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1 **The sodium/proton exchanger SLC9C1 (sNHE) is essential for human sperm motility and**
2 **fertility.**

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19

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21

22 **Running Title** : *SLC9C1* mutation in human asthenozoospermia

23

24 **Conflict of interest Statement**

25 The authors declare no conflict of interest.

26

27

28 **Abstract**

29 Asthenozoospermia, defined by the absence or reduction of sperm motility, constitutes
30 the most frequent cause of human male infertility. This pathological condition is caused by
31 morphological and/or functional defects of the sperm flagellum, which preclude proper sperm
32 progression. While in the last decade many causal genes were identified for asthenozoospermia
33 associated with severe sperm flagellar defects, the causes of purely functional
34 asthenozoospermia are still poorly defined. We describe here the case of an infertile man,
35 displaying asthenozoospermia without major morphological flagellar anomalies and carrying a
36 homozygous splicing mutation in *SLC9C1* (sNHE), which we identified by whole-exome
37 sequencing. *SLC9C1* encodes a sperm-specific sodium/proton exchanger, which in mouse
38 regulates pH homeostasis and interacts with the soluble Adenylyl Cyclase (sAC), a key
39 regulator of the signalling pathways involved in sperm motility and capacitation. We
40 demonstrate by means of RT-PCR, immunodetection and immunofluorescence assays on
41 patient's semen samples that the homozygous splicing mutation (*c.2748+2T>C*) leads to in-
42 frame exon skipping resulting in a deletion in the cyclic nucleotide-binding domain of the
43 protein. Our work shows that in human, similar to mouse, *SLC9C1* is required for sperm
44 motility. Overall, we establish a homozygous truncating mutation in *SLC9C1* as a novel cause
45 of human asthenozoospermia and infertility.

46

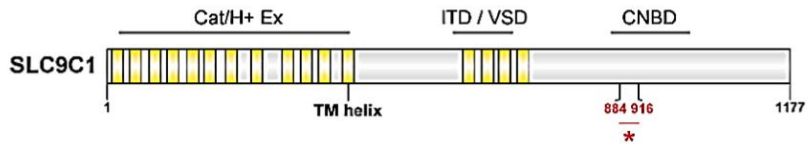
47 **Key words:** male infertility; asthenozoospermia; gene mutation; whole-exome sequencing;
48 *SLC9C1* - sNHE; ion channel.

49

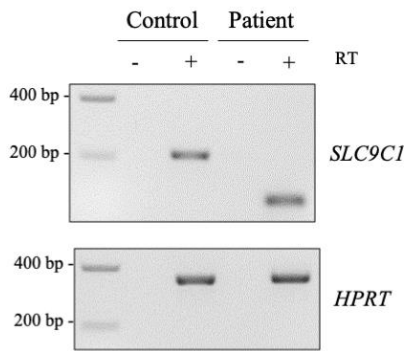
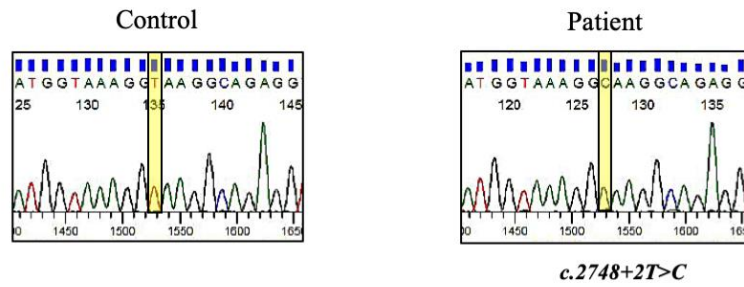
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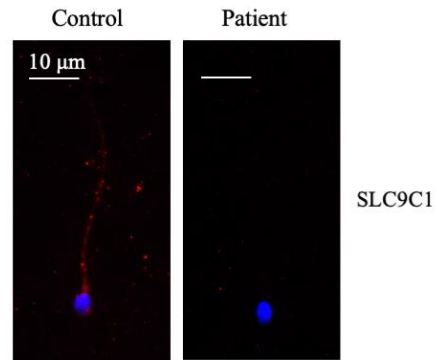
SLC9C1 (sNHE): Sperm specific Sodium/Proton Exchanger



Cat/H+ Ex: Ion exchange domain
ITD/VSD: Voltage sensing domain
CNBD: Cyclic Nucleotide Binding Domain



In-frame exon skipping



**In-frame protein truncation
 p. del 884-916**

54 **Introduction**

55 Asthenozoospermia, defined by the absence or reduction of sperm motility, is observed
56 in nearly 80% of male infertility cases, either alone or in association with other sperm defects¹.
57 This pathophysiological condition is induced by structural defects of the sperm flagellum and/or
58 functional alterations impairing flagellar beating and sperm progression. Among the structural
59 defects, a remarkable condition described as Multiple Morphological Abnormalities of sperm
60 Flagella (MMAF) results from defective assembly of the flagellum and leads to the presence in
61 the ejaculate of spermatozoa with short, absent, coiled, bent or irregular tails. This phenotype
62 of asthenoteratozoospermia was previously identified as ‘dysplasia of the fibrous sheath’, ‘short
63 tails’ or ‘stump tails’²⁻⁴, and in the last decade, numerous mutations causing this phenotype
64 were identified (AK7, ARMC2, MAATS1, CEP135, *CFAP43-44-65-69-70-251*, *DNAH1-2-6-*
65 *8-17*, *QRICH2*, *SPEF2*, *TTC21A*, *TTC29*)^{5,6}. Besides MMAF, some asthenoteratozoospermic
66 patients with milder morphological defects such as defective annulus and abnormal
67 mitochondrial sheath were also described to carry mutations (*TEKT2*, *SEPT12* or
68 *SLC26A8*)^{1,7,8}. Importantly, asthenozoospermia is also detected in the complete absence of
69 morphological defects, suggesting the involvement of functional defects in activation and/or
70 regulation of flagellar beating. To date, such cases of functional asthenozoospermia remain
71 poorly characterized, and only a few mutations were identified in genes encoding for proteins
72 with enzymatic properties (*GALNTL5*), seminal component (*SEMG1*) and ion channels
73 (*CATSPER1-2-ε*, *SLC26A3*, *VDAC2*)^{1,9-12}. Overall, the knowledge obtained by studying those
74 sperm pathological phenotypes provides evidence that, in addition to proper morphology and
75 structure, sperm fertilization potential relies on proper activation of the signalling pathways
76 regulating motility, metabolism and energy production.

