressive motility (%)</td>
<td>6.0; 3.00</td>
<td>5.0; 5.00</td>
<td>0.039*</td>
</tr>
<tr>
<td>Immotile (%)</td>
<td>42.0; 70.00</td>
<td>45.0; 11.00</td>
<td>0.213</td>
</tr>
<tr>
<td>Morphology (%) normal</td>
<td>8.0; 4.00</td>
<td>5.0; 5.00</td>
<td>0.001*</td>
</tr>
<tr>
<td>Round cells (×10⁶/mL)</td>
<td>13.2; 2.00</td>
<td>1.0; 1.00</td>
<td>0.197</td>
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<tr>
<td>Neutrophils (×10⁶/mL)</td>
<td>0.0; 0.00</td>
<td>0.0; 0.00</td>
<td>0.855</td>
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<tr>
<td>Acrosome integrity (%)</td>
<td>80.1; 5.71</td>
<td>73.4; 9.35</td>
<td>0.008*</td>
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<tr>
<td>DAB I (%)</td>
<td>4.5; 7.80</td>
<td>2.0; 12.30</td>
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<tr>
<td>DAB II (%)</td>
<td>72.8; 14.01</td>
<td>69.9; 9.97</td>
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<td>DAB III (%)</td>
<td>15.5; 7.80</td>
<td>15.0; 13.30</td>
<td>0.083</td>
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<tr>
<td>DAB IV (%)</td>
<td>2.0; 9.00</td>
<td>4.0; 2.80</td>
<td>0.010*</td>
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</table>

Groups were compared by Mann–Whitney (values expressed in median; interquartile range), unless otherwise noted. *Statistically significant difference. Unpaired Student’s t-test. Values expressed in mean; standard derivation.

Table 2 Seminal and functional analysis (substudy II)

<table>
<thead>
<tr>
<th></th>
<th>Pre-varicocelectomy (n = 22)</th>
<th>Post-varicocelectomy (n = 22)</th>
<th>p</th>
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<tbody>
<tr>
<td>Age (years)*</td>
<td>29.7; 6.90</td>
<td>29.9; 6.51</td>
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<tr>
<td>Volume (mL)</td>
<td>2.1; 1.50</td>
<td>3.2; 1.4</td>
<td>0.002*</td>
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<tr>
<td>Sperm concentration</td>
<td>27.1; 19.90</td>
<td>25.2; 22.99</td>
<td>0.618</td>
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<tr>
<td>(×10⁹/mL)*</td>
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<tr>
<td>Total count (×10⁶)</td>
<td>31.4; 77.25</td>
<td>42.6; 138.00</td>
<td>0.355</td>
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<tr>
<td>Progressive motility (%)</td>
<td>45.0; 22.00</td>
<td>43.0; 28.00</td>
<td>0.499</td>
</tr>
<tr>
<td>Non-progressive motility (%)</td>
<td>5.0; 5.00</td>
<td>5.0; 3.00</td>
<td>0.709</td>
</tr>
<tr>
<td>Immotile (%)</td>
<td>49.0; 29.00</td>
<td>48.0; 29.00</td>
<td>0.702</td>
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<tr>
<td>Morphology (%) normal</td>
<td>3.0; 4.00</td>
<td>5.0; 3.00</td>
<td>0.018*</td>
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<td>Round cells (×10⁶/mL)</td>
<td>0.8; 1.20</td>
<td>0.6; 1.10</td>
<td>0.471</td>
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<tr>
<td>Neutrophils (×10⁶/mL)</td>
<td>0.0; 0.00</td>
<td>0.0; 0.00</td>
<td>0.207</td>
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<tr>
<td>Acrosome integrity (%)</td>
<td>75.6; 10.05</td>
<td>74.5; 9.20</td>
<td>0.328</td>
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<tr>
<td>DAB I (%)</td>
<td>2.0; 2.00</td>
<td>2.5; 3.30</td>
<td>0.030*</td>
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<tr>
<td>DAB II (%)</td>
<td>71.3; 10.49</td>
<td>70.7; 9.81</td>
<td>0.929</td>
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<tr>
<td>DAB III (%)</td>
<td>22.1; 8.13</td>
<td>21.9; 9.64</td>
<td>0.747</td>
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<tr>
<td>DAB IV (%)</td>
<td>4.9; 3.66</td>
<td>4.8; 2.45</td>
<td>0.841</td>
</tr>
</tbody>
</table>

Groups were compared by a paired Wilcoxon signed rank test for Pre- and Post-varicocelectomy samples groups (values expressed in median; interquartile range), unless otherwise noted. *Statistically significant difference. Unpaired Student’s t-test. Values expressed in mean; standard derivation.

