

# Quantification of CatSper I expression in human spermatozoa and relation to functional parameters

L. Tamburrino<sup>1</sup>, S. Marchiani<sup>1</sup>, E. Vicini<sup>2</sup>, B. Muciaccia<sup>2</sup>, M. Cambi<sup>1</sup>, S. Pellegrini<sup>3</sup>, G. Forti<sup>1</sup>, M. Muratori<sup>1</sup>, and E. Baldi<sup>1,\*</sup>

<sup>1</sup>Department of Biomedical Experimental and Clinical Sciences, Center of Excellence DENOthe, University of Florence, Viale Pieraccini 6, Florence I-50139, Italy <sup>2</sup>Fondazione Pasteur Cenci Bolognietti, Department of Anatomy, Histology, Forensic Medicine and Orthopedic, Section of Histology, Sapienza University of Rome, Italy <sup>3</sup>Centro Procreazione Assistita 'Demetra' of Florence, Via Caccini 18, Florence I-50139, Italy

\*Correspondence address. E-mail: elisabetta.baldi@unifi.it

Submitted on December 17, 2014; resubmitted on April 2, 2015; accepted on April 20, 2015

**STUDY QUESTION:** Is CatSper I expression in human spermatozoa related to semen parameter values and sperm functions?

**SUMMARY ANSWER:** CatSper I expression is positively related to progressive and hyperactivated (HA) motility,  $[Ca^{2+}]_i$  responsiveness to progesterone but not the acrosome reaction (AR).

**WHAT IS KNOWN ALREADY:** The role of cationic channel of sperm (CatSper) in sperm functions is clear in animal models but less defined in human sperm cells. Current knowledge is mostly based on low specificity CatSper inhibitors showing agonistic and toxic effects on human spermatozoa and is thus of little help in clarifying the role of the CatSper channel in human sperm functions.

**STUDY DESIGN, SIZE, DURATION:** CatSper I protein expression was evaluated in 115 men undergoing semen analysis for couple infertility. CatSper I expression was related to routine semen parameters, motility kinematic parameters and basal and progesterone-stimulated  $[Ca^{2+}]_i$  and the AR.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** CatSper I expression was evaluated ( $n = 85$  normozoospermic,  $n = 30$  asthenozoospermic patients) by immunofluorescence coupled to flow cytometry leading to quantitative measurement of the percentage of ejaculated sperm cells expressing the protein. Semen analysis was evaluated according to World Health Organization guidelines. Kinematic parameters were evaluated by a computer-aided sperm analysis system.  $[Ca^{2+}]_i$  was measured by a spectrofluorimetric method in fura-2-loaded spermatozoa. The AR was evaluated in live sperm cells by fluorescent-labeled lectin.

**MAIN RESULTS AND THE ROLE OF CHANCE:** CatSper I protein expression in spermatozoa was reduced in asthenozoospermic men (mean  $\pm$  SD:  $53.0 \pm 15.5\%$ ,  $n = 30$  versus  $67.9 \pm 17.1\%$  in normozoospermic,  $n = 85$ ,  $P < 0.01$ ) and was significantly correlated with progressive ( $r = 0.36$ ,  $P < 0.001$ ), total ( $r = 0.35$ ,  $P < 0.001$ ) and HA ( $r = 0.41$ ,  $P < 0.005$ ) motility. In addition to a higher percentage of spermatozoa not expressing CatSper I, asthenozoospermic men showed a large number of spermatozoa with immunofluorescent signal localized outside the principal piece compared with those in normozoospermia. A significant positive correlation was found between CatSper I protein expression and the increase of  $[Ca^{2+}]_i$  in response to progesterone ( $r = 0.36$ ,  $P < 0.05$ ,  $n = 40$ ) but not with basal  $[Ca^{2+}]_i$ . No correlation was found with the AR, either basal or in response to progesterone.

**LIMITATIONS, REASONS FOR CAUTION:** The study is partly descriptive. Furthermore, we cannot rule out the possibility that some round cells remain after a single round of 40% density gradient centrifugation or that this step may have removed some defective or slow swimming sperm, and therefore this preparation may not be representative of the entire sperm sample. Although our data suggest that CatSper I may be a useful marker for infertility, and a possible contraceptive target, any clinical application is limited without further research.

**WIDER IMPLICATIONS OF THE FINDINGS:** Our results demonstrate an association of CatSper I expression with human sperm progressive and HA motility and provide preliminary evidence that lack of expression or mislocalization of CatSper I in spermatozoa may be involved in the pathogenesis of asthenozoospermia. However, mechanistic studies are needed to confirm that the correlations between CatSper I expression and sperm functions are causative.

**STUDY FUNDING/COMPETING INTEREST(S):** Supported by grants from Ministry of University and Scientific Research (PRIN project to E.B. and FIRB project to S.M.) and by Regione Toscana (to G.F.). L.T. was recipient of a grant from Accademia dei Lincei (Rome, Italy). The authors have no conflicts of interest to declare.

**Key words:** CatSper / asthenozoospermia / sperm motility / hyperactivation

## Introduction

The cationic channel of sperm (CatSper) is a well-characterized calcium channel in mammalian spermatozoa: it is a sperm-specific (Quill *et al.*, 2001), pH-sensitive (Kirichok *et al.*, 2006), voltage-gated calcium channel (Quill *et al.*, 2001), located in the principal piece of the flagellum (Ren *et al.*, 2001; Smith *et al.*, 2013; Tamburrino *et al.*, 2014). In view of the key role of calcium in most sperm functions necessary for successful fertilization, CatSper appears as an interesting target for the development of new therapeutic strategies for treatment of male infertility. In human (but not mouse) sperm cells CatSper is strongly activated by progesterone (Lishko *et al.*, 2011; Strunker *et al.*, 2011), the female steroid hormone released by cumulus cells surrounding the egg, involved in the fertilization process (Baldi *et al.*, 2009, 2011). Since men with a reduced sperm-responsiveness to progesterone show reduced natural (Tesarik and Mendoza, 1992; Falsetti *et al.*, 1993) and assisted reproduction (Krausz *et al.*, 1996; Jacob *et al.*, 1998) fertility, an impaired CatSper expression or function leading to a reduced ability to initiate the calcium signaling cascade and the acrosome reaction (AR) process can be hypothesized. However, while substantial progress has been achieved in understanding the physiological role of CatSper in mouse sperm functions, the situation is still confused concerning human spermatozoa.

In the mouse, knockout of any of the CatSper family of genes leads to infertility because of decreased maintenance of motility and lack of achievement of HA motility (Ren *et al.*, 2001; Qi *et al.*, 2007; Jin *et al.*, 2007). Moreover, although initial studies in CatSper-null mice did not support an involvement of CatSper in the tyrosine phosphorylation increase during sperm capacitation (Carlson *et al.*, 2003; Quill *et al.*, 2003), a recent study (Chung *et al.*, 2014) analyzing the spatial organization of CatSper in the flagellum in association with signaling activation demonstrated a clear involvement of the channel in such a function. More recently, a role for CatSper in rheotaxis has been also proposed (Miki and Clapham, 2013). Conversely, the involvement of CatSper in the AR of mouse sperm cells has been excluded on the basis of similar percentages of the AR in response to an ionophore in both wild-type and CatSper-null spermatozoa (Xia *et al.*, 2007). In human spermatozoa, the role of CatSper in HA is still controversial, as *in vitro* studies where the channel has been stimulated either with progesterone or by raising intracellular pH did not lead to increased hyperactivation parameters in all samples (Alasmari *et al.*, 2013a,b; Tamburrino *et al.*, 2014), and incubation with available CatSper inhibitors led to inconsistent results (Sagare-Patil *et al.*, 2012, 2013; Tamburrino *et al.*, 2014). Similarly, for progesterone-stimulated ARs, contrasting results have been reported on the effects of CatSper inhibitors (Sagare-Patil *et al.*, 2012, 2013; Tamburrino *et al.*, 2014). Inconsistencies in the results with CatSper inhibitors might be due to their poor specificity. Both NNC55-0396 and Mibefradil also block human sperm  $K^+$  currents (Mansell *et al.*, 2014) and evoke  $Ca^{2+}$  responses themselves, by rising intracellular pH, therefore acting as partial CatSper agonists (Brenker *et al.*, 2012; Tavares *et al.*,

2013). In addition, we have demonstrated a toxic effect of Mibefradil on human spermatozoa at the concentrations used to inhibit CatSper (Tamburrino *et al.*, 2014). Conversely, the reason behind reduced or absent responses to CatSper stimuli in some men is unclear. Few men with CatSper1 or CatSper2 mutations that lead to a truncated protein have been described in the literature. These men show reduced or absent sperm motility and frequent oligozoospermia, although the two phenotypes are not severe or have not been evaluated or reported for all the subjects with the deletions (Avidan *et al.*, 2003; Avenarius *et al.*, 2009; Hildebrand *et al.*, 2010; Jaiswal *et al.*, 2014). In one of these patients, showing severe asthenoteratozoospermia due to tail abnormalities and abnormal motility patterns, no basal or progesterone-stimulated  $Ca^{2+}$  currents were detectable (Smith *et al.*, 2013). We recently demonstrated that a higher percentage of spermatozoa expresses CatSper1 after swim-up selection and that a positive correlation exists between CatSper1 protein expression, evaluated by western blot analysis, and the percentage of progressive motility, although such a result was obtained in a small number of subjects (Tamburrino *et al.*, 2014). Interestingly, in non-motile mouse spermatozoa, the CatSper channel is degraded or mislocalized (Chung *et al.*, 2014). Overall these studies suggest that low, absent or mislocalized CatSper expression in spermatozoa may be involved in determining an asthenozoospermic phenotype and low HA and could explain the lack of response or reduced responses of spermatozoa to CatSper stimuli in some subjects (Alasmari *et al.*, 2013a,b; Tamburrino *et al.*, 2014).

To better understand the role of CatSper in human sperm functions, we directly investigated the relationships between the percentage of sperm cells expressing CatSper1 protein, as evaluated by immunofluorescence staining coupled to flow cytometry, sperm motility and HA, as well as basal and progesterone-stimulated intracellular calcium levels and AR. In addition, we re-examined CatSper1 localization in spermatozoa from normo- and asthenozoospermic men by fluorescence microscopy.

