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DOI:

[10.1093/humrep/dey278](https://doi.org/10.1093/humrep/dey278)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Brown, S, Miller, M, Lishko, PV, Lester, D, Publicover, S, Barratt, C & Martins da Silva, S 2018, 'Homozygous in-frame deletion in CATSPERE in a man producing spermatozoa with loss of CatSper function and compromised fertilizing capacity', *Human Reproduction*, vol. 33, no. 10, pp. 1812–1816.
<https://doi.org/10.1093/humrep/dey278>

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Checked for eligibility: 25/09/2018

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Homozygous in-frame deletion in *CATSPERE* in a man producing spermatozoa with loss of
CatSper function and compromised fertilizing capacity

Sean G. Brown¹

Melissa R. Miller²

Polina V. Lishko²

Douglas H. Lester¹

Stephen J. Publicover⁴

Christopher L.R. Barratt³

Sarah Martins Da Silva³

¹School of Science, Engineering & Technology, Abertay University, Dundee, DD11HG, UK.

² Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

³Reproductive and Developmental Biology, School of Medicine, Ninewells Hospital and
Medical School, University of Dundee, Dundee, DD19SY, UK.

Assisted Conception Unit, Ninewells Hospital Dundee, DD19SY, UK.

⁴School of Biosciences, the University of Birmingham, Birmingham, B15 2TT, UK.

Correspondence: Christopher L.R. Barratt, c.barratt@dundee.ac.uk and Polina V. Lishko,
lishko@berkeley.edu

Running Title: Rare *CATSPERE* variant is associated with loss of CatSper function

Key words: Calcium signalling/infertility/CatSper/spermatozoa/mutation

23

24 **Study question:** Does a man (patient 1) with a previously described deficiency in CatSper
25 channel function have a mutation in *CATSPERE* and/or *CATSPERZ*?

26 **Summary answer:** Patient 1 possess a homozygous in-frame 6-bp deletion in exon 18
27 (c.2393_2398delCTATGG, rs761237686) of *CATSPERE*.

28 **What is known already:** CatSper is the principal calcium channel of mammalian
29 spermatozoa. Spermatozoa from patient 1 had a specific loss of CatSper function and were
30 unable to fertilise at in vitro fertilization (IVF). Loss of CatSper function could not be
31 attributed to genetic abnormalities in coding regions of seven CatSper subunits (Williams *et*
32 *al.*, 2015). Two additional subunits (CatSper-epsilon (*CATPSERE*) and CatSper-zeta
33 (*CATSPERZ*) were recently identified (Chung *et al.*, 2017), and are now proposed to
34 contribute to the formation of the mature channel complex.

35 **Study design, size, duration:** This was a basic medical research study analyzing genomic
36 data from a single patient (patient 1) for defects in *CATSPERE* and *CATSPERZ*.

37 **Participants/materials, setting, methods:** The original exome sequencing data for patient
38 1 was analysed for mutations in *CATSPERE* and *CATSPERZ*. Sanger sequencing was
39 conducted to confirm the presence of a rare variant.

40 **Main results and the role of chance:** Patient 1 is homozygous for an in-frame 6-bp
41 deletion in exon 18 (c.2393_2398delCTATGG, rs761237686) of *CATSPERE* that is
42 predicted to be highly deleterious.

43 **Limitations, reasons for caution:** The nature of the molecular deficit caused by the
44 rs761237686 variant and whether it is exclusively responsible for the loss of CatSper
45 function remain to be elucidated.

46 **Wider implications of the findings:** Population genetics are available for a significant
47 number of predicted deleterious variants of CatSper subunits. The consequence of
48 homozygous and compound heterozygous forms on sperm fertilisation potential could be

significant. Selective targeting of CatSper subunit expression maybe a feasible strategy for the development of novel contraceptives.

Study funding/competing interest(s): This study was funded by project grants from the MRC (MR/K013343/1, MR/012492/1), Chief Scientist Office/NHS research Scotland. This work was also supported by NIH R01GM111802, Pew Biomedical Scholars Award 00028642 and Packer Wentz Endowment Will to P.V.L.

