

Mas receptor signalling supports spermatogenesis and protection against diabetic testicular injury in mice

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Article

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Abstract

Background

The angiotensin-(1–7)/Mas receptor (MasR) axis has been implicated in male reproduction, but its pharmacological modulation in metabolic testicular injury remains incompletely characterised. We combined human sperm analyses, genetic and pharmacological loss-of-function experiments, and AVE0991 treatment in a diabetic mouse model.

Methods

Sperm samples from 10 normozoospermic and 10 asthenozoospermic men were analysed in assay-specific subsets. Acute AVE0991 responses were examined ex vivo. Reproductive endpoints were assessed in global *Mas1*-null mice, adult wild-type mice receiving A779 (0.05 mg/kg/day), and streptozotocin-induced diabetic mice receiving AVE0991 (0.6 mg/kg/day) for six weeks. AVE0991 was also administered to non-diabetic *Mas1*-null mice. Continuous endpoints were analysed using t-tests or analysis of variance with multiplicity adjustment where applicable; mating outcomes were descriptive.

Results

MasR protein abundance and immunofluorescence signal were lower in the small asthenozoospermic sperm subsets. RNA assays showed lower relative *MAS1* signals, with interpretation limited by RNA composition and assay specificity. AVE0991 at 0.1 or 1 nM transiently increased progressive motility, whereas 10 nM showed a decrease. A779 attenuated the motility response and the calcium response. AVE0991 was also associated with fewer acrosome-reacted spermatozoa and higher phosphorylated PI3K and AKT signals. *Mas1*-null mice and A779-treated wild-type mice showed impaired seminiferous architecture and reduced sperm parameters. In diabetic mice, AVE0991 partially improved histology and sperm concentration and motility without restoring testicular AKT phosphorylation. No comparable improvement was evident in the non-diabetic knockout arm.

Conclusions

The findings support a role for MasR signalling in normal spermatogenesis and suggest a preclinical benefit of AVE0991 in diabetic testicular injury. The human findings are exploratory. The study does not establish a PI3K/AKT-mediated protective mechanism or definitively demonstrate receptor dependence of the diabetic treatment response.

1. Background

Male reproductive dysfunction contributes substantially to infertility, and population studies have documented declining sperm counts [1, 2]. Clinical assessment must account for heterogeneous acquired and genetic causes, while treatments that improve underlying sperm production or function remain limited [3, 4, 5]. Defining modifiable regulators of the testicular and sperm microenvironments is therefore relevant to the development of new therapeutic approaches.

Redox regulation is important to germ-cell integrity and epididymal sperm maturation [6, 7]. The concept of male oxidative stress infertility has provided a framework for considering oxidative injury in otherwise unexplained cases [8]. Antioxidant trials and systematic reviews, including the 2019 Cochrane review and its 2022 update, have nevertheless left uncertainty about the magnitude and reliability of clinical benefit [9, 10, 11]. These findings motivate investigation of defined biological targets without assuming that a redox-related intervention necessarily improves fertility.

The renin–angiotensin system (RAS) is present in the male reproductive tract [12, 13]. Angiotensin-(1–7) acts through the G protein-coupled Mas receptor, encoded by *MAS1* in humans and *Mas1* in mice [14]. The classical angiotensin II/AT1 receptor pathway regulates vascular, inflammatory and growth-related responses [15]; the ACE2/angiotensin-(1–7)/Mas axis can counterbalance selected actions of that pathway and has effects extending beyond the cardiovascular and renal systems [16, 17]. MasR has been identified in human testis and spermatozoa, and angiotensin-(1–7) has been associated with changes in human sperm motility and AKT phosphorylation [18, 19]. Leal and colleagues previously described disrupted spermatogenesis in Mas-deficient mice and after chronic A779 treatment in rats [20].

The present study extends that work by combining adult pharmacological antagonism and germline deletion in mice with AVE0991 treatment in a streptozotocin-induced diabetic model. Human sperm analyses provide clinical context and exploratory evidence of acute pharmacological responses. Our principal question was whether these complementary approaches support a role for MasR in spermatogenic function and whether AVE0991 improves testicular endpoints under diabetic conditions. We evaluated the phenotypic and molecular responses without assuming that acute signalling in human sperm explains chronic effects in mouse testis.

2. Materials and Methods

2.1 Clinical cohorts and ethics

The Ethics Committee of Beijing Chao-Yang Hospital approved all human protocols (Approval 2023-Ke-759), in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

The semen cohort comprised men with asthenozoospermia (AS, n = 10) and normozoospermic donors (NC, n = 10) recruited between January and December 2022 (age ≥ 18 years; no known reproductive genetic disorder, cryptorchidism/orchitis, active genital infection or recent gonadotoxic/hormonal

treatment). Samples were obtained by masturbation after five days of ejaculatory abstinence and liquefied at 37°C for 30 min. The original clinical group labels were retained. The motility limits used in the original records were progressive motility < 32% and/or total motility < 40% for AS, corresponding to the fifth-edition WHO reference limits [21]. All NC participants had progressive motility ≥ 32% and total motility ≥ 40%, and all listed sperm concentrations exceeded 15 × 10⁶/mL. The NC label denotes the recorded normozoospermic comparator group; it does not imply that every semen parameter was independently verified as within its reference interval or that fertility was established. These motility limits were used to describe the original grouping, not as a stand-alone diagnosis of fertility or infertility. Detailed morphology measurements and systematic information on body mass index, smoking and comorbidities were unavailable for adjustment. Cohort characteristics are summarised in Table 1, and participant-level records are provided in Supplementary Table S2.

Table 1
Clinical cohort characteristics

Characteristic	NC (n = 10)	AS (n = 10)
Age (years)	34.80 ± 6.96	33.20 ± 2.49
Semen volume (mL)	4.36 ± 2.25	4.35 ± 0.96
Sperm concentration (×10 ⁶ /mL)	61.42 ± 13.80	55.90 ± 14.92
Total sperm count (×10 ⁶ /ejaculate)	270.26 ± 149.97	242.08 ± 73.25
Progressive motility (%)	42.34 ± 9.74	21.73 ± 4.43
Total motility (%)	53.32 ± 11.28	29.51 ± 6.93

Values are mean ± sample SD from 20 participant-level records. NC and AS denote the recorded normozoospermic comparator and asthenozoospermic groups, respectively. The original grouping was retained; the AS motility limits were progressive motility < 32% and/or total motility < 40%, corresponding to the fifth-edition WHO reference limits [21]. The NC label does not establish fertility or confirm that every semen parameter was within its reference interval. Total sperm count uses the recorded participant-level values. Means and sample SDs were calculated from the listed records and rounded to two decimal places. No baseline hypothesis tests are reported.