## Materials and Methods

### Chemicals

Human tubal fluid (HTF) medium and human serum albumin (HSA) were purchased from Celbio (Milan, Italy). Bovine serum albumin (BSA) was purchased from ICN Biomedicals (Solon, OH, USA). PureSperm was obtained from Nidac International (Mölnådal, Sweden). Paraformaldehyde (PFA) was obtained from Merck Chemicals (Milan, Italy). Rabbit anti-CatSper1 polyclonal antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Ig)G was obtained from Southern Biotech (Birmingham, AL, USA). Fura 2/AM, TOTO-3 dye and Alexafluor 488 goat anti-rabbit secondary antibody, LI0120 LIVE/DEAD<sup>®</sup> Fixable Far Red Dead Cell Stain Kit were obtained from Molecular Probes (Life Technology, Monza, Italy). Anti-human acrosin antibody was obtained from Biosonda Corp. (Santiago, Chile).

Donkey Cy3-conjugated anti-mouse secondary antibody was obtained from Jackson ImmunoResearch (Suffolk, UK). Propidium iodide (PI) was obtained from Calbiochem (Nottingham, UK). Rabbit serum was obtained from Signet Laboratories (Hayward, CA, USA). Normal goat serum (NGS), normal donkey serum (NDS), progesterone, FITC-labeled *Arachis hypogea* (peanut) lectin, digitonin, EGTA and Triton were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

## Semen collection and preparation

Semen samples were obtained [according to World Health Organization (WHO) criteria (WHO, 2010)] from a total of 115 consecutive patients undergoing routine semen analysis for couple infertility, in the Andrology laboratory of the University of Florence, Italy, after obtaining written informed patient's consent to allow use of the remaining semen after completion of the analysis for research purposes. Semen parameters were assessed by optical microscopy and samples were divided into two groups: normozoospermic ( $n = 85$ ) and asthenozoospermic ( $n = 30$ ), on the basis of progressive (a + b) sperm motility  $<32\%$  and viability  $>58\%$ , as the fifth centile of reference values of WHO (WHO, 2010). Patients' age and standard semen parameter values for the two groups of men are shown in Table I. The two groups of patients were comparable in age but differed in semen quality. Asthenozoospermic men showed a reduced motility (both progressive and hyperactivated (HA)), a lower sperm concentration and total number per ejaculate and a worse sperm morphology than normozoospermic men. Since all the subjects included in the study were male partners of infertile couples of unknown fertility potential they are indicated as sub-fertile.

In experiments to evaluate motility by computer-aided sperm analysis (CASA), intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) and AR, semen samples were selected by a 40% gradient fraction of PureSperm, prepared in HTF with 10% HSA and centrifuged at 500g for 30 min at 26°C to eliminate the round cells and debris present in semen that interfere with analysis by CASA (ESHRE, 1998). The resulting 40% pellet was collected and washed in HTF–10% HSA medium. Each sample was carefully evaluated under microscopy for effective reduction of germ cells and leukocytes and for maintenance of the initial motility, or otherwise discarded. After selection, the percentage progressive motility was closely correlated with the same parameter at the beginning of sample processing ( $r = 0.78$ ,  $P < 0.001$ ,  $n = 60$ ), although the number of recovered sperm did not allow us to perform AR and  $[Ca^{2+}]_i$  experiments in all the samples.

## Immunofluorescence staining and detection of CatSperI

The percentage of CatSperI-expressing sperm cells was determined by immunofluorescence staining coupled to flow cytometry in all the 115 samples. A total of  $10 \times 10^6$  spermatozoa from whole semen were fixed in PFA [500  $\mu$ l, 4% w/v in phosphate-buffered saline (PBS), pH 7.4] for 30 min at room temperature. After being washed twice in 1% (v/v) NGS–PBS, sperm cells were permeabilized with 0.1% (v/v) Triton X-100 in 100  $\mu$ l 0.1% (w/v) sodium citrate for 4 min in ice. Then, samples were split into two aliquots and subsequently incubated (1 h at room temperature) either with anti-CatSperI antibody (4  $\mu$ g/ml, test sample) or rabbit serum (4  $\mu$ g/ml, the negative control). After being washed twice in 1% NGS–PBS, spermatozoa were incubated in the dark for 60 min with goat anti-rabbit IgG-FITC (dilution 1:100 in 1% NGS–PBS). Samples were washed twice, re-suspended in 300  $\mu$ l PBS, stained with 4.5  $\mu$ l PI (50  $\mu$ g/ml in PBS) and incubated in the dark for 15 min at room temperature. An additional aliquot of sperm suspension was prepared with the same procedure as for the test sample but omitting the PI staining and used for instrumental compensation.

Samples were acquired using a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA, USA) equipped with a 15-mW argon ion laser for excitation. Green fluorescence of FITC-conjugated goat anti-mouse IgG was revealed by an FL-1 (515–555-nm wavelength band) detector; red fluorescence of PI was detected by an FL-2 (563–607-nm wavelength band) detector. We acquired 8000 nucleated events in the gate (i.e. the events labeled with PI) of the characteristic forward scatter/side scatter region of sperm cells (Muratori et al., 2008). A marker was established in the CatSperI axis dot plot of the negative control, including 99% of total events. This marker was translated in the corresponding test sample and all the events beyond the marker were considered positive for CatSperI. The mean fluorescence intensity (MFI) of CatSperI in sperm was calculated as the ratio between the mean intensity of cells of the test sample and the mean intensity of cells of the corresponding isotype negative control.

To determine CatSperI localization, labeled sperm samples were examined in a fluorescence microscope (Carl Zeiss, Axiolab A1 FL, Milan, Italy), equipped with filter set 15, 44 and 49 by an oil immersion  $\times 100$  magnification objective. Images were captured by an AxioCam ICm1 camera with Axiovision 4.8 (provided by Carl Zeiss, Milan, Italy) and edited by Adobe Photoshop CS 2 (Adobe Systems, Inc., San Jose, CA, USA). Localization of the CatSperI signal was evaluated in 200 spermatozoa from 10 asthenozoospermic and 10 normozoospermic subjects.

**Table I** Average age and semen characteristics in normozoospermic (N) and asthenozoospermic (A) patients.

Parameters	N ( $n = 85$ )	A ( $n = 30$ )	P value
Age	34.8 $\pm$ 7.5	38.2 $\pm$ 8.9	n.s.
Progressive motility (%)	57.2 $\pm$ 12.4	20.1 $\pm$ 7.2	<0.001
Immotile sperm (%)	33.7 $\pm$ 11.0	65.1 $\pm$ 11.7	<0.001
Total motility (%)	66.4 $\pm$ 10.8	34.9 $\pm$ 11.7	<0.001
Hyperactivated motility (%)	3.0 (0 $\div$ 21)	1.0 (0 $\div$ 3)	<0.005
Sperm concentration ( $\times 10^6$ /ml)	74.0 (8.8 $\div$ 330)	19.3 (6.5 $\div$ 138.0)	<0.001
Sperm number ( $\times 10^6$ /ejaculate)	299.4 (40.7 $\div$ 1067)	56.3 (13.6 $\div$ 515.6)	<0.001
Volume (ml)	4.0 $\pm$ 3.1	3.4 $\pm$ 1.6	n.s.
Sperm morphology (%)	5.5 $\pm$ 3.5	3.1 $\pm$ 2.5	<0.005

Sperm number, sperm concentration and hyperactivated motility did not follow a normal distribution and are expressed as median (minimum  $\div$  maximum). Statistical comparisons were made using the Mann–Whitney *U*-test. All other parameters follow a normal distribution and are expressed as mean  $\pm$  SD. Statistical comparisons were made by the *t*-test.

For experiments of CatSper I detection in live sperm cells, after being washed with HTF medium, fresh semen samples were incubated for 1 h at room temperature in 500  $\mu$ l PBS with Live/Dead Fixable Far Red Dead Cell Stain Kit (LI10120, diluted 1:10 000), washed and processed as described above and acquired using FACSSaria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a violet laser, a blue laser and a red laser for excitation, respectively, at 405, 488 and 633 nm (Muratori *et al.*, 2015).

### Assessment of sperm motility by CASA

Immediately after 40% PureSperm selection, sperm motility was analyzed by the CASA system (Hamilton Thorn Research, Beverly, MA, USA) as described previously (Luconi *et al.*, 2005; Tamburrino *et al.*, 2014) in the 60 samples (including 18 from asthenozoospermic men) which maintained the initial motility characteristics after PureSperm selection (see above). Average path velocity (VAP,  $\mu$ m/s), straight line velocity (VSL,  $\mu$ m/s), curvilinear velocity (VCL,  $\mu$ m/s), amplitude of lateral head displacement (ALH,  $\mu$ m), beat cross frequency (BCF, Hz), linearity of progression (LIN, %), percentages of sperm cells with total, progressive and rapid motility (sperm with VAP  $\geq$  25  $\mu$ m/s), percentages of sperm cells with non-progressive motility (static) and hyperactivated spermatozoa (HA) were recorded. The threshold values for HA cells identification were manually set (VCL > 150  $\mu$ m/s, ALH > 7  $\mu$ m, LIN < 50%; Mortimer *et al.*, 1998). A minimum of 100 motile cells and 5 fields were analyzed for each aliquot. All analyses were performed at 37°C.