Introduction

Human CatSper is a highly complex progesterone-sensitive calcium channel that is expressed in the principle piece of the sperm flagellum (Lishko & Mannowetz 2018). While evidence from CatSper knock-out mice implicate it as essential for male fertility (Ren *et al.*, 2001; Chung *et al.*, 2011; Qi *et al.*, 2007), attempts to identify equivalent naturally-occurring mutations in infertile men have produced equivocal results. Large genomic deletions and compounding issues with spermatogenesis in such patients result in multiple sperm defects making it impossible to conclude that loss of CatSper *per se* was sufficient to cause infertility (Avidan *et al.*, 2003; Smith *et al.*, 2013).

In a previous study we used progesterone-mediated calcium influx as a 'marker' of CatSper function to screen for patients with 'normal' semen parameters but failure of CatSper function (Williams *et al.*, 2015). We reported that spermatozoa from one man (patient 1) had a stable lesion in CatSper function and failed to fertilise at IVF. Specifically, spermatozoa from patient 1 failed to produce any CatSper-related ion currents and failed to respond with calcium influx when stimulated with progesterone (William *et al.*, 2015). Of particular note was that we did not observe any genetic abnormalities that could result in the reported phenotype in any of the coding regions of CatSper subunits.

Recently two new CatSper subunits have been identified: CatSper-epsilon and CatSper zeta. Based on mouse gene knockout studies, CatSper-zeta has been confirmed to have a role in mouse fertilisation competence (Chung *et al.*, 2017). However, the importance of CatSper-epsilon remains to be verified. A lesion in this subunit may correlate with failed CatSper function. We report here that indeed the exome sequence analysis revealed a homozygous in-frame deletion in the putative extracellular coding region of *CATSPERE* and hypothesize that it is the cause of loss of CatSper conductance and subfertility in patient 1.

Methods

Analysis was conducted on genomic DNA and sequencing data obtained previously (Williams *et al.*, 2015). Patient 1 is of white European ethnicity from non-consanguineous parents.

Bioinformatics

Normal *CATSPERE* genomic Sanger SCF trace (<https://trace.ncbi.nlm.nih.gov/Traces/home/>) was compared with a Sanger sequencing SCF file generated from patient 1 DNA (<http://bioedit.software.informer.com/7.2/>). CatSper-epsilon evolutionary distant orthologues (<http://www.uniprot.org/> and <https://www.ncbi.nlm.nih.gov/gene/>) were aligned (<https://www.ebi.ac.uk/Tools/msa/muscle/>). The generated CLUSTAL multiple sequence alignment was imported and edited (<http://www.softpedia.com/get/Science-CAD/GeneDoc.shtml>). A search for conserved structural domains within CatSper-epsilon (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) indicated that residues I699-P902 align well (E value = $1.65e-23$) with the pfam15020 conserved extracellular CatSper-delta super family domain. Therefore, this sequence was aligned with the corresponding CatSper-delta sequence (K515-Q718) to calculate sequence similarity. A pathogenicity score was generated (http://provean.jcvi.org/seq_submit.php).

Sanger sequencing

To confirm the *CATSPERE* variant in patient 1 genomic DNA, exon 18 was amplified by PCR using ThermoPrime ReddyMix PCR Master Mix (Thermoscientific) and the bespoke primers (F: CATCCAGCTGTCAAAAGACAC, R:CTACCCACTGCTGCCTTATTC) under the following conditions; 95°C for 10 minutes, followed by a program of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds for 35 cycles, and ending with a 10 minute extension at 72°C. The expected 431bp amplicon, was confirmed by electrophoresis. The remaining PCR amplicon was purified using a QIAquick PCR Purification Kit (Qiagen).

