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1 **Full title: Phenotypic Continuum and Poor ICSI Prognosis in patients**
2 **harboring *HENMT1* variants**

3 **Short title:** Phenotypic continuum of *HENMT1* variants

4
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39 **Key words**

40 Infertility, piwi pathway, piRNA, spermatozoa, azoospermia, teratozoospermia

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71 **Abstract**

72 Background: Small RNAs interacting with PIWI (piRNAs) play a crucial role in regulating
73 transposable elements and translation during spermatogenesis and are essential in male germ
74 cell development. Disruptions in the piRNA pathway typically lead to severe spermatogenic
75 defects and thus male infertility. The *HENMT1* gene is a key player in piRNAs primary
76 biogenesis and dysfunction of HENMT1 protein in meiotic and haploid germ cells resulted in
77 the loss of piRNA methylation, piRNA instability, and TE de-repression. *Henmt1*-knockout
78 mice exhibit a severe oligo-astheno-teratozoospermia (OAT) phenotype, whereas patients
79 with *HENMT1* variants display more severe azoospermia phenotypes, ranging from meiotic
80 arrest to hypospermatogenesis. Through whole-exome sequencing (WES) of infertile patient
81 cohorts, we identified two new patients with variants in the *HENMT1* gene presenting
82 spermatozoa in their ejaculate, providing us the opportunity to study spermatozoa from these
83 patients.

84 Objectives: Investigate the spermatozoa of two patients harboring a *HENMT1* variant to
85 determine whether or not these scarce spermatozoa could be used with assisted reproductive
86 technologies.

87 Materials and Methods: *HENMT1* variants identified by WES were validated through Sanger
88 sequencing. Comprehensive semen analysis was conducted, and sperm cells were subjected to
89 transmission electron microscopy for structural examination, *in situ* hybridization for
90 aneuploidy assessment, and aniline blue staining for DNA compaction status. Subsequently,
91 we assessed their suitability for *in vitro* fertilization using intracytoplasmic sperm injection
92 (IVF-ICSI).

93 Results: Our investigations revealed a severe OAT phenotype similar to *knockout* mice,
94 revealing altered sperm concentration, mobility, morphology, aneuploidy and nuclear
95 compaction defects. Multiple IVF-ICSI attempts were also performed, but no live births were
96 achieved.

97 Discussion: We confirm the crucial role of *HENMT1* in spermatogenesis and highlight a
98 phenotypic continuum associated with *HENMT1* variants. Unfortunately, the clinical outcome
99 of these genetic predispositions remains unfavourable, regardless of the patient's phenotype.

100 Conclusion: Presence of spermatozoa is insufficient to anticipate ICSI pregnancy success in
101 *HENMT1* patients.

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106 INTRODUCTION

107 Infertility has emerged as a pressing global concern, impacting approximately 50 million
108 couples worldwide who face challenges in natural reproduction despite 12 months of regular
109 and unprotected intercourse¹. This condition is clinically heterogeneous and characterized by
110 a complex etiology. Consequently, despite its widespread prevalence, nearly 40% of infertile
111 couples receive a diagnosis of unexplained or idiopathic infertility². While acknowledging the
112 multifaceted nature of infertility, it is crucial to recognize the significant role played by
113 genetic defects in its manifestation. Studies suggest that up to half of idiopathic cases of male
114 infertility may be linked to yet-to-be-identified genetic abnormalities³. Despite the diagnostic
115 yield reaching up to 50% for certain rare phenotypes with known genetic causes⁴, some severe
116 form of male infertility like azoospermia, defined by the absence of sperm in the ejaculate,
117 continues to present a diagnostic yield below 20%⁵⁻⁶. As researchers explore different
118 transmission models⁷ and treatment approaches⁸, the identification of pathogenic variants
119 within the genes associated with different molecular pathways in the testis remains of
120 paramount importance for genetic and reproductive counselling, diagnostic precision, and
121 potential future personalized medicine. Notably, the piRNA-pathway has gained considerable
122 attention, and studies involving knockout mice and patients with bi-allelic variants in piRNA
123 biogenesis-related genes consistently reveal severe disruptions in spermatogenesis,
124 highlighting the pathway's critical role in male fertility (see⁹⁻¹¹ for review).

125 PIWI-interacting ribonucleic acids, or piRNAs, constitute a highly diverse class of small,
126 single-stranded noncoding RNAs characterized by lengths ranging from 23 to 32 nucleotides.
127 They bind to specific PIWI-clade members (from P-element Induced-Wimpy testis) of the
128 Argonaute protein family¹², and encompass over 8.5 million distinct sequences in human and
129 68.5 million in mouse¹³. According to their temporal expression patterns and sequence
130 content, piRNAs are classified into fetal, pre-pachytene, and pachytene classes¹⁴⁻¹⁵. Fetal
131 piRNAs are expressed in prospermatogonia and interact with PIWIL2 and PIWIL4¹⁵. Pre-
132 pachytene piRNAs, detected in testes before germ cells enter the pachytene stage of meiotic
133 prophase I, interact with PIWIL2 and are primarily involved in controlling the transposable
134 elements¹⁵⁻¹⁶. Pachytene piRNAs, which are first produced when germ cells enter the
135 pachytene stage of meiosis¹⁵, account for approximately 95% of piRNAs in the adult testis¹²,
136 associate with PIWIL1 and PIWIL2¹⁵, and are mainly involved in regulating post-meiotic
137 gene expression¹⁷⁻¹⁹.

138 Knockout mice for piRNA biogenesis-related genes predominantly exhibit a phenotype of
139 non-obstructive azoospermia (NOA) characterized by germ cell maturation arrest at meiosis²⁰⁻
140 ²⁵ or round spermatids stages²⁶⁻²⁸. In contrast, human patients present a more complex
141 phenotypic continuum, ranging from Sertoli cells-only syndrome with absence of sperm
142 production¹¹, through azoospermia due to meiotic arrest²⁹ or round spermatid arrest³⁰, to
143 cryptozoospermia³¹ and extreme and severe oligozoospermia³²⁻³³ where spermatozoa are
144 present in the ejaculate. This phenotypic continuum suggests that *in-vitro* fertilization with
145 intracytoplasmic sperm injection (IVF-ICSI) could be an effective medical response for some
146 of the patients, but it remains uncertain whether the few sperm cells produced are suitable for
147 assisted medical procreation and compatible with a successful pregnancy^{11,34}.

