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The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction

Lara Tamburrino, Sara Marchiani, Federico Minetti, Gianni Forti, Monica Muratori, and Elisabetta Baldi*

Department of Biomedical, Experimental and Clinical Sciences, Centre of Excellence DeNothe, University of Florence, Florence, Italy

*Correspondence address. Department of Biomedical, Experimental and Clinical Sciences, Unit of Clinical Pathophysiology, University of Florence, viale Pieraccini 6, I-50139 Florence, Italy. Tel: +390554271486; Fax: +390554271371; E-mail: elisabetta.baldi@unifi.it

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STUDY QUESTION: Does CatSper have a role in the achievement of human sperm motility and in the Progesterone (P)-induced acrosome reaction (AR)?

SUMMARY ANSWER: CatSper1 expression is associated with human sperm progressive motility and the P-induced AR; it may have a role in the pathogenesis of asthenozoospermia.

WHAT IS KNOWN ALREADY: Knockout mice for any of the Catsper family genes fail to acquire hyperactivated motility and are infertile. CatSper channels mediate P-induced Ca^{2+} influx in human sperm. The role of CatSper in human sperm hyperactivated/activated motility and in asthenospermia is less clear. A few men with *CatSper* mutations have been described but the phenotype regarding sperm motility has not been well established.

STUDY DESIGN, SIZE, DURATION: The effects of two Catsper inhibitors, NNC55-0396 (NNC, 10 and 20 μM) and Mibefradil (Mib, 30 and 40 μM), were tested on human sperm motility parameters and the P-induced AR. Catsper1 protein expression was evaluated in unselected and swim-up selected sperm samples and in sperm from normo- and astheno-zoospermic subjects.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Semen sample kinematic parameters were analysed by a CASA system. A fluorescent-labelled lectin was used to evaluate P-induced AR in live sperm by fluorescence microscopy. CatSper1 protein expression was determined by western blot analysis and by flow cytometry. Intracellular calcium concentrations ($[Ca^{2+}]_i$) were evaluated by a spectrofluorimetric method following sperm loading with the calcium-sensitive probe fura 2/AM.

MAIN RESULTS AND THE ROLE OF CHANCE: CatSper1 protein was localized in the tail of human sperm. CatSper1 expression was higher in swim up selected than unselected sperm both when measured by western blot or by flow cytometry (52.7 ± 15.8% versus 27.2 ± 9.01%, $n = 7$, $P < 0.01$). Basal and P-stimulated $[Ca^{2+}]_i$ were significantly higher in swim-up selected compared with unselected sperm. CatSper1 expression (western blot analysis) was found to be decreased in sperm from asthenozoospermic ($n = 10$) compared with those from normozoospermic ($n = 9$) men (intensity values relative to β-actin: 244.4 ± 69.3 versus 385.8 ± 139.5, $P < 0.01$). A positive correlation was found between CatSper1 protein expression and the percentage of sperm with progressive motility ($n = 19$, $r = 0.59$, $P = 0.007$). NNC (10 μM) and Mib (30 μM) significantly reduced the percentage of sperm with progressive motility and several kinematic parameters but did not affect the percentage of hyperactivated sperm. Their effects were the same whether they were added to swim-up selected and capacitated sperm or were added to the swim-up medium. Mib was found to have a slight but significant effect on sperm viability at both concentrations tested. P-stimulated AR was significantly reduced by both inhibitors ($P < 0.05$). Overall, our results indicate that, in human sperm, CatSper channel expression and function are associated with progressive motility and may be involved in the pathogenesis of asthenozoospermia.

LIMITATIONS, REASONS FOR CAUTION: In general, studies evaluating the effect of inhibitors have the limitation of the specificity of the molecules. We show here that Mib may have toxic effect on human sperm. Although most of the parameters have been evaluated in live sperm, the toxic effect could have contributed to the observed decreases. More studies are necessary to evaluate further the role of CatSper1 in asthenozoospermia.

WIDER IMPLICATIONS OF THE FINDINGS: In view of the involvement in P-induced AR and of the evidence of a role in the pathogenesis of astenozoospermia, CatSper channels may represent a promising target for the development of new drugs for the treatment of male infertility and for non-hormonal contraception.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants from Ministry of University and Scientific Research (Prin project to E.B. and FIRB project to S.M) and Regione Toscana (to G.F.). The authors have no conflicts of interest to declare.

Key words: CatSper / male infertility / sperm motility / acrosome reaction

Introduction

CatSper is a pH-sensitive voltage-gated calcium channel exclusively expressed in the testis and in sperm (Quill *et al.*, 2001). The CatSper channel is formed by four subunits (named CatSper1, 2, 3 and 4), all of which are essential for its function, leading to the development of sperm hyperactivated motility and fertility in male mice (Qi *et al.*, 2007). In view of the exclusive expression of CatSper in sperm, the protein is a potential target for development of new contraceptive strategies as well as for treatment, *in vivo* and *in vitro*, of male infertility.

Although the role of CatSper in fertility and development of sperm hyperactivated motility has been clearly demonstrated in mice (Ren *et al.*, 2001), its role in human fertility is less clear. Inactivating mutations of CatSper1 and 2 have been found in a few men (reviewed in Hildebrand *et al.*, 2010) but the relationship between CatSper mutations and the phenotype is not obvious. Sperm motility was very variable among these men (from 50 to 0%) and the ability of their sperm to achieve hyperactivation (HA) or capacitation has not been clearly established (Hildebrand *et al.*, 2010). A recent study found that only few sperm were motile in the ejaculate of a subject with CatSper2 mutation (Smith *et al.*, 2013). In addition, new *in vitro* studies questioned the involvement of CatSper in induction of HA in human sperm (Alasmari *et al.*, 2013a,b) evidencing possible species differences in the role of the channel in sperm functions. Thus, the link between CatSper and human sperm motility remains ambiguous.

Two breakthrough papers in 2011 demonstrated, by employing the patch clamp technique, that CatSper channels mediate calcium influx in response to progesterone (P) in human sperm (Lishko *et al.*, 2011; Strünker *et al.*, 2011). The channel is activated by the steroid from pico- to micro-molar concentrations and the intracellular calcium elevations induced by P are greatly reduced by CatSper inhibitors (Lishko *et al.*, 2011; Strünker *et al.*, 2011). However, whether CatSper channels are involved in the several actions of P described in sperm (Baldi *et al.*, 2009) has not been clarified so far. In particular, micro-molar concentrations of P have been shown to induce the acrosome reaction (AR) and P has been indicated as the molecule that mediates, at sub-nanomolar concentrations, sperm chemotaxis in the proximity of the oocyte (Teves *et al.*, 2006; Blengini *et al.*, 2011). The effect of P on sperm motility is less clear (Baldi *et al.*, 2009). Indeed, although some studies have demonstrated a positive effect on forward and hyperactivated motility (Uhler *et al.*, 1992; Jaiswal *et al.*, 1999; Sagare-Patil *et al.*, 2012), others have not fully confirmed such findings (Wang *et al.*, 2001; Luconi *et al.*, 2004b; Alasmari *et al.*, 2013a,b).

P is present throughout the entire female genital tract reaching micromolar concentrations in the cumulus oophorus (Osman *et al.*, 1989; Munuce *et al.*, 2006), a key structure in the fertilization process. Recent work has demonstrated that in mouse oocytes the cumulus oophorus is the site where sperm undergo the AR before oocyte penetration (Bedford 2011; Jin *et al.*, 2011; Sun *et al.*, 2011) leading to the hypothesis that P could be the physiological inducer of AR during mammalian fertilization (Baldi *et al.*, 2011; Jin *et al.*, 2011). Our group has previously demonstrated that the AR and intracellular calcium accumulation in response to P are variable and are highly correlated with sperm fertilizing ability (Krausz *et al.*, 1995, 1996) being reduced in subfertile (oligoasthenospermic) subjects (Falsetti *et al.*, 1993). In this light, the elucidation of the role of CatSper in the AR in response to P and sperm HA appears crucial.