Assays were performed on subsets of this cohort: sperm RNA sequencing (n = 3 per group), targeted RT-qPCR (n = 6 per group), Western blotting (n = 3 per group), and immunofluorescence (n = 3 per group). Each Western blot lane and immunofluorescence data point represents an independent donor. Subsets were defined by available RNA and protein yield after preparation, without reference to measured outcomes. The protein and immunofluorescence comparisons were exploratory; assay-specific subsets were not treated as independent clinical cohorts.

Human testicular tissue was examined as a single illustrative case pair rather than as a cohort. Tissue with preserved spermatogenesis was obtained from non-tumorous parenchyma distal to the lesion in

one man undergoing orchiectomy, with active spermatogenesis confirmed histologically. Tissue with spermatogenic failure was obtained during diagnostic sperm extraction in one man with severe oligozoospermia. Detailed pretreatment and exclusion criteria for this case pair were not fully documented. Three non-consecutive sections were examined per case. One portion of each specimen was snap-frozen and the remainder was fixed in modified Davidson's fluid [22]. Because only one case was available in each category, these sections are presented solely as a representative illustration of MasR localisation in seminiferous epithelium; fields within a section are technical observations of a single individual, so no between-group quantification was performed and no statistical test was applied.

2.2 Animals, ethical oversight and in vivo intervention

Animals and husbandry

Male C57BL/6J mice aged 8–10 weeks were obtained from GemPharmatech (Nanjing, China). Heterozygous *Mas1*^{+/-} mice (Strain T015753, GemPharmatech) were interbred to generate homozygous *Mas1*^{-/-} offspring. Genotypes were confirmed by PCR of tail DNA, and only homozygous males were used. Animals were housed under specific pathogen-free conditions at 22 ± 2°C and 50–60% relative humidity, with a 12-h light/dark cycle and ad libitum access to food and water, with no more than five animals per cage. All procedures complied with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (Approval JLHK-20240604-01).

Welfare, allocation and exclusions

General health, coat condition, posture, spontaneous activity, food and water intake were monitored daily; body weight was recorded weekly. Humane endpoints were inability to reach food or water, a persistently moribund appearance, or a loss of at least 20% of peak body weight. Animals reaching an endpoint were killed immediately and excluded from analysis. Animals were allocated after genotype confirmation and, for diabetic groups, after confirmation of hyperglycaemia, using a computer-generated random sequence. Investigators performing histological scoring, acrosome scoring and hormone assays were blinded to group allocation; investigators performing surgery, dosing and sample collection were not blinded. Prespecified exclusions were death before completion, failure to meet the diabetic criterion, documented pump failure, and wound dehiscence.

Diabetes induction

Type 1 diabetes was induced by intraperitoneal injection of streptozotocin (Sigma-Aldrich, Cat# S0130) at 50 mg/kg/day on three consecutive days (cumulative dose 150 mg/kg), using a repeated low-dose regimen adapted from Furman [23]. Streptozotocin was dissolved in 0.1 M sodium citrate buffer (pH 4.5) immediately before use and injected within 15 min of preparation after a 4-h fast. Control animals received an equal volume of citrate buffer. Tail-vein glucose was measured beginning seven days after the final streptozotocin injection. Animals with fasting glucose ≥ 16.7 mmol/L on two measurements 48 h apart were considered diabetic, and the six-week intervention began after the second confirmatory measurement.

Experimental groups

Animals were allocated to seven groups: (1) NC, wild-type mice receiving vehicle; (2) A779, wild-type mice receiving A779 at 0.05 mg/kg/day; (3) KO, *Mas1*^{-/-} mice receiving vehicle; (4) T1DM, diabetic mice receiving vehicle; (5) T1DM + AVE0991, diabetic mice receiving AVE0991 at 0.6 mg/kg/day; (6) KO + AVE0991, *Mas1*^{-/-} mice receiving AVE0991 at 0.6 mg/kg/day; and (7) T1DM + AVE0991 + LY294002. Group 7 contributed molecular endpoints only. The KO and KO + AVE0991 groups were non-diabetic; the design did not include a diabetic *Mas1*-null treatment comparison. The NC and KO labels denote the comparator groups used in the respective figure sets; cross-figure comparisons were not treated as additional independent replication. The study components are outlined in Fig. 1.

A1, Human semen cohort: ten recorded normozoospermic comparators, NC, and ten men with asthenozoospermia, AS, retaining the fifth-edition WHO motility grouping described in Section 2.1. Subsets per group comprised three donors for RNA sequencing, six for *MAS1* RT-qPCR, and three each for MasR Western blotting and immunofluorescence. A2, Separate illustrative testicular case pair, one case per category. B1, Vehicle or AVE0991 at 0.1, 1 or 10 nM; CASA at 0, 10, 30 and 60 min. B2, A779 at 1 or 10 nM for 15 min before AVE0991 at 0.1 nM, with vehicle, agonist-alone and A779-alone comparators. B3, Acrosomal status after 60 min. B4, Fluo-4 AM calcium imaging after a 60-s baseline, with AVE0991 at 0.1 nM, ATP at 100 μ M, vehicle or A779 pretreatment; acquisition at 1 Hz. B5, GAPDH-normalised protein signals after 30 min of AVE0991 at 0.1 nM; no A779 arm. Motility used five donors per comparison; acrosome and calcium assays used three per experiment; protein assays used three per condition. C, *Mas1* deletion or six-week A779 infusion at 0.05 mg/kg/day. D1, Streptozotocin at 50 mg/kg/day intraperitoneally for three days; fasting glucose $\geq$ 16.7 mmol/L confirmed twice, 48 h apart, before intervention. D2, Non-diabetic comparators. D3, LY294002 at 25 mg/kg intraperitoneally every 48 h, days 1–41; molecular endpoints only. All AVE0991 groups received 0.6 mg/kg/day for six weeks via one subcutaneous ALZET 2006 pump. Main sperm/hormone endpoints used six animals per group; tissue/molecular endpoints used three. Mating summaries were descriptive. Icons do not denote sample size; donors and animals were biological units.

Continuous infusion

A779 (MedChemExpress, Cat. HY-P0216) and AVE0991 sodium salt (MedChemExpress, Cat. HY-15778A) were delivered by continuous subcutaneous infusion using ALZET osmotic minipumps (Model 2006; DURECT Corporation, Cupertino, CA, USA; nominal reservoir volume 200 μ L, nominal flow rate 0.15 μ L/h, nominal duration 42 days). Pumps were implanted dorsally under isoflurane anaesthesia after a single subcutaneous dose of meloxicam (5 mg/kg).