148 The *HENMT1* gene, also known as Hen1 methyltransferase homolog 1, is a key player in
149 piRNAs primary biogenesis, the pathway generating most of piRNAs in adult germ cells³⁵,
150 where piRNAs are processed from long non-coding RNAs transcribed from genomic piRNA
151 clusters (see^{12,36} for review). It encodes an enzyme responsible for catalyzing the 2'-O-
152 methylation at the 3' end of piRNAs, a modification that contributes to their stability and
153 functionality³⁷⁻³⁹. In line with the other essential genes involved in the piwi pathway, previous
154 investigations revealed that dysfunction of HENMT1 protein in meiotic and haploid germ
155 cells resulted in piRNA instability, the loss of piRNA methylation, and TE de-repression (in
156 mice), leading to severe phenotype in both human³⁴ and mouse³⁷ models. *Henmt1*-knockout
157 mice exhibit a severe oligo-astheno-teratozoospermia (OAT) phenotype characterized by a
158 majority of pinhead sperm with stumpy tails lacking a mitochondrial sheath in the midpiece of
159 the sperm tail³⁷. In humans, three *HENMT1* variants were reported in three men displaying
160 azoospermia of varying severity. Two patients harboring homozygote missense variants,
161 c.226G>A;p.Gly76Arg and c.400A>T;p.Ile134Leu, respectively exhibited meiotic³⁴ and
162 round spermatid arrest¹¹. In contrast, a patient harboring a homozygous loss-of-function
163 variant c.456C>G; p.Tyr152Ter exhibited hypospermatogenesis with positive testis sperm
164 retrieval³⁴.

165 Through whole-exome sequencing of a cohort of azoospermic men, we previously identified³⁴
166 two of the cited patients (P0109_p.Gly76Arg and P0272_p.Tyr152Ter). Given the broad
167 phenotypic continuum observed so far, we extended our search for variants to a cohort of
168 teratozoospermic men. This strategy allowed us to identify two new patients with variants in
169 the *HENMT1* gene: a novel biallelic loss-of-function variant c.100C>T;p.Gln34Ter and the
170 already uncovered c.456C>G;p.Tyr152Ter variant. Spermatozoa were present in the ejaculate

171 of both patients, providing a unique opportunity to study them, characterize their nuclear
172 quality, and assess their potential in IVF-ICSI.

173

174 **MATERIALS AND METHODS**

175 **Patient recruitment**

176 Patient P0582 and a fertile control were respectively recruited at the Hôpital Jeanne de
177 Flandre - Lille University Hospital (France) and Grenoble-Alpes University Hospital
178 (France). Patients P0109, P0272 and P1021 were recruited at the “Clinique des Jasmins” in
179 Tunis (Tunisia). Informed consent was obtained from all individuals participating in the study
180 according to local protocols and the principles of the Declaration of Helsinki. The study was
181 approved by local ethics committees, and samples were then stored in the Centre de
182 Ressources Biologiques Germethèque (certification under ISO-9001 and NF-S 96–900)
183 according to a standardized procedure, or were part of the Fertithèque collection declared to
184 the French Ministry of Health (DC-2015–2580) and the French Data Protection Authority
185 (DR-2016–392).

186 **Exome sequencing and bioinformatic analysis**

187 Both entire cohort of respectively 245 azoospermic and 105 teratozoospermic patients
188 underwent data processing following our established protocol⁴⁰. Briefly, coding regions along
189 with intron/exon boundaries were enriched using the Exon V6 kit from Agilent Technologies
190 (Wokingham, UK), followed by sequencing on an Illumina HiSeq X platform by a contracted
191 service provider (Novogene, Cambridge, UK). Exome data were analyzed using an in-house
192 developed bioinformatics pipeline, comprising two modules available on GitHub under the
193 GNU General Public License v3.0: <https://github.com/ntm/grexome-TIMC-Primary> and
194 <https://github.com/ntm/grexome-TIMC-Secondary>, as previously detailed⁴¹. Variants with a
195 minor allele frequency exceeding 1% in gnomAD v2.0 or 3% in the 1000 Genomes Project
196 phase 3 were excluded, and only variants predicted to have high impact (e.g., stop-gain or
197 frameshift variants) by the Variant Effect Predictor v110⁴² were further examined.

198 **Sanger sequencing**

199 The previous candidates and the newly identified *HENMT1* variants all underwent validation
200 through Sanger sequencing conducted on an ABI 3500XL instrument (Applied Biosystems)
201 and data analysis was conducted using SeqScape software (Applied Biosystems).

202 **Minigene splicing reporter assay**

203 To further validate the deleterious impact of the previously identified c.226G>A;p.Gly76Arg
204 variant and to better characterize its impact on RNA splicing, we performed a minigene assay.

205 DNAs from the proband patient, carrying the identified homozygous splicing variant of
206 HENMT1 c.226G>A and DNA from a fertile control, WT for the variant c.226G, were
207 amplified using a CloneAmp HiFi PCR Premix (#639298, Takara). Primers sequences (5'-3'),
208 forward (intron 3): CCTACAGCGCACGCGTGAATGGGAAAACACTGTGTGTACG and,
209 reverse (intron 4): GTTGCTTTCCGTCGACATGGTTCAAATGCAGGCGG (Figure S1A).
210 The 1360 bp amplicon (c.151-335_263+356) was inserted between MluI and Sall restriction
211 sites of the pCineo minigene vector as previously described⁴³, using ProLigation-Free Cloning
212 Kit (#E086/E087, Abm) (Figure S1B). Constructed vectors were transformed in Escherichia
213 coli DH5 α - competent cells (#44-0097, Invitrogen) for amplification. DNA sequences of
214 amplicons cloned into pCineo vector, WT and mutant plasmids were checked by DNA
215 sequencing (3500xl Genetic Analyzer and SeqScape3 software, Thermo Fisher Scientific).
216 HEK 293T cells were purchased from the American Type Culture Collection (ATCC, USA)
217 and grown in DMEM medium (#31966021) supplemented with 10% fetal bovine serum
218 (#10270106), penicillin-streptomycin (respectively 100 U/L and 100mg/L, #15140122) all
219 from ThermoFisherScientific. Cell cultures were incubated at 37°C and 5% CO₂ in a
220 humidified incubator. The day before transfection, 2.105 cells were plated in a 6-well culture
221 plate and transiently transfected with 2 μ g of plasmids (empty pTB2 (control), pTB2- WT
222 (WT) and pTB2- mutant) using calcium phosphate (#631312, Takara) according to the
223 manufacturer's instructions. After 48h of incubation, cells were harvested with trypsin-EDTA
224 (#15400054, ThermoFisherScientific) and total RNA was extracted (NucleoSpin RNA,
225 #740955.50, Macherey Nagel). First-strand cDNA was synthesized from 1 μ g of extracted
226 total RNA (SuperScript™ III First-Strand Synthesis SuperMix, #11752050,
227 ThermoFisherScientific). The resulting cDNA was amplified by PCR using vector- specific
228 primers surrounding the cloning site thank to CloneAmp HiFi PCR Premix (#639298,
229 Takara). The PCR products were analyzed on 1% agarose gel. The target DNA bands were
230 gel-cut, purified (PCR Clean up, #740609-250, Macherey Nagel) and sequenced to identify
231 mutation impact on splicing process.

232 **Semen Analysis**

233 Semen samples were obtained by masturbation after three to four days of sexual abstinence
234 and were incubated for 30 min at 37°C for liquefaction. Parameters evaluated according to
235 World Health Organization (WHO) guidelines⁴⁴ were: ejaculate volume, pH and viscosity,
236 and sperm cells concentration, vitality, motility and morphology.