77 Sperm functional maturation occurs after spermiation during the transit within the
78 epididymis, where sperm cells acquire the ability to move forward, and ultimately within the

79 female genital tract, where they acquire complete fertilization potential through a process
80 known as capacitation¹³. Numerous ion channels and transporters have been identified at the
81 surface of sperm cells and conduct complex fluxes between the male and female genital tract
82 milieus and the sperm cytoplasm¹⁴. These ion exchanges activate several biochemical and
83 electrophysiological changes that are essential for sperm motility and fertilization potential¹⁵.
84 Among the changes occurring within the female genital tract (i.e. capacitation), were described
85 alkalinization of the cytoplasm, membrane hyperpolarization and protein phosphorylation
86 cascades induced by the activation of soluble adenylate cyclase (sAC) and Protein Kinase A
87 (PKA), which specifically target flagellar components required for sperm fertilization
88 potential¹⁶.

89 In the present study, by performing Whole-Exome Sequencing (WES) on a patient
90 presenting a very low progressive motility but no detectable ultrastructural defects of the
91 axoneme, we identified a homozygous pathogenic mutation in *SLC9C1*. This gene, also called
92 *sNHE*, encodes a sperm-specific member of the Na⁺/H⁺ exchanger family involved in the
93 regulation of pH and cell volume in a wide range of tissues¹⁷. We demonstrate here that *SLC9C1*
94 is critical for human sperm motility and that mutations in *SLC9C1* are a novel cause of human
95 asthenozoospermia.

96 **Materials & methods**

97 **Subject and biological samples**

98 The study was approved by the Comité de Protection des Personnes CPP Ile de France III
99 (record number CPP02748) and performed in accordance with ethical guidelines (Declaration
100 of Helsinki). All patient and control individuals included in the study gave their informed
101 consent prior to their inclusion in the study. Semen samples were obtained by masturbation
102 after 2-7 days of sexual abstinence. Semen parameters were evaluated according to the World
103 Health Organization (WHO) guidelines¹⁸ and David's classification¹⁹.

104

105 **WES analysis and Sanger sequencing**

106 WES analysis was performed on genomic DNA extracted from a blood sample, as previously
107 described²⁰. In brief, the enrichment of coding regions together with intron/exon boundaries
108 was performed with Exon V5 kit (Agilent Technologies, Wokingham, UK). Sequencing was
109 performed with Illumina HiSeq 2000 at the Genoscope (Evry, France). Exome-seq data were
110 analysed using a bioinformatics pipeline developed in-house as previously described²¹. The
111 homozygous mutation in *SLC9CI* identified by WES was confirmed by Sanger sequencing
112 using ABI 3130XL and SeqScape software (Applied Biosystems; Foster City, CA, USA).
113 Primer sequences (5'-3'): F: TCCAACCACTTCTAAAATGTTGT, R:
114 TGCATTTATAAATAACACTGCCTGGT.

115

116 **Transmission electron microscopy analysis**

117 Sperm cells were collected from fresh ejaculate and washed with M2 medium (Sigma-Aldrich
118 Co. Ltd; Irvine, UK) by centrifugation at 300g/RT for 10 minutes. Sperm cells were then fixed

119 and embedded for semi-thin sections as previously described²². All sections were examined
120 with a JEOL 1011 electron microscope (Jeol Ltd; Tokyo, Japan). Images were acquired with
121 Digital Micrograph software coupled to a Gatan Erlangshen CCD camera.

122

123 **RT-PCR analysis**

124 Total RNA from control and patient spermatozoa (800-1000ng) was extracted using
125 NucleoSpin RNA kit (Macherey-Nagel; Düren, Germany) and used for reverse transcription
126 with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher
127 Scientific; Waltham, MA, USA) as previously described²². Amplicons were gel purified and
128 sequenced (Eurofins Scientifics, France). Results were analysed with BioEdit software (Ibis
129 Therapeutics; Carlsbad, CA, USA).

130 Primer sequences (5'-3'): **SLC9C1-F**: TTATCAGGCCTCTTACTGTTG, **SLC9C1-**
131 **R**: TGATCAATCCCTAAACCTGGC; **HPRT-F**: CCTGCTGGATTACATTAAAGCACTG,
132 **HPRT-R**: GTCAAGGGCATATCCAACAACAAAC.

133

134 **Immunofluorescence assay**

135 Slides were prepared by spreading 10 µL of fresh semen sample onto a Superfrost Plus slide
136 (Menzel Glasbearbeitungswerk, GmbH & Co. KG; Braunschweig, Germany), followed by
137 fixation 10 minutes in PBS 4% paraformaldehyde. Immunostaining was performed as
138 previously described²², using commercial antibodies, which specificity was not validated.

139 Primary antibodies : SLC9C1 rabbit polyclonal antibody raised against the central region
140 (Invitrogen PA5-104160; 1:100 dilution); SLC9C1 rabbit polyclonal antibody raised against
141 the C-terminal region (Sigma SAB2106664; 1:100 dilution); mouse monoclonal anti- α -Tubulin

142 Sigma T9026 (1:500 dilution). Secondary antibodies: goat polyclonal anti-rabbit IgG Alexa
143 Fluor 568 (Invitrogen A-11029; 1:500 dilution) and goat polyclonal anti-mouse IgG Alexa
144 Fluor 488 (Invitrogen A-28175; 1:500 dilution).

145

146 **Western blot analysis**

147 10 million sperm cells from control individual and patient were washed in M2 medium and next
148 in PBS. Sperm cells were then subjected to sonication (20s on, 60s off, 15 cycles) in RIPA
149 buffer [150 mM NaCl, 1% NP40 (Sigma-Aldrich Co.; St. Louis, MO, USA), 0.5% DOC, 0.1%
150 SDS, 25 mM TrisHCL, cOmplete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)].
151 Samples were kept on ice for 30 minutes and centrifuged 13000g at 4°C for 15 minutes. The
152 supernatant was collected and denatured in Laemmli sample buffer, prior to SDS-PAGE [8%
153 acrylamide/bisacrylamide (40%, 37.5:1)] and transfer to nitrocellulose membranes. After a
154 blocking step in PBS 0.1% Tween, 5% BSA or milk for SLC9C1 and β -Tubulin detection,
155 respectively, incubation was performed with primary and secondary antibodies. Primary
156 antibodies: rabbit polyclonal anti-SLC9C1 Invitrogen (PA5-104160; 1:500 dilution) and mouse
157 monoclonal anti- β -Tubulin clone AA2 (Sigma 05-661; 1:2500 dilution). Secondary antibodies:
158 rabbit polyclonal anti-mouse Ig coupled to Horse Radish Peroxydase (HRP) (Dako; 1:1000
159 dilution), swine polyclonal anti-rabbit Ig coupled to HRP (Dako; 1:1000 dilution).