Pump-fill concentrations were calculated as 0.347 mg/mL for A779 and 4.167 mg/mL for AVE0991 to target 0.05 and 0.6 mg/kg/day, respectively, at a design body weight of 25 g and the nominal flow rate of 0.15 μ L/h. These are nominal dose calculations, not measured systemic exposures. A779 was formulated in sterile 0.9% sodium chloride; AVE0991 was formulated in 10% (v/v) dimethyl sulfoxide and

90% (v/v) polyethylene glycol 300. Vehicle administration is described according to the experimental protocol.

Pumps were primed in sterile saline at 37°C for 60 h before implantation. A single pump per animal covered the six-week intervention. Because infusion delivered a fixed mass per unit time, the dose expressed per kilogram varied inversely with body weight. No measured exposure range or quantitative equivalence between in vivo dosing and ex vivo concentrations is reported.

The 42-day intervention spans approximately one mouse spermatogenic cycle and overlaps with epididymal transit. It therefore exposes germ cells across multiple developmental stages but does not establish that every cauda epididymal spermatozoon collected at endpoint was exposed from the spermatogonial stage onward.

PI3K inhibition

LY294002 (MedChemExpress, Cat# HY-10108) was dissolved in dimethyl sulfoxide and diluted in sterile saline to a final dimethyl sulfoxide concentration of 5% (v/v). It was administered by intraperitoneal injection at 25 mg/kg every 48 h from day 1 through day 41, for 21 doses.

Fertility testing and blood collection

Each male was co-housed with two wild-type C57BL/6J females aged 8–10 weeks for seven days. Pregnancy proportion was defined as the number of pregnant females divided by the number of females paired. Litter size denotes offspring per delivered litter; Figs. 5G and 6G each include eight litters per group. Exact mating denominators and sire-to-litter links were not available for analysis; the plotted pregnancy proportions and litter-size summaries are therefore reported descriptively. Litters from the same sire are clustered, and no independent-litter statistical test is used to infer treatment effects on fertility.

At the end of the intervention, mice were anaesthetised with isoflurane and killed by decapitation. Trunk blood was collected between 09:00 and 11:00. Serum was separated within 30 min and stored at – 80°C in single-use aliquots.

Sample size and experimental unit

Serum hormones and cauda epididymal sperm concentration and motility were assessed in six animals per group. Histological, immunohistochemical, testicular RT-qPCR and Western blot endpoints were assessed in three animals per group. The LY294002 subgroup contributed molecular endpoints from three animals. The animal was the experimental unit; tubules, sections, fields and cells were nested technical observations.

2.3 Human sperm preparation and ex vivo pharmacology

Semen was liquefied at 37°C for 30 min. Spermatozoa were prepared by centrifugation at $300 \times g$ for 10 min and washed twice in Hanks' balanced salt solution. Prepared spermatozoa ($1 \times 10^7/\text{mL}$) were incubated in capacitation medium at 37°C in 5% CO_2 .

Cells were treated with AVE0991 at 0.1, 1 or 10 nM or with A779 at 1 or 10 nM. In antagonism experiments, spermatozoa were pre-incubated with A779 for 15 min before addition of AVE0991 at 0.1 nM. An A779-only condition at 1 nM was included. Motility was recorded at 0, 10, 30 and 60 min ($n = 5$ independent donors per comparison). AVE0991 at 0.1 nM was used in the subsequent acrosome-reaction, calcium-imaging and signalling experiments.

AVE0991 was prepared as a 10 mM stock in dimethyl sulfoxide (Sigma-Aldrich, Cat. D2650, sterile-filtered BioReagent), and A779 as a 10 mM stock in sterile water. Intermediate stocks were prepared by serial dilution in capacitation medium immediately before use. The final dimethyl sulfoxide concentration was matched across conditions and did not exceed 0.0002% (v/v) in any condition. The selected concentrations constitute a narrow-range screen; no EC_{50} , curve shape, maximal effect or therapeutic window was inferred.

2.4 Computer-assisted sperm analysis

Sperm motility was assessed using a WLJY-9000 colour sperm quality analyser (WeiLi New Century Science and Technology Development Co., Beijing, China) with the manufacturer's reusable 10- μm ruby counting chamber. The chamber and stage were maintained at 37°C. Five microlitres of sample was loaded and allowed to settle for 30 s.

Fields were acquired using a 10 $\times$ phase-contrast objective at 30 frames/s, with 30 frames recorded per field and a minimum track length of 15 frames. At least five non-overlapping fields and two independent aliquots were analysed per sample at each time point; aliquot results were averaged before biological-unit analysis. Grade a and grade b were defined by straight-line velocity thresholds of $\geq 25 \mu\text{m/s}$ and 5 to $< 25 \mu\text{m/s}$, respectively. The instrument-specific grade labels were retained: grades a + b were mapped to progressive motility (PR), and grades a + b + c to total motility (PR plus non-progressive motility). These instrument categories were not used to reclassify the clinical cohort. Debris, round cells, aggregates and incomplete tracks were excluded. Donors and animals were the statistical units; fields and spermatozoa were not counted as independent replicates.

The 10- μm chamber may constrain flagellar movement relative to deeper chambers; therefore, no velocity or lateral-head-displacement parameters are interpreted. Because the instrument's upper velocity detection limit is 180 $\mu\text{m/s}$, only total motility is reported for mouse spermatozoa. Mouse sperm concentration and motility were measured in cauda epididymal spermatozoa released into pre-warmed medium.

2.5 Acrosome reaction

Acrosomal status was assessed using a dual-fluorescence kit containing PNA-rhodamine and Hoechst 33258 (GENMED, Cat# GMS14015.1.2). Spermatozoa were exposed to AVE0991 at 0.1 nM, A779 at 1 nM applied 15 min before AVE0991, or vehicle for 60 min before fixation. Uniform bright acrosomal-cap fluorescence indicated an intact acrosome; patchy or absent fluorescence indicated an acrosome-reacted state. The acrosome-reaction rate was reported without subtraction of the spontaneous rate. At least 200 spermatozoa across at least ten fields were scored per donor in three independent donors by an observer blinded to treatment. Per-donor percentages were used for comparison.

2.6 Live-cell calcium imaging

Purified spermatozoa were loaded with Fluo-4 AM (2 μ M; Invitrogen) and 0.05% Pluronic F-127 for 30 min at 37°C in the dark, washed, and immobilised on poly-L-lysine-coated coverslips in a laminar-flow chamber. Time-lapse confocal images were acquired at 1 Hz using a Zeiss LSM 880 confocal microscope with a 40 $\times$ oil objective (NA 1.3). Fluo-4 was excited at 488 nm and emission was collected at 505–530 nm. Baseline fluorescence was recorded for 60 s before perfusion. Cells were challenged with AVE0991 at 0.1 nM, ATP at 100 μ M as a positive control, or vehicle; A779 at 1 nM was applied 15 min before AVE0991 in inhibition experiments.