### Evaluation of intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) in capacitated spermatozoa

$[Ca^{2+}]_i$  was evaluated as previously described (Krausz *et al.*, 1995, 1996; Tamburrino *et al.*, 2014) in 40 samples (including 10 from asthenozoospermic men) selected by PureSperm (see above). Briefly, after 40% PureSperm selection, spermatozoa were capacitated for 3 h in HTF–10% HSA medium and incubated for 30 min at 37°C with Fura2/AM for 45 min, washed, resuspended in FM buffer (125 mM NaCl, 10 mM KCl, 2.5 mM CaCl<sub>2</sub>, 0.25 mM MgCl<sub>2</sub>, 19 mM sodium lactate, 2.5 mM sodium pyruvate, 20 mM HEPES/NaOH) and further incubated for 30 min.  $[Ca^{2+}]_i$  was measured by using a spectrofluorimetric method with a Perkin–Elmer LS50B instrument equipped with a fast rotary filter shuttle for alternate 340 and 380 nm excitation. Spermatozoa were stimulated with progesterone 10  $\mu$ M which was added directly in the cuvette. Fluorescence measurements were converted to  $[Ca^{2+}]_i$  by determining maximal fluorescence (F<sub>max</sub>) with digitonin (0.01% (w/v) final concentration) followed by minimal fluorescence (F<sub>min</sub>) with 10 mM EGTA, pH 10.  $[Ca^{2+}]_i$  was calculated according to Grynkiewicz *et al.* (1985) assuming a dissociation constant of Fura 2 for calcium of 224 nM. Each sample was evaluated in triplicate and reported results are referred to the mean values of at least two determinations.  $\Delta [Ca^{2+}]_i$  was calculated as the difference between progesterone-stimulated and basal  $[Ca^{2+}]_i$ .

### Evaluation of the AR

AR was evaluated in 35 samples (including 9 from asthenozoospermic men) selected by 40% PureSperm. Spermatozoa ( $10 \times 10^6$ /ml) were capacitated in HTF–10% HSA for 3 h and stimulated for 1 h with progesterone (10  $\mu$ M) dissolved in dimethyl sulfoxide (DMSO) at an initial concentration of 0.1 M and diluted in HTF/HSA medium. Control samples were incubated with DMSO (0.0001%, v/v). Sperm AR was evaluated through the use of the fluorescent probe FITC-labeled *Arachis hypogea* (peanut) lectin as previously described (Falsetti *et al.*, 1993; Krausz *et al.*, 1995; Tamburrino *et al.*, 2014). In order to evaluate AR only in live spermatozoa, at the end of the incubation period spermatozoa were washed and re-suspended in 0.5 ml of hypo-osmotic swelling medium (WHO, 2010) for 1 h at 37°C, washed

again and finally re-suspended in 50  $\mu$ l ice-cold methanol. The sperm suspension was layered on a slide, air-dried at room temperature and stored at –20°C. On the day of evaluation, the slides were stained with FITC-labeled *Arachis hypogea* (peanut) lectin and fluorescence was observed in a fluorescence microscope. A total of 200 curled-tail (viable) spermatozoa per slide were scored for the AR. The difference between the AR percentage in the progesterone-induced and control was considered to be the percentage of spermatozoa responsive to the progesterone stimulation (acrosome reaction following progesterone challenge, ARPC).

### Immunohistochemistry and confocal microscopy

Double-immunostaining for CatSper I and acrosin was performed on human testicular biopsies as described (Muciaccia *et al.*, 2005, 2013). Briefly, surgical testicular samples were obtained from two heart-beating organ donors at the hospital Policlinico Umberto I at the Sapienza University of Rome, Italy. Biopsies were fixed in 10% (v/v) buffered formalin and embedded in paraffin. Three to five- $\mu$ m-thick paraffin sections were serially collected and mounted on polylysine-coated slides. Dewaxed and rehydrated sections were incubated in 10 mM citrate buffer (pH 6.0) in a microwave oven for antigen retrieval at 750 W, three times for 5 min. After blocking non-specific binding with 5% (v/v) NGS plus 5% (v/v) NDS, sections were incubated with monoclonal anti-acrosin (diluted 1:1000) and polyclonal anti-CatSper I (diluted 1:100) primary antibodies overnight at 4°C. After being washed, samples were incubated with secondary antibodies: Alexafluor 488 goat anti-rabbit (diluted 1:1000) and donkey Cy3-conjugated anti-mouse (diluted 1:300), for 2 h at room temperature. Negative control experiments were performed by using rabbit serum instead of primary antibody. Nuclei were stained using TOTO3 dyes (1:2000). Slides were observed with a Leica laser scanning microscope TCS SP2 equipped with three lasers (Leica, Wetzlar, Germany). Each channel was acquired separately by using specific laser lines to avoid a bleed-through of the fluorochromes. Photomicrographs were acquired with LAS AF Leica Confocal Software (Leica, Wetzlar, Germany).

### Statistical analysis

Statistical analysis was performed using the Statistical package for the Social Sciences version 20.0 (SPSS, Chicago, IL, USA) for Windows. The Kolmogorov–Smirnov test was used to test the data distribution. Data are expressed as mean  $\pm$  SD when normally distributed and as median with min and max values when non-normally distributed. For non-normally distributed parameters, differences between groups were evaluated by the Mann–Whitney non-parametric tests; for normally distributed parameters unpaired two-sided Student's t-test was used. For normally distributed parameters correlations were assessed by Pearson's correlation tests, whereas for all other parameters Spearman's regression analysis was used. Multiple linear regression analysis was applied whenever appropriate. A P-value of <0.05 was considered significant in all the regression and statistical analyses.

A receiver operating characteristic (ROC) curve was prepared in order to evaluate the accuracy of the percentage of spermatozoa expressing CatSper I in predicting the occurrence of HA.

## Results

### CatSper I protein expression in spermatozoa from normozoospermic and asthenozoospermic men

The cytometric protocol revealed a significantly different percentage of CatSper I-expressing spermatozoa between asthenozoospermic ( $n = 30$ ) and normozoospermic ( $n = 85$ ) men (respectively, mean  $\pm$  SD:

$53.02 \pm 15.5\%$ , and  $67.9 \pm 17.1\%$ ,  $P < 0.001$ ). Similar results were obtained when the MFI of CatSper1 expression was considered (data not shown).

### CatSper1 protein expression and semen parameter values

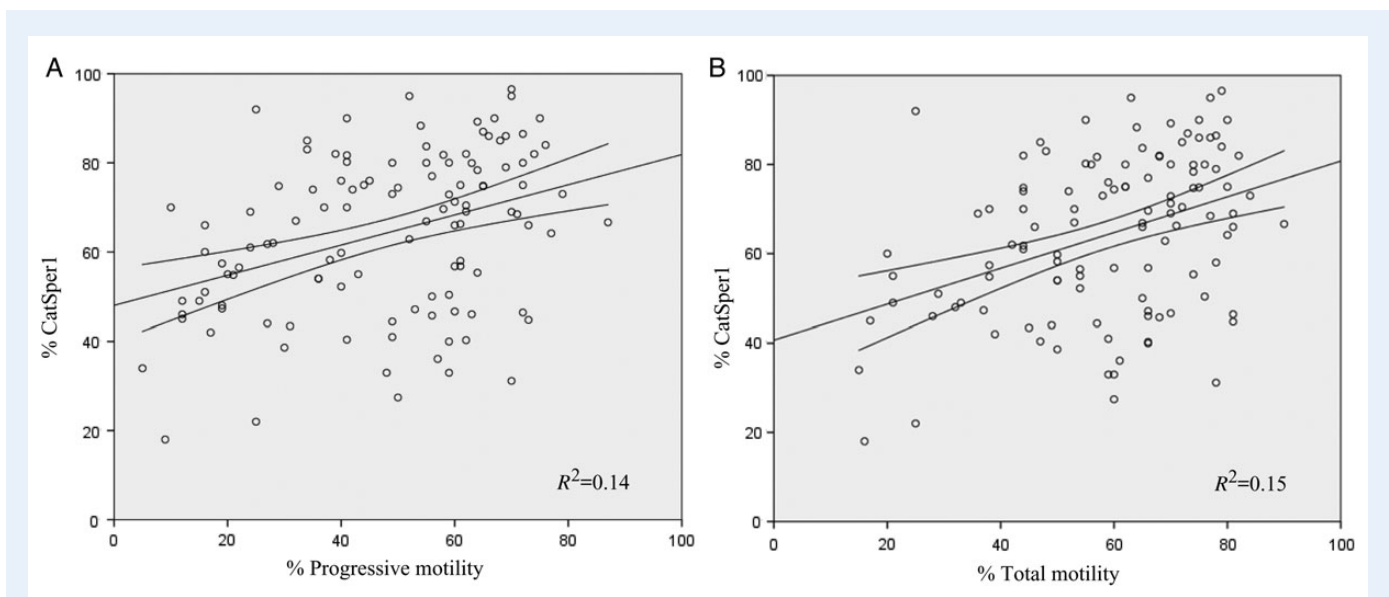
To investigate if CatSper1 expression was related to testicular function and sperm quality, we correlated the percentage of sperm cells expressing CatSper1 with the values of standard semen parameters (univariate analysis). Positive correlations between CatSper1 expression and progressive and total motility (Fig. 1A and B and Table II), and a negative correlation with the percentage of immotile spermatozoa (Table II) were found. CatSper1 expression was also positively associated with sperm concentration and total number/ejaculate, and negatively correlated with patient age (Table II). Similar results were obtained when MFI of CatSper1 expression was considered (data not shown). Age was negatively associated with total motility ( $r = -0.26$ ,  $P < 0.01$ ). After multiple regression analysis including age, sperm concentration and motility, correlations between CatSper1 expression and progressive (adjusted  $r = 0.3$ ,  $P < 0.005$ ) and total (adjusted  $r = 0.3$ ,  $P < 0.001$ ) motility and age (adjusted  $r = -0.18$ ,  $P < 0.05$ ) remained significant. Although such correlations were highly significant, a high percentage of spermatozoa expressing CatSper1 were immotile (the intercept of the regression line occurs at  $\sim 40\%$  CatSper1 expression, Fig. 1B). To understand whether such a discrepancy may be due to expression of the channel in dead spermatozoa, we used the probe LI0120, which binds to dead cells in a stable manner allowing evaluation of CatSper1 in both LI0120-negative (live) and -positive (dead) sperm (Marchiani et al., 2011; Muratori et al., 2015). Although CatSper1 was prevalently expressed in live spermatozoa ( $53.3 \pm 14\%$ ,  $n = 3$ ),  $20.4 \pm 0.2\%$  of dead sperm cells also expressed the protein explaining, at least in part, the occurrence of patients with high CatSper1 expression and low motility.