The present study was undertaken to evaluate CatSper1 expression and localization in human sperm, its role in activated and hyperactivated motility and in P-induced AR. In addition, we have evaluated CatSper1 expression in high motility sperm fractions and in asthenozoospermic men.

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 from Nidacon International (Mölndal, Sweden). Paraformaldehyde (PFA) was obtained by Merck Chemicals (Milan, Italy). NNC55-0396 (NNC) was from Tocris Bioscience RND Systems Company (Bristol, UK). Coomassie brilliant dye was from Bio-Rad Laboratories (Hercules, CA, USA). Rabbit anti-Catsper polyclonal antibodies were from Bioss (Woburn, MA, USA) and from Santa Cruz Biotechnology (Dallas, TX, USA). Fura 2/AM was from Molecular Probes (Life Technology, Monza, Italy).

Normal goat serum (NGS), progesterone (P), mibefradil (Mib), FITC-labelled *Arachis hypogea* (peanut) lectin, phosphatase inhibitor cocktail, protease inhibitor cocktail, skim milk powder, digitonin, EGTA, anti-rabbit IgG peroxidase-conjugated secondary antibody, anti-B actin primary antibody and anti-mouse IgG peroxidase-conjugated secondary antibody were from Sigma Chemical Co. (St. Louis, MO, USA).

Semen collection and preparation

Semen samples were obtained [according to WHO criteria (WHO, 2010)] from subjects undergoing routine semen analysis for couple infertility in the Andrology laboratory of the University of Florence after informed patient's consent. Sperm morphology, motility and viability were assessed by optical microscopy, according to WHO criteria (WHO, 2010).

In some experiments, sperm were selected by swim-up procedure in HTF-10% HSA medium as previously reported (Krausz *et al.*, 1995; Luconi *et al.*, 2004a).

For western blot and calcium experiments, semen samples were washed by centrifugation (500 g, 30 min, 26°C) through a layer of 40% PureSperm, prepared in HTF-10% HSA medium. The resulting pellet was collected and washed in HTF-10% HSA medium. Each sample was checked at optical microscope for effective elimination of germ cells and leucocytes and the motility of the prepared sperm was evaluated to be sure that initial motility conditions were maintained after selection. Samples that failed to meet these criteria were discarded. For experiments comparing CatSper1 expression in swim-up selected and unselected sperm, a western blot was performed directly on extracts of selected sperm and on an aliquot of the whole sample prepared by centrifugation through 40% PureSperm as described above.

Immunofluorescence staining and detection of CatSper1 by flow cytometry

Immunofluorescence staining of CatSper1 was performed on swim-up selected sperm and sperm from whole semen (unselected) of the same ejaculate. Ten million sperm were fixed in PFA [500 μl, 4% w/v in phosphate-buffered saline (PBS), pH 7.4] for 30 min at room temperature. After washing twice with 1% NGS-PBS, sperm were permeabilized with 0.1% Triton X-100 in 100 μl of 0.1% sodium citrate for 4 min in ice. Then, samples were split into two aliquots subsequently incubated (1 h at room temperature) with either anti-CatSper1 antibody (20 μg/ml Bioss and 1 μg/ml Santa Cruz, in 1% NGS-PBS, test sample) or rabbit serum (20 μg/ml, in 1% NGS-PBS) for the negative control. After washing twice with 1% NGS-PBS, sperm 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 μl of PBS, stained with 4.5 μl of propidium iodide (PI; 50 μg/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 for test sample but omitting the PI staining and used for instrumental compensation.

Samples were acquired using FACScan flow cytometer (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 labelled with PI) of the characteristic forward scatter/side scatter (FSC/SSC) region of sperm (Muratori *et al.*, 2008). A marker was established in the CatSper axis dot plot of 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 CatSper1.

Samples were also examined using a fluorescence microscope (Carl Zeiss, Axiolab A1 FL, Milan, Italy), equipped with filter set 15, 44 and 49 by an oil immersion ×100 magnification objective. Images were captured by an AxioCam ICm1 camera using Axiovision 4.8 (provided by Canon, Japan) and edited by Adobe Photoshop CS 2 (Adobe Systems, Inc., San Jose, CA, USA).

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

$[Ca^{2+}]_i$ was evaluated as previously described (Krausz *et al.*, 1995, 1996). Briefly, sperm from the same ejaculates were selected by swim-up or washed through 40% PureSperm and capacitated for 3 h in HTF-10% HSA medium. They were incubated for 30 min at 37°C with Fura2/AM (2 μM, final concentration) for 45 min, washed, resuspended in FM buffer (125 mM NaCl, 10 mM KC1, 2.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 19 mM sodium lactate, 2.5 mM sodium pyruvate, 20 mM HEPES/NaOH, pH 7.6) and further incubated for 30 min. $[Ca^{2+}]_i$ was measured using a spectrofluorimetric method with a Perkin-Elmer LS50B instrument equipped with a fast rotary filter shuttle for alternate 340 and 380 nm excitation. Sperm were stimulated with 10 μM P which was added directly in the cuvette. Fluorescence measurements were converted to $[Ca^{2+}]_i$, by determining maximal fluorescence (F_{max}) with digitonin (0.01% final concentration) followed by minimal fluorescence (F_{min}) with 10 mM EGTA, pH 10. $[Ca^{2+}]$ was calculated according to Grynkiewicz *et al.* (1985) assuming a dissociation constant of Fura 2 for calcium of 224 nM. Each sample (swim-up and PureSperm selected) was evaluated in triplicate. Reported basal $[Ca^{2+}]_i$ represents the mean level of 30 s record before addition of P. P-stimulated $[Ca^{2+}]_i$ was evaluated at maximum peak level after addition of the steroid.

Assessment of sperm motility by computer-assisted semen analysis

Sperm were selected by swim-up and capacitated in HTF-10% HSA for 3 h. Time course experiments were performed to establish the incubation time for P. Results of these experiments (Supplementary data, Fig. S1) indicate that, when a positive effect of P (1 pM and 10 μM) on HA was found, it was maintained for 15 min and thus, this time was chosen for subsequent experiments. No effects were observed for progressive motility with both P concentrations (Supplementary data, Fig. S1). Swim-up selected sperm were divided into equal aliquots (20×10^6/ml each one) and incubated with P (1 pM, 10 μM), Mib (30 and 40 μM) and NNC (10 and 20 μM) for 15 min at 37°C.

Sperm viability was evaluated by eosin-nigrosin staining (WHO, 2010) after 15 min and 1 h treatment with both concentrations of NNC and Mib.

In another set of experiments, whole semen samples were divided into three equal aliquots and selected by swim-up in HTF-10% HSA, HTF-10% HSA supplemented with Mib (30 μM) and HTF-10% HSA supplemented with NNC (10 μM).

Sperm motility was analysed by a computer-assisted semen analysis (CASA) system (Hamilton Thorn Research, Beverly, MA, USA) as described previously (Luconi *et al.*, 2005, 2004a). Average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), linearity of progression (LIN, %), percentages of sperm with progressive and rapid motility, percentages of sperm with non-progressive motility (static) and hyperactivated sperm (sort fraction, SF) were recorded. The threshold values to identify hyperactivated cells were manually set (VCL >150 μm/s, amplitude of lateral head displacement (ALH) >7 μm, linearity (LIN) <50% (Mortimer *et al.*, 1998)). A minimum of 100 cells and 5 fields were analysed for each aliquot. All analyses were performed at 37°C.