Calcium responses were displayed as relative fluorescence trajectories for vehicle, ATP, AVE0991 and A779 pre-treatment conditions. Thirty spermatozoa were examined per donor in three independent donors. Cells were nested within donors; the displayed trajectories were interpreted descriptively and were not used to infer population-level response amplitudes, responder fractions or a causal link to motility.

2.7 Histology and immunostaining

Testes were fixed in modified Davidson's fluid, embedded in paraffin and sectioned at 5 μ m. Two pathologists blinded to group allocation independently scored at least 50 seminiferous tubule cross-sections per animal from three non-consecutive sections using the modified Johnsen score [24]. Observer scores were averaged and tubule scores were then averaged per animal. PLZF⁺ cells were counted in at least 30 round tubule cross-sections per animal and reported as cells per tubule cross-section.

Sections and spermatozoa were incubated overnight at 4°C with antibodies against MasR (1:100; Alomone Labs #AAR-013) and PLZF (1:100; Abclonal #A5863) [25]. Signals were detected using HRP-DAB or fluorophore-conjugated secondary antibodies and quantified as integrated optical density or mean fluorescence intensity in Image-Pro Plus 6.0. Samples compared with one another were stained in the same run and imaged using identical acquisition settings.

MasR immunoreactivity in *Mas1*-null testicular tissue was examined alongside wild-type tissue as a genetic negative control. Signal in knockout tissue was at or near the detection floor. Integrated optical density was reported without background subtraction.

2.8 Serum hormone assays

Serum testosterone was measured by liquid chromatography–tandem mass spectrometry on a SCIEX Triple Quad 6500 + system using an isotope-labelled internal standard.

Serum LH and FSH were measured using mouse-specific sandwich ELISAs (Invitrogen Mouse Luteinizing Hormone ELISA Kit, Cat# EEL121; Invitrogen Mouse FSH ELISA Kit, Cat# EEL097). The stated analytical sensitivities were 0.19 ng/mL for LH and 0.94 ng/mL for FSH, with assay ranges of 0.31–20 ng/mL and 1.56–100 ng/mL, respectively. All testosterone and gonadotrophin concentrations are reported in ng/mL.

Samples were measured in duplicate in a single run for each analyte, with treatment groups balanced across the plate and the operator blinded to group allocation. Standard curves, quality controls and duplicate coefficients of variation were reviewed before unblinding. Values below the assay range were identified in the figure and were not treated as exact measurements. Because LH secretion is pulsatile, the terminal single-time-point measurement is interpreted as a spot concentration rather than a measure of pulsatile secretion.

2.9 Western blotting

Tissues and purified spermatozoa were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was determined by BCA assay. Twenty micrograms of protein was resolved on 10% SDS-PAGE gels and transferred to 0.45- μ m PVDF membranes. Membranes were blocked in 5% (w/v) bovine serum albumin in TBST for 1 h at room temperature and incubated overnight at 4°C with antibodies against MasR, PI3K p85 (CST #4292), phospho-PI3K p85 Tyr458/p55 Tyr199 (CST #17366), AKT (CST #9272), phospho-AKT Ser473 (CST #9271) and GAPDH, all at 1:1000.

Signals were acquired using a Tanon 5200 imaging system and quantified in ImageJ 1.53. Total and phosphorylated proteins were detected on parallel membranes loaded with matched aliquots of the same lysates. In the human sperm experiments, total and phosphorylated signals were each normalised to GAPDH, as displayed in Fig. 4. In mouse testis, phosphorylation was expressed as the p-AKT/total AKT ratio (Fig. 6I, J). Human spermatozoa were exposed to AVE0991 at 0.1 nM or vehicle for 30 min before lysis. Each lane in the human experiment represented an independent donor. The displayed human Western blots do not include an A779 pre-treatment arm.

2.10 RNA sequencing

Total RNA was extracted from purified spermatozoa using TRIzol and assessed using an Agilent 2100 Bioanalyzer. Libraries were sequenced by Shanghai Weihuan Biotech on an Illumina NovaSeq 6000 platform using paired-end 150-bp reads. Differential expression was analysed using DESeq2 [26] with adjusted $P < 0.05$ and $|\log_2 \text{fold change}| > 1$. Data deposition and access arrangements are described in the Availability of data and materials section.

The groups showed a marked and asymmetric shift in RNA composition. Of 41,167 tested genes, 19,278 (46.8%) met the differential-expression thresholds: 14,756 were lower and 4,522 were higher in AS

libraries. Sperm-associated transcripts were lower, whereas somatic and housekeeping transcripts were relatively higher. Gene-level results are therefore interpreted as relative abundance within sequenced libraries, not as absolute transcript change per spermatozoon. These patterns raise concerns about compositional confounding; the proportion of differentially expressed genes alone does not prove that normalisation failed.

2.11 Targeted RT-qPCR

RNA was extracted using TRIzol and reverse-transcribed using Takara PrimeScript RT Master Mix. Amplification used PowerUp SYBR Green Master Mix (Applied Biosystems, Cat# A25742) on an Applied Biosystems QuantStudio 5 instrument. Supplementary Table S1 lists the primer sequences, accession identifiers and amplicon lengths for the mouse *Mas1* assay, the 12-gene mouse panel, mouse *Gapdh*, and the human *MAS1* and *ACTB* assays. Technical triplicates were averaged before biological-unit analysis. Mouse target Cq values were normalised to *Gapdh*. Relative abundance was calculated using the $2^{-\Delta\Delta Cq}$ method, with ΔCq defined as target Cq minus *Gapdh* Cq and $\Delta\Delta Cq$ as the sample ΔCq minus the mean ΔCq of the NC group.

Figures 5I and 6K show a targeted 12-gene testicular RT-qPCR panel, with one group-summary column per group. Functional annotations are descriptive groupings, not enrichment results or evidence of cell-type-specific expression. For each gene, animal-level relative abundance was averaged within each group. The resulting group means were centred by subtracting their mean and divided by their population standard deviation across the displayed groups, using a variance divisor of three for Fig. 5I and five for Fig. 6K. Averaging preceded standardisation, and each figure was scaled separately.

Human *MAS1* Cq values were referenced to *ACTB* and are shown as $-\Delta Cq$. Amplification efficiency, melt-curve, no-template and no-reverse-transcriptase controls were unavailable, and the primer design did not distinguish cDNA from genomic DNA. In view of these limitations [27], the measurements are presented as descriptive assay signals, without fold-change conversion or an inferential comparison of continuous Cq values. Samples with no amplification by cycle 40 were displayed at their individual assay boundaries and were not assigned measured expression values.

2.12 Statistical analysis

The donor or animal was the biological experimental unit. Fields, tubules, cells and technical replicate wells were summarised within each biological unit. Clinical baseline data are reported as mean $\pm$ sample SD without hypothesis tests. Endpoints with three biological units per group were exploratory; all available individual values are displayed.