As mentioned, a recent report (Chung et al., 2014) demonstrated that CatSper may be absent or mislocalized in immotile mouse sperm. To evaluate whether CatSper1 localization differed between asthenozoospermic and normozoospermic subjects, we evaluated the subcellular localization of anti-CatSper1 staining by fluorescent microscopy in spermatozoa from 10 subjects for each category. In some cells, the staining was punctuated and localized exclusively in the principal piece (example in Fig. 2A) while in other cells, the staining was localized in different sperm structures, such as, but not exclusively, the midpiece (examples in Fig. 2B). We next calculated the percentage of cells showing the two different staining patterns in the total CatSper1-positive spermatozoa. We found that the percentage of spermatozoa showing

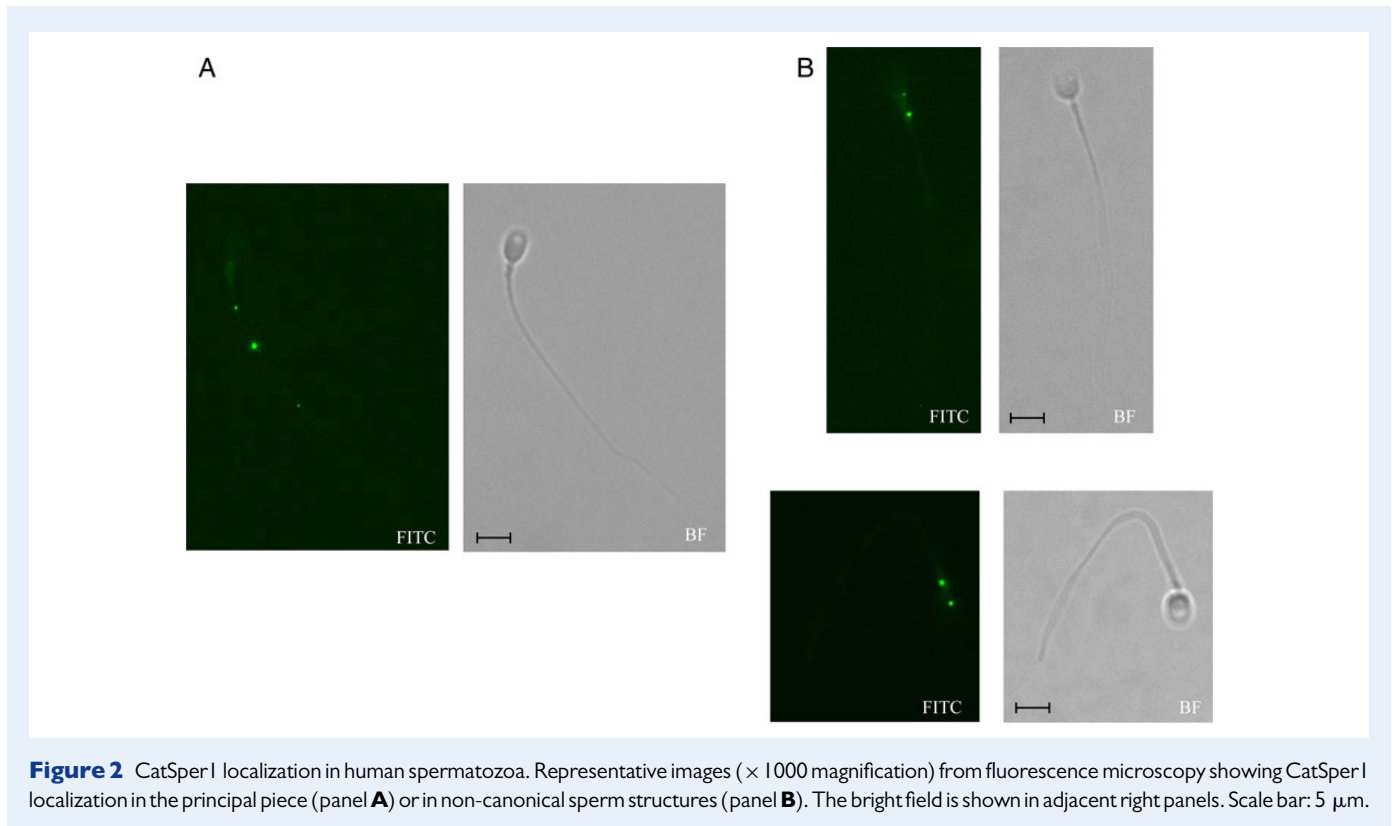
**Table II** Correlations between cationic channel of sperm (CatSper)1 expression and semen parameter values ( $n = 115$ ).

Parameters	Correlation coefficients	P value
Age	-0.25	<0.005
Progressive motility	0.37	<0.001
Immotile sperm	-0.41	<0.001
Total motility	0.39	<0.001
<i>In situ</i> motility	-0.09	n.s.
Sperm concentration	0.25	<0.01
Sperm number	0.22	<0.05
Volume	-0.05	n.s.
Sperm normal morphology	-0.06	n.s.

Correlations were assessed by Pearson's correlation analysis for all parameters except for *in situ* motility, sperm concentration and sperm number where Spearman's correlation analysis was used.



**Figure 1** Scatterplots of correlations between percentages of cationic channel of sperm (CatSper)1-expressing human spermatozoa and progressive (A) and total (B) motility. 95% Confidence intervals of the correlations and  $R^2$  values for linear regression analysis are shown.



**Figure 2** CatSper I localization in human spermatozoa. Representative images ( $\times 1000$  magnification) from fluorescence microscopy showing CatSper I localization in the principal piece (panel **A**) or in non-canonical sperm structures (panel **B**). The bright field is shown in adjacent right panels. Scale bar: 5  $\mu\text{m}$ .

CatSper I exclusively in the principal piece was higher in normozoospermic subjects ( $49.9 \pm 7.7\%$  versus  $36.7 \pm 8.7\%$  in asthenozoospermic subjects,  $P < 0.05$ ).

The occurrence of different patterns of expression of CatSper I in ejaculated spermatozoa prompted us to investigate CatSper I localization in developing human germ cells. Testicular sections obtained from heart-beating donors, where all the steps of germ cell differentiation are present, were co-stained with anti-CatSper I and anti-acrosin antibodies (Fig. 3A). Acrosin detection allows the identification of the different steps of acrosome biogenesis in haploid spermatids (Muciaccia *et al.*, 2013). Interestingly, CatSper I partially co-localizes with acrosin in round spermatids from step 2 to step 7 (Fig. 3B). In step 8 spermatids, CatSper I immunostaining decreases in the acrosomal region but in favorable sections CatSper I immunostaining was found at the caudal pole of the nucleus close to the base of the developing flagellum (Fig. 3C). In elongating spermatids, CatSper I immunostaining in the acrosomal region became undetectable (Fig. 3B, step 10).

### CatSper I expression and CASA parameter values

The relationship between CatSper I and HA was evaluated by measuring sperm motion parameters by CASA in sperm samples from 60 men. CatSper I expression was correlated with most CASA parameter values (Table III and Fig. 4). In particular, VCL (Fig. 4A) and LIN (Fig. 4B), two main parameters defining HA (Mortimer *et al.*, 1998), were, respectively, positively and negatively correlated with CatSper I expression, which was positively correlated with HA (Fig. 4C). A positive correlation was also present between CatSper I expression and percentage total, progressive and rapid motility as evaluated by CASA (Table III).

After adjustment for patient age and sperm concentration, most correlations between CatSper I expression and CASA motility parameters were maintained (Table III).

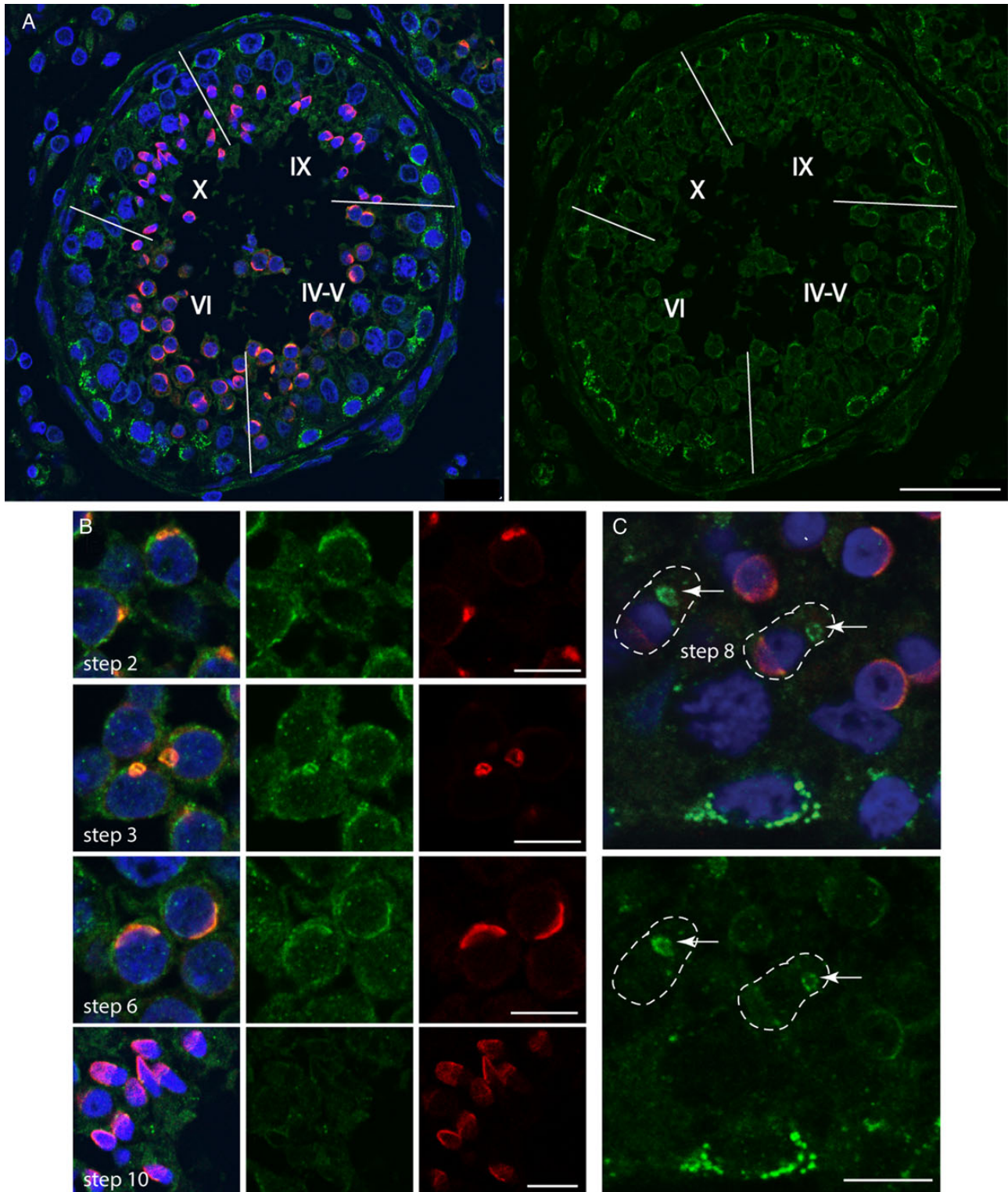
To determine the accuracy of CatSper I expression in predicting the % HA of the sample, we used ROC as a binary classifier system. A value of 10% HA was chosen from the study of Alasmari *et al.* (2013a) showing that at this threshold HA was able to distinguish between donors and sub-fertile patients. At a threshold of 75.5%, CatSper I expression predicted the occurrence of 10% HA with an accuracy of  $83.7 \pm 6.2\%$  ( $P < 0.01$ ) showing a sensitivity and a specificity of 83 and 73%, respectively (Fig. 4D). Conversely, other parameters that can be evaluated by routine semen analysis could not predict achievement of HA status (Table IV).