237 **Transmission Electron Microscopy**

238 Transmission Electron Microscopy (TEM) experiments were performed like previously⁴⁵
239 using sperm cells from fertile control and patient P0582. After fixation in 2.0% v/v
240 glutaraldehyde in phosphate buffer (pH 7.4), the sperm pellet underwent a 15 minutes wash in
241 fresh buffer containing 4% w/v sucrose and was subsequently embedded in 2% agar. Post-
242 fixation involved the use of 1% osmic acid in phosphate buffer. Following fixation, small
243 pieces of agar containing spermatozoa were dehydrated through a graded series of ethanol.
244 Subsequently, these pieces were further embedded in Epon resin (Polysciences Inc.,
245 Warrington, PA, USA). Sections were then cut using a Reichert OmU2 ultramicrotome
246 (Reichert-Jung AG, Vienna, Austria) equipped with a diamond knife. Ultrathin sections (70
247 nm) were collected on Parlodion 0.8%/isoamyl acetate-coated 100 mesh Nickel grids (EMS,
248 Fort Washington, PA, USA) and counterstained with 2% uranyl acetate and lead citrate before
249 observation. Examination of the sections was conducted using a Zeiss transmission electron
250 microscope 902 (Leo, Rueil-Malmaison, France), and images were acquired utilizing a Gatan
251 Orius SC1000 CCD camera (Gatan France, Grandchamp, France).

252 **Immunostaining**

253 Immunofluorescence (IF) experiments were performed on fertile control and patient P0582.
254 Sperm cells were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde for 30
255 seconds at room temperature, washed two time in PBS and spotted onto 0.1% poly L-lysine
256 pre-coated slides (Thermo Fisher Scientific, Waltham, MA, USA). After attachment, sperm
257 were washed 2x5 minutes with 0.1% (v/v) Triton X-100-DPBS (Triton X-100; Sigma-Aldrich
258 Co., Ltd., Irvine, UK) at room temperature. Slides were then blocked 30 minutes in 2% normal
259 serum–0.1% (v/v) Triton X-100-DPBS (normal goat or donkey serum; GIBCO, Thermo
260 Fisher Scientific) and incubated overnight at 4°C with the primary antibodies: polyclonal
261 rabbit anti-HENMT1 (AB121991, Abcam (Cambridge, UK), 1:100) and monoclonal mouse
262 anti-acetylated- β -tubulin (AB61601, Abcam (Cambridge, UK), 1:400). Washes were
263 performed with 0.1% (v/v) Tween 20–DPBS, followed by 1 h incubation at room temperature
264 with secondary antibodies (Dylight 488 and Dylight 549, Jackson ImmunoResearch®, 1:1000)
265 and counterstained with 5 mg/mL Hoechst 33342 (Sigma-Aldrich). Appropriate controls
266 without primary antibodies were performed for each experiment. Fluorescence images were
267 captured with a confocal microscope (Zeiss LSM 710).

268 **Aniline blue staining**

269 Aniline blue coloration was performed on ejaculated spermatozoa from patients P0582 and
270 fertile control. After washing twice with 5 ml of PBS 1 \times , a small portion of semen samples
271 were fixed with a 3% glutaraldehyde solution in PBS 1 \times for 30 min at room temperature.

272 Slides were then incubated in a succession of baths: 5 min in water, 10 min in 5% aniline blue
273 diluted in 4% acetic acid solution, twice for 2 min in water, 2 min in 70%, 90% and 100%
274 ethanol solutions and finally for 2 min in toluene. Slides were then analyzed using a
275 transmitted light microscope at 100× objective with oil. Dark blue cells were considered as
276 positive, when lightly and very lightly stained cells were considered as negative.

277 **Hybridization *in situ* fluorescence**

278 FISH was performed on ejaculated spermatozoa from patients P0582 and fertile control.
279 Sperm cells were prepared for hybridization like previously described⁴⁶. Then, two
280 spermFISH experiments were performed using a mix of 18 spectrum blue, X spectrum green
281 and Y spectrum orange probes and a mix of 13 spectrum green and 21 spectrum orange
282 probes. Scoring was performed with a device (METAHER Metasystems®) previously
283 validated for spermFISH analysis⁴⁶ with additional verification of two trained users according
284 to strict criteria⁴⁷.

285

286 **RESULTS**

287 **Variant identification**

288 Whole exome sequencing was performed on both azoospermic and teratozoospermic infertile
289 men cohorts. In the azoospermia cohort, we identified two patients harboring bi-allelic
290 pathogenic variants in *HENMT1* we previously flagged as candidate variants³³. Patient P0109
291 carried a missense variant (c.226G>A;p.Gly76Arg) affecting an evolutionarily conserved
292 glycine at position 76 (Figure 1A-B), while patient P0272 carried a truncating variant
293 (c.456C>G;p.Tyr152Ter). In the teratozoospermia cohort, another individual (P0582) was
294 discovered carrying a homozygous truncating c.100C>T;p.Gln34Ter variant in exon 2 of
295 *HENMT1* (NM_001102592.2), and a second one (P1021) with the same variant as P0272
296 (c.456C>G;p.Tyr152Ter), findings corroborated by Sanger sequencing (Figure 1A).

297 We then performed a minigene assay to evaluate the deleterious impact of the p.Gly76Arg
298 variant of P0109 and to characterize its effect on splicing. RT-PCR was conducted on RNA
299 extracted from both non-transfected and transfected cells containing either the wild-type or
300 mutant *HENMT1* sequence in the minigene construct. In HEK cells transfected with the wild-
301 type minigene, RT-PCR produced a 622 bp amplicon indicative of normal splicing (Figure
302 S1C). In contrast, cells transfected with the mutant minigene generated a smaller 509 bp
303 amplicon, which, upon Sanger sequencing, was found to contain exclusively exonic
304 sequences from the vector, confirming aberrant splicing due to exon 4 skipping (Figure S1D).

305 The presence of spermatozoa in patient's ejaculate opens avenues for employing assisted
306 reproduction technologies in their treatment.

307

308 **Patient characterization**

309 Patient P0582, a man of 43 years during the initial examination, sought consultation for
310 infertility with his 27 years old wife at the Reproductive Biology laboratory-CECOS of Lille
311 University Hospital (France). Patient P1021, a man of 37 years during the initial examination,
312 sought consultation for infertility with his 34 years old wife at the Clinique des Jasmins of
313 Tunis (Tunisia). Medical examinations of both wives revealed no apparent abnormalities.
314 Both couples originated from Algeria and were born to unrelated parents. P1021 reported a
315 family history of infertility, with paternal and maternal cousins affected. Physical
316 examinations of both men revealed no abnormalities, and neither reported exposure to
317 tobacco, drugs, medical treatments, or any reprotoxic environments. Three sperm analyses
318 were conducted during their treatment, all diagnosing oligo-astheno-terato-necrozoospermia
319 (Table 1). Spermocytogram revealed 100% of morphological abnormalities with a
320 predominance of microzoospermic/pinhead, globocephalic/round and
321 macrozoospermic/multiple sperm, along with flagellum abnormalities (Figure 1C). These
322 findings were subsequently confirmed by TEM (Figure 1D), with no normal forms observed
323 across all samples. Notably, many immature germ cells and isolated flagella were present in
324 the ejaculate of both men (Table 1, Figure 1C).