Evaluation of AR

To test the effect of inhibition of CatSper on P-stimulated AR, sperm (10×10^6/ml) selected by swim-up and capacitated in HTF-HSA 10% for 3 h were stimulated for 1 h with P 10 μM, Mib 30 μM, NNC 10 μM, P (10 μM) + Mib (30 and 40 μM), P (10 μM) +NNC (10 and 20 μM). P was dissolved in dimethyl sulphoxide (DMSO) at an initial concentration of 0.1 M and diluted in HTF/HAS medium. Control samples were incubated with DMSO 0.0001%. Sperm AR was evaluated with the fluorescent probe, FITC-labelled *A. hypogea* (peanut) lectin, as previously described (Falsetti *et al.*, 1993; Krausz *et al.*, 1995). In order to evaluate AR only in live sperm, at the end of the incubation period sperm were washed and re-suspended in 0.5 ml of hypo-osmotic swelling medium for 1 h at 37°C and washed again and finally re-suspended in 50 μl of 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-labelled *A. hypogea* (peanut) lectin and fluorescence was observed under a fluorescent microscope. The AR was evaluated on a total of 200 sperm per slide. Only curly-tailed (viable) sperm were scored. Results are reported as % acrosome-reacted sperm in the different experimental conditions.

SDS-PAGE and western blot analysis

Protein extraction and Western analysis were performed as described previously (Muratori *et al.*, 2004; Luconi *et al.*, 2005). Briefly, after centrifugation through 40% PureSperm (see above), sperm samples were washed in PBS supplemented with 1 mm Na_3VO_4, centrifuged and resuspended in RIPA buffer [20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 1 mM sodium orthovanadate, 1% Triton –X100] supplemented with phosphatase inhibitor cocktail (diluted 1:100) and protease inhibitor cocktail (diluted 1:100). The protein concentration was measured (Coomassie brilliant dye) and volumes of cell lysates containing 35 μg protein were mixed with an equal volume of 2X Laemmli's reducing sample buffer [62.5 mM Tris pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 2.5% pyronin and 200 mM dithiothreitol], boiled at 95°C for 5 min and loaded onto 8% polyacrylamide–bisacrylamide gels. After SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred to nitrocellulose membrane (Whatman GmbH, Dassel, Germany) and cut with scissor at 70 kDa molecular weight based on molecular weight markers (Prestained Protein SHARPMASS V PLUS, Euroclone, Milan, Italy). The membranes were incubated for 1 h in 5% milk blocking buffer in TTBS solution (Tris-buffered saline containing 0.1% Tween 20, pH 7.4). The membranes with molecular weight >70 kDa were incubated overnight with the primary anti-CatSper antibodies (both diluted 1:500 in fatty acid-free BSA 1% in TTBS), then washed and incubated with anti-rabbit IgG peroxidase-conjugated secondary antibody (diluted 1:2000 in BSA 1% in TTBS) for 2 h. As control for lane loading the membranes with molecular weight <70 kDa were probed with anti-B actin primary antibody (diluted 1:1000 in 1% BSA in TTBS) and incubated with anti-mouse IgG peroxidase-conjugated secondary antibody (diluted 1:2000 in 1% BSA in TTBS), 1 h at room temperature. Proteins were revealed by Immobilon Western, chemiluminescent HRP substrate (Millipore, MA, USA). Image acquisition was performed using Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories). Densitometric analysis of western blots performed with ImageJ (software developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were analysed by the SPSS 20.0 software package (Chicago, IL, USA).

Data were assessed by the Kolmogorov–Smirnov test and were normally distributed. Data are expressed as mean ± SD. Statistical comparisons were made using ANOVA followed by Student's *t*-test. Correlation between CatSper1 expression and sperm motility was performed by linear regression analysis using Pearson's product moment correlation coefficient.

Results

Catsper expression in human sperm

Western blot analysis of pooled human sperm proteins using the anti-CatSper1 antibody from Bioss reveals the presence of a band at about 90 kDa corresponding to the molecular weight of the CatSper1 subunit (Fig. 1A, left panel). Another band was present at the molecular weight of ~45 kDa which might correspond to a different CatSper subunit (probably 3, see Kanwal *et al.*, 2011). Similar results were obtained with the anti-CatSper1 antibody from Santa Cruz (Fig. 1B, left panel).

By immunofluorescence the anti-CatSper1 antibody localizes to the principal piece of the tail of unselected sperm (Fig. 1A, right panels) in agreement with previous studies in animal (Ren *et al.*, 2001) and human (Smith *et al.*, 2013). The staining reveals a patchy and punctate pattern in the tail (Fig. 1A, arrows). Similar results were obtained with the Santa Cruz antibody (Fig. 1B). As can be observed, the staining was not present in all sperm (the arrows in the bright field evidence negative sperm, Fig. 1), suggesting inhomogeneous expression of CatSper channels in the ejaculate. This result prompted us to evaluate whether sperm selection by swim-up enhances the percentage of sperm showing positive staining to CatSper1. To this end, we evaluated the percentage of sperm expressing CatSper1 by immunofluorescence coupled to flow cytometry, using the anti-CatSper1 antibody from Bioss. This technique combines the use of PI and immunofluorescence staining allowing us to evaluate CatSper1 expression solely in the sperm population, eliminating all the other interferents (Muratori *et al.*, 2005, 2008). Figure 2A shows the dot plot of the negative control (left panel) and of unselected (medium panel) and swim-up selected (right panel) sperm from a typical experiment. As can be observed, a dramatic shift to the right of the sperm population occurs after swim-up selection (Fig. 2A) demonstrating that a much higher proportion of swim-up selected sperm expresses CatSper1. The average percentages of CatSper1 expressing sperm in unselected semen and following swim-up selection, as calculated based on the marker set in the negative control, in 7 samples are shown in Fig. 2B. A significant increase of the percentage of CatSper1 expressing sperm was observed. Western analysis of swim-up selected and 40% PureSperm purified sperm (in order to eliminate round cells present in the ejaculate but maintaining a similar motility of the unselected sample) confirmed the increase in CatSper1 expression after selection (Fig. 2C).

In order to evaluate whether the different CatSper1 levels observed in swim-up and 40% PureSperm washed sperm are functionally associated with different basal or P-stimulated $[Ca^{2+}]_i$, the latter parameters were evaluated in the two sperm preparations from eight subjects. As shown in Fig. 2D (right panel), basal $[Ca^{2+}]_i$ values were significantly lower in PureSperm prepared sperm compared with swim-up selected sperm from the same ejaculate. Similarly, average peak increase in response to P was lower in PureSperm purified sperm (Fig. 2D, right panel). An example of a typical calcium experiment is shown in the left panel of Fig. 2D. Interestingly, in the eight subjects, basal and P-stimulated $[Ca^{2+}]_i$ activities in 40% PureSperm washed sperm were significantly related to progressive motility of the samples ($r = 0.71$, $P = 0.04$ and $r = 0.81$, $P = 0.01$, respectively).