160

161 **Results**

162 The patient presented in this study first consulted for primary infertility in 2016 at the
163 age of 31, after two years of natural conception failure, and was followed at the reproductive
164 biology centre of the Cochin hospital (Paris, France). Apart from a non-evolutive chronic
165 hepatitis B diagnosed in 2008, no urogenital disease, neither lifestyle factors nor exposures
166 potentially deleterious for fertility were reported. The couple benefited from intra-marital ICSI
167 performed with fresh ejaculated sperm cells but frozen oocytes because the man failed to
168 produce a semen sample on the day of oocytes pick-up of the first ICSI attempt. Seven out of
169 nine of the thawed oocytes were injected and 3 zygotes were observed 18 hours after ICSI.
170 After a prolonged embryo culture, 3 embryos with a good quality, according to the Istanbul
171 consensus 2011 classification²³, were obtained. Embryo transfer scheduled on day 5 was
172 postponed due to a high endometrial thickness; two of the embryos were frozen at day 5 and
173 the third one at day 6. To date, only one thawed embryo was transferred but the resulting
174 pregnancy ended with a miscarriage after 5 weeks and 3 days.

175 The successive semen analyses performed over 3 years on the patient revealed a severe
176 alteration of sperm progressive motility, which never exceeded 5% (normal value > 32%),
177 contrasting with normal sperm counts and vitality rates (Table 1). The mean percentage of
178 typical spermatozoa was slightly decreased but the patient did not present any sperm
179 abnormalities indicative of a MMAF phenotype (the percentage of spermatozoa with short,
180 absent and irregular calibre of flagella being under the lower reference values¹⁹) (Table 1).
181 However, semen analyses indicated a significant proportion of spermatozoa with an excess
182 residual cytoplasm and disorganised midpiece with occurrences of plasma membrane lysis
183 (Figure 1A). In addition, flagellar bending was noticed together with some head anomalies (thin
184 head) (Table 1). Acrosomal staining performed with fluorescent peanut agglutinin (PNA) on
185 sperm sample from the patient indicated an intact acrosome on most of the spermatozoa; hence

186 the rate of PNA negative sperm cells, indicative of premature acrosomal reaction, was 8.8%
187 and 5.4% for control and patient, respectively. The procedure of sperm selection with density
188 gradient centrifugation slightly increased the fraction of progressive spermatozoa (5-25%) but
189 also induced an increase of the number of isolated sperm heads. We found that the percentage
190 of isolated sperm heads remained very low in control samples defined by normal sperm
191 parameters, including morphology according to WHO¹⁹ (1% and 3% before and after selection,
192 respectively), while it significantly increased in patient sample after density gradient
193 centrifugation (2% and 33% before and after selection, respectively). Importantly, we noted
194 that flagellar bending and breaking points on sperm from the patient were not limited to the
195 connective piece but were also present along the flagella (Supplementary Figure S1). Overall,
196 these observations suggested an important fragility of the sperm tail, amplified by the selection
197 procedure.

198 We performed Transmission Electron Microscopy (TEM) analysis to precisely examine
199 the sperm ultrastructure of the patient. In line with the observations by optic microscopy, we
200 detected the presence of midpiece defects, in particular, an excess of residual cytoplasm around
201 the mitochondrial sheath and plasma membrane fragility illustrated by plasma membrane lysis
202 (Figure 1B). Importantly, no alteration of the axonemal structure was observed in patient's
203 sperm as quantification indicated a percentage of normal transversal sections (9+2
204 conformation) close to that recorded in semen from control individuals (Patient: 84%; Control
205 mean: 84.5%, n=4). Overall, the patient presented in this study was classified as a case of
206 asthenozoospermia with a severe reduction of the proportion of progressive sperm, membrane
207 and flagellar fragility but no major defects of the sperm flagellum ultra-structure and assembly.

208 We thus subsequently performed WES analysis of this patient in search for genetic
209 variants potentially involved in the infertility phenotype. After processing and filtering the data,
210 we identified homozygous variants with high predicted impact on the encoded proteins in three

211 genes: *c.2748+2T>C* in *SLC9C1*, *c.3583C>T* in *ABCB5* and *c.9del* in *ZNF891*. Among those,
212 the variant *c.2748+2T>C* in *SLC9C1* appeared as the best candidate considering the pattern
213 and abundance of expression of the three genes and the frequency of the variants in the
214 population according to public databases (Supplementary Table 1). Indeed, in contrast to the
215 two other candidates, *SLC9C1* was shown to be preferentially expressed in the testis (Illumina
216 Body Map; The Human Protein Atlas database; ReproGenomics Viewer) and the encoded
217 protein was previously detected in human sperm proteome²⁴ (Supplementary Table 1). The
218 homozygous variant *c.2748+2T>C* identified in *SLC9C1* impairs a consensus sequence of the
219 splice donor site located downstream of exon 22 and was associated with a low allele frequency
220 in the gnomAD database (4.6×10^{-5} , chr3:112180562 A>G (GRCh38.p12), rs2007949663-
221 111899409-A-G). The presence of this homozygous variant in the patient was also consistent
222 with the familial history of infertility and the known consanguinity of his parents (cousins). The
223 proband originated from West Africa (Guinea Conakry); he has two brothers, one of them
224 known to be infertile, and two fertile sisters (Figure 2A). In addition, the proband has one cousin
225 who also displays male infertility; as it was not possible to define if the cousin was from
226 maternal or paternal side, this individual is not included in the genealogic tree (Figure 2A).
227 Unfortunately, we could not obtain biological samples from the family members. In
228 conclusion, considering all the above arguments, we retained the *c.2748+2T>C* variant
229 identified in *SLC9C1* by WES, which we also confirmed by Sanger sequencing on DNA sample
230 from the proband (Figure 2B).

231 *SLC9C1*, also known as *sNHE* or *SLC9A10*, is located on chromosome 3 and comprises
232 29 exons (Figure 2C). Alternative splicing produces five transcript variants, three of which
233 encoding proteins, and among them transcript *SLC9C1-201* encodes for the longer isoform of
234 135 kDa (Figure 2C). The full *SLC9C1* protein isoform comprises the ion exchange domain
235 (Cat/H⁺ ex), which is characteristic of *SLC9* family members and is generally formed by ten to

236 twelve transmembrane (TM) helices (Figure 2C). The protein also exhibits a unique C-terminal
237 tail with four additional TM helices showing similarity to a Voltage-Sensing Domain
238 (ITD/VSD) and a consensus sequence for a Cyclic Nucleotide-Binding Domain (CNBD),
239 which is composed of several α -helices and β -sheets and protrudes towards the cytoplasmic
240 side^{25,26}. These two latter features are typical of ions channels, which, unlike solute carriers, are
241 not activated by ion gradients but display a more sophisticated regulatory mechanism dependent
242 on the cell status¹⁴.