325

326 **Patient care**

327 For P0582, three IVF-ICSI cycles resulted in the collection of 29 oocytes, 21 of which were
328 injected. This led to the formation of 11 zygotes and the development of 10 embryos, of
329 which eight were transferred. Despite achieving two biochemical pregnancies, no live births
330 occurred (Table 2). For P1021, one IVF-ICSI attempt was conducted with the addition of
331 calcium ionophore. Eleven oocytes were collected, eight were injected, resulting in four
332 zygotes, all of which developed into embryos. This led to two embryo transfers, but no live
333 birth occurred.

334

335 **Variant effect on protein**

336 *HENMT1* is located on chromosome 1 and contains eight exons encoding a predicted 393-
337 amino acid protein (Q5T8I9). To demonstrate the influence of the candidate variant on protein
338 expression and localization, we conducted immunofluorescence experiments using an anti-

339 HENMT1 antibody. The results displayed a strong signal in the neck and post-acrosomal
340 region of fertile control sperm, accompanied by a faint signal along the entire flagellum. In
341 contrast, no signal was detected in the patient's sperm cells, suggesting the absence or
342 truncation of the protein (Figure 2). Due to the patients' phenotype, very few spermatozoa
343 were available initially, and at this point, only a few spermatozoa from P0582 remained. The
344 negative IVF-ICSI outcomes, along with prior observations of nuclear abnormalities in
345 *Henmt1* knockout mice, prompted us to examine the nuclear quality of these scarce
346 spermatozoa.

347

348 **Nuclear analysis**

349 Nuclear morphology was quantitatively assessed using Nuclear Morphology Analysis
350 Software⁴⁸ (version 2.1, https://bitbucket.org/bmskinner/nuclear_morphology/wiki/Home),
351 disclosing a notable enlargement and dysmorphia in P0582's sperm nucleus compared to the
352 control sperm (Figure S2). Considering that this may be associated with aneuploidy, we
353 conducted sperm fluorescent *in situ* hybridization (spermFISH) targeting chromosomes 13,
354 18, 21, X, and Y. The findings revealed a substantial rise in diploid sperm cells (16%
355 compared to less than 1% in fertile controls) and an average aneuploidy increase of 2% across
356 all examined chromosomes (Figure 3A). Extrapolation from these data suggests that the
357 overall sperm aneuploidy for this patient could potentially reach up to 65%.

358 In the light of this result, we hypothesized that this condition might be associated with
359 substantial DNA compaction defects. Subsequently, we conducted blue aniline coloration,
360 which use the capacity of this acidic dye to binds to lysine residues to discriminate between
361 lysine-rich histones and arginine- and cysteine-rich protamines⁴⁹⁻⁵⁰. All patient's sperm cells
362 displayed a positive dark blue staining, indicating abnormal histone presence (Figure 3B).

363

364 **DISCUSSION**

365 In the present work, we report two new patients harboring bi-allelic truncating variants in
366 *HENMT1*, which results in extreme oligo-astheno-teratozoospermia phenotype. Our
367 investigations revealed altered sperm concentration, mobility, morphology, nuclear DNA
368 compaction and increased sperm aneuploidy. Multiple IVF-ICSI were conducted, yet no live
369 births ensued despite two biological pregnancies.

370 The reproductive phenotypes associated with variants in piRNA biogenesis-related genes
371 exhibit only partial overlap between mice and humans with notable phenotypic differences for
372 some genes¹¹. Most of the time, human spermatogenesis seems to allow further progression,

373 as seen in TDRD9 or FKBP6, where knockout mice exhibit meiotic arrest^{21,32} while patients
374 display severe oligozoospermia^{11,32}. HENMT1 has been, until now, an exception to this
375 pattern (as well as GPAT2^{11,51}, PIWIL2⁵²⁻⁵³ and PLD6⁵⁴⁻⁵⁵) with Henmt1-knockout mice
376 displaying an OAT phenotype³⁷ and patients displaying the more severe phenotype
377 azoospermia^{11,34}. We report here on two new patients with an OAT phenotype that is very
378 similar to the one observed in mice, thus extending the phenotypic continuum of HENMT1 in
379 men (see Table 3). It should be noted that while P1021's sperm morphology closely resembles
380 the knockout mice one, characterized by a predominance of pinhead sperm and stumpy tails,
381 P0582 exhibits a higher proportion of macrocephalic heads. Although both forms indicate
382 altered spermatogenesis, this underscores once again the intricacy of the impairments
383 resulting from piwi pathway alterations³⁴.

384 The origin of this continuum may lie in the nature of the different variants, which could affect
385 the protein in various ways—from complete loss to truncation to a compromised version of
386 the protein. Interestingly, the three patients with loss-of-function variants, which likely result
387 in at least a severe truncation of the protein, exhibited a less severe phenotype than the
388 patients with missense variants, where a less compromised protein is expected. This suggests
389 that the complete absence of the protein might be less harmful than the presence of a
390 malfunctioning protein. However, the clinical impact of genetics proves even more complex,
391 as patients with the same p.Tyr152Ter variant exhibited phenotypes of varying severity. It is
392 known that the same genetic variant can produce a spectrum of phenotypes in different
393 individuals, ranging from no detectable clinical symptoms to severe disease, even among
394 relatives⁵⁶. These variants of variable expressivity, in which the same genotype can cause a
395 wide range of clinical symptoms across a spectrum have already been reported in the context
396 of male infertility⁵⁷. This variability is believed to be driven by multiple factors, including
397 common genetic variants, regulatory region variants, epigenetic modifications, environmental
398 influences, and lifestyle⁵⁶.

399 Beyond the phenotype, the key question revolves around the potential for a therapeutic
400 response in patients. Although the observed phenotypes fall short of the severity associated
401 with azoospermia, it still presents a distinctive challenge. Indeed, the therapeutic approach for
402 severe OAT phenotype involves IVF-ICSI, necessitating the selection of sperm with the most
403 normal morphology possible. However, neither of the two patients exhibited any
404 morphologically normal heads. Pinhead sperm, which possess minimal to no DNA content,
405 and macrocephalic sperm which contain excessive DNA, are both incompatible with
406 successful pregnancies⁵⁸⁻⁶⁰. Given the impracticality of using these sperm for injection, the

407 operator has no choice but to utilize the remaining globocephalic spermatozoa (which explain
408 the use of the artificial oocyte activation treatment during P1021 IVF-ICSI attempt).
409 However, globozoospermic sperm have been associated with altered genome packaging,
410 DNA damage and epigenetic modifications⁶¹⁻⁶³ compromising embryo development and
411 successful pregnancy. The aneuploidy increase and the DNA compaction anomalies we
412 reported here for P0582 underscore the need for future studies to investigate the presence of
413 genetic and epigenetic anomalies in the sperm of HENMT1 variant carriers and their potential
414 transmission to the offspring.

415 As of now, the medical counseling and management for carriers of HENMT1 variants appear
416 to hold an unfavorable prognosis. No successful IVF treatments have been reported for these
417 carriers, and concerns persist regarding the potential risk of transmission of genetic and
418 epigenetics anomalies. Moreover, the variability observed among individuals emphasizes the
419 need for caution in applying findings from one patient to another. A broader representation of
420 carrier patients in the literature is required, as a successful pregnancy report would
421 significantly shift the current pessimistic prognosis. Without additional case descriptions, it is
422 also premature to extrapolate this unfavorable prognosis to all carriers of deleterious variants
423 in piRNA biogenesis-related genes, despite the absence of documented successful pregnancies
424 in carrier patients thus far.