Effect of CatSper1 inhibitors on sperm motility parameters

NNC and Mib were added to 3 h capacitated swim-up selected sperm and incubated for further 15 min before evaluating sperm motion parameters by CASA. As can be observed in Fig. 3A, at the concentration of 10 μM, NNC significantly reduced the sperm motion parameters VAP, VSL, and LIN and the percentage of progressive and rapid motile sperm. Similar effects were observed with 40 and 30 μM Mib (Fig. 3A). No significant effects were observed on ALH or VCL (not shown). At 20 μM concentration, NNC significantly reduced LIN and the percentage of progressive and rapid motile sperm (Fig. 3A). Both inhibitors had no significant effect on the average percentage of hyperactivated sperm (% HA, Fig. 3A). However, when single cases were considered, NNC (10 μM) decreased the % HA in 7 samples out of the 14 analysed, although in some of the samples the effect was minimal (Supplementary data, Fig. S2A shows individual data). Similarly, Mib (30 μM) decreased the % HA in 6/10 samples (Supplementary data,

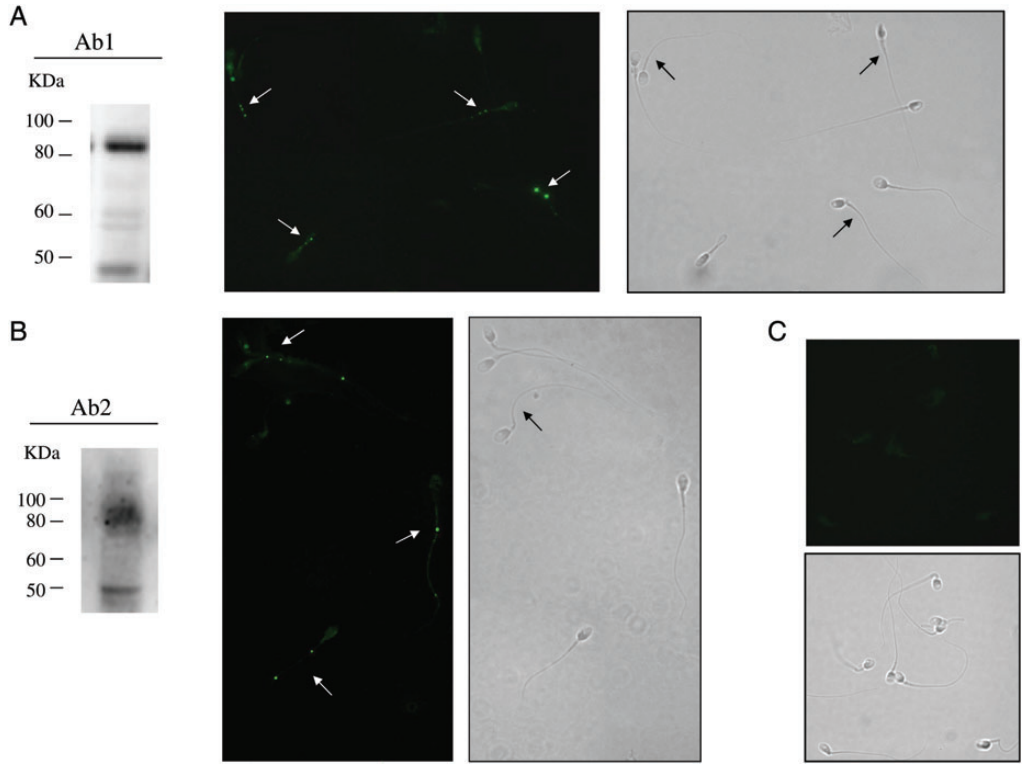

Figure 1 Expression of CatSper1 in human sperm. (**A**) Western blot analysis of 40% PureSperm washed sperm (left) and immunofluorescence analysis of Catsper1 expression in unselected sperm (right) revealed with anti-CatSper1 antibody from Bioss (Ab 1). (**B**) Western blot analysis of 40% PureSperm washed sperm (left) and immunofluorescence analysis of CatSper1 expression in unselected sperm (right) revealed with anti-CatSper1 antibody from Santa Cruz (Ab 2). White arrows indicate CatSper1-positive sperm. Right panels show the corresponding bright fields. Black arrows indicate sperm negative to CatSper1 staining. Representative of at least three similar experiments for both (A) and (B). (**C**) Negative control obtained using rabbit serum instead of primary antibody.

Fig. S2B). In the paper by Alasmari *et al.* (2013b), it was shown that the effect of NNC on spontaneous HA was seen in samples where the % HA before treatment was high. In agreement with these results, we found that average basal HA in samples where NNC was effective was higher compared with those where it was not (mean ± SD: 19.8 ± 3.9 versus 8.6 ± 2.8, $P < 0.001$). Overall, these data suggest a marginal involvement of CatSper in HA of human sperm. To evaluate whether inhibitors were more effective on HA if added from the beginning of the capacitation process, Mib (30 μM) and NNC (10 μM) were added directly to the HTF-10% HSA medium used for swim-up selection (1 h) and motility evaluated by CASA after selection. As can be observed in Fig. 3B, the addition of Mib (30 μM) and NNC (10 μM) to the swim-up medium determined a decrease of progressive and rapid sperm motility and an increase of static (non-progressive) motility. In addition, VAP, VSL, BCF and sort fraction were significantly decreased by Mib, whereas NNC showed a significant effect on VSL, BCF, LIN and a modest, non-significant, decrease of the average % HA (SF, Fig. 3B). No significant effects were observed on ALH and VCL (not shown). In particular, when single cases were considered, NNC decreased the % HA in 7 samples out of the 14 analysed (Supplementary data, Fig. S2C) and Mib in 8/11 (Supplementary data, Fig. S2D). As for experiments performed after swim-up selection (see above), basal HA was higher in samples responsive to NNC (18.19 ± 13.1 versus 8.6 ± 6.1) although statistical significance has not been reached in this case ($P = 0.09$).

To exclude that the effects of the two CatSper inhibitors on human sperm motility were due to toxicity of the molecules, we evaluated their effect on sperm viability after incubation for 15 and 60 min. As can be observed in Fig. 3C, Mib, at 30 and 40 μM concentrations, significantly reduced sperm viability at both 15 and 60 min incubation, whereas no significant effects were observed with 10 and 20 μM NNC.

As mentioned in the introduction, the occurrence of a stimulatory effect of P on human sperm motility is controversial (Baldi *et al.*, 2009). We have here evaluated the effect of P on sperm motility parameters using two different concentrations, one (10 μM) similar to that found in the cumulus oophorus (Osman *et al.*, 1989; Munuce *et al.*, 2006) and known to induce AR (Baldi *et al.*, 2009) and the other (1 pM), which has been demonstrated to induce chemotaxis and hypothesized to act in the proximity of the fertilization site to guide sperm to reach the oocyte (Teves *et al.*, 2006). In our hands, both concentrations of P (tested at 15 min) did not alter average sperm motion parameters of swim-up selected 3 h capacitated sperm in either the absence or presence of Mib and NNC (results not shown). Only occasionally (in 5/14 samples, see Supplementary data, Fig. S2E for individual data) 10 μM P showed a positive effect on sperm motion parameters and on sort