### CatSper I expression and $[\text{Ca}^{2+}]_i$

A significant correlation was found between CatSper I expression and progesterone-stimulated  $[\text{Ca}^{2+}]_i$ , both in terms of peak concentration ( $r = 0.36$ ,  $P < 0.05$ ) and  $\Delta$  increase ( $r = 0.36$ ,  $P < 0.05$ ,  $n = 40$ ; Fig. 5). Both correlations were maintained after adjustment for patient age and sperm concentration (progesterone-stimulated  $[\text{Ca}^{2+}]_i$ , adjusted  $r = 0.39$ ,  $P < 0.05$ ;  $\Delta$  increase, adjusted  $r = 0.42$ ,  $P < 0.01$ ). Conversely, no correlation was found between CatSper I expression and basal  $[\text{Ca}^{2+}]_i$  ( $r = 0.23$ ,  $P = 0.18$ ) although the latter was highly significantly correlated with progesterone-stimulated  $[\text{Ca}^{2+}]_i$  ( $r = 0.87$ ,  $P < 0.001$ ,  $n = 40$ ).

### CatSper I and the AR

The involvement of CatSper in the AR was examined by directly investigating the relation of basal and progesterone-stimulated AR with



**Figure 3** CatSper1 protein expression in the human testis. Formalin-fixed paraffin sections were stained with anti-CatSper1 (green), anti-acrosin (red) and TOTO-3 (blue, to detect nuclei), and single Z-sections were acquired at the confocal microscope. Merging of the pictures is shown in the left column in **(A)** and **(B)** and the upper panel in **(C)**. **(A)** In the normal human seminiferous epithelium CatSper1 expression is high in spermatogonia and decreases in meiotic cells and haploid cells. Roman numerals indicate the germ cell associations of the cycle of the seminiferous epithelium (identification of the stages according to [Muciaccia et al. 2013](#)). **(B)** In spermatids, CatSper1 partially co-localizes with acrosin in round spermatids from step 2 to step 7 (examples of step 2, 3 and 6 are shown) whereas such co-localization is lost from early elongating spermatids and thereafter during spermiogenesis. **(C)** In step 8 round spermatids, Catsper1 localizes at the posterior pole of the nucleus (white arrow) at the base of the developing flagellum. Dashed lines highlight the cellular profiles. Scale bars: 50  $\mu\text{m}$  in **(A)**, 10  $\mu\text{m}$  in **(B)** and **(C)**. Pictures are representative of two samples from two testis.

**Table III** Correlations between CatSper I expression and CASA parameter values ( $n = 60$ ).

CASA parameters	$r$	$P$ value	Adj. $r$	Adj. $p$
VAP	0.29	<0.05	0.28	<0.05
VSL	0.21	n.s.	0.19	n.s.
VCL	0.37	<0.001	0.37	<0.01
ALH	0.25	0.06	0.26	0.06
BCF	0.17	n.s.	0.15	n.s.
LIN	-0.27	<0.05	-0.29	<0.05
HA	0.41	<0.05	0.44	<0.001
Total motility	0.35	<0.01	0.33	<0.05
Progressive motility	0.36	<0.01	0.24	n.s.
Rapid	0.34	<0.01	0.32	<0.05
Static motility	-0.35	<0.01	-0.32	<0.05

Correlations were assessed by Pearson's correlation analysis for all parameters except for progressive, static motility and HA motility where univariate Spearman's regression analysis was used. Adj  $r$  and Adj  $p$  are, respectively, correlation coefficient and  $P$  value adjusted for sperm concentration and patient age.

VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity of progression.

CatSper I protein expression in 35 subjects. No correlation was found between CatSper I expression and either basal ( $r = -0.22$ ,  $P = 0.19$ ) or progesterone-induced AR ( $r = -0.14$ ,  $P = 0.4$ ) and ARPC ( $r = 0.08$ ,  $P = 0.6$ ). In the 32 samples where  $[Ca^{2+}]_i$  and AR were concomitantly evaluated, a significant correlation was found between the percentage of progesterone-stimulated AR and progesterone-stimulated  $[Ca^{2+}]_i$  ( $r = 0.49$ ,  $P < 0.01$ ). Conversely, basal AR did not show any correlation with basal  $[Ca^{2+}]_i$  ( $r = 0.29$ ,  $P = 0.14$ ).

## Discussion

The role of CatSper in human sperm pathophysiology is still unclear. Most of our current knowledge derives from studies where CatSper activation was obtained after stimulation with progesterone (Lishko *et al.*, 2011), with stimuli increasing intracellular pH (such as 4-AP) (Alasmari *et al.*, 2013b) or with low specificity CatSper antagonists (Strunker *et al.*, 2011; Sagare-Patil *et al.*, 2013; Tamburrino *et al.*, 2014). However, no studies so far have attempted to evaluate a direct relationship between CatSper expression and sperm functions. Here, by employing a large sample size we elucidated the relationship between CatSper I protein expression and the main sperm functions where the channel has been hypothesized to be involved.

In our study, expression of the CatSper I subunit has been assumed to be a measure of the occurrence of the CatSper channel in spermatozoa. Indeed, although functional CatSper channel is formed by four homologous subunits (CatSper 1–4) and at least three auxiliary subunits (CatSper  $\beta$ ,  $\gamma$  and  $\delta$ ) (Ren and Xia 2010; Chung *et al.*, 2011), studies in the mouse demonstrate that lack of even one of the CatSper subunits leads to complete absence of the channel in mature sperm (Qi *et al.*, 2007). Studies in men with a CatSper2 gene deletion (Smith *et al.*, 2013), demonstrating lack of CatSper currents and of expression of

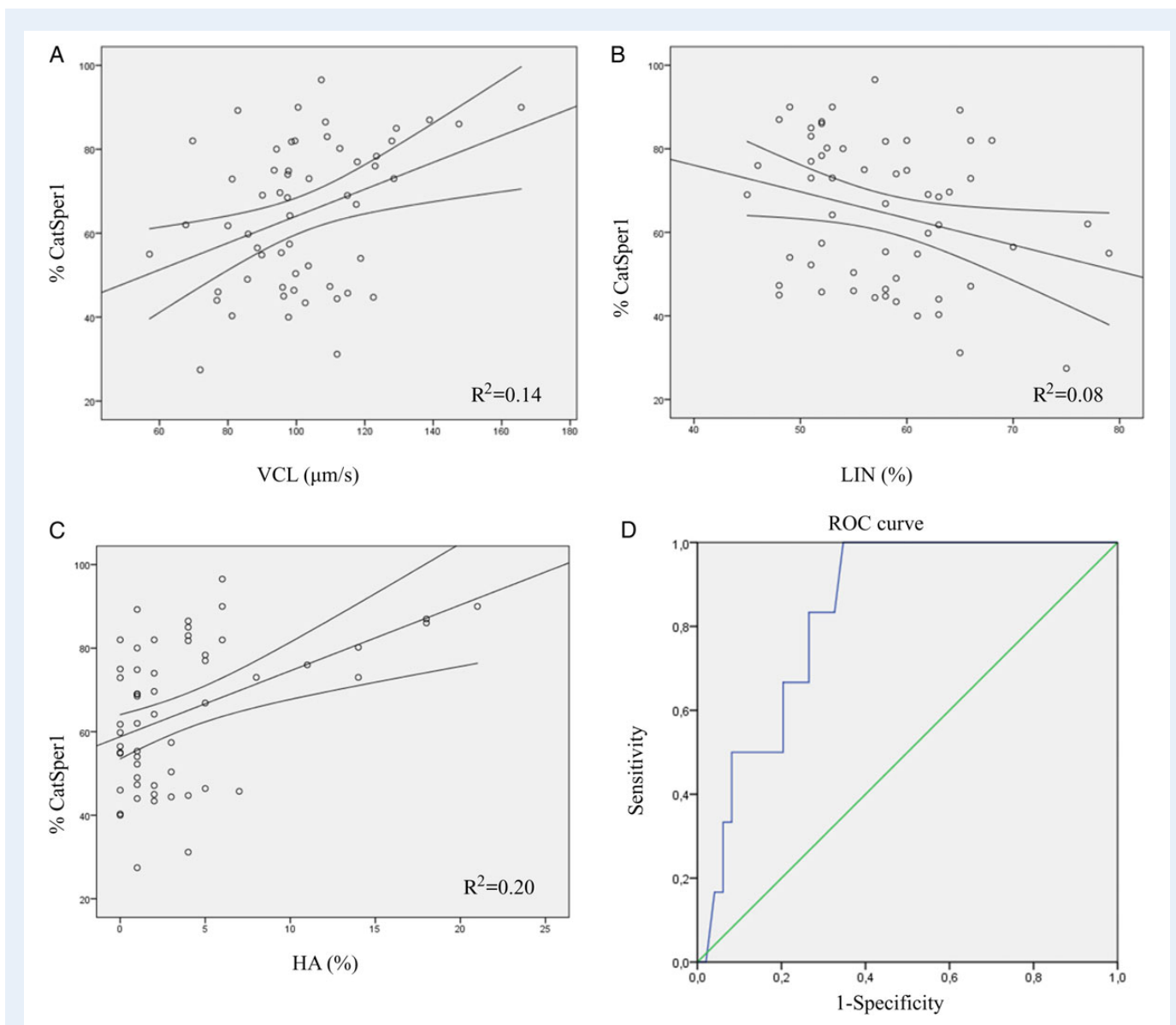
the CatSper  $\beta$  subunit in their sperm, suggest that all subunits are required for channel expression and its function also in humans.