Results

Sequence variations, all of which are reported in the Ensembl genome browser database (GRCh37; <http://grch37.ensembl.org>), were identified in both *CATSPERE* (c1orf101) and *Z* loci from patient 1 (figure 1). All intronic variations are predicted to be benign. However, patient 1 is homozygous for a highly deleterious (pathogenicity score of -11.3) in-frame 6-bp deletion in exon 18 (c.2393_2398delCTATGG, rs761237686) of *CATSPERE* which, if translated, would cause the loss of two amino acids in the extracellular domain (p.Met799_Ala800del) in isoform 1 of CatSper-epsilon (figure 2).

Discussion

CatSper is a highly complex channel that consists of at least nine subunits and gene knock-out studies demonstrate that it is essential for male fertility in mouse (Ren *et al.*, 2001; Chung *et al.*, 2011, 2017; Liu *et al.*, 2007; Qi *et al.*, 2007). Identification of genetic abnormalities in *CATPSER1* and *CATSPER2* genes in subfertile men is consistent with similar importance of this channel in human spermatozoa. However, semen samples in these cases had multiple abnormalities thus impaired fertility could not be conclusively

125 attributed to the exclusive loss of CatSper (Avidan *et al.*, 2003; Smith *et al.*, 2013). In
126 contrast, we reported a case of a stable lesion in CatSper function in sperm from patient 1
127 that failed to fertilise at IVF but had normal motility and concentration (Williams *et al.*, 2015).
128 Genetic analysis revealed no significant changes in CatSper coding regions. However, in
129 light of the recent identification of two new channel auxiliary subunits (CatSper-epsilon and
130 CatSper-zeta, Chung *et al.*, 2017) we re-examined the genetics of this patient and now
131 report the presence of an in-frame microdeletion in the putative extracellular coding region of
132 *CATSPERE*. Our analysis shows a homozygous 6-bp frameshift deletion in exon 18 of
133 *CATSPERE*.(Figure 1 and 2)

134 CatSper-epsilon is predicted to be a single transmembrane spanning protein type II with a
135 topology similar to CatSper-gamma and delta that localises specifically to the plasma
136 membrane in the same distinct quadrilateral arrangement shown for other subunits (Chung
137 *et al.*, 2017) supporting the premise that it is an integral part of the mature CatSper signalling
138 complex. Interestingly, the deleted amino acids are present within a pfam1502 conserved
139 CatSper-delta superfamily domain. Alignment of corresponding extracellular sequences of
140 human CatSper-epsilon and delta indicates they have a high sequence homology (52%
141 identical/similar amino acids) which may indicate a stabilising function on channel assembly
142 like that demonstrated for mouse CatSper-delta which is critical for channel expression and
143 male fertility (Chung *et al.*, 2011, Figure 3).

144 Determining the effect of the frameshift mutation on CatSper channel biogenesis during
145 human spermatogenesis, as well as determining the stoichiometry of subunits and precise
146 composition of the channel complex, is critical future work. Destabilisation of the channel
147 complex may manifest through impaired CatSper-epsilon protein production due to transcript
148 nonsense-mediated decay if the variant causes mis-splicing of exon 18. Alternatively, if the
149 erroneous CatSper-epsilon transcript is translated, a highly conserved methionine (figure 3)
150 and alanine will be absent in the putative extracellular domain of the protein which is
151 predicted to be highly deleterious to its conformation (PROVEAN pathogenicity score of -

11.3) and potentially to channel assembly. As has been shown recently using super-resolution microscopy, the absence of even a single CatSper subunit can result in the ultimate destabilisation of the whole channel complex, and disorganize the precise nanodomain organization of the whole flagellum (Chung et al., 2017). Our data add to a growing body of evidence to suggest an association between aberrations in CatSper genes and impaired fertility that may not manifest an overt phenotypical defect (Williams *et al.*, 2015) and therefore can cause unexplained infertility. Since heterologous expression of the functional CatSper channel complex cannot yet be achieved despite the decades of failed attempts by many research groups, the study of this channel is limited to its natural expression system – the mature spermatozoon. Therefore, the human genetics studies provide a valuable insight on the channel putative composition and the role of its subunits.