Scatter panels in Figs. 2D,F, 3D, 4B,C,E,F, 5C,F, 6C,J, S1F and S2F show individual biological-unit values with a mean line and SD error bars. Motility time courses in Fig. 3A,B are presented as mean $\pm$ SEM. All boxplots in Figs. 5E,G,H, 6E,F,G,H, S1A,B,E and S2A,B,E show the median, the 25th–75th percentile interval, and whiskers extending to the minimum and maximum, with individual observations overlaid.

Human protein and fluorescence signals in Fig. 2D,F were compared using two-sided Welch t-tests. Acrosome-reaction percentages in Fig. 3D were compared using one-way repeated-measures ANOVA with Tukey-adjusted comparisons, with donor as the matched unit. The four human sperm protein endpoints in Fig. 4B,C,E,F were analysed using two-sided paired t-tests, with Holm adjustment across the four comparisons. Mouse endpoints in Figs. 5C,E,F,H, 6C,E,F,H,J, S1A,B,E,F and S2A,B,E,F were analysed using one-way ANOVA followed by Tukey-adjusted comparisons. These analyses used untransformed animal-level values. Statistical analyses were performed in R 4.4.1 and GraphPad Prism 10.

Histological measurements were summarised per animal after averaging across observers and tubules. Human *MAS1* assay-detection proportions were compared using a two-sided Fisher exact test; this test does not assess the difference in continuous – ΔCq values.

Human sperm motility time courses, calcium traces, litter sizes and pregnancy proportions are interpreted descriptively. No treatment-by-time or independent-litter inference is reported. Statistical annotations for the retained inferential comparisons are * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and ns, $P \geq 0.05$; ns does not establish equivalence.

2.13 AI assistance

The authors reviewed and edited the AI-assisted content and take responsibility for the final manuscript and schematic.

3. Results

3.1 MasR protein signals are lower in asthenozoospermic spermatozoa

The clinical cohort included 10 men per group, with lower progressive and total sperm motility in AS participants (Table 1). Sperm RNA sequencing showed lower *MAS1* relative abundance in AS ($\log_2$ fold change – 1.80; adjusted $P = 0.0072$; Fig. 2A). Targeted RT-qPCR detected *MAS1* in 6/6 NC and 3/6 AS samples (Fig. 2B). Western blotting showed lower MasR abundance in AS spermatozoa (Fig. 2C, D). Immunofluorescence identified staining in the acrosomal and neck regions and showed a lower mean signal in AS samples (Fig. 2E, F). Testicular immunohistochemistry illustrated MasR localisation in seminiferous epithelium in the available case pair (Fig. 2G).

A, MA plot of 41,167 genes in AS versus NC sperm libraries; $n = 3$ donors/group. Orange and blue denote higher and lower relative abundance in AS at adjusted $P < 0.05$ and $|\log_2$ fold change| > 1 ; grey denotes other genes. *MAS1*, selected a priori, has baseMean 18.6, $\log_2$ fold change – 1.80 and adjusted $P = 0.0072$. The dashed line marks the genome-wide median $\log_2$ fold change of – 0.52. B, *MAS1* assay signals relative to *ACTB*, expressed as – ΔCq ; $n = 6$ donors/group. Filled circles indicate detection by cycle 40; open triangles indicate non-detection at donor-specific assay boundaries, *ACTB* Cq minus 40, rather than measured expression values. The dashed line marks the mean assay boundary. Detection

frequencies were 6/6 NC and 3/6 AS; two-sided Fisher exact $P = 0.182$. C,D, Western blot and GAPDH-normalised MasR abundance; $n = 3$ donors/group, one lane/point per donor. MasR and GAPDH regions were cropped from the same source image and separated by white space. Both strips were horizontally flipped to display NC before AS, corresponding to original source lanes 6–1. No individual lanes were independently rearranged or images from different sources combined. The complete source image in its original orientation and lane annotations are provided in the Supplementary Information. The approximately 45-kDa band was not identified by deglycosylation or mass spectrometry. E,F, MasR immunofluorescence and donor-level intensity; $n = 3$ /group, with ≥ 10 fields averaged per donor. G, Illustrative testicular MasR immunohistochemistry from one preserved-spermatogenesis case and one spermatogenic-failure case; no between-case test was performed. Scale bars: 50 μm in E and G. D,F show mean $\pm$ SD; two-sided Welch t-tests. In D,F, $**P < 0.01$ for the bracketed comparisons; the Fisher test in B applies only to detection frequency. Protein/fluorescence comparisons are exploratory.

3.2 Ex vivo MasR activation alters human sperm motility and associated signalling

AVE0991 at 0.1 or 1 nM transiently increased progressive motility, with the largest differences at 10–30 min and a decline toward baseline by 60 min; 10 nM showed lower motility (Fig. 3A). Pre-incubation with A779 at 1 or 10 nM attenuated the response to AVE0991 at 0.1 nM. A779 alone at 1 nM showed no clear reduction in baseline motility (Fig. 3B).

After 60 min, AVE0991 at 0.1 nM reduced the proportion of acrosome-reacted spermatozoa, and A779 pre-treatment shifted values toward vehicle levels (Fig. 3C, D).

A, Progressive motility after DMSO vehicle or AVE0991 at 0.1, 1 or 10 nM. B, A779 at 1 or 10 nM for 15 min before AVE0991 at 0.1 nM, with vehicle, AVE0991-alone and A779-alone at 1 nM as comparators. CASA measurements were obtained at 0, 10, 30 and 60 min in five independent donors per comparison. Values are percentages of each donor's baseline, set to 100%, rather than absolute percentages of motile cells. A,B show mean $\pm$ SEM. Time courses are descriptive. C,D, Representative acrosomal staining and percentages of acrosome-reacted spermatozoa after 60 min of vehicle, AVE0991 at 0.1 nM, or A779 pretreatment at 1 nM followed by AVE0991 at 0.1 nM. At least 200 spermatozoa across at least ten fields were scored per donor in three donors. Points are donor-level percentages; spontaneous reaction was not subtracted. Scale bars: 50 μm in C. Cells and fields are nested observations. D shows mean $\pm$ SD; one-way repeated-measures ANOVA with Tukey-adjusted comparisons across donor-matched conditions. For bracketed comparisons in D, $*P < 0.05$, $**P < 0.01$ and ns, $P \geq 0.05$. Acrosome comparisons are exploratory.

AVE0991 increased GAPDH-normalised phosphorylated AKT and PI3K signals, while total-protein signals were similar between conditions (Fig. 4A–F). Calcium fluorescence increased with AVE0991 and the ATP positive control; responses were smaller after A779 pre-treatment and with vehicle (Fig. 4G).