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contributing to an infertile state, is not as straightforward. In fact, while the World Health Organization demonstrated, in 1992, that varicocoele is more than twice as prevalent in infertile men than in a group of known fertility (‘The influence of varicocoele on parameters of fertility in a large group of men presenting to infertility clinics. World Health Organization’, 1992), up to 85% of men with varicocoeles may be fertile (Sylora & Pryor, 1994).

Bearing this in mind, it would seem logical to infer that increasing comprehension of the biological mechanisms underlying varicocoele would advance our capability of properly identifying an infertility-defining varicocoele. Under this assumption, we have recently identified cysteine-rich secretory protein 3 (CRISP-3) as a protein that is highly increased in seminal plasma of adolescents with varicocoele when semen quality is altered (Del Giudice et al., 2016). In fact, these values were close to 90-fold increased when compared to values observed in adolescents without varicocoele, or with varicocoele and normal semen in that study. In this study, we wished to verify whether this was merely related to the initial establishment of varicocoele, or whether this protein would present a differential expression pattern in adults emulating those observed in adolescents. Moreover, we wished to verify whether surgical correction of varicocoeles would affect CRISP-3 values as well.

CRISP-3, a member of the cysteine-rich secretory proteins (CRISPs) family (Eberspaecher et al., 1995), is a 245 amino acid-long protein (‘CRISP-3 Gene—GeneCards | CRIS3 Protein | CRIS3 Antibody’) encoded by the CRISP-3 gene and presents a N-glycosylation site at position 239 (‘CRISP-3—Cysteine-rich secretory protein 3—Proteomics’). In vivo, both the unglycosylated form [29 kDa] and the glycosylated form [31 kDa] are observed (Udby et al., 2002a, 2005a; ‘CRISP-3 Gene—GeneCards | CRIS3 Protein | CRIS3 Antibody’).

In the present study, as was the case for our previous study with adolescents with varicocoele (Del Giudice et al., 2016), our results showed that both 29 (unglycosylated) and 31 kDa (glycosylated) isoforms of CRISP-3 are highly overexpressed in the seminal plasma of men with varicocoele, with a 67.5-fold and 5.2-fold increase when compared to controls, respectively. Furthermore, we demonstrated that varicocelectomy is capable of decreasing 5.6-fold the 29 kDa isoform, and 4.3-fold the 31 kDa isoform, when compared to the CRISP-3 levels in the seminal plasma prior to surgery.
CRISP-3 mRNA and protein expression has been observed throughout the secretory epithelium from the epididymis to the prostate gland (Udby et al., 2005b). Although CRISP-3 has been shown to participate in reproduction, both its localization to neutrophils and its presence in exocrine secretions demonstrate its role in innate immunity, defense response, and chronic inflammation (Udby et al., 2002b, 2005a; Liao et al., 2003). Its overexpression in the varicocele group and its decrease after varicocelectomy may also reflect a possible inflammatory state in the testicular tissue, albeit of a more chronic nature than our results in adolescents would suggest (Del Giudice et al., 2016). Interestingly, other studies from our group as well as from other groups have associated varicocele with an inflammatory testicular environment (Camargo et al., 2013; Del Giudice et al., 2013; Zylbersztejn et al., 2013; Fraczek & Kurpisz, 2015). It seems, thus, that an inflammatory environment is a typical molecular phenotype of varicocele-derived male infertility, and our results from this study support this concept.

Accordingly, CRISP-3 upregulation is associated with the downregulation of ANXA1, an anti-inflammatory gene, leading to the establishment of an inflammatory state (Pathak et al., 2016). Additionally, CRISP-3 has been found to be upregulated in prostate cancer and its recurrence, and in prostate cancer metastatic activity, conditions which have been demonstrated to be associated with inflammation (Kosari et al., 2002; Bjartell et al., 2007; Dahlman et al., 2010; Grupp et al., 2013; Al Bashir et al., 2014). Finally, it was previously suggested that CRISP-3 may modulate the activity of other genes either at the expression or at the functional level which, in turn, may control cell invasion in prostate cancer (Pathak et al., 2016). This suggested gene expression modulation is important because alterations in gene expression can alter sperm function and quality (Castaneda et al., 2017; Ratnam et al., 2017). Indeed, in our study, varicocele was associated with a decrease in seminal and functional quality of spermatozoa, when compared with controls group. We also observed that varicocelectomy leads to restoration of seminal variables and sperm functional traits.

Moreover, inflammation and upregulation of inflammatory proteins have been shown to determine testicular alterations, thus resulting in altered semen parameters (GuaZZone et al., 2009). In the present study, we observed low mitochondrial activity in men with varicocele, and higher mitochondrial activity after varicocelectomy. While inflammatory pathways may be activated by diverse stimuli, mitochondrial dysfunction is a point of convergence downstream of these stimuli, in that inflammatory agents interact with mitochondria and alter their functional status (LóPez-Armada et al., 2013). Indeed, in semen, chronic prostatitis has been shown to determine the release of mitochondrial proteins (Hu et al., 2007) while incubation of semen with infectious agents leads to loss of mitochondrial membrane potential (Fraczek et al., 2012). Therefore, mitochondria are important modulators of inflammatory activity in response to cellular stress and mitochondrial damage, and the inflammation process leads to mitochondrial dysfunction (West, 2017).