425 In summary, further studies are required to establish guidelines for managing patients with
426 mutations in HENMT1 or other piwi pathway genes. Nonetheless, our findings offer valuable
427 initial insights to inform patients, despite the unfavorable prognosis.

428

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431

432 **Authors' roles**

433 Z.W., A-L.B., C.C. and G.M. analysed the data and wrote the manuscript; C.Ca., M.B., V.S.,
434 N.T-M., Z-E.K., F.P., C.C. and G.M. performed and analysed the genetic data; Z.W., E.F., J-
435 P.H. an G.M. performed the sperm analysis and the IF experiments; A-L.B. and A.B.
436 performed the electron microscopy experiments; A-L.B., P.P., S.F.B.M., R.Z. and Z-E.K.
437 provided clinical samples and data; C.C. and G.M. designed the study, supervised all
438 molecular laboratory work, had full access to all of the data in the study and took
439 responsibility for the integrity of the data and its accuracy. All authors contributed to the
440 report.

441

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447

448 **Conflict of interest**

449 The authors declare no conflict of interest.

450

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633

634 **Figure legends**

635

636 Figure 1. (A) Electropherograms from Sanger sequencing indicating the presence of the
637 c.100C>T variant in patient P0582 and c.456C>G variant (NM) in patient P1021
638 (NM_001102592.2), as well as the two previously identified variants c.226G>A and
639 c.456C>G in patients P0109 and P0272, respectively. The positions of these observed variants
640 are indicated on the structure of the canonical transcript of *HENMT1*, and their impact on the
641 HENMT1 protein is visually represented using modeling from SWISS-MODEL for the
642 truncated and mutated proteins. (B) Conservation and alignment of *HENMT1* sequences from
643 various orthologs surrounding the missense variant identified in the patient P0109. (C) Light
644 microscopy analysis of spermatozoa obtained from a fertile control individual and patient
645 P0582. All spermatozoa from patient P0582 exhibited abnormal morphology, including
646 macrocephalic multi-flagellated sperm (white arrow), globozoospermic sperm (black arrow)
647 and pinhead sperm (blue arrow) along numerous immature germ cells (far right panel). Scale
648 bar = 10 μ m. (D) Transmission electron microscopy analysis of sperm cells from patient
649 P0582, revealing abnormal sperm with multiple abnormal nuclei.

650

651 Figure 2. HENMT1 immunostaining of sperm cells obtained from a fertile control individual
652 and patient P0582 with the c.100C>T variant. Sperm cells were subjected to labeling using an
653 anti-HENMT1 antibody (red), an anti-acetylated tubulin antibody (green), and DAPI (blue)
654 for nuclear staining. In the fertile control, the HENMT1 signal was detected with high
655 intensity at the neck and post-acrosomal region of sperm heads, and a discrete signal
656 throughout the entire length of the flagellum. Conversely, in all sperm from patient P0582,
657 regardless of their morphology, the HENMT1 signal was completely absent. No antibody
658 signals were detected in the negative control. Scale bars: 10 μ m.

659

660 Figure 3. (A) spermFISH analysis of P0582 spermatozoa using a mix of 18 spectrum blue, X
661 spectrum green and Y spectrum orange probes and a mix of 13 spectrum green and 21
662 spectrum orange probes. Histograms display the results for each experiment (left) and some
663 illustrations are presented (right). (B) Control and patient P0582 spermatozoa were stained
664 with aniline blue. In contrast to the control, all of the patient's sperm exhibited a strong blue
665 staining.

666

667 Figure S1. Minigene Splicing Reporter Assay for HENMT1 Exon 4. (A) Amplification
668 Strategy for HENMT1 Exon 4. The genomic HENMT1 sequence encompassing exon 4 was
669 amplified, including flanking intronic regions, to facilitate splicing analysis. (B) Insertion
670 Strategy into the pCineo Minigene Vector. HENMT1 exon 4 was inserted into the pCineo
671 minigene vector in place of exon 9 of the CASQ2 gene. This replacement allows assessment
672 of exon 4 splicing within the minigene construct. (C) RT-PCR Gel Electrophoresis. Results of
673 RT-PCR show a smaller band in cells transfected with the mutated minigene compared to the
674 control, indicating a splicing alteration. (D) Sanger Sequencing of RT-PCR Products.
675 Chromatogram from Sanger sequencing confirms in HENMT1_exon 4 skipping, as
676 demonstrated by the direct junction of CASQ2 exons 8 and 10 in cells transfected with the
677 mutated minigene.

678

679 Figure S2. Nuclear morphology analysis of control (blue) and P0582 (yellow) sperm head
680 obtained thanks to the Nuclear morphology software (version 1.19.2,
681 https://bitbucket.org/bmskinner/nuclear_morphology/wiki/Home), according to the analysis
682 method described in Skinner et al., 2019 (doi:10.1093/biolre/ioz013). Consensus nuclear
683 outlines (left panel) and angle profiles (bottom) of control and patient P0582. The x axis
684 represents an index of the percentage of the total perimeter as measured counterclockwise
685 from the apex of the sperm nucleus and the y axis represents the interior angle measured
686 across a sliding window centered on each index location. Statistical significance of
687 differences between populations were assessed by the software, applying a Mann-Whitney U
688 test with Bonferroni multiple testing correction. p-values were considered significant when
689 inferior to 0.05.

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TABLES

Table 1. Detailed semen parameters of patients P0582 and P1021.

Date	P0582			P1021			Normal range
	17/09/2018	20/11/2018	18/11/2020	30/01/2023	08/07/2023	14/07/2023	
Specimen characteristics							
Abstinence duration (days)	3	3	4	4	7	4	2-7
Volume (mL)	4,5	0,87	5,1	3	5	1,2	>1,5
pH	7,9	8,1	7,9	na	na	na	>7,2
Viscosity	weak	weak	normal	normal	normal	normal	
Numeration							
Sperm count (10 ⁶ /ml)	0,3	0,185	0,8	0,14	0,2	0,2	>15
Total numeration (10 ⁶ /ejaculate)	1,35	0,161	4,08	0,42	1	0,24	>39
Round cells (10 ⁶ /ml)	2,65	2,1	1,9	0,28	0,66	na	<5
Polynuclear (10 ⁶ /ml)	1,64	0,42	0,67	na	na	na	<1
Motility							
Progressive sperm (%)	0	0	0	0	1	0	>32
Non progressive sperm (%)	0	0	0	0	1	0	P+NP>40
Immotile sperm (%)	100	100	100	100	99	100	<60
Other tests							
Vitality (% alive)	10	10	26	0	5	4	>58
Morphology							
Normal (%)	0	0	0	0	0	0	>4
Abnormal (%)	100	100	100	100	100	100	<96
<hr/>							
Head anomaly (%)	na	na	100	100	100	na	
<i>Elongated (%)</i>	na	na	5	4	0	na	
<i>Thinned (%)</i>	na	na	0	0	0	na	
<i>Microcephalic/Pinhead (%)</i>	na	na	19	64	70	na	
<i>Globocephalic/Round (%)</i>	na	na	34	40	50	na	
<i>Macrocephalic/Multiple (%)</i>	na	na	47	16	20	na	
<i>Abnormal base (%)</i>	na	na	65	36	48	na	
<i>Malformed acrosome (%)</i>	na	na	100	100	100	na	
Intermediate piece anomaly (%)	na	na	38	28	34	na	
<i>Cytoplasmic residue (%)</i>	na	na	11	4	8	na	
<i>Thin (%)</i>	na	na	0	0	0	na	
<i>Angulation (%)</i>	na	na	28	24	26	na	
Flagellum anomaly (%)	na	na	95	74	92	na	
<i>Absent (%)</i>	na	na	5	0	0	na	