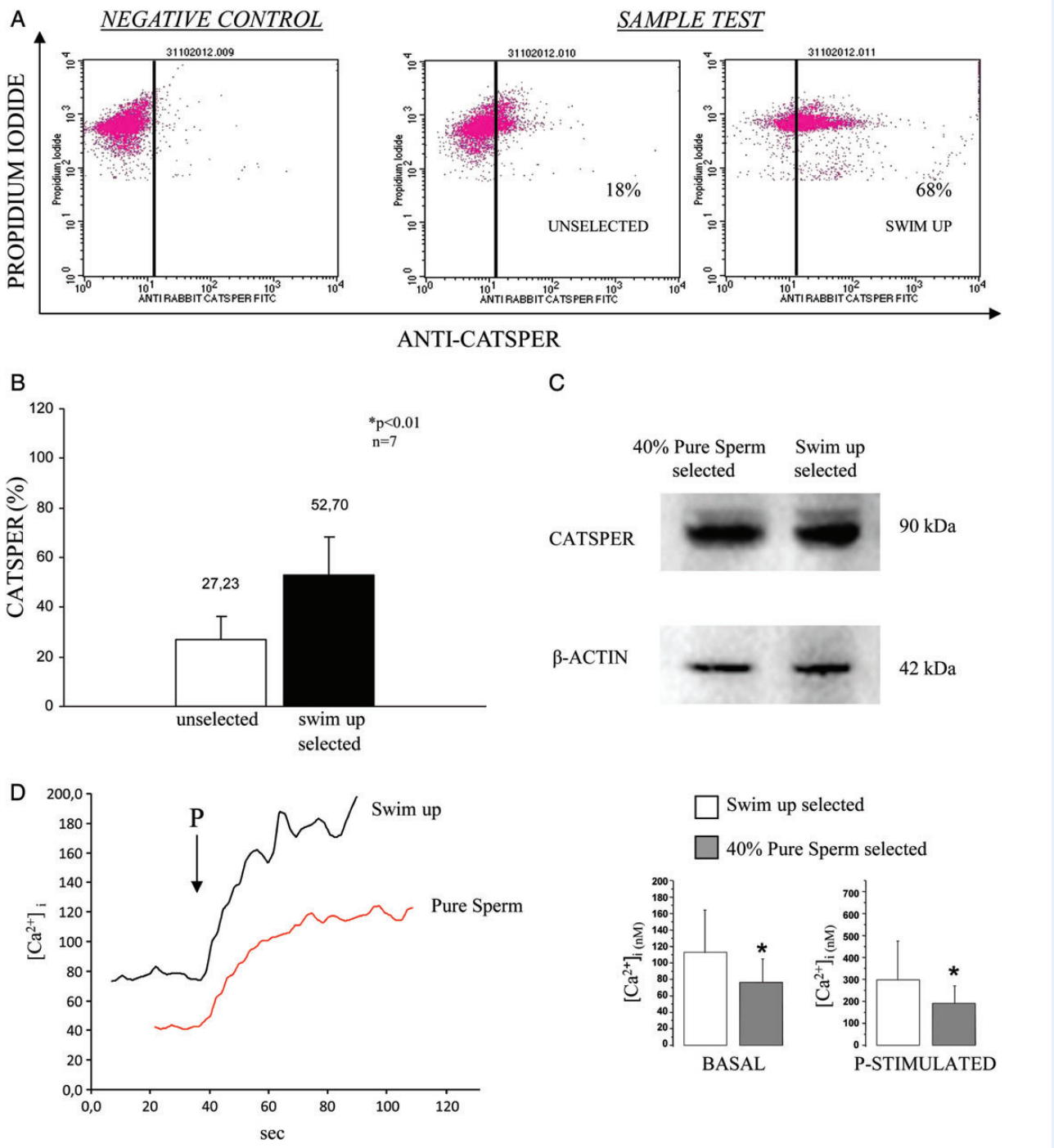

Figure 2 CatSper1 expression in swim-up selected human sperm. (**A**) Typical dot plots of negative control (left) and the corresponding CatSper1 test sample in unselected sperm (middle) and swim-up selected sperm (right). (**B**) Histograms of mean (± SD) percentages of CatSper1 expressing sperm in seven unselected and swim-up selected samples. *$P < 0.01$ respect to unselected. (**C**) Western blot analysis of CatSper1 expression in swim-up selected versus semen prepared by 40% PureSperm to eliminate germ cells and leukocytes. Representative of two similar experiments. (**D**) Typical $[Ca^{2+}]_i$ changes in response to P (10 μM) in fura-2-loaded 40% PureSperm washed and swim-up selected sperm are shown in the left panel. Histograms of mean (± SD) basal and P-stimulated $[Ca^{2+}]_i$ levels in 40% PureSperm washed and swim-up selected samples ($n = 8$, *$P < 0.05$) are showed in the right panel.

fraction, whereas at 1 pM, an effect was observed in 7/14 samples (Supplementary data, Fig. S2F). Interestingly, in these samples, the effect of P was reverted by NNC (results not shown). There was correspondence between the increasing effect of P and the decreasing effect of 10 μM NNC on HA only in two of the responsive samples to 10 μM P (Supplementary data, Fig. S2E, evidenced in red) and in four among those responding to 1 pM (Supplementary data, Fig. S2F, evidenced in red).