243 To investigate the molecular consequence of the mutation identified in *SLC9C1*, we
244 performed RT-PCR analysis on semen sample from control and patient individuals using
245 forward and reverse primers localised in exons 21 and 23, respectively. The PCR amplicon
246 obtained for control individual was observed at the expected size of 217 bp while product
247 amplified from the patient sample was considerably smaller (Figure 2D). The experimental
248 normalization, operated by analysis of the housekeeping gene *HPRT*, did not reveal any
249 difference in *SLC9C1* transcripts amount between the patient and control individual.
250 Sequencing of *SLC9C1* RT-PCR amplicons indicated the total absence of exon 22 in the
251 transcripts from the patient (Figure 2E). This exon encodes for a region included in the Cyclic
252 Nucleotide-Binding Domain (Figure 2C) and comprises 99 bp. Such exon skipping would
253 therefore cause a 33 amino acids in-frame deletion (corresponding to amino acids 884-916).
254 These results confirm the deleterious impact of the identified variant.

255 We next performed immunofluorescence assays with two antibodies raised against
256 different epitopes of the SLC9C1 protein: a so-called ‘Central antibody’, which recognizes
257 amino-acids 600-750 of the protein, spanning the VSD, upstream from the deletion and a so-
258 called ‘C-Terminal antibody’ targeting amino-acids 882-932, in the CNBD and mainly
259 coinciding with the deleted sequence. We first observed that both antibodies detected the
260 SLC9C1 protein along the sperm flagellum with a signal intensity decreasing towards the tail

261 end. Importantly, in consistence with RT-PCR data which predicted the deletion of amino acids
262 884-916, immunofluorescence assays performed with the ‘Central antibody’ marked SLC9C1
263 protein in spermatozoa from both control and patient samples (Figure 3A), whereas the ‘C-
264 Terminal antibody’ whose epitope largely overlaps with the deleted region only marked the
265 control sperm (Figure 3A).

266 Lastly, we performed western blot immunodetection from control and patient sperm
267 protein extracts. Using the ‘Central antibody’ we detected a band at the expected molecular
268 weight of the SLC9C1 longer isoform (135 kDa) in both control and patient sperm samples
269 (Figure 3B). Technical limits of the gel electrophoresis did not permit to evidence the small
270 size difference between the normal and the deleted protein but this data firmly confirm the
271 presence of the SLC9C1 truncated protein in sperm cells from the studied patient.

272 **Discussion**

273 In conclusion, we characterize an infertile patient displaying functional
274 asthenozoospermia caused by a homozygous splice mutation in *SLC9C1*, which generates a 33
275 amino-acid deletion within the Cyclic Nucleotide-Binding Domain (CNBD). This protein
276 domain is considered to be involved in the interaction and reciprocal modulation between
277 *SLC9C1* (sNHE) and the soluble Adenylate Cyclase (sAC) in mouse and sea urchin sperm^{26,27},
278 the latter being a key element for the regulation of sperm motility and capacitation.

279 In mice, the disruption of *Nhe-1* did not impact male fertility²⁸ while disruption of
280 *Slc9c1* (*sNhe*) as well as *Nha1* and *Nha2* (single and double conditional KO mice) all resulted
281 in a phenotype of null sperm motility. Interestingly, these phenotypes were significantly
282 rescued by sperm incubation with cAMP analogues, confirming that Na⁺/H⁺ family members
283 are required for the activation of sperm motility and capacitation through regulation of the
284 cAMP-PKA signalling pathway^{25,29}. *SLC9C1* (sNHE) was previously described to only localise
285 to the principal piece of the mouse sperm flagellum²⁵. Zhang et al.³⁶ proposed the same
286 localisation in human sperm cells, although the staining was weak and quite heterogeneous.
287 Our immunostaining assays carried out with two different antibodies suggest a distinct flagellar
288 profile in human sperm. Hence, while we detected a prominent localisation of sNHE in the
289 principal piece of murine spermatozoa (Supplementary Figure S2), in human sperm, *SLC9C1*
290 was detected along the whole length of the flagellum.

291 Regarding, the molecular mechanisms of action, it is well established that intracellular
292 pH regulation is fundamental at different steps of sperm maturation and capacitation³⁰, and
293 Na⁺/H⁺ exchangers (NHE) play a remarkable role in sperm alkalinisation³¹. *SLC9C1* (sNHE)
294 was shown to be required for pH homeostasis and suggested to [promote CatSper activation](#)
295 [following Slo3-mediated sperm hyperpolarization](#) during mouse sperm capacitation^{25,32}. In

296 humans, to date no information is available regarding sNHE possible implication in sperm pH
297 homeostasis. Some elements have pointed a role of the proton channel Hv1^{33,34}, although so far,
298 no mutation in *Hv1* were reported in human asthenozoospermia. Our study therefore sheds light
299 on the essential role of sNHE and a likely similar function in pH homeostasis in humans.
300 Importantly, our findings are in line with a recent comparative genomic analysis, [which](#)
301 [identified the molecular trio sNHE-sAC-CatSper as an evolutionary conserved machinery for](#)
302 [the regulation of sperm flagellar beating in Metazoa](#)³⁵.

303 The involvement of *SLC9C1* in human sperm physiology has been poorly investigated,
304 and so far only weak evidence was provided regarding its dysfunction in human
305 asthenozoospermia^{17,36,37}. The mutation in *SLC9C1* we described here constitutes a solid
306 demonstration of SLC9C1 involvement in the regulation of human sperm motility and
307 fertilization potential. We show that this mutation has a drastic impact on sperm progression;
308 however sperm motility was not totally abrogated as observed in *Slc9c1* KO mouse model²⁵.
309 This difference might be explained by the nature of the mutations in those two models; the KO
310 mouse model totally lacks the protein while the patient carries a truncated protein, which could
311 preserve partial functionality. In addition, while in patient sperm we observed flagellar
312 angulation, excess residual cytoplasm and overall a flagellar fragility upon sperm selection,
313 such defects were not reported in the KO mouse model. These observations in patient sperm
314 are compatible with a dysregulation of the ion equilibrium and overall cell volume regulation,
315 which were previously described to depend on channels and aquaporins activity³⁸.