Spermatozoa received vehicle or AVE0991 at 0.1 nM for 30 min before protein analysis. A, Western blots for total AKT and phospho-AKT Ser473, with corresponding GAPDH controls. B,C, GAPDH-normalised total and phospho-AKT signals. D, Total and phosphorylated PI3K p85 blots with GAPDH controls. E,F, Corresponding GAPDH-normalised PI3K signals. Each condition contains three independent donors. Total and phosphorylated proteins were measured on parallel membranes with matched lysate aliquots; values are separate GAPDH-normalised signals, not phospho/total ratios. B,C,E,F show mean $\pm$ SD; two-sided paired t-tests with Holm adjustment across the four protein endpoints. G, Relative calcium-fluorescence trajectories with vehicle, ATP at 100 μ M, AVE0991 at 0.1 nM, or A779 at 1 nM for 15 min before AVE0991 at 0.1 nM. Thirty spermatozoa per donor were examined in three donors; cells are not independent biological replicates. The horizontal axis shows recording time in seconds. Traces are descriptive. Protein panels contain no A779 arm. For bracketed protein comparisons, * $P < 0.05$ and ns, $P \geq 0.05$. All experiments are exploratory.

3.3 *Mas1* deletion and adult A779 treatment are associated with impaired spermatogenesis

Global *Mas1*-null males showed seminiferous tubule vacuolisation and germ-cell shedding, lower modified Johnsen scores, and fewer PLZF-positive spermatogonia than wild-type controls (Fig. 5A–D, F). Cauda epididymal sperm concentration and total motility were also lower (Fig. 5E, H). The targeted 12-gene testicular panel showed lower relative signals for several reproduction-related genes, including deleted in azoospermia-like (*Dazl*), tektin 1 (*Tekt1*), glutathione peroxidase 4 (*Gpx4*) and lactate dehydrogenase C (*Ldhc*) (Fig. 5I). Serum FSH was higher in the knockout group, whereas LH concentrations were similar and testosterone was modestly lower (Supplementary Figure S1A, B, E).

Adult wild-type mice receiving A779 for six weeks showed abnormalities in the same direction, generally of intermediate magnitude between NC and knockout animals. Descriptive mating summaries showed smaller litter-size values in A779 and knockout groups (Fig. 5G) and pregnancy proportions of 87.5% in NC, 50.0% in A779 and 37.5% in KO groups (Supplementary Figure S1C).

Groups are wild-type vehicle controls, NC; wild-type mice receiving A779 at 0.05 mg/kg/day for six weeks; and non-diabetic *Mas1*-null mice receiving vehicle, KO. A,B, Low- and high-magnification haematoxylin and eosin sections. C, Modified Johnsen score: each point is the animal-level mean across ≥ 50 tubule cross-sections from three non-consecutive sections, averaged across two blinded pathologists; $n = 3$ animals/group. D, Promyelocytic leukaemia zinc finger protein, PLZF, immunohistochemistry. E, Cauda epididymal sperm concentration; $n = 6$ /group. F, PLZF-positive cells per tubule cross-section, averaged across ≥ 30 round cross-sections per animal. Each point represents one animal; $n = 3$ /group. G, Descriptive offspring counts per delivered litter; $n = 8$ litters/group. Sire-to-litter linkage was unavailable; points are not assumed to represent independent sires. H, Total cauda epididymal sperm motility; $n = 6$ /group. I, Targeted 12-gene testicular RT-qPCR panel, shown as row-standardised group summaries; $n = 3$ /group. Functional annotations are descriptive, not enrichment results. Colours are scaled within each gene and separately from Fig. 6K. Scale bars: 500 μ m in A; 50 μ m

in B and D. C,F show mean $\pm$ SD. E,G,H show the median, interquartile range and minimum–maximum whiskers, with individual observations. C,E,F,H were analysed using one-way ANOVA with Tukey-adjusted comparisons. In C,E,F,H, *P < 0.05 and **P < 0.01 for bracketed comparisons. No inferential comparison is reported for G. Tissue endpoints are exploratory.

3.4 AVE0991 partially improves testicular endpoints in diabetic mice

Streptozotocin-induced diabetic mice showed seminiferous disorganisation and lower sperm concentration and total motility. Six weeks of AVE0991 administration was associated with higher Johnsen scores, more PLZF-positive spermatogonia and improved sperm parameters compared with untreated diabetic mice, although values remained below those of non-diabetic controls (Fig. 6A–F, H). Descriptive mating summaries showed larger litter-size values in the treated diabetic group (Fig. 6G). Pregnancy proportions were 87.5%, 50.0%, 75.0%, 37.5% and 37.5% in NC, T1DM, T1DM + AVE0991, KO and KO + AVE0991 groups, respectively (Supplementary Figure S2C).

Diabetic mice showed lower testosterone and LH and higher FSH than non-diabetic controls; AVE0991 shifted these concentrations toward control values (Supplementary Figure S2A, B, E). The non-diabetic *Mas1*-null mice receiving AVE0991 showed no clear improvement over the untreated knockout group in the displayed reproductive endpoints.

Testicular p-AKT/total AKT ratios remained below control values after AVE0991 treatment, including in the displayed LY294002 subgroup (Fig. 6I, J). The 12-gene panel showed a shift toward NC values for several genes in AVE0991-treated diabetic mice, whereas the non-diabetic knockout profiles remained comparatively similar with and without AVE0991 (Fig. 6K). These are descriptive within-gene patterns. Testicular MasR immunoreactivity remained low after AVE0991 (Supplementary Figure S2D, F).

Main groups are wild-type vehicle controls, NC; diabetic vehicle-treated mice, T1DM; T1DM + AVE0991 at 0.6 mg/kg/day for six weeks; non-diabetic KO; and non-diabetic KO + AVE0991. A,B, Haematoxylin and eosin sections. C, Modified Johnsen score. D, PLZF immunohistochemistry. E, Cauda epididymal sperm concentration. F, PLZF-positive cells per tubule cross-section. C,F use three animals/group after averaging nested observations within each animal. G, Descriptive offspring counts per delivered litter; n = 8 litters/group. Sire-to-litter linkage was unavailable, and points are not assumed to represent independent sires. H, Total cauda epididymal sperm motility. E,H use six animals/group. I,J, Testicular Western blots and p-AKT/total AKT ratios; n = 3/group. Molecular group order is NC, T1DM, T1DM + AVE0991, T1DM + AVE0991 + LY294002, KO, and KO + AVE0991. The LY294002 subgroup contributed molecular endpoints only. K, Targeted 12-gene panel, shown as row-standardised group summaries; n = 3/group. Functional annotations are descriptive; colour intensities must not be quantitatively compared between figures. The KO arms do not constitute a diabetic knockout rescue experiment. Scale bars: 500 μ m in A; 50 μ m in B and D. C,J show mean $\pm$ SD. E,F,G,H show the median, interquartile range and minimum–maximum whiskers, with individual observations. C,E,F,H,J were analysed using one-way ANOVA with Tukey-adjusted comparisons. In C,E,F,H,J, *P < 0.05, **P < 0.01 and ns, P $\geq$ 0.05, for

bracketed comparisons. No inferential comparison is reported for G. Tissue/molecular endpoints are exploratory.