<i>Short (%)</i>	<i>na</i>	<i>na</i>	<i>13</i>	<i>4</i>	<i>16</i>	<i>na</i>
<i>Irregular size (%)</i>	<i>na</i>	<i>na</i>	<i>47</i>	<i>48</i>	<i>64</i>	<i>na</i>
<i>Coiled (%)</i>	<i>na</i>	<i>na</i>	<i>7</i>	<i>0</i>	<i>2</i>	<i>na</i>
<i>Multiple (%)</i>	<i>na</i>	<i>na</i>	<i>23</i>	<i>22</i>	<i>10</i>	<i>na</i>

Table 2. ICSI outcomes with sperm cells from the patient P0582 and P121.

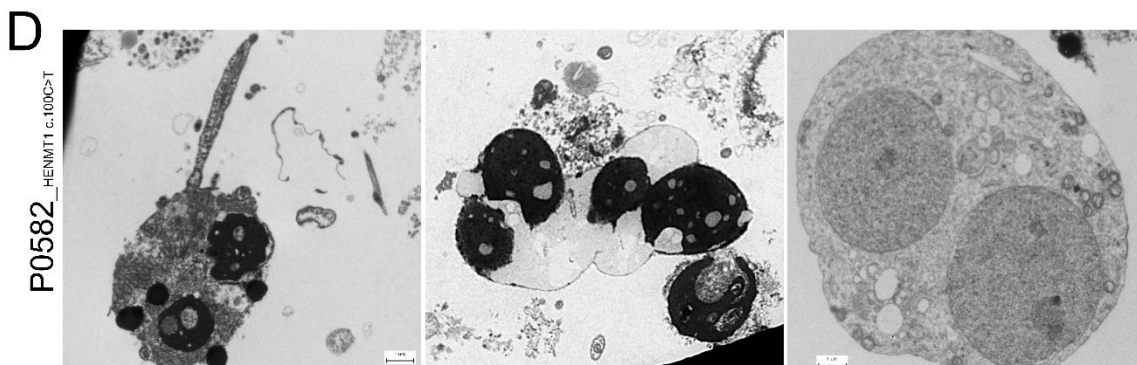
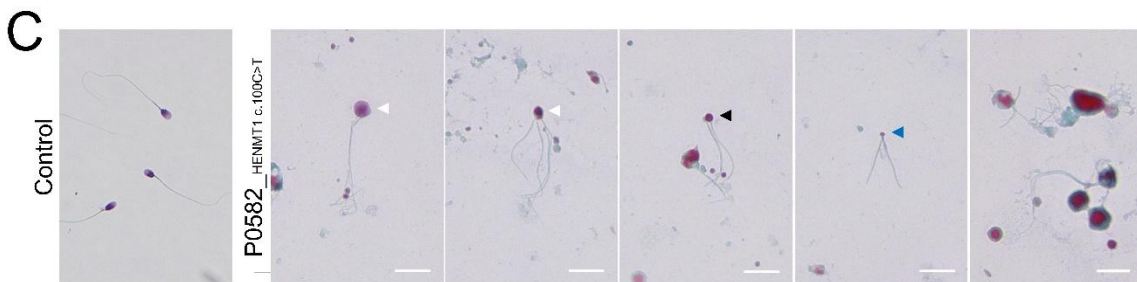
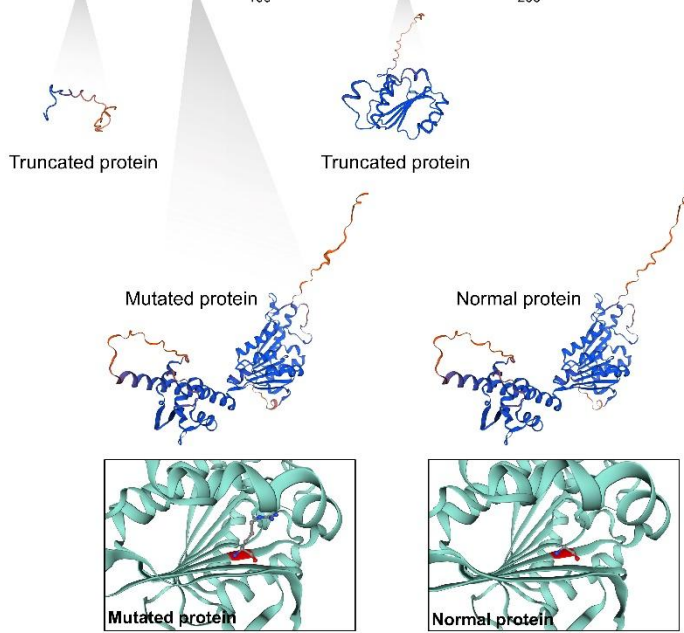
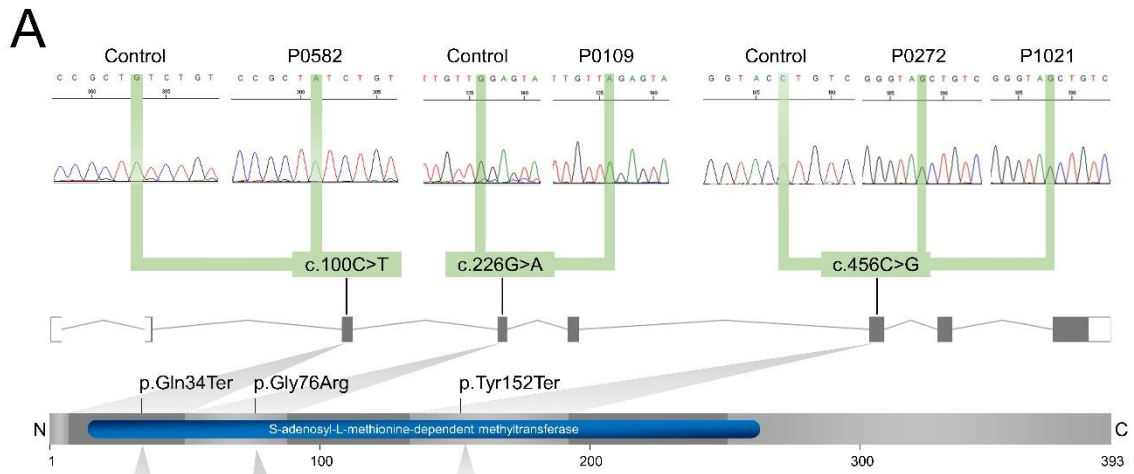
IVF-ICSI	P0582 n°1	P0582 n°2	P0582 n°3	P1021 n°1
Date	10/2019	05/2021	11/2022	07/2023
Use of calcium ionophore	No	No	No	Yes
Collected oocytes	14	11	4	11
Injected oocytes	8	10	3	8
Zygotes	3	6	2	4
Unfertilised	4	4	1	3
Lysed	1	0	0	1
Day-2/3 embryos	3	6	1	4
Day-2/3 embryos with <10% fragmentation and expected cell stage	1	2*	0	2
Transfers at D2 or D3 (fresh or frozen)	3	2	0	2
Transfer at blastocyst stage (fresh or frozen)	0	2	1**	0
Biochemical pregnancy	0	1	1	0
Ultrasound pregnancy	0	0	0	0
Childbirth	0	0	0	0