316 Overall, this article provides a new evidence of the physiological importance of ion
317 exchange regulation and pH homeostasis in the control of sperm motility and fertilization
318 potential. The first and most remarkable evidence was provided for the CATSPER channel, for
319 which mutations (*CATSPER 1-2-ε* subunits) were identified in several asthenoteratozoospermic
320 individuals with altered fertilizing capacity and/or sperm with coiled flagella^{12,39}. More

321 recently, pathogenic variants affecting members of the SLC26 family of anion exchangers,
322 namely *SLC26A3* and *SLC26A8* (*TAT1*), were also shown to impair the functionality of the
323 Cystic Fibrosis Transmembrane conductance Regulator (CFTR) channel, causing
324 asthenozoospermia, associated with defective capacitation, midpiece and annulus
325 disorganization in the case of *SLC26A8*^{11,40,41}. The voltage-dependent anion channels *VDAC2*
326 *and 3* were also identified with pathogenic defects leading to idiopathic asthenozoospermia ⁴²
327 ⁹. The specific location of all those ion channels/transporters at the plasma membrane of sperm
328 cells, constitutes an interesting cellular background for potential therapeutic strategies to
329 improve sperm motility in asthenozoospermic men.

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336

337 **Author's roles**

338 AT designed the study. ED and CP recruited the patient, performed clinical analysis and
339 characterization. AC and PS carried out the ICSI procedure for the patient and his partner.
340 NTM, CC and PR performed exome and bioinformatics analysis. EC did experimental work
341 (RT-PCR, Sequencing, Immunofluorescence, Immunoblotting, MO analysis), data
342 quantification and analysis. AT did TEM analysis. MW, LS and PL contributed in setting up
343 the experimental conditions. EC and AT performed data illustrations. EC and AT analysed the
344 data and wrote the manuscript. MW, AC, PL, CC, CA, PR, NTM, CP and ED did critical
345 reading of the manuscript.

346

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352 and AT).

353

354 **Data Availability Statement**

355 The data that support the findings of this study are available from the corresponding author,
356 [AT], upon reasonable request.

357

358

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- 483

	General Semen Characteristics						Flagellar defects					Midpiece defects	Head defects		Acrosome defects	
	Volume (ml)	Sperm concentration (10 ⁶ /mL)	Total Motility	Progressive Motility	Vitality	Typical forms	Absent	Short	Irregular	Coiled	Bent	Excess residual cytoplasm	Tapered	Thin	Post-acrosomal	Acrosomal
	3.9	117.5	25	5	49	26	1	1	1	5	19	14	0	3	53	35
	3.4	52	30	5	62	23	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3.5	74	35	5	82	10	3	3	0	5	10	1	3	36	22	78
	2.4	73	15	0	80	15	0	2	0	3	6	10	2	25	45	80
	3.8	103	25	5	67	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
mean	3.4	83.9	26	4	68	18.5	1.3	2	0.3	4.3	11.6	8.3	1.7	21.3	40	64.3
Reference limits ^a	1.5	15	40	32	58	23	5	1	2	17	13	4	3	14	42	60
	(1.4-1.7)	(12-16)	(38-42)	(31-34)	(55-63)	(20-26)	(4-6)	(0-2)	(1-3)	(15-19)	(11-15)	(3-5)	(2-4)	(12-16)	(39-45)	(57-63)

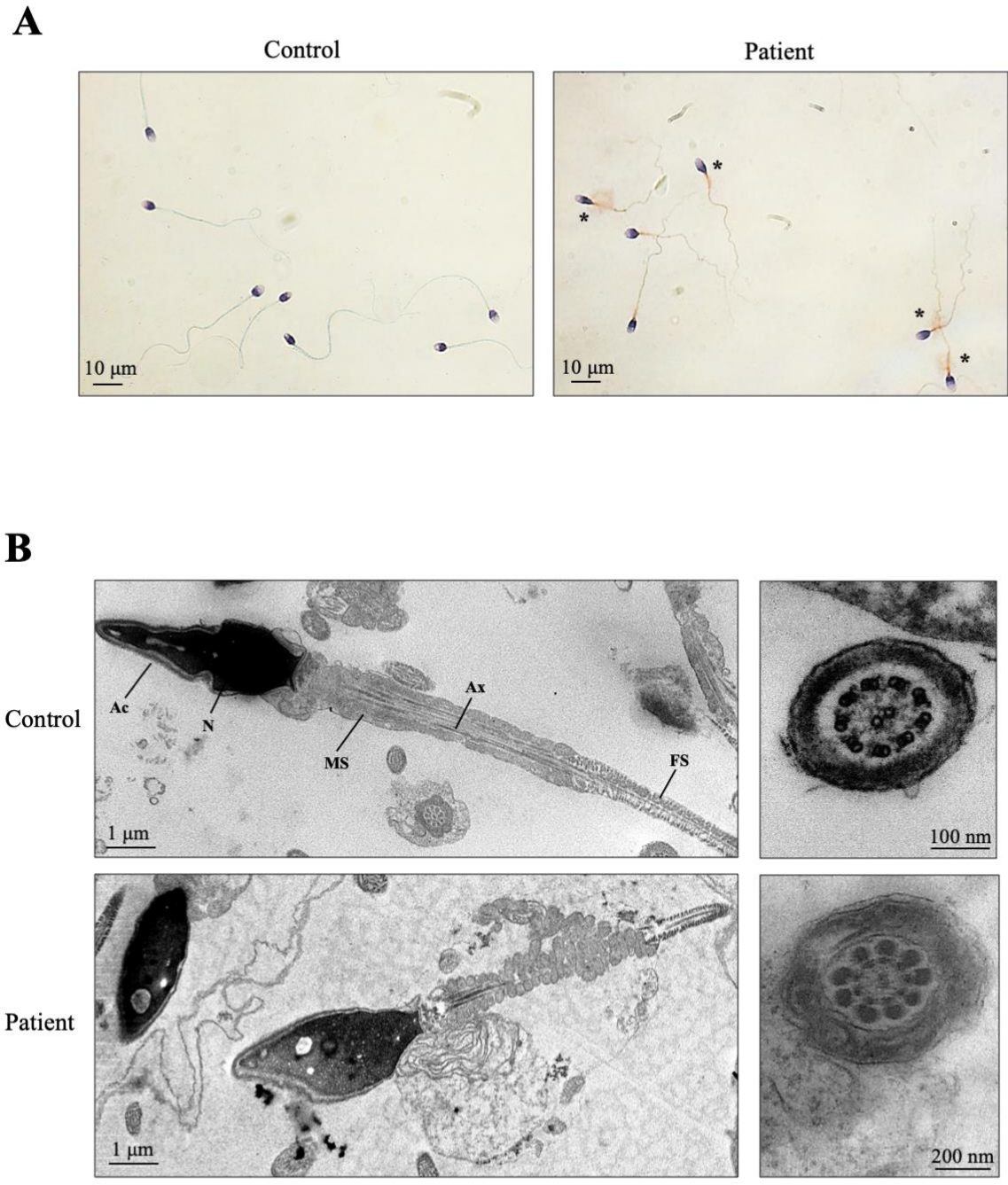
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485 **Table 1** – Semen parameters and sperm morphological defects (flagellum, head and acrosome) of the patient carrying a mutation in *SLC9C1* gene
486 Five semen evaluations were performed for the patient over 3 years. Values are expressed in percent, unless specified otherwise. ND: not
487 determined.