On the other hand, CRISP-3 has also been suggested to participate in gamete fusion and fertilization, because of its localization to the acrosome and tail of capacitated spermatozoa, and to the equatorial region of acrosome-reacted spermatozoa, although no alteration in oocyte penetration was observed when blocking its effect by the use of an anti-CRISP-3 antibody. The two isoforms (29 and 31 kDa) can be observed in ejaculated spermatozoa, and the 31 kDa isorm is released during capacitation, whereas the 29 kDa isorm remains attached to the sperm membrane even after capacitation and acrosome reaction (Da Ros et al., 2015). Therefore, one can hypothesize that the 31 kDa isorm could be important for modulation of sperm capacitation, while the 29 kDa isorm is important for gamete fusion per se. Interestingly, in our study, patients with varicocele presented lower acrosome integrity. While this may have been brought upon by the same mechanisms that decreased sperm mitochondrial activity (Fraczek & Kurpisz, 2007), it should be noted that this acrosomal damage could lead to the release of both CRISP-3 isoforms to the seminal plasma. The fact that CRISP-3 presents a calcium-binding domain and an ion channel regulator domain (Gibbs et al., 2006) further supports the association between CRIPS3 levels and acrosome integrity.

Taking into consideration that higher levels of both isoforms were observed in the seminal plasma of men with varicocele, it is possible to suggest two pathways that can be altered in varicocele. The first one is that the inflammation events in varicocele lead to the increased CRISP-3 levels, which in turn activate or potentiate downstream pro-inflammatory events that determine decreased semen or sperm functional quality. The second is that an acrosomal or mitochondrial dysfunction might be associated with the release of these proteins to the seminal plasma instead of their attachment to the sperm membrane, which may be related to the observed infertility (Fraczek et al., 2012; Fraczek & Kurpisz, 2015).

In this study, we observed medium to high effect sizes, which demonstrates that differences were indeed quite high. Because effect size is directly related to sample overlap (Jacob Cohen, 1988), our results indicate that CRISP-3 is in essence of a discerning capability between presence and absence of varicocele. While it is obvious that varicocele is diagnosed by a physical examination, and not by detection of a molecular marker in semen, these results offer support for an interpretation that indeed seminal plasma molecular profiles reflect the presence or absence of varicocele, as we and others have previously demonstrated (Benoff et al., 2009; Del Giudice et al., 2013, 2016; Zylbersztejn et al., 2013; Agarwal & Esteves, 2016; Belardin et al., 2016). Mapping these profiles is a first step in constructing the panorama of the seminal molecular milieu in humans, and this study demonstrates that inflammation is a portion of this panorama.

Taken together, our data along with previous studies (Benoff et al., 2009; Del Giudice et al., 2013, 2016; Zylbersztejn et al., 2013; Agarwal & Esteves, 2016; Belardin et al., 2016) strengthen the link between CRISP-3 and its inflammatory function in varicocele. It remains to be seen which pathways are affected by this protein, and which are other regulators of these pathways. Even with no significant improvements in semen quality in all patients after varicocelectomy, with our study it is possible to observe that there is an important effect in the protein levels after the surgery. These levels may indicate the beginning of restoration of inflammatory/non-inflammatory pathways. The better knowledge of these pathways and CRISP-3 can influence in the clinical practice, because if we know the correct levels of this protein, we can monitor the efficacy of the surgery on decreasing the seminal inflammatory state.
We therefore conclude, based on our results that, in the presence of varicocele, there is a marked increase in seminal CRISP-3 levels, especially in the unglycosylated form. Surgical intervention (varicocelectomy) decreases CRISP-3 levels and improves semen quality.

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DISCLOSURE
The authors have no conflict of interest to disclosure.

AUTHORS’ CONTRIBUTIONS
Larissa Belardin: Conception and design of the work; acquisition, analysis, and interpretation of the data; drafting and revising the work; and final approval of the version to be published. Mariana Camargo: Design of the work; acquisition and interpretation of the data for the work; revising the work; and final approval of the version to be published. Paula Intasqui: Acquisition and interpretation of the data for the work; revising the work; and final approval of the version to be published. Mariana Antoniassi: Interpretation of the data for the work; revising the work; and final approval of the version to be published. Renato Fraietta: Acquisition of the data for the work; revising the work; and final approval of the version to be published. Ricardo Bertolla: Conception and design of the work; analysis and interpretation of the data; drafting and revising the work; and final approval of the version to be published.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Original uncropped digitized image containing the entire Western blot membrane.