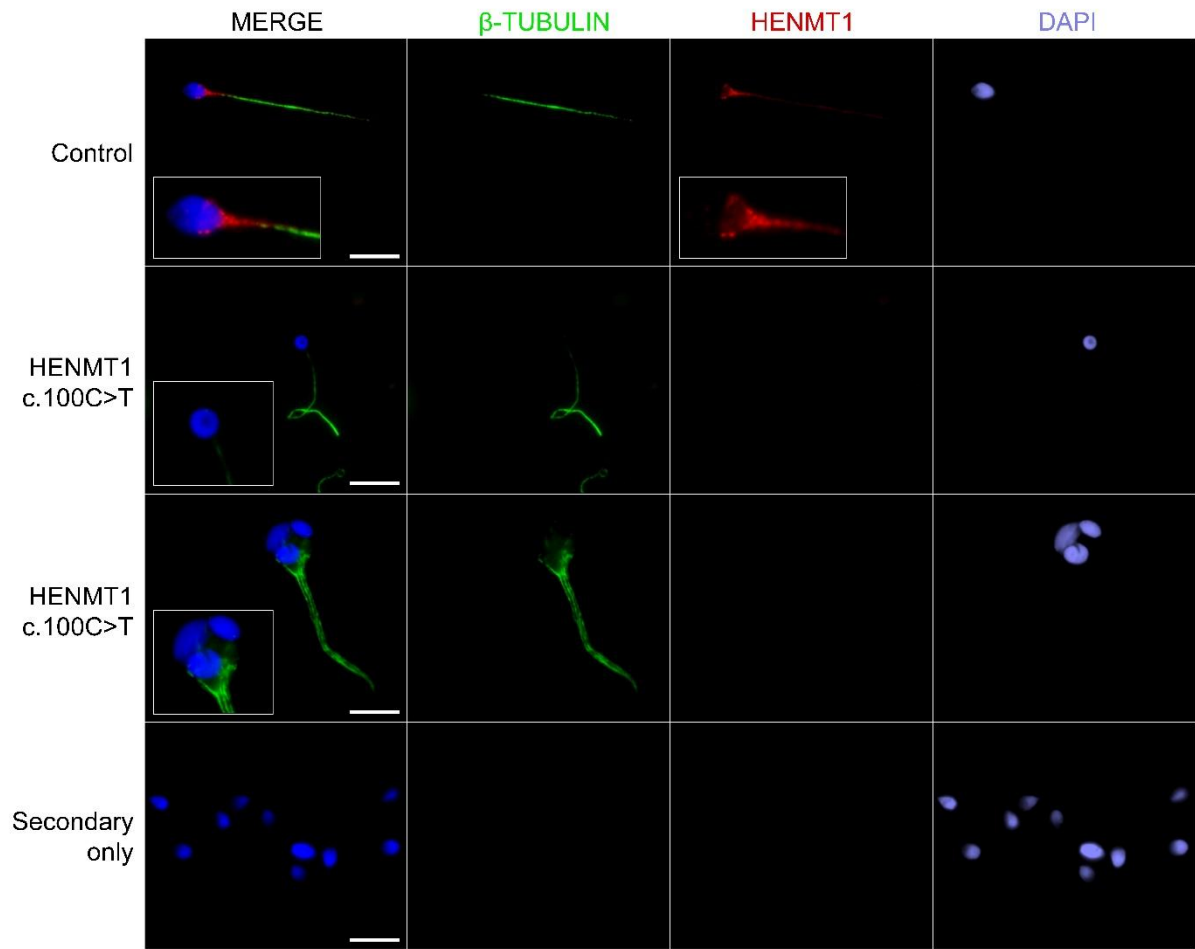
* of the four supernumerary that were kept in culture until Day-6 because they exhibited 4 cell on Day 3, two embryos evolved until the blastocyst stage, graded B4BB according to Gardner's classification (Gardner and Schoolcraft, 1999). The two blastocysts were frozen for subsequent embryo transfer. ** the embryo was transferred at the morula compacted stage at Day-5

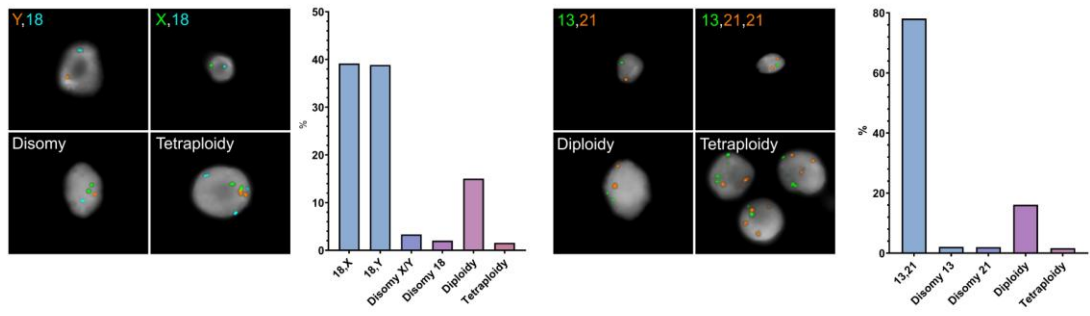
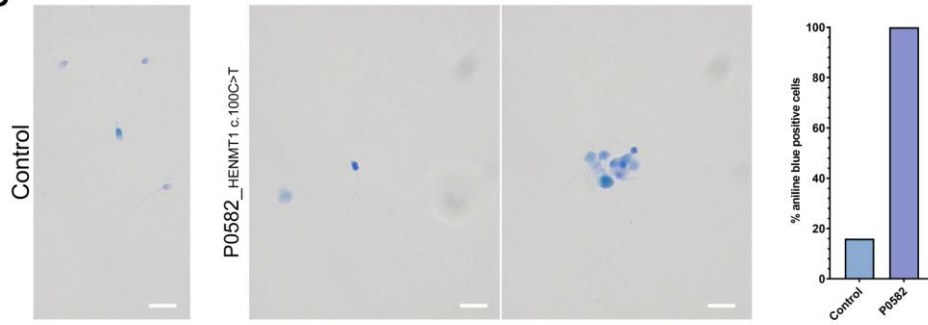
Table 3. Patients' summary.