488 ^a Lower or upper reference limits (5th centiles and their 95% confidence intervals) according to the World Health Organization (WHO) standards
489 and the distribution range of morphologically abnormal spermatozoa observed in fertile individuals^{18,19}; in bold characters: abnormal values.

490

Figure 1



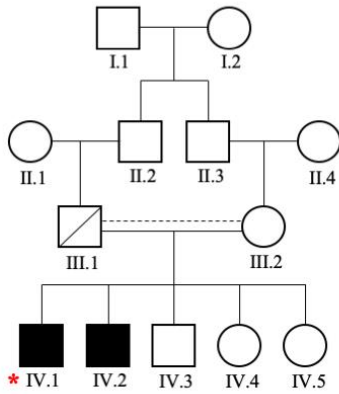
493 **Figure 1 – Morphological and ultrastructural anomalies of spermatozoa from the patient.**

494 (A) Morphology of spermatozoa from the patient (right) compared to spermatozoa from a
495 control individual (left). Semen analysis showed a fraction of spermatozoa carrying
496 disorganised midpiece with residual cytoplasm and plasma membrane lysis (*). Scale bars
497 represent 10 μm . (B) Transmission Electron Microscopy analysis of semen sample from a
498 control individual (top) and from the patient (bottom), showing flagellar longitudinal and
499 transversal sections. Spermatozoa from the patient showed residual cytoplasm associated with
500 plasma membrane lysis and irregular mitochondrial sheath. The transversal sections of
501 spermatozoa from the patient showed normal ultra-structure with the (9+2) pattern. Scale bars
502 represent 1 μm , 100 and 200 nm. *Ac*, acrosome; *N*, nucleus; *MS*, mitochondrial sheath; *Ax*,
503 axoneme; *FS*, fibrous sheath.

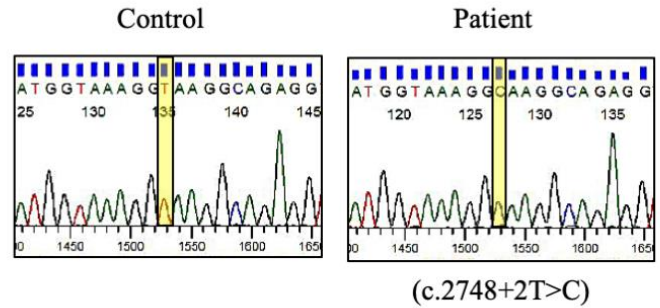
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Figure 2

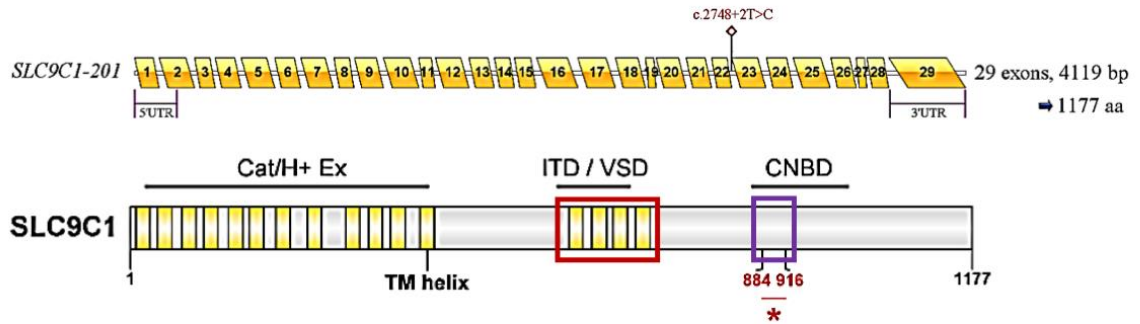
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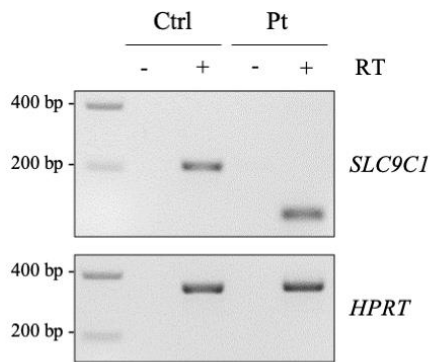
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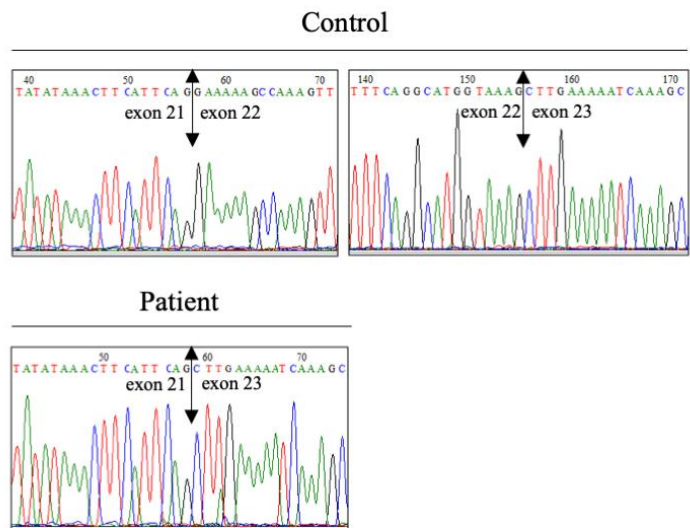
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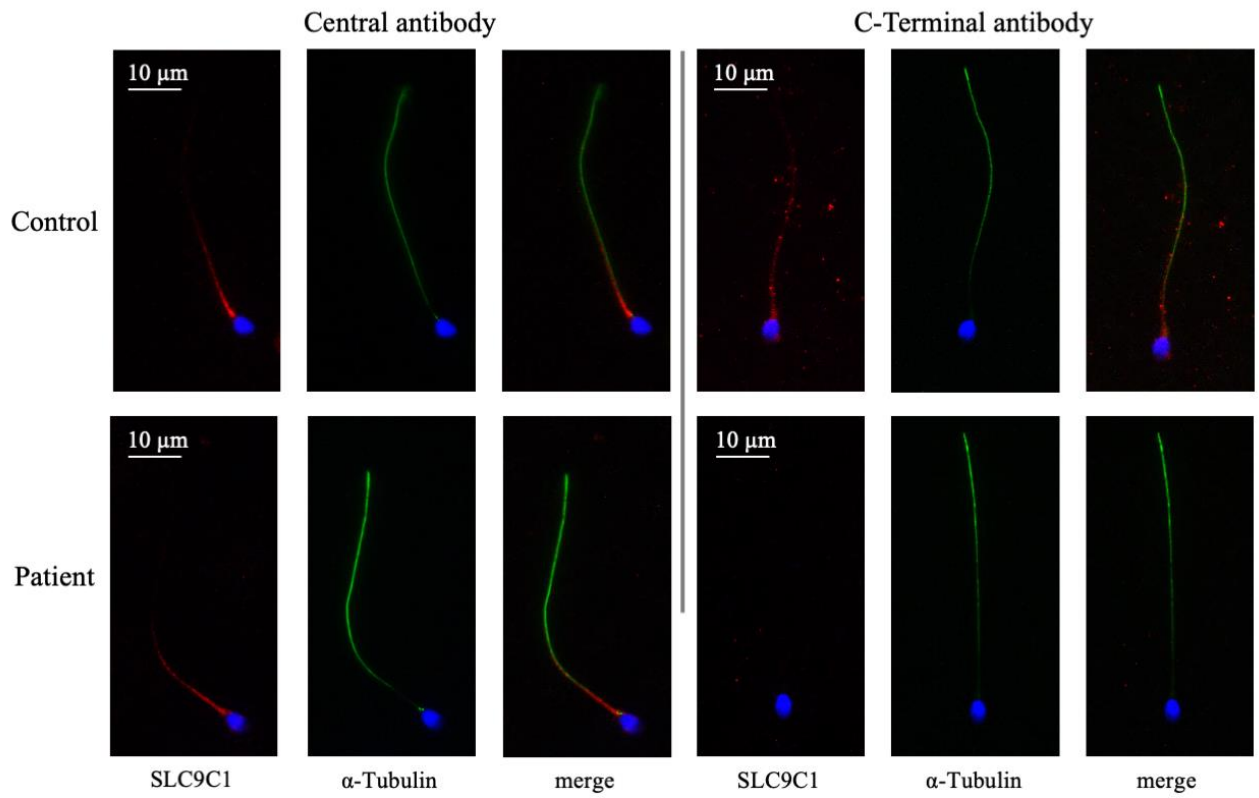