These initial observations show an association between a CatSper-epsilon variant and loss of CatSper channel function. Proof that this variant is the exclusive reason for the loss of channel function and fertilisation competence will require further evidence. Generation of an equivalent *CATSPERE* mouse model is a conventional strategy and is potentially useful but a fundamental issue is that nothing is known about the molecular regulation of assembly or processing of human CatSper during spermatogenesis and storage/transport through the epididymis therefore species comparisons maybe flawed. *In vitro* recombinant studies to examine the expression and stability of the variant human protein have merit but only if functional expression is feasible (see above). An alternative approach is use human genetic studies to investigate the channel putative composition and the role of its subunits. However, due to the low frequency of homozygous males in the population (approximately 1 in 500000 men. Ensembl/gnomAD) finding an identical case by screening, allowing replication of our study (Williams et al., 2015), is unlikely. An effective strategy may require studies involving a multi-centre collaborative effort (Barratt *et al.*, 2017; 2018) to identify sentinel men through phenotypic screening (Kelly *et al.*, 2018) and/or clinical outcomes and perform genetic

analysis and *in vitro* experiments (e.g targeted quantitative proteomics and high-resolution imaging of CatSper *in situ*, Chung *et al.*, 2017).

In summary, we describe the first reported case of a man with a homozygous in-frame deletion in *CATSPERE* (r761237686) which may cause infertility through loss of mature CatSper channel function in spermatozoa. However, the precise molecular deficit remains to be elucidated and compounding genetic errors cannot be ruled out.

Figure legends

Figure 1. Sequence variation summary information for *CATSPERE* and *CATSPERZ* from patient 1. The exome sequencing identification, of a homozygous pathogenic 6bp deletion (CTATGG, rs761237686) in *CATSPERE* exon18, of patient 1 (c.2393_2398del). This deletion (indicated by a red line below exon18, A), if translated, results in loss of a Methionine (M) and an Alanine (A) residue (p.Met799_Ala800del) in the CatSper-epsilon protein. In addition to the 6bp pathogenic deletion in exon 18 the position and genotype of 6 non-pathogenic intronic flanking SNPs are shown in the 22 exon of *CATSPERE*. The position of 4 non-pathogenic, highly variable, intronic SNPs and 1bp large homopolymeric 13/ 14 bp T tract in/del (rs10572994), are also indicated by red lines on the diagram.

Figure 2. Sanger sequencing conformation of the initial exome sequencing results from patient 1. Highlighted is the position of the 6bp CTATGG deletion (c.2393_2398del), in the normal trace (A) and the subsequent re-joining event, between the flanking Adenine and Cytosine bases shown in Patient 1's trace (B). The normal sequence shows the position of Met 799 and Ala 800 amino acids that would be deleted if the variant protein is expressed in patient 1 (p.Met799_Ala800del).

203

204 Figure 3. Alignment of a truncated region of CatSper-epsilon protein sequences
205 (corresponding to 769aa- 931aa of human CatSper-epsilon). Five selected evolutionary
206 distant species were compared to the Human sequence using the EBI MUSCLE programme.
207 Stars (*) indicate evolutionary conserved amino acids. The box with the blue lettering
208 indicates the evolutionary conservation of the predicted deleted MA region in CatSper-
209 epsilon of the patient 1 (p.Met799_Ala800del). The box containing red lettering illustrates a
210 high density of hydrophobic amino acids that is the predicted transmembrane domain of the
211 CatSper-epsilon orthologous proteins. The Uniprot or Genbank Accession numbers (Acc
212 No.) for the different CatSper-epsilon proteins are given.

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216 Authors' roles

217 SGB proposed the project and conducted the molecular biology. PVL, MRM and DHL
218 conducted bioinformatic analysis and identified the lesion. SGB, SJP, CLRB and SMDS
219 obtained funding for the study. All authors contributed to the writing and approval of the final
220 manuscript.

221 Acknowledgements

222 We acknowledge Hannah Williams, Steven Mansell, Stuart Wilson, Wardah Alasmari and
223 Keith Sutton for their role in the original study.