The lower percentage of spermatozoa expressing CatSper I in asthenozoospermic patients and the positive correlations between expression of the channel subunit and motility clearly indicate an involvement of CatSper in the pathogenesis of asthenozoospermia, as previously suggested (Nikpoor *et al.*, 2004; Li *et al.*, 2007; Bhilawadikar *et al.*, 2013; Tamburrino *et al.*, 2014). Since progressive motility is fundamental for penetration of the cervical barrier, our results highlight the importance of CatSper in this process (Alasmari *et al.*, 2013b). The reason for lower expression of CatSper I in asthenozoospermic men and non-motile spermatozoa is presently unclear but may be related to disturbances in spermatogenesis leading to anomalies in the production or increased degradation of the proteins forming the channel, or their assembly. The recent identification of heterozygous mutations of CatSper1-4 genes in 6 of 30 men with asthenozoospermia (which have not been found in normozoospermic men) (Visser *et al.*, 2011) suggests that alterations in channel expression or function may also be due to mutations. Moreover, we show here that CatSper I protein is predominant in live spermatozoa (as only 20% of dead ones express the channel), suggesting that processes, such as apoptosis, leading to sperm death may also result in a lack of expression, degradation or incorrect assembly of the channel. On the other hand, the occurrence of CatSper I in 20% of dead spermatozoa might explain, at least in part, the subjects with high CatSper I expression and low sperm motility. However, sperm motility is highly influenced by several other factors (for example oxidative stress producing membrane damage and altered mitochondrial function), which could explain reduced motility in spite of an elevated percentage of CatSper I-expressing spermatozoa. Furthermore, it has recently been shown that in non-motile mouse spermatozoa, CatSper domains may not only be degraded but also are no longer confined to the principal piece of the tail (Chung *et al.*, 2014). Here, by examining CatSper I localization in a large number of spermatozoa from asthenozoospermic and normozoospermic men, we found that although CatSper I was mostly expressed in the principal piece, non-canonical localization of the protein was also observed and this was higher in asthenozoospermic men. This result indicates that, besides a lower percentage of sperm cells expressing CatSper I, patients with low sperm motility may also display a non-functional localization. Indeed, at least in mouse spermatozoa, such incorrect localization does not allow CatSper-mediated signaling and tubule assembly necessary for motility (Chung *et al.*, 2014). The reason for incorrect localization of CatSper signal is presently unclear. We show here, by immunofluorescence confocal analysis, that during the last phases of sperm differentiation, CatSper I expression moves from the head to the base of the flagellum in testicular specimens with ongoing spermatogenesis. It can be hypothesized that abnormalities during spermiogenesis may lead to a lack or defective reorganization of CatSper I resulting in a malfunctioning and low motility spermatozoon.

Our study also reveals the presence of subjects with low CatSper I expression and elevated motility. In the seven men with CatSper genes deletion so far reported in the literature (reviewed in Hildebrand *et al.*, 2010), sperm motility ranged from 0 to 50%. Clearly, these results suggest that alternative pathways may be activated in spermatozoa that rescue sperm motility in the absence of CatSper.

We found a negative correlation between CatSper I expression and the patients' age, sperm concentration and total sperm number, the





**Figure 4** Scatterplots of correlations between percentages of CatSper1-expressing human spermatozoa and curvilinear velocity (VCL, panel **A**), linearity of progression (LIN, panel **B**) and hyperactivation (HA, panel **C**) as assessed by CASA. 95% Confidence intervals of the correlations (upper and lower lines) and  $R^2$  values for linear regression analysis are shown. (Panel **D**) ROC curve of the ability of CatSper1 protein expression to predict 10% HA. The area under the ROC curve is 0.83. The optimal cutoff for the prediction of HA is 75.5%, with 83% sensitivity and 73% specificity.

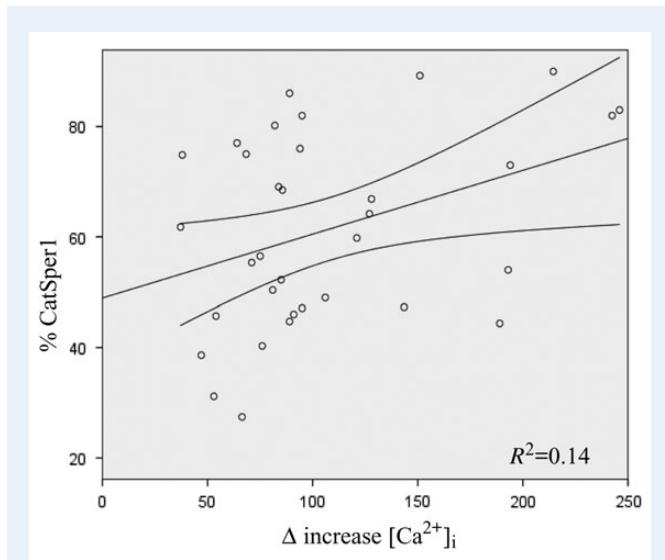
**Table IV** ROC curve analysis of the accuracy of routine semen parameters in predicting HA  $\geq$  10%.

Semen parameters	AUC (% $\pm$ SE)	P value
Progressive motility	61 $\pm$ 9.5	n.s.
Total motility	61 $\pm$ 9.3	n.s.
Sperm concentration	72 $\pm$ 9.0	n.s.
Sperm number	72 $\pm$ 8.1	n.s.
Morphology	49 $\pm$ 14	n.s.

A threshold value of 10% HA was chosen on the basis of the study of Alasmari et al. (2013a).

AUC, area under the curve, percentage value.

latter two consistent with the frequent oligozoospermic phenotype observed in CatSper-deleted men (Hildebrand et al., 2010). However, after multiple linear regression analysis including progressive sperm motility, patients' age and sperm concentration or number, only age and motility maintained significant correlations, indicating that correlations with sperm number and concentration are biologically irrelevant. A negative correlation of CatSper1 with patients' age suggests that mechanisms of regulation of its expression in the testis might be altered with aging. Although a decrease in human semen quality with age is highly debated (Bujan et al., 1996; Kidd et al., 2001; Eskenazi et al., 2003), in our particular cohort sperm motility was negatively associated with age. Aging may cause degenerative alterations in the germinal epithelium affecting sperm quality (Kidd et al., 2001). We speculate that such alterations may also affect CatSper1 expression. The CatSper1 gene promoter contains



**Figure 5** Scatterplot of correlation between percentages of CatSper1-expressing human spermatozoa and  $\Delta [Ca^{2+}]_i$ .  $\Delta [Ca^{2+}]_i$  was calculated as the difference between progesterone-stimulated and basal  $[Ca^{2+}]_i$ . 95% Confidence intervals of the correlations and  $R^2$  values for linear regression analysis are shown.

multiple binding sites for transcription factors (Mata-Rocha *et al.*, 2013, 2014) including members of the SOX gene family of fundamental importance for testicular development and male fertility (Jiang *et al.*, 2013). Studies in rodents demonstrate that CatSper gene expression is increased by agents such as selenium and Vitamin E (Mohammadi *et al.*, 2009, 2013) and modulated by drugs that affect sperm quality (Wang *et al.*, 2013; Amini *et al.*, 2014; Park *et al.*, 2014), suggesting that expression of the channel is regulated in the testis by oxidative status and lifestyle habits. It can be speculated that such factors are involved in down-regulating CatSper1 expression in the aging male and in men with asthenozoospermia.

Whereas studies in CatSper knockout (KO) mice show a clear involvement of the channel in sperm HA, studies in men are less clear on this. Alasmari *et al.* (2013a,b) reported that CatSper activation was not sufficient to achieve HA and that the failure of HA in response to stimulation was more common in IVF patients than in donors. Similarly, after stimulation with progesterone, we detected an increase in HA in only a few subjects (Tamburrino *et al.*, 2014). Here, we found a significant correlation between CatSper1 expression and basal (spontaneous) HA levels, which indicates an involvement of the channel in the process of the development of HA. Such a finding is not surprising in view of the role of calcium in the HA process (Suarez, 2008) and suggests that failure to achieve HA in sub-fertile subjects may be related to poor, absent or mislocalized expression of the channel on spermatozoa. Importantly, we show here that the percentage of spermatozoa expressing CatSper1 (but none of the parameters evaluated by routine semen analysis) predicts with high accuracy, sensitivity and good specificity, the ability of the sample to reach a level of HA of 10% which distinguishes between donors (young men with no known fertility problems) and sub-fertile subjects (Alasmari *et al.*, 2013a), reinforcing involvement of the CatSper channel in establishment of HA in human spermatozoa. HA is related to natural fertility (Peedicayil *et al.*, 1997; Mazzilli *et al.*, 2001)

and predicts results of IVF (Wang *et al.*, 1993; Sukcharoen *et al.*, 1995; Hirano *et al.*, 2001). Conversely, routine semen analysis is poorly predictive of both natural and *in vitro* fertilization (Liu and Baker, 2002; Leushuis *et al.*, 2014). Further studies are needed to understand the relation of sperm CatSper expression with natural and assisted fertilization.