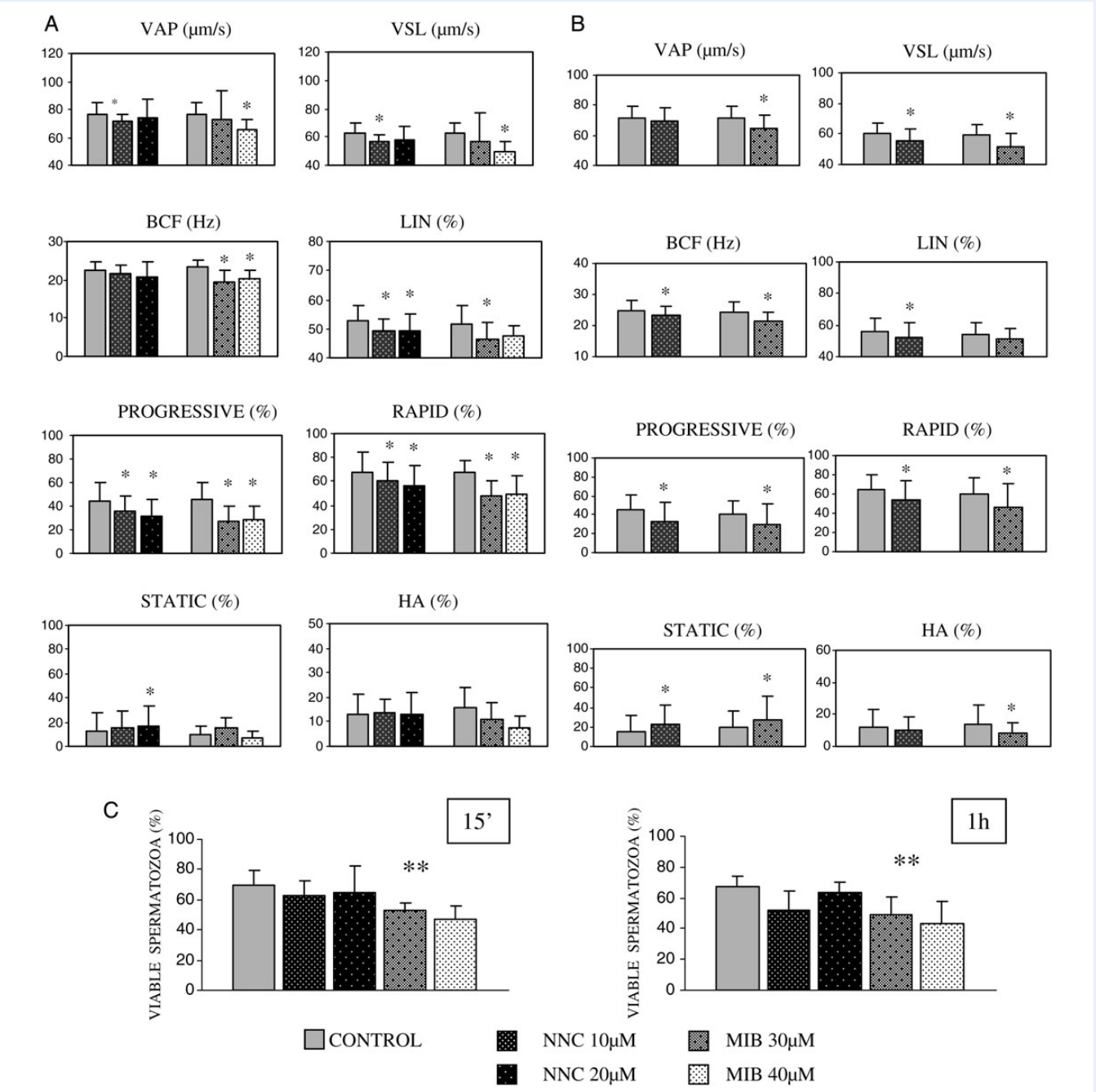

Figure 3 Effects of CatSper inhibition on sperm motility and viability. (**A**) Kinematic parameters as evaluated by CASA of 3 h capacitated swim-up selected sperm incubated for 15 min with 30 and 40 μM Mib ($n = 10$) and 10 and 20 μM NNC ($n = 14$), $^*P < 0.05$ versus control. (**B**) Kinematic parameters as evaluated by CASA sperm selected by swim-up in the presence of 30 μM Mib ($n = 11$) and 10 μM NNC ($= 14$), $^*P < 0.05$. (**C**): Histograms of mean ($\pm$ SD) percentages of viable sperm after 15′ (left panel) and 1 h (right panel) incubation with Mib (30 and 40 μM) and NNC (10 and 20 μM). $n = 5$, $^*P < 0.05$ versus control.

Effect of CatSper1 inhibitors on sperm AR

We have previously reported that several calcium channels inhibitors, including Mib, do not affect the P-stimulated AR (Bonaccorsi *et al.*, 2001). However, in our previous study, Mib was tested in a range of concentrations (1–10 μM) which does not appear to affect P-stimulated CatSper channels (Strünker *et al.*, 2011; Brenker *et al.*, 2012). Here we have tested the effect of 30 and 40 μM Mib and 10 and 20 μM NNC on the P-induced AR by pre-incubating 3 h capacitated swim-up selected sperm with the two inhibitors before addition of P (10 μM). AR was evaluated only in the live sperm (see materials and methods); thus, the toxic

effect of Mib (Fig. 3C) should not influence the net results. As can be observed in Fig. 4, P stimulated AR in basal conditions, in agreement with previous data (Falsetti *et al.*, 1993; Krausz *et al.*, 1995; Bonaccorsi *et al.*, 2001). When the experiments were carried out in the presence of 20 and 10 μM NNC, the stimulatory effect of P was decreased to an insignificant level (Fig. 4). Mib also inhibited P-stimulated AR at 40 μM but not at 30 μM (Fig. 4). It should be noted that the treatment with both antagonists determined, *per se*, an increase of acrosome-reacted sperm (Fig. 4).

Catsper1 expression in sperm from asthenospermic men

The fact that not all the sperm in an ejaculate express CatSper1 (Fig. 1) and that its expression is enhanced after swim-up selection (Fig. 2) indicates that immotile sperm may express lower levels of the protein or fail to express it at all. To test this hypothesis we have evaluated CatSper1 expression by western blot in normozoospermic and asthenozoospermic men, selected based on a total sperm motility $<32\%$ and a viability $>60\%$, as for the fifth percentile of reference values of WHO V edition (WHO, 2010). As can be observed in Fig. 5A, CatSper1 expression appears to be lower in sperm from asthenozoospermic men respect to normozoospermic. In particular, after normalization for β-actin expression in the same blot, levels of CatSper1 were found to be significantly lower in sperm from 10 asthenozoospermic men compared with those from 9 normozoospermics (Fig. 5B). When levels of normalized CatSper1 expression were correlated with sperm progressive motility in the samples, a significant positive correlation was found ($r = 0.59$, $P = 0.007$, Fig. 5C).

Discussion

The main finding of the present study regards the evidence that CatSper1 expression is associated with human sperm progressive motility and may be involved in the pathogenesis of asthenozoospermia. In addition, we show evidence that CatSper channels are involved in P-stimulated AR.

The essential role of CatSper channels in mouse sperm fertility has been clearly demonstrated (Ren *et al.*, 2001; Shukla *et al.*, 2012). In this species, indeed, knockout of any of the genes that encode for the proteins that form CatSper determines lack of expression of the channel in the mature sperm and of the ability to achieve hyperactivated motility (Carlson *et al.*, 2005; Qi *et al.*, 2007). In human, the role of CatSper channels in sperm motility is less clear. Mutations of CatSper1 and CatSper2 leading to deletion of the protein have occasionally been found in the population (Avidan *et al.*, 2003; Avenarius *et al.*, 2009) but the association between lack of CatSper1 or 2 and sperm motility in these subjects is not clear (Hildebrand *et al.*, 2010). Although a recent study using the patch clamp technique has demonstrated the absence of CatSper currents in sperm from one man carrying CatSper2 mutations (Smith *et al.*, 2013), the relationship between CatSper mutations and asthenozoospermia remains to be clarified, because the percentage of motile sperm in the ejaculates of subjects with mutations was variable and HA has not been evaluated (Hildebrand *et al.*, 2010). We show here strong evidence that CatSper expression is associated with human sperm progressive motility. Indeed, we found that CatSper1 is heterogeneously expressed in an ejaculate: not all the sperm (even from normozoospermic men) express detectable CatSper1 protein in their tails. Of interest, we found that a much higher percentage of sperm expresses CatSper1 after swim-up selection. These results suggest that CatSper1 expression is required for migration in the upper medium. In addition, we provide here evidence that higher CatSper expression in swim-up selected sperm is functionally associated with higher basal and P-stimulated $[Ca^{2+}]_i$ levels, indicating greater activity of the channel in the motile sperm population. Most importantly, we found that sperm from asthenozoospermic men express, on average, lower levels of CatSper1 and that expression of the protein is highly correlated with the percentage of progressive motility of the semen samples. Similarly, a positive correlation was found between basal and P-stimulated calcium levels and progressive motility. Although these data need to be confirmed in a higher number of subjects, they are highly indicative of a link between expression and function of CatSper channels and the motility of a spermatozoon and suggest an involvement of these channels

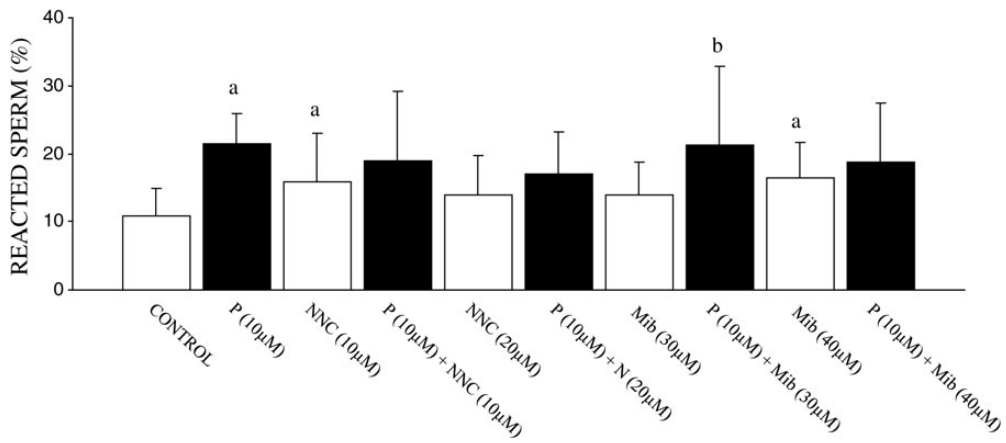

Figure 4 Effects of CatSper inhibition on progesterone (P)-induced acrosome reaction. Swim-up selected 3 h capacitated sperm were incubated for 1 h with P (10 μM) in the presence or absence of 10 and 20 μM NNC and 30 and 40 μM Mib or with only the two inhibitors. $n = 11$; $^aP < 0.01$ versus Control, $^bP < 0.05$ or versus 30 μM Mib.