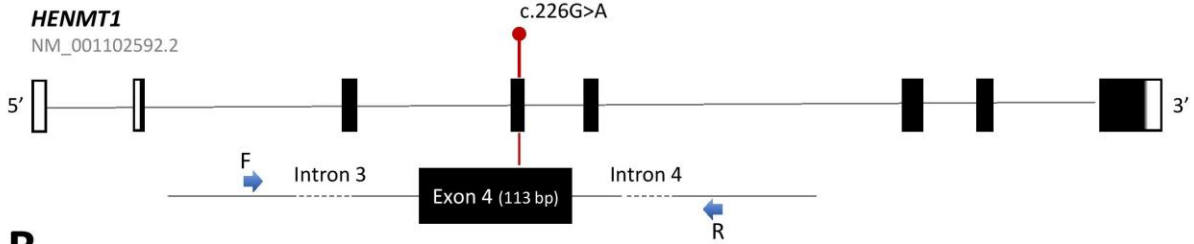
Patient	Variant	Consequence	Exon	Phenotype	Testis sperm retrieval	Childbirth	Reference
P0582	c.100C>T; p.Gln34Ter	stop gained	2/7	Severe OAT	-	No	This study
P0109	c.226G>A; p.Gly76Arg	missense	3/7	Azoospermia (MeA)	Negative	No	Kherraf et al., 2022
M3079	c.400A>T ; p.Ile134Leu	missense	5/7	Azoospermia (RsA)	Negative	No	Stallmeyer et al., 2024
P0272	c.456C>G; p.Tyr152Ter	stop gained	5/7	Azoospermia (Hypo)	Positive	No	Kherraf et al., 2022
P1021	c.456C>G; p.Tyr152Ter	stop gained	5/7	Severe OAT	-	No	This study

MeA : Meiotic arrest ; RsA : Round spermatid arrest ; Hypo : Hypospermatogenesis ; OAT : Oligo-astheno-teratozoospermia

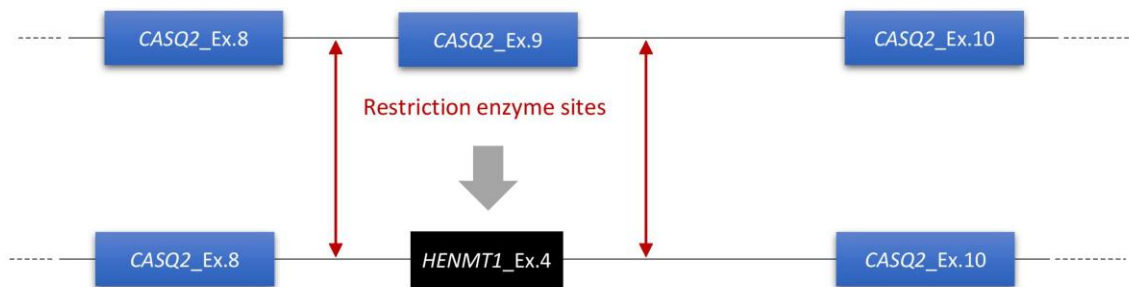
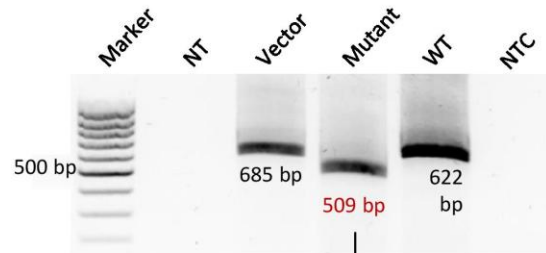
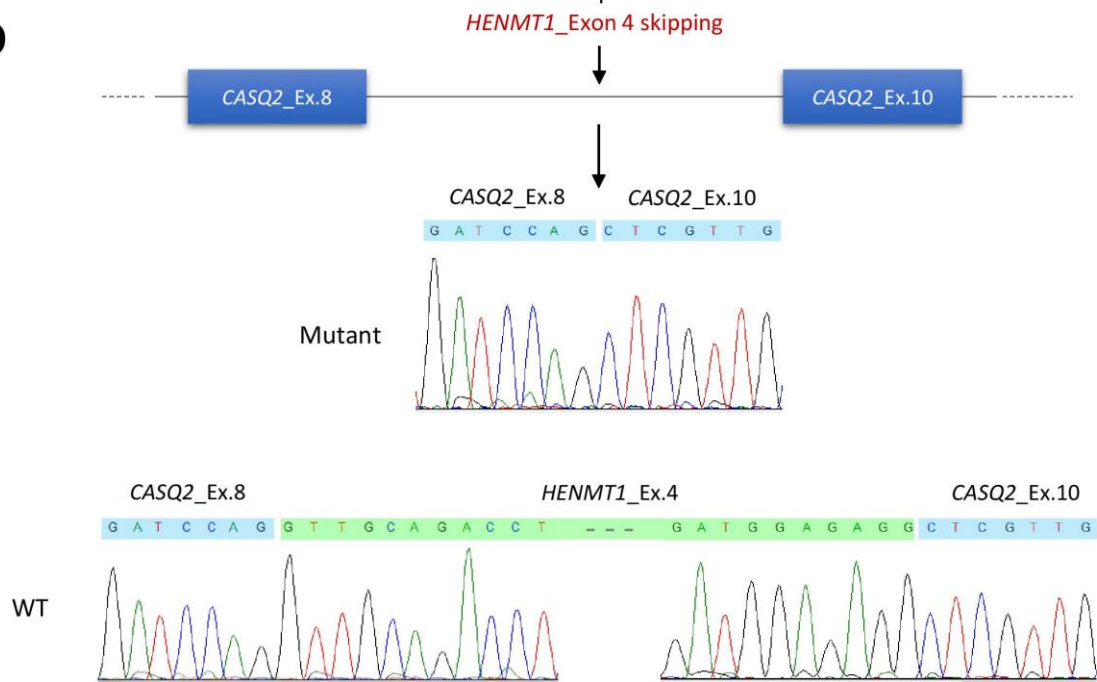


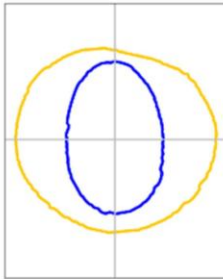


A**B**

A**B**

pCI-neo Mammalian Expression Vector

**C****D**



Parameters	Control	Patient	Statistical values
Area 95% CI (μm^2)	$66,40 \pm 0,63$	$3186,83 \pm 104,78$	$>0,0001$
Perimeter 95% CI (μm^2)	$31,95 \pm 0,18$	$57,02 \pm 5,15$	$>0,0001$
Max feret mean 95% CI	$11,87 \pm 0,08$	$18,66 \pm 1,68$	$>0,0001$
Circularity 95% CI	0,81	0,87	$>0,0001$
Min diameter 95% CI (μm)	$7,14 \pm 0,04$	$15,11 \pm 1,23$	$>0,0001$
Ellipticity 95% CI	$0,66 \pm 0,01$	$0,97 \pm 0,03$	$>0,0001$
Aspect ratio 95% CI	$1,56 \pm 0,01$	$1,05 \pm 0,03$	$>0,0001$
Elongation 95% CI	$0,21 \pm 0,01$	$0,02 \pm 0,01$	$>0,0001$