224

225 Funding

226 This study was funded by project grants from the MRC (MR/K013343/1, MR/012492/1)
227 (S.G.B, S.J.P, C.L.R.B.), Tenovus Scotland and Chief Scientist Office/NHS research
228 Scotland (S.M.S.S) and NIH R01GM111802, Pew Biomedical Scholars Award 00028642
229 and Packer Wentz Endowment Will to P.V.L.

230

231 Conflict of interest

232 C.L.R.B is the editor-in-chief of Molecular Human Reproduction, has received lecturing fees
233 from Merck and Ferring and is on the Scientific Advisory Panel for Ohana BioSciences.

234 C.L.R.B was chair of the World Health Organisation Expert Synthesis Group on Diagnosis of
235 Male infertility (2012-2016)

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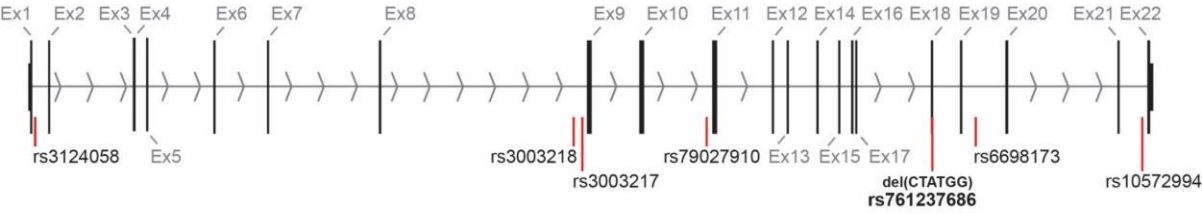
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 273 compromise fertilizing capacity of human spermatozoa. *Hum Reprod* 2015;**30**:2737-2746.

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Gene	Position	Consequence	Reference Allele	Mutant Allele	Reference Amino Acid Sequence	Mutant Amino Acid Sequence	Zygosity	SNP ID
C1orf101/CATSPERE	chr1: 244624887	intronic SNP	T	C	N/A	N/A	-/+	rs3124058
C1orf101/CATSPERE	chr1: 244715411	intronic SNP	G	C	N/A	N/A	+/+	rs3003218
C1orf101/CATSPERE	chr1: 244715439	intronic SNP	T	C	N/A	N/A	+/+	rs3003217
C1orf101/CATSPERE	chr1: 244735593	intronic SNP	G	T	N/A	N/A	+/+	rs79027910
C1orf101/CATSPERE	chr1: 244773784	intronic SNP	T	C	N/A	N/A	+/+	rs6698173
C1orf101/CATSPERE	chr1: 244769086-244769091	deletion	CTATGG	-	TMAQ	TQ	+/+	rs761237686
C1orf101/CATSPERE	chr1: 244803206	intronic deletion	T	-	N/A	N/A	+/+	rs10572994
C11orf20/CATSPERZ	chr11: 64070940	intronic	T	C			-/+	rs11231746

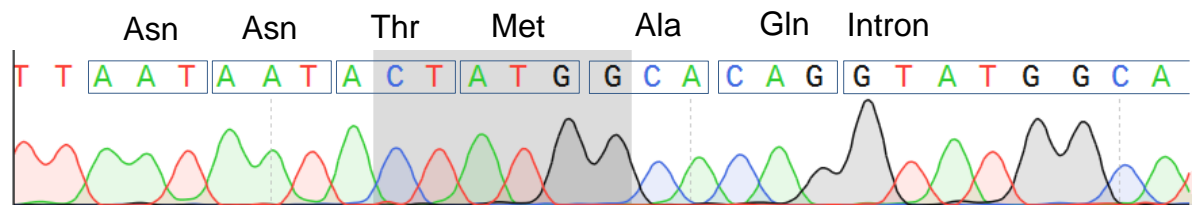
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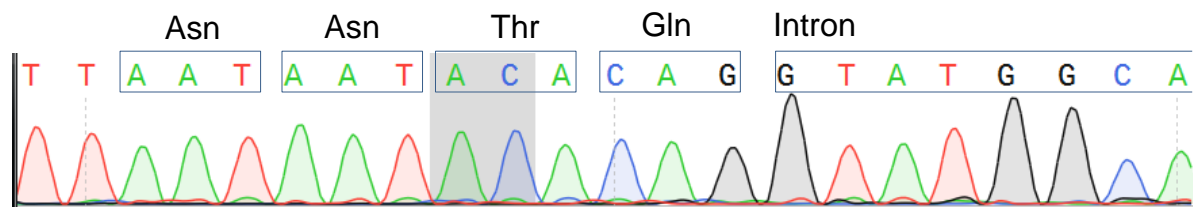
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A. Normal.