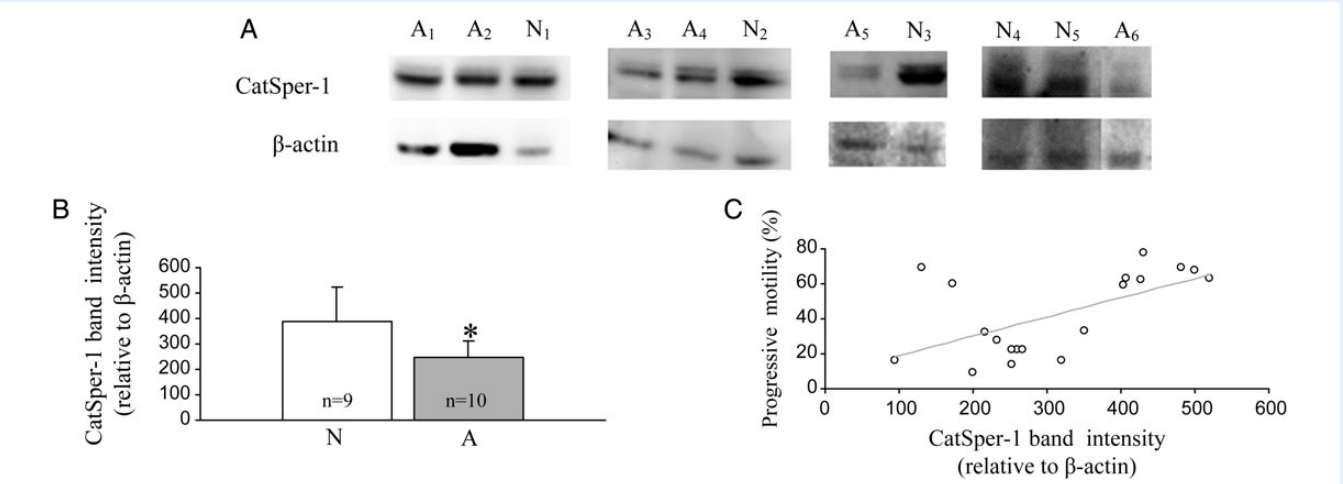

Figure 5 CatSper1 expression in sperm from normo- and asthenozoospermic men. (**A**) Western blot analysis of CatSper1 expression. Semen was prepared by 40% PureSperm to eliminate germ cells and leukocytes, from normo- (N) and asthenozoospermic (A) men. Membranes were blotted with anti-CatSper1 (Bioss, upper blots) and with anti-actin (lower blots) antibody as described in materials and methods. (**B**) Mean (±SD) CatSper1 levels in normo- and astheno-zoospermic men after normalization for β-actin expression. *$P < 0.01$. (**C**) Correlation between CatSper1 expression in sperm and progressive motility of the different samples ($n = 19$, $r = 0.59$, $P = 0.007$).

in the pathogenesis of asthenozoospermia, providing a possible explanation for the reduced $[Ca^{2+}]_i$ increase in response to P observed in oligoasthenospermic subjects (Falsetti *et al.*, 1993). Recently, a study reported that expression of CatSper2 was reduced in sperm from oligoasthenozoospermic men (Bhilawadikar *et al.*, 2013). Furthermore, Nikpoor *et al.* (2004) have shown that CatSper mRNA expression in the testis is highly variable among individuals and levels were higher in biopsies from men with non-obstructive azoospermia. Overall, these results indicate that alterations of CatSper genes and proteins regulation may occur during spermatogenesis leading to different levels of expression of the functional channel in mature sperm and consequent alterations of motility. It remains to be explained whether the reduced CatSper1 expression in asthenozoospermic men is associated with a reduction of number of CatSper channels/cell, absence of the channel in a high percentage of sperm or both. Our results in swim-up selected versus unselected sperm (Fig. 2) and fluorescence microscopy analysis suggest that there may be sperm not expressing CatSper1 at all in their tails. It would be interesting, in future studies, to evaluate CatSper expression in testis biopsies of asthenozoospermic men and its relation to levels of reproductive hormones, in the intent of demonstrating if and how CatSper proteins expression is regulated.

An important question concerns whether CatSper channels are involved in human sperm HA, as *in vitro* studies in men seem to question such a function in human and equine sperm, suggesting species specificity (Alasmari *et al.*, 2013a,b; Loux *et al.*, 2013). Here we show that *in vitro* treatment with the CatSper inhibitor NNC significantly affected several sperm kinematic parameters and reduced total progressive and rapid motility. However, the pattern of HA, as determined by CASA, was not affected in all the samples, and on average the inhibitor had no significant effect, whether it was added to swim-up sperm after selection and capacitation or to the swim-up medium during selection. A clear effect on HA was observed in some samples; generally these had higher basal HA. Although it must be considered that evaluation of HA in human sperm is tricky as the cells may rapidly move from a non-HA to a HA state and vice versa (Mortimer *et al.*, 1997; Pacey *et al.*, 1997), overall, our results suggest a role of CatSper in sperm activated (progressive) motility rather than HA. In particular, we did not observe any effect of the inhibitors on average values of ALH or VCL which should increase in HA (Mortimer and Swan, 1995). Similar to our results, the recent studies of Alsmari *et al.* (2013a,b) demonstrated that activation of CatSper with alkalinization or P had negligible effects on HA. It must be mentioned that a reduction in sperm motion parameters was observed also with Mib, another widely used CatSper inhibitor (Strünker *et al.*, 2011; Brenker *et al.*, 2012; Sagare-Patil *et al.*, 2012). However, we show here that, at the concentration used to inhibit CatSper, Mib is toxic for human sperm (Fig. 3C), and thus, results obtained using this inhibitor should consider this fact even when the parameters evaluated were only in live sperm (such as ALH, BCF and HA).

Another controversy in the literature concerns whether P is able to stimulate activated/HA motility (Baldi *et al.*, 2009). In our hands, P, either at high concentrations (like those found in the cumulus mass surrounding the oocyte) or at low concentrations (which have been demonstrated to stimulate sperm chemotaxis), did not stimulate, on average, sperm motion parameters, showing enhancement of hyperactivated motility only in some of the samples. In the recent paper by Alasmari *et al.* (2013a), P was found to be poorly effective in inducing HA in donor sperm, and was demonstrated to be almost ineffective in IVF/ICSI patients. It is possible that the effect of P on sperm motility may depend on levels of expression of CatSper channels in sperm; further experiments are needed to verify this hypothesis.