As expected, by evaluating the relationship between CatSper1 expression and basal and progesterone-stimulated  $[Ca^{2+}]_i$  accumulation, a positive correlation was found with the initial peak of  $[Ca^{2+}]_i$  in response to the steroid. However, the correlation with progesterone response was not as tight as expected if CatSper were the only pathway involved in the process. On the other hand, the  $[Ca^{2+}]_i$  in response to the steroid is not totally blunted by CatSper inhibitors (Strunker *et al.*, 2011) even at concentrations higher than those that completely blocked the CatSper current (Lishko *et al.*, 2011), suggesting involvement of other pathways (Publicover and Barratt, 2011). Furthermore, in our hands, no correlation was found between CatSper1 expression and basal  $Ca^{2+}$ , suggesting that CatSper is not the main player for establishing or maintaining  $[Ca^{2+}]_i$  in unstimulated conditions. By evaluating CatSper currents, Lishko *et al.* (2011) demonstrated that human sperm currents were overall smaller and reached similar values to those of the mouse only after stimulation with progesterone, indicating low channel activation in basal conditions. Conversely, in mouse sperm progesterone is not effective, underlying a striking difference between CatSper activation in the two species (Miller *et al.*, 2014). Most likely, release of  $Ca^{2+}$  from intracellular stores located in the neck and in the acrosome (Publicover and Barratt, 2011) or other pathways or channels located in the membrane are involved in maintaining basal calcium in human spermatozoa (Marquez *et al.*, 2007; Darszon *et al.*, 2011). On the other hand, considering the different lengths of the sperm journey in the female genital tracts of the mouse and human, a high activation of CatSper, which may lead to excessive HA, might be unfavorable thus requiring a further activation in the proximity of the oocyte.

We found no correlation between the percentage of CatSper1-expressing spermatozoa and either basal or stimulated AR, in contrast with previous results (Sagare-Patil *et al.*, 2012; Tamburrino *et al.*, 2014) showing a significant, but incomplete, effect of CatSper inhibition on progesterone-stimulated AR. Moreover the inhibitors themselves showed an effect *per se* on the AR (Tamburrino *et al.*, 2014). Lack of correlation between AR and CatSper1 expression is in agreement with results in CatSper KO mouse spermatozoa, showing no alterations of either basal or ionophore-stimulated AR (Xia *et al.*, 2007). In addition, the location of CatSper1 in the tail (Smith *et al.*, 2013; Tamburrino *et al.*, 2014) and lack of co-localization with acrosin in late phases of spermiogenesis, where immunostaining for CatSper1 is detected in the region of the caudal pole of the nucleus, close to the base of the developing flagellum (present study), also exclude a direct involvement of the channel in the AR. However this result appears to contrast with the fact that progesterone-stimulated AR is highly dependent on the increase of  $[Ca^{2+}]_i$  induced by the steroid (Baldi *et al.*, 2009) and that such an increase shows significant associations both with CatSper1 expression and progesterone-stimulated AR (this study, Krausz *et al.*, 1995). To reconcile these apparently contrasting results it has recently been demonstrated (Sanchez-Cardenas *et al.*, 2014) by concomitant evaluation of AR and  $[Ca^{2+}]_i$  modifications in live human spermatozoa that the initial calcium peak in response to progesterone is not sufficient to trigger the AR. Indeed, in most sperm cells undergoing AR in response to the steroid (representing ~12% of the total number, as in our

study) the initial peak is followed by a second peak occurring about 10 min later that immediately precedes AR initiation. The nature and the mechanisms of this second peak of  $[Ca^{2+}]_i$  induced by progesterone is unclear. One hypothesis is that it might be related to influx of  $Ca^{2+}$  from fusion pores generated during AR initiation (Sanchez-Cardenas et al., 2014). Clearly, such a mechanism, if involved, would be CatSper-independent, as also suggested by the lack of correlation between AR and CatSper1 expression in this study.

One limitation of our study is the fact that CASA motility, AR and  $[Ca^{2+}]_i$  were evaluated after 40% PureSperm selection of the samples, possibly not reflecting the whole sperm sample. However, all the samples were carefully evaluated by microscopy before performing the experiments and only those samples where initial motility characteristics were maintained after selection were kept, as indicated by the significant relationship between pre- and post-selection motility.

In conclusion, our study demonstrates a clear implication of the CatSper channel in progressive and HA motility of human spermatozoa and provides preliminary evidence that lack of expression or mislocalization of CatSper1 in spermatozoa may be involved in the pathogenesis of asthenozoospermia. Finally, our data reinforce the concept that the CatSper channel represents a possible target for the development of new human contraceptives.

## Acknowledgements

We thank Drs Erminio Filimberti, Selene Degl'Innocenti and Maria Grazia Fino (AOUC Careggi) for semen analysis. We thank Dr Roberto Zonefrati (Department of Surgery and Translational Medicine, University of Florence) for help with calcium experiments.

## Authors' roles

L.T. performed the experiments, collected and analyzed the data and was responsible for manuscript preparation. S.M. was involved in flow cytometry analysis. E.V. and B.M. performed immunofluorescence experiment and confocal microscopy analysis, helped in the interpretation of results. M.C. contributed to set up and perform experiments in live and dead spermatozoa. S.P. contributed in patient enrollment. G.F. contributed to the final review of the manuscript. M.M. was involved in the interpretation of flow cytometric data and helped in the statistical analysis. E.B. designed and coordinated the study and wrote the manuscript. All the authors made substantial contributions in critically revising the article.

## Funding

The study was supported by grants from Italian Ministry of University and Scientific Research (PRIN project to E.B. and FIRB to S.M.) and Regione Toscana (to G.F.).

## Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## References

- Alasmari W, Barratt CL, Publicover SJ, Whalley KM, Foster E, Kay V, Martins da Silva S, Oxenham SK. The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. *Hum Reprod* 2013a; **28**:866–876.
- Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, Ramalho-Santos J, Kirkman-Brown J, Michelangeli F, Publicover S et al.  $Ca^{2+}$  signals generated by CatSper and  $Ca^{2+}$  stores regulate different behaviors in human sperm. *J Biol Chem* 2013b; **288**:6248–6258.
- Amini M, Shirinbayan P, Behnam B, Roghani M, Farhoudian A, Joghataei MT, Koruji M. Correlation between expression of CatSper family and sperm profiles in the adult mouse testis following Iranian Kerack abuse. *Andrology* 2014; **2**:386–393.
- Avenarius MR, Hildebrand MS, Zhang Y, Meyer NC, Smith LL, Kahrizi K, Najmabadi H, Smith RJ. Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet* 2009; **84**:505–510.
- Avidan N, Tamary H, Dgany O, Cattán D, Pariente A, Thulliez M, Borot N, Moati L, Barthelme A, Shalmon L et al. CATSPER2, a human autosomal nonsyndromic male infertility gene. *Eur J Hum Genet* 2003; **11**:497–502.
- Baldi E, Luconi M, Muratori M, Marchiani S, Tamburrino L, Forti G. Nongenomic activation of spermatozoa by steroid hormones: facts and fictions. *Mol Cell Endocrinol* 2009; **308**:39–46.
- Baldi E, Luconi M, Krausz C, Forti G. Progesterone and spermatozoa: a long-lasting liaison comes to definition. *Hum Reprod* 2011; **26**:2933–2934.
- Bhilawadikar R, Zaveri K, Mukadam L, Naik S, Kamble K, Modi D, Hinduja I. Levels of Tektin 2 and CatSper 2 in normozoospermic and oligoasthenozoospermic men and its association with motility, fertilization rate, embryo quality and pregnancy rate. *J Assist Reprod Genet* 2013; **30**:513–523.
- Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Kräling M, Müller A, Kaupp UB, Strünker T. The CatSper channel: a polymodal chemosensor in human sperm. *EMBO J* 2012; **31**:1654–1665.
- Bujan L, Mansat A, Pontonnier F, Mieuisset R. Time series analysis of sperm concentration in fertile men in Toulouse, France between 1977 and 1992. *BMJ* 1996; **312**:471–472.
- Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF. CatSper1 required for evoked  $Ca^{2+}$  entry and control of flagellar function in sperm. *Proc Natl Acad Sci USA* 2003; **100**:14864–14868.
- Chung JJ, Navarro B, Krapivinsky G, Krapivinsky L, Clapham DE. A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. *Nat Commun* 2011; **2**:153.
- Chung JJ, Shim SH, Everley RA, Gygi SP, Zhuang X, Clapham DE. Structurally distinct  $Ca^{2+}$  signaling domains of sperm flagella orchestrate tyrosine phosphorylation and motility. *Cell* 2014; **157**:808–822.
- Darszon A, Nishigaki T, Beltran C, Treviño CL. Calcium channels in the development, maturation, and function of spermatozoa. *Physiol Rev* 2011; **91**:1305–1355.
- ESHRE Andrology Special Interest Group. Guidelines on the application of CASA technology in the analysis of spermatozoa. *Hum Reprod* 1998; **13**:142–145.
- Eskenazi B, Wyrobek AJ, Slotter E, Kidd SA, Moore L, Young S, Moore D. The association of age and semen quality in healthy men. *Hum Reprod* 2003; **18**:447–454.
- Falsetti C, Baldi E, Krausz C, Casano R, Failli P, Forti G. Decreased responsiveness to progesterone of spermatozoa in oligozoospermic patients. *J Androl* 1993; **14**:17–22.
- Grynkiewicz G, Poenie M, Tsien RY. A generation of Ca indicator with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**:3440–3450.
- Hildebrand MS, Avenarius MR, Fellous M, Zhang Y, Meyer NC, Auer J, Serres C, Kahrizi K, Najmabadi H, Beckmann JS et al. Genetic male