B. Patient 1. c.2393_2398delCTATGG (p.Met799_Ala800del)



282

283

Human : LFDDNGYVKDVEANFIVWEIHGRDDYSFNNTMA-QSGCLHEAQTWKSME
Rat : LYDENGFIKIVDANFILWEVHGRNDYMYNSTMQ-QNGCINEAQTWDIMIE
Mouse : LYDENGFIKIVDANFILWEIHGRNDYTFNSTME-QNGCINEAQTWDSMIE
Chameleon : LYMGERAIGTVEANYVLWEMNGRNDFNYNSTMEQQVRCLNTAQTWQKAIE
Salmon : LYDGDNYVRNVDANFIVWDRFGRKDYSFNATMR-QVACLHESQTFWSMLT
Brachiopod : LYDGEEFVRPVTGNFILWEEQGRTDYSYNATMK-QAGCHKVAQTWSQIRD
* * * * *

Human : L---NKHL-P--LEEVWGPENYKHCFSY--AIGKPGDLNQPYEIIINSSNG
Rat : E---NPGV-P--MEDIWGPQNYRPCFSY--AIGTPGDLSQPYEIIINYSNK
Mouse : E---NPDI-P--LDDVWGPQNYRPCFSY--AIGKPGDLGQPYEILNYSNK
Chameleon : KINRTSSLTPDEVESLWGPARNYRSCFDS--QVDEIANLDTPEILNHSGM
Salmon : G---GKSL----EEAWGPENYRTCFKV--SPGKLENLDQPYEIMNRSSK
Brachiopod : E---QGMLTD--WGQGWGPWNYRSCFEETNTVIDSSLLQRPYQILNSTGV
* * * * *

Human : NHIFWPMGHSGMYVFRVKILDPNYSFCNLMTAMFAIETFGLIPS----PS
Rat : NALKWSSSYAAMYVYRLKVLDPNYSFCNLTTYFAIESLGQIPSVFPDSS
Mouse : NHIKWPMTYAGMYVYRLKILDPNYSFCNLTTIFAIESLGMIPR----SS
Chameleon : NSIIWPLYYNGIYLFRLRILDPNYSFCKLNTFFAVRTVGIIER----PR
Salmon : NFLTFSQVDSATYVFNVKILDPNYSFCDLHAVFAVQTYGITIP----KY
Brachiopod : SWLQFPNTHDSMYTFRARIVDPNYSFCDLEISFAVQTYGAQHP----ED
* * * * *

Human : VYLVASFVLMMLFFFTILV-LSYFRYMR : **Q5SY80**
Rat : IYLVAAALVF----SSCHILSHLSYFWYSK : **A0A0G2K0P3**
Mouse : VYLVAAALIFVLMMLTFISILV-LSYFWYLK : **P0DP43**
Chameleon : WLPVAAWITVIMILLLSVLL-FTYFTYVK : **H9G914**
Salmon : QHLTTYVAIVFTIFSLCILG-YSYCRYVT : **A0A1S3SNN3**
Brachiopod : LTVTMITVGGIMGAVLLGLL-GSYFVYRK : **XP_013382013**
* *

Transmembrane domain