Finally, we show here evidence that CatSper channels may be involved in P-stimulated AR of human sperm. Indeed, we found that the effect of P on AR is blunted in the presence of NNC at the same concentrations that inhibit P-stimulated calcium influx of ~80% (Strünker *et al.*, 2011). Despite the fact that, at 30 μM, Mib was almost as efficient as NNC in

inhibiting calcium influx stimulated by the steroid (Strünker *et al.*, 2011), it was less potent, in our hands, than NNC in inhibiting P-induced AR. Moreover, although AR is evaluated only in live sperm, we cannot exclude that the toxic effect of Mib may have contributed to the reduced response to P observed in its presence. It should be noted that both NNC and Mib *per se* increased the percentage of acrosome-reacted sperm (Fig. 4). This unexpected effect might be related to a stimulation of $[Ca^{2+}]_i$ increase (Strünker *et al.*, 2011; Brenker *et al.*, 2012). Although our results indicate an involvement of CatSper channels in P-induced AR, it should be noted that they are located in the tail (Fig. 1), whereas AR occurs in the head of the spermatozoon. It has been shown that calcium may propagate from the tail to the head after CatSper stimulation in mouse sperm (Xia *et al.*, 2007), and thus, a similar mechanism might be prospected for human sperm. However, other pathways have been shown to be involved in the induction of AR by P (Baldi *et al.*, 2009) which may contribute with CatSper signalling in the process. It has been recently shown that not all the calcium signalling activated by P can be explained by CatSper (Lefièvre *et al.*, 2012). In addition, the plateau phase of calcium increase in response to P is still present after treatment of Mib and NNC (Strüunker *et al.*, 2011) and thus, even calcium signalling does not appear to be completely blunted.

In conclusion, we demonstrated an involvement of CatSper1 in human sperm progressive motility and in P-stimulated AR. In particular we have shown here evidence that CatSper1 expression and function are associated with progressive sperm motility. The involvement of CatSper channels in P-induced AR and astenozoospermia may open new scenarios both for the treatment of male infertility as well as for contraceptive purposes.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

L.T. performed the experiments, collected and analysed the data and was responsible for manuscript preparation. S.M. was involved in set-up of western blot and flow cytometry analysis. F.M. contributed to the initial set-up of the sperm motility and AR experiments. E.B. designed and coordinated the study and wrote the manuscript. 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. All the authors made substantial contributions in critically revising the article.

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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.

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, Cattan 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. Review.

Baldi E, Luconi M, Krausz C, Forti G. Progesterone and spermatozoa: a long-lasting liaison comes to definition. *Hum Reprod* 2011;**26**: 2933–2934.

Bedford JM. Site of the mammalian sperm physiological acrosome reaction. *Proc Natl Acad Sci USA* 2011;**108**:4703–4704.

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.

Blengini CS, Teves ME, Uñates DR, Guidobaldi HA, Gatica LV, Giojalas LC. Human sperm pattern of movement during chemotactic re-orientation towards a progesterone source. *Asian J Androl* 2011;**13**:769–773.

Bonaccorsi L, Forti G, Baldi E. Low-voltage-activated calcium channels are not involved in capacitation and biological response to progesterone in human sperm. *Int J Androl* 2001;**24**:341–351.

Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Krähling M, Müller A, Kaupp UB, Strünker T. The CatSper channel: a polymodal chemosensor in human sperm. *EMBO J* 2012;**31**:1654–1665.

Carlson AE, Quill TA, Westenbroek RE, Schuh SM, Hille B, Babcock DF. Identical phenotypes of CatSper1 and CatSper2 null sperm. *J Biol Chem* 2005;**280**:32238–32244.

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 a Ca^{2+} 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.

Jaiswal BS, Tur-Kaspa I, Dor J, Mashiach S, Eisenbach M. Human sperm chemotaxis: is progesterone a chemoattractant? *Biol Reprod* 1999; **60**:1314–1319.

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. *Proc Natl Acad Sci USA* 2011;**108**:4892–4896.

Kanwal S, Iqbal H, Parveen A, Zaman Khattak J, Mir A. CatSper ion channels: bioinformatics analysis in Homo sapiens. *Afr J Biotechnol* 2011; **10**:9017–9022.

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.

Lefièvre L, Nash K, Mansell S, Costello S, Punt E, Correia J, Morris J, Kirkman-Brown J, Wilson SM, Barratt CL *et al.* 2-APB-potentiated channels amplify CatSper-induced Ca(2+) signals in human sperm. *Biochem J* 2012;**448**:189–200.

Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature* 2011;**471**:387–391.

Loux SC, Crawford KR, Ing NH, González-Fernández L, Macías-García B, Love CC, Varner DD, Velez IC, Choi YH, Hinrichs K. CatSper and the Relationship of Hyperactivated Motility to Intracellular Calcium and pH Kinetics in Equine Sperm. *Biol Reprod* 2013;**89**:123.

Luconi M, Carloni V, Marra F, Ferruzzi P, Forti G, Baldi E. Increased phosphorylation of AKAP by inhibition of phosphatidylinositol 3-kinase enhances human sperm motility through tail recruitment of protein kinase A. *J Cell Sci* 2004a;**117**:1235–1246.

Luconi M, Francavilla F, Porazzi I, Macerola B, Forti G, Baldi E. Human spermatozoa as a model for studying membrane receptors mediating rapid nongenomic effects of progesterone and estrogens. *Steroids* 2004b; **69**:553–559.

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.

Mortimer ST, Swan MA. Kinematics of capacitating human spermatozoa analysed at 60 Hz. *Hum Reprod* 1995;**10**:873–879.

Mortimer ST, Schëväert D, Swan MA, Mortimer D. Quantitative observations of flagellar motility of capacitating human spermatozoa. *Hum Reprod* 1997;**12**:1006–1012.

Mortimer ST, Swan MA, Mortimer D. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Hum Reprod* 1998;**13**:2139–2146.

Munuce MJ, Quintero I, Caille AM, Ghersevich S, Berta CL. Comparative concentrations of steroid hormones and proteins in human peri-ovulatory peritoneal and follicular fluids. *Reprod Biomed Online* 2006; **13**:202–207.

Muratori M, Porazzi I, Luconi M, Marchiani S, Forti G, Baldi E. AnnexinV binding and merocyanine staining fail to detect human sperm capacitation. *J Androl* 2004;**25**:797–810.

Muratori M, Marchiani S, Forti G, Baldi E. Sperm ubiquitination positively correlates to normal morphology in human semen. *Hum Reprod* 2005; **20**:1035–1043.

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.

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.

Osman RA, Andria ML, Jones AD, Meizel S. Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Commun* 1989;**160**:828–833.

Pacey AA, Ladbrook MB, Barratt CL, Cooke ID. The potential shortcomings of measuring hyperactivated motility by computer-aided sperm analysis when sperm motion is multiphasic. *Hum Reprod Update* 1997;**3**:185–193.

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.

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.

Shukla KK, Mahdi AA, Rajender S. Ion channels in sperm physiology and male fertility and infertility. *J Androl* 2012;**33**:777–788.

Smith JF, Syritsyna O, Fellous M, Serres C, Mannowetz N, Kirichok Y, Lishko PV. Disruption of the principal, progesterone-activated sperm 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 Ca^{2+} influx in human sperm. *Nature* 2011;**471**:382–386.

Sun TT, Chung CM, Chan HC. Acrosome reaction in the cumulus oophorus revisited: involvement of a novel sperm-released factor NYD-SP8. *Protein Cell* 2011;**2**:92–98.

Teves ME, Barbano F, Guidobaldi HA, Sanchez R, Miska W, Giojalas LC. Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertil Steril* 2006;**86**:745–749.

Uhler ML, Leung A, Chan SY, Wang C. Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. *Fertil Steril* 1992;**58**:1191–1198.

Wang Y, Storeng R, Dale PO, Abyholm T, Tanbo T. Effects of follicular fluid and steroid hormones on chemotaxis and motility of human spermatozoa *in vitro*. *Gynecol Endocrinol* 2001;**15**:286–292.

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 Ca^{2+} entry into mouse sperm triggers a tail-to-head propagation. *Biol Reprod* 2007;**77**:551–559.