4. Discussion

Spermatogenesis depends on a regulated testicular microenvironment that can be disrupted by diabetes and associated metabolic changes [28]. Against this background, the present study links genetic and adult pharmacological loss of MasR function with impaired spermatogenesis and identifies a partial phenotypic benefit of AVE0991 in diabetic mice. The contribution is an extension of the rodent findings of Leal and colleagues, who already examined Mas deficiency and chronic A779 exposure [20]. The added diabetic intervention provides a disease-related context in which the pathway may be pharmacologically modifiable.

The biological rationale involves the contrasting actions of RAS pathways. Angiotensin II/AT1 signalling can promote inflammatory and growth-related responses, whereas the angiotensin-(1–7)/Mas axis has counter-regulatory effects in several tissues [15, 16, 17]. Ferreira and colleagues reported beneficial effects of AVE0991 in a rat myocardial-infarction model, providing independent evidence of its activity outside the reproductive system [29]. In the present study, AVE0991 improved testicular architecture and sperm parameters in diabetic mice. The absence of a comparable improvement in non-diabetic *Mas1*-null mice is compatible with receptor involvement, but the design did not include a diabetic knockout treatment comparison. Systemic vascular, metabolic and endocrine contributions therefore remain possible; these data do not establish exclusive receptor dependence or cardiovascular safety.

The human observations connect this experimental framework with previous clinical and ex vivo findings. Reis and colleagues identified angiotensin-(1–7) and Mas in human testis [18], while Valdivia and colleagues described Mas-associated effects on sperm motility and AKT phosphorylation [19]. Our lower sperm protein signals and acute responses to AVE0991 are consistent with involvement of this axis in sperm function. However, the reduction in acrosome-reacted spermatozoa differs from the absence of an acrosome-reaction effect reported by Valdivia and colleagues [19]. Ligand, incubation and scoring differences may contribute. A lower acrosome-reaction percentage cannot by itself be interpreted as improved fertilising capacity.

The divergence between acute sperm signalling and chronic testicular responses is also informative. AVE0991 increased phosphorylated PI3K and AKT signals in human sperm, but did not restore testicular p-AKT/total AKT in diabetic mice. Species, cell composition, exposure duration and disease context differ between these experiments. Redox injury and epididymal antioxidant regulation provide relevant biological background [6, 7], and broader Mas-related tissue responses have been described [16, 17]. Oxidative stress, inflammation and blood–testis barrier integrity were not measured here, however. They remain candidate explanations for investigation rather than established mechanisms of the observed benefit; the molecular-only LY294002 subgroup cannot resolve this question.

Adult A779 exposure produced a milder phenotype than lifelong *Mas1* deletion. This may reflect exposure or blockade differences, developmental effects, or compensation, rather than identifying a specific pubertal requirement. Developmental programming of the angiotensin-(1–7) axis has been discussed in other organ systems, but that literature cannot establish a testis-specific developmental mechanism in these mice [30]. Reduced PLZF-positive cell counts are consistent with loss of cells carrying an undifferentiated spermatogonial marker [25]. *Dazl* and *Gpx4* provide further biological context because their disruption can impair gametogenesis or male fertility [31, 32]. Changes in the selected transcript panel nevertheless cannot distinguish regulation within surviving cells from altered germ-cell composition. Prior treatment effects on epididymal or mature sperm also remain possible.

The principal limitations concern biological sample size, clinical characterisation and mechanism. The human protein subsets and mouse tissue endpoints were small, the testicular specimens comprised one illustrative case per category, and detailed clinical covariates and morphology were unavailable. Sperm RNA composition and incomplete RT-qPCR specificity controls prevent a conclusion of *MAS1*-specific transcriptional downregulation; lower relative RNA abundance also does not prove global RNA-pool depletion. The original human samples were unavailable for further assay validation. Time courses and calcium traces lacked a donor-resolved inferential analysis, and three AVE0991 concentrations do not define an EC₅₀ or therapeutic window. Mating summaries lacked sire-level analysis of clustered litters. Global deletion and systemic dosing do not establish cell autonomy, while streptozotocin toxicity and hyperglycaemia were not separated by an insulin-treated or glycaemia-matched comparison. Longitudinal glucose, body weight and pharmacokinetic outcomes were not available, and terminal hormone measurements cannot establish preserved Leydig-cell function.

These findings support the angiotensin-(1–7)/Mas axis as a candidate non-hormonal target for further study in metabolic reproductive dysfunction [12, 28]. AVE0991 partially improved testicular histology and sperm parameters in this diabetic model, but the study does not establish restored fertility, a defined intracellular protective pathway or clinical efficacy. Cell-specific genetic models, matched diabetic knockout comparisons and identification of the chronic downstream response would clarify the contribution of this pathway to testicular function.

5. Conclusions

MasR signalling contributes to spermatogenic function, and AVE0991 partially improves testicular histology and sperm parameters in streptozotocin-induced diabetic mice. Human sperm observations provide exploratory supporting context. The intracellular mechanism, the receptor dependence of the diabetic treatment response and the implications for human fertility remain unresolved.

Abbreviations

ACTB

β-actin

AKT
protein kinase B
ANOVA
analysis of variance
AS
asthenozoospermia
CASA
computer-assisted sperm analysis
Cq
quantification cycle
FSH
follicle-stimulating hormone
GAPDH
glyceraldehyde-3-phosphate dehydrogenase
H&E
haematoxylin and eosin
IHC
immunohistochemistry
IQR
interquartile range
KO
knockout
LH
luteinising hormone
MasR
Mas receptor
NC
control comparator
PI3K
phosphoinositide 3-kinase
PLZF
promyelocytic leukaemia zinc finger protein
PR
progressive motility
RT-qPCR
reverse-transcription quantitative polymerase chain reaction
SD
standard deviation
SEM
standard error of the mean

STZ
streptozotocin
T1DM
type 1 diabetes mellitus
TM
total motility
WHO
World Health Organization
WT
wild type.

Declarations

Ethics approval and consent to participate

Human protocols were approved by the Ethics Committee of Beijing Chao-Yang Hospital under approval number 2023-Ke-759, and written informed consent was obtained from participants. Animal procedures were approved by the Institutional Animal Care and Use Committee under approval number JLHK-20240604-01.

Consent for publication

Not applicable. The manuscript contains no identifiable individual information or images.