- infertility and mutation of CATSPER ion channels. *Eur J Hum Genet* 2010; **18**:1178–1184.
- Hirano Y, Shibahara H, Obara H, Suzuki T, Takamizawa S, Yamaguchi C, Tsunoda H, Sato I. Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. *J Assist Reprod Genet* 2001; **18**:213–218.
- Jacob A, Hurley I, Mandel FS, Hershlag A, Cooper GW, Benoff S. Human sperm non-nuclear progesterone receptor expression is a novel marker for fertilization outcome. *Mol Hum Reprod* 1998; **4**:533–542.
- Jaiswal D, Singh V, Dwivedi US, Trivedi S, Singh K. Chromosome microarray analysis: a case report of infertile brothers with CATSPER gene deletion. *Gene* 2014; **542**:263–265.
- Jiang T, Hou CC, She ZY, Yang WX. The SOX gene family: function and regulation in testis determination and male fertility maintenance. *Mol Biol Rep* 2013; **40**:2187–2194.
- Jin J, Jin N, Zheng H, Ro S, Tafolla D, Sanders KM, Yan W. Catsper3 and Catsper4 are essential for sperm hyperactivated motility and male fertility in the mouse. *Biol Reprod* 2007; **77**:37–44.
- Kidd SA, Eskenazi B, Wyrobek AJ. Effects of male age on semen quality and fertility: a review of the literature. *Fertil Steril* 2001; **75**:237–248.
- Kirichok Y, Navarro B, Clapham DE. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated  $Ca^{2+}$  channel. *Nature* 2006; **439**:737–740.
- Krausz C, Bonaccorsi L, Luconi M, Fuzzi B, Criscuoli L, Pellegrini S, Forti G, Baldi E. Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with in-vitro fertilization. *Hum Reprod* 1995; **10**:120–124.
- Krausz C, Bonaccorsi L, Maggio P, Luconi M, Criscuoli L, Fuzzi B, Pellegrini S, Forti G, Baldi E. Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Hum Reprod* 1996; **11**:1661–1667.
- Leushuis E, van der Steeg JW, Steures P, Repping S, Bossuyt PM, Mol BW, Hompes PG, van der Veen F. Semen analysis and prediction of natural conception. *Hum Reprod* 2014; **29**:1360–1367.
- Li HG, Ding XF, Liao AH, Kong XB, Xiong CL. Expression of CatSper family transcripts in the mouse testis during post-natal development and human ejaculated spermatozoa: relationship to sperm motility. *Mol Hum Reprod* 2007; **13**:299–306.
- Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal  $Ca^{2+}$  channel of human sperm. *Nature* 2011; **471**:387–391.
- Liu DY, Baker HW. Evaluation and assessment of semen for IVF/ICSI. *Asian J Androl* 2002; **4**:281–485.
- Luconi M, Porazzi I, Ferruzzi P, Marchiani S, Forti G, Baldi E. Tyrosine phosphorylation of the a kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate. *Biol Reprod* 2005; **72**:22–32.
- Mansell SA, Publicover SJ, Barratt CL, Wilson SM. Patch clamp studies of human sperm under physiological ionic conditions reveal three functionally and pharmacologically distinct cation channels. *Mol Hum Reprod* 2014; **20**:392–408.
- Marchiani S, Tamburrino L, Giuliano L, Nosi D, Sarli V, Gandini L, Piomboni P, Belmonte G, Forti G, Baldi E et al. Sumo1-ylation of human spermatozoa and its relationship with semen quality. *Int J Androl* 2011; **34**:581–593.
- Marquez B, Igotz G, Suarez SS. Contributions of extracellular and intracellular  $Ca^{2+}$  to regulation of sperm motility: release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. *Dev Biol* 2007; **303**:214–221.
- Mata-Rocha M, Alvarado-Cuevas E, Hernández-Sánchez J, Cerecedo D, Felix R, Hernández-Reyes A, Tesoro-Cruz E, Oviedo N. Molecular cloning and analysis of the Catsper1 gene promoter. *Mol Hum Reprod* 2013; **19**:336–347.
- Mata-Rocha M, Hernández-Sánchez J, Guarneros G, de la Chesnaye E, Sánchez-Tusié AA, Treviño CL, Felix R, Oviedo N. The transcription factors Sox5 and Sox9 regulate Catsper1 gene expression. *FEBS Lett* 2014; **588**:3352–3360.
- Mazzilli F, Rossi T, Delfino M, Dondero F, Makler A. A new objective method for scoring human sperm hyperactivation based on head axis angle deviation. *Int J Androl* 2001; **24**:189–196.
- Miki K, Clapham DE. Rheotaxis guides mammalian sperm. *Curr Biol* 2013; **23**:443–452.
- Miller MR, Mansell SA, Meyers SA, Lishko PV. Flagellar ion channels of sperm: similarities and differences between species. *Cell Calcium* 2014 [Epub ahead of print].
- Mohammadi S, Movahedin M, Mowla SJ. Up-regulation of CatSper genes family by selenium. *Reprod Biol Endocrinol* 2009; **7**:126.
- Mohammadi S, Jalali M, Nikravesh MR, Fazel A, Ebrahimzadeh A, Gholamin M, Sankian M. Effects of vitamin-E treatment on CatSper genes expression and sperm quality in the testis of the aging mouse. *Iran J Reprod Med* 2013; **11**:989–998.
- Mortimer ST, Swan MA, Mortimer D. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Hum Reprod* 1998; **13**:2139–2146.
- Muciaccia B, Padula F, Vicini E, Gandini L, Lenzi A, Stefanini M. Beta-chemokine receptors 5 and 3 are expressed on the head region of human spermatozoon. *FASEB J* 2005; **19**:2048–2050.
- Muciaccia B, Boitani C, Berloco BP, Nudo F, Spadetta G, Stefanini M, de Rooij DG, Vicini E. Novel stage classification of human spermatogenesis based on acrosome development. *Biol Reprod* 2013; **89**:1–10.
- Muratori M, Marchiani S, Tamburrino L, Tocci V, Failli P, Forti G, Baldi E. Nuclear staining identifies two populations of human sperm with different DNA fragmentation extent and relationship with semen parameters. *Hum Reprod* 2008; **23**:1035–1043.
- Muratori M, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C, Forti G, Baldi E. Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity and oxidative stress. *Mol Med* 2015 [Epub ahead of print].
- Nikpoor P, Mowla SJ, Movahedin M, Ziaee SA, Tiraihi T. CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility. *Hum Reprod* 2004; **19**:124–128.
- Park EH, Kim do R, Kim HY, Park SK, Chang MS. Panax ginseng induces the expression of CatSper genes and sperm hyperactivation. *Asian J Androl* 2014; **16**:845–851.
- Peedicayil J, Deendayal M, Sadasivan G, Shivaji S. Assessment of hyperactivation, acrosome reaction and motility characteristics of spermatozoa from semen of men of proven fertility and unexplained infertility. *Andrologia* 1997; **29**:209–218.
- Publicover S, Barratt C. Reproductive biology: progesterone's gateway into sperm. *Nature* 2011; **471**:313–314.
- Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, Kirichok Y, Ramsey IS, Quill TA, Clapham DE. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci USA* 2007; **104**:1219–1223.
- Quill TA, Ren D, Clapham DE, Garbers DL. A voltage-gated ion channel expressed specifically in spermatozoa. *Proc Natl Acad Sci USA* 2001; **98**:12527–12531.
- Quill TA, Sugden SA, Rossi KL, Doolittle LK, Hammer RE, Garbers DL. Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc Natl Acad Sci USA* 2003; **100**:14869–14874.
- Ren D, Xia J. Calcium signaling through CatSper channels in mammalian fertilization. *Physiology (Bethesda)* 2010; **25**:165–175.
- Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham DE. A sperm ion channel required for sperm motility and male fertility. *Nature* 2001; **413**:603–609.

- Sagare-Patil V, Galvankar M, Satiya M, Bhandari B, Gupta SK, Modi D. Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa. *Int J Androl* 2012;**35**:633–644.
- Sagare-Patil V, Vernekar M, Galvankar M, Modi D. Progesterone utilizes the PI3K-AKT pathway in human spermatozoa to regulate motility and hyperactivation but not acrosome reaction. *Mol Cell Endocrinol* 2013; **374**:82–91.
- Sánchez-Cárdenas C, Servín-Vences MR, José O, Treviño CL, Hernández-Cruz A, Darszon A. Acrosome reaction and  $\text{Ca}^{2+}$  imaging in single human spermatozoa: new regulatory roles of  $[\text{Ca}^{2+}]_i$ . *Biol Reprod* 2014; **91**:67.
- Smith JF, Syritsyna O, Fellous M, Serres C, Mannowetz N, Kirichok Y, Lishko PV. Disruption of the principal progesterone-activated sperm  $\text{Ca}^{2+}$  channel in a CatSper2-deficient infertile patient. *Proc Natl Acad Sci USA* 2013; **110**:6823–6828.
- Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced  $\text{Ca}^{2+}$  influx in human sperm. *Nature* 2011; **47**:382–386.
- Suarez SS. Control of hyperactivation in sperm. *Hum Reprod Update* 2008; **14**:647–657.
- Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Hum Reprod* 1995; **10**:2928–2937.
- Tamburrino L, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. *Hum Reprod* 2014;**29**:418–428.
- Tavares RS, Mansell S, Barratt CL, Wilson SM, Publicover SJ, Ramalho-Santos J. *p,p'*-DDE activates CatSper and compromises human sperm function at environmentally relevant concentrations. *Hum Reprod* 2013;**28**:3167–3177.
- Tesarik J, Mendoza C. Defective function of a nongenomic progesterone receptor as a sole sperm anomaly in infertile patients. *Fertil Steril* 1992; **58**:793–797.
- Visser L, Westerveld GH, Xie F, van Daalen SK, van der Veen F, Lombardi MP, Repping S. A comprehensive gene mutation screen in men with asthenozoospermia. *Fertil Steril* 2011;**95**:1020–1024.e1–9.
- Wang C, Lee GS, Leung A, Surrey ES, Chan SY. Human sperm hyperactivation and acrosome reaction and their relationships to human in vitro fertilization. *Fertil Steril* 1993;**59**:1221–1227.
- Wang Y, Shi J, Li L, Liu D, Li L, Tang C, Li J. Adverse effects of 2,2',4,4'-tetrabromodiphenyl ether on semen quality and spermatogenesis in male mice. *Bull Environ Contam Toxicol* 2013;**90**:51–54.
- World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Cambridge, UK: Cambridge University Press, 2010.
- Xia J, Reigada D, Mitchell CH, Ren D. CATSPER channel-mediated  $\text{Ca}^{2+}$  entry into mouse sperm triggers a tail-to-head propagation. *Biol Reprod* 2007;**77**:551–559.