506 **Figure 2 – Pedigree of the patient, description of the *c.2748+2T>C* mutation in *SLC9CI***
507 **and analysis of the transcript in sperm from the patient.**

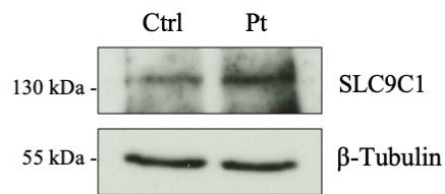
508 (A) Pedigree of the infertile patient with functional asthenozoospermia. The proband (IV.1) is
509 indicated by the red asterisk. Consanguinity between parents (III.1 and III.2) is indicated by the
510 dashed line. One of the brothers of the proband (IV.2) is also known to be infertile; the second
511 one (IV.3) has not manifested desire of parenthood yet. The two sisters of the proband (IV.4 –
512 IV.5) are fertile and had children. A cousin not illustrated in the pedigree was also reported by
513 the proband to be infertile. (B) Electropherograms of *SLC9CI* Sanger sequencing for patient
514 and control individual. The *c.2748+2T>C SLC9CI* mutation identified by WES was confirmed
515 by Sanger sequencing. (C) (top) Linear structure of *SLC9CI* main transcript (*SLC9CI-201*)
516 showing the exons (yellow boxes), according to the gene description available from Ensembl
517 database. The localization of the mutation is pointed by a stick [figure obtained with IBS tool
518 of DOG software⁴³]. (bottom) Linear structure of SLC9C1 protein (SLC9C1-201 isoform). The
519 yellow boxes represent transmembrane (TM) helices and the horizontal bars indicate all
520 functional domains: Cat/H⁺ Ex, Cation/H⁺ Exchanger domain; ITD / VSD, Ion Transport-like
521 Domain / Voltage-Sensing Domain; CNBD, Cyclic Nucleotide-Binding Domain. Red and
522 purple boxes highlight the epitopes of the two antibodies used in the study, the ‘Central
523 antibody’ and the ‘C-Terminal antibody’ respectively. The region between amino-acids 884
524 and 916 is encoded by exon 22 [figure obtained with DOG software⁴⁴]. (D) RT-PCR analysis
525 of a semen sample from the patient carrying the *c.2748+2T>C* mutation. The expected
526 amplicon size is 217 bp for *SLC9CI* transcript and 352 bp for *HPRT*. RNA samples treated in
527 absence of Reverse Transcriptase (- RT) constituted negative experimental controls. (E)
528 Electropherogram of *SLC9CI* transcript sequencing for a control individual and the patient. In
529 the sample from the patient, sequencing indicates the absence of exon 22 due to the
530 *c.2748+2T>C* mutation, which abrogates intron 22 splice donor site.

Figure 3

A



B

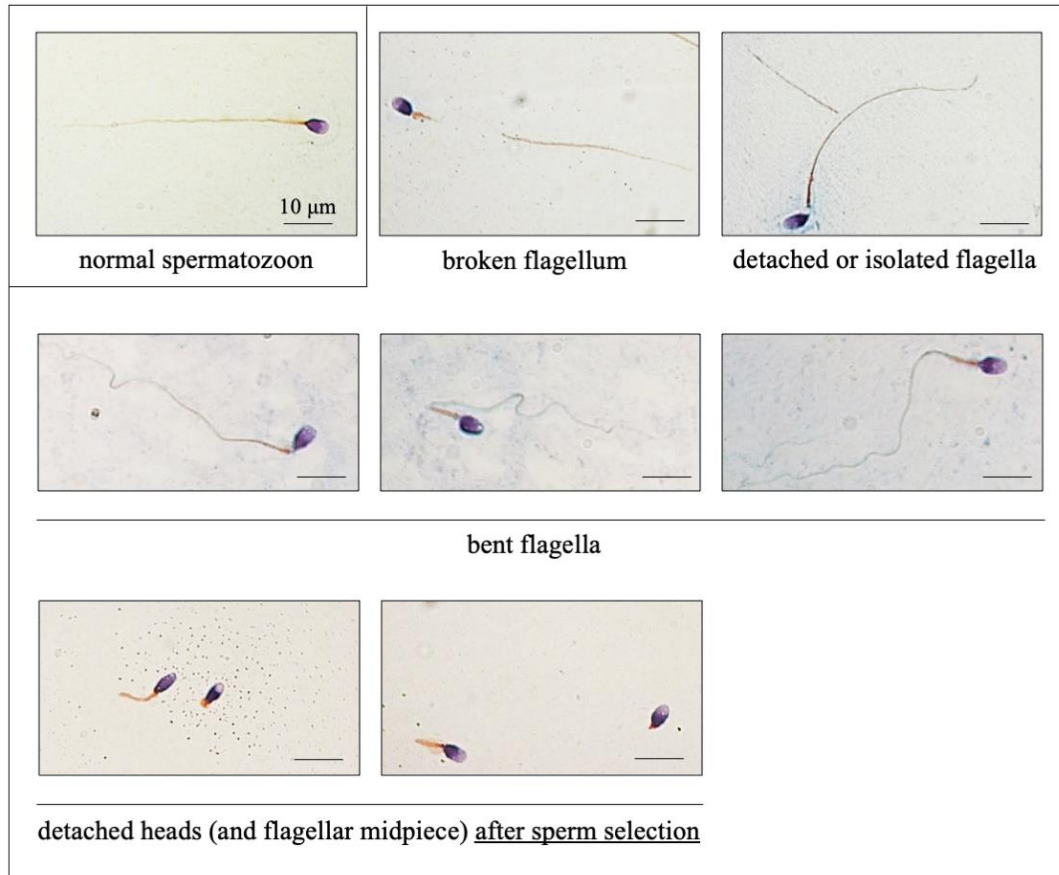


532 **Figure 3 – Analysis of SLC9C1 protein in sperm from the patient carrying the**
533 ***c.2748+2T>C* mutation.**

534 (A) SLC9C1 protein detection (red) by immunofluorescence assay in sperm cells from control
535 individual (top) and patient (bottom). Two different antibodies were used: one antibody directed
536 against the central region of SLC9C1 and a second antibody directed against the C-Terminal
537 region and including exon 22 coding region. Co-staining with α -Tubulin antibody (green) was
538 also performed. The protein localises at the midpiece and principal piece of sperm flagella, with
539 a decreasing gradient towards the end of the tail. The Central antibody allowed to confirm
540 presence of SLC9C1 in patient spermatozoa, while absence of signal of the C-Terminal
541 antibody confirmed an abnormal protein lacking part of the CNBD. Scale bars represent 10 μ m.
542 (B) Immunoblotting detection of SLC9C1 protein (135 kDa) in sperm lysate from control
543 individual (left) and patient (right). β -Tubulin (55 kDa) was used for normalization.

544

Figure S1



546

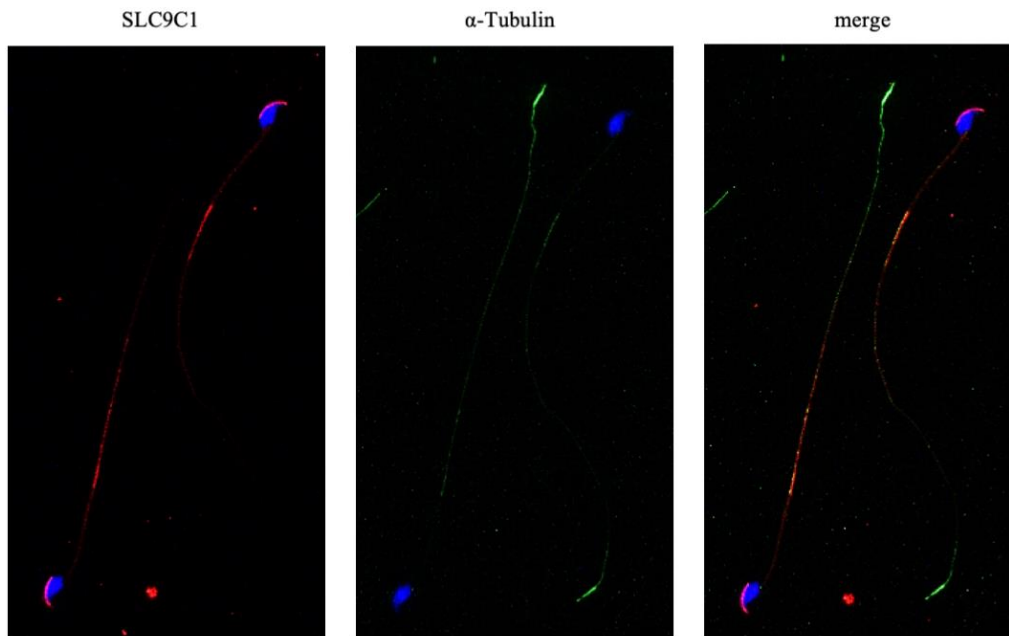
547 **Figure S1 – Details of the flagellar morphological anomalies observed in sperm from the**
548 **patient.**

549 Catalogue of the sperm flagellar defects observed in semen from the patient carrying the
550 *c.2748+2T>C* mutation, compared to a normal spermatozoon (top, left corner). Various
551 flagellar angulation and break points were observed at the level of the connecting piece or along
552 the flagellum, suggesting a fragility of the sperm tail. This phenotype was even more evident
553 after sperm selection with density gradient centrifugation. Scale bar represents 10 μm.

554

555

Figure S2



556

557 **Figure S2 – SLC9C1 detection in mouse spermatozoa.**

558 Immunofluorescence detection of SLC9C1 protein on mouse spermatozoa using the antibody
559 directed against the central region of SLC9C1, co-staining with α -Tubulin (green). SLC9C1
560 (red) preferentially localises at the principal piece of the flagella. Scale bar represents 10 μ m.

Gene	Testis/all tissue gene expression ratio	Variant	Frequency (gnomAD database)	Nature of the variant	Predicted effect at protein level	Detection in sperm proteome (Wang <i>et al.</i> , 2013)
<i>SLC9C1</i>	12.71	c.2748+2T>C	4.576 10 ⁻⁵	Splice donor variant	-	+
<i>ABCB5</i>	2.37	c.3583C>T	0.0008645	Stop gained	p.Gln1195Ter	-
<i>ZNF891</i>	1.71	c.9del	0.003403	Frameshift variant	p.Met4TrpfsTer9	-

Supplementary Table 1 – Homozygous variants with high pathogenic impact proposed for the patient by bioinformatic analysis of WES data.