Competing interests

All authors declare no competing interests.

Supplementary material

Supplementary Figures S1 and S2 and Tables S1 and S2 are included below.

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Author Contribution

X.-Y.B. performed the main experiments, curated and analysed the data, prepared the figures and wrote the first draft. T.A. contributed to methodology and data analysis. Z.S. and L.F. contributed to experiments and data collection. G.C. contributed to methodology and experimental work. Q.L. conceived and supervised the study and revised the manuscript. All authors reviewed and approved the final manuscript.

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Not applicable.

Data Availability

Deposition of the raw human sperm RNA-sequencing data in GSA-Human, part of the Genome Sequence Archive at the National Genomics Data Center, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences, is in progress under submission identifier subHRA033074. The permanent HRA accession and dataset-specific link are pending. The submission is being prepared for controlled access through <https://ngdc.cncb.ac.cn/gsa-human>, subject to approval by the designated Data Access Committee. Clinical cohort summaries are included in Table 1, primer information in Supplementary Table S1, and participant-level clinical records in Supplementary Table S2.

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Figures

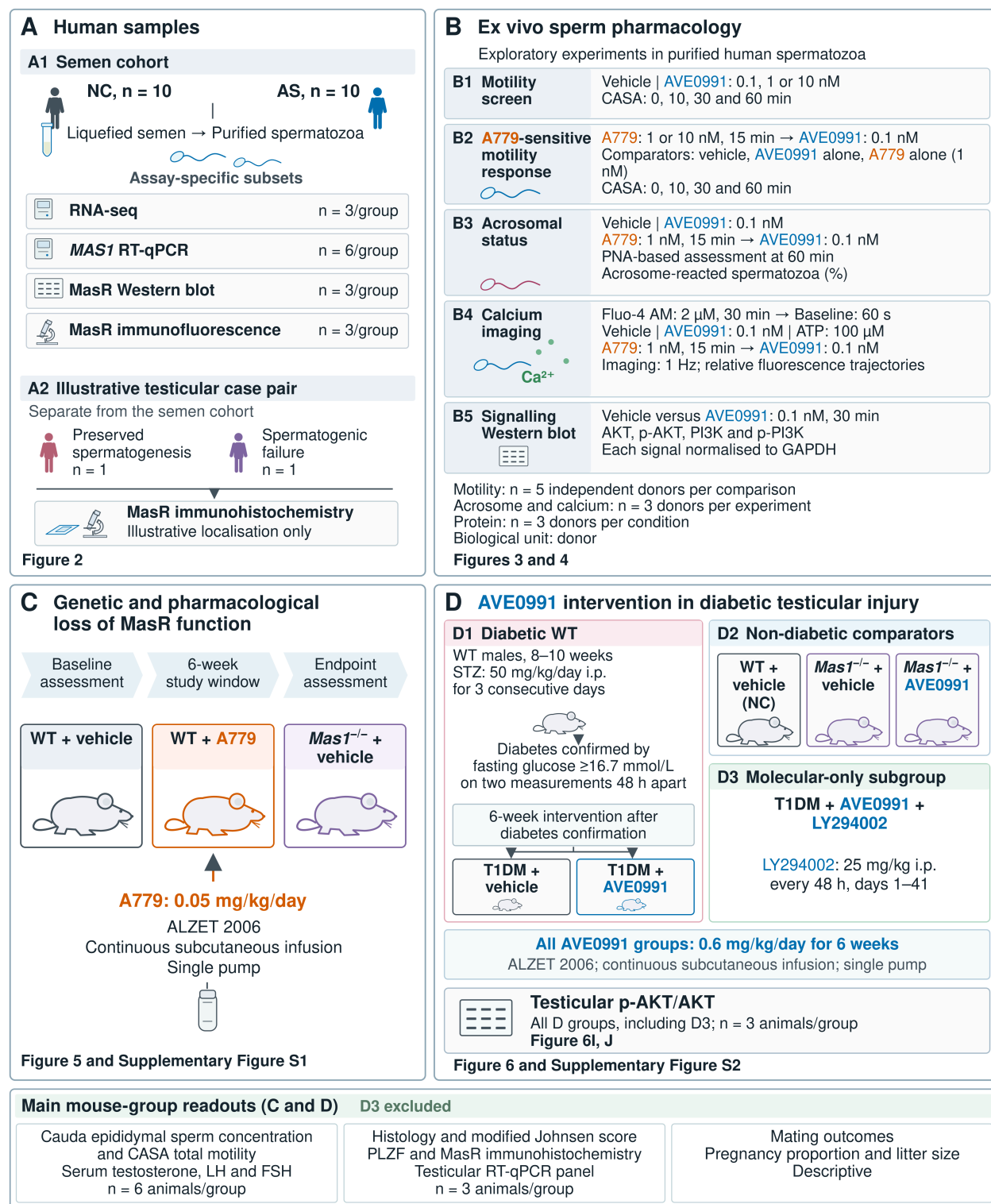


Figure 1

Study design

A1, Human semen cohort: ten recorded normozoospermic comparators, NC, and ten men with asthenozoospermia, AS, retaining the fifth-edition WHO motility grouping described in Section 2.1. Subsets per group comprised three donors for RNA sequencing, six for MAS1 RT-qPCR, and three each

for MasR Western blotting and immunofluorescence. A2, Separate illustrative testicular case pair, one case per category. B1, Vehicle or AVE0991 at 0.1, 1 or 10 nM; CASA at 0, 10, 30 and 60 min. B2, A779 at 1 or 10 nM for 15 min before AVE0991 at 0.1 nM, with vehicle, agonist-alone and A779-alone comparators. B3, Acrosomal status after 60 min. B4, Fluo-4 AM calcium imaging after a 60-s baseline, with AVE0991 at 0.1 nM, ATP at 100 μ M, vehicle or A779 pretreatment; acquisition at 1 Hz. B5, GAPDH-normalised protein signals after 30 min of AVE0991 at 0.1 nM; no A779 arm. Motility used five donors per comparison; acrosome and calcium assays used three per experiment; protein assays used three per condition. C, *Mas1* deletion or six-week A779 infusion at 0.05 mg/kg/day. D1, Streptozotocin at 50 mg/kg/day intraperitoneally for three days; fasting glucose $\geq$ 16.7 mmol/L confirmed twice, 48 h apart, before intervention. D2, Non-diabetic comparators. D3, LY294002 at 25 mg/kg intraperitoneally every 48 h, days 1–41; molecular endpoints only. All AVE0991 groups received 0.6 mg/kg/day for six weeks via one subcutaneous ALZET 2006 pump. Main sperm/hormone endpoints used six animals per group; tissue/molecular endpoints used three. Mating summaries were descriptive. Icons do not denote sample size; donors and animals were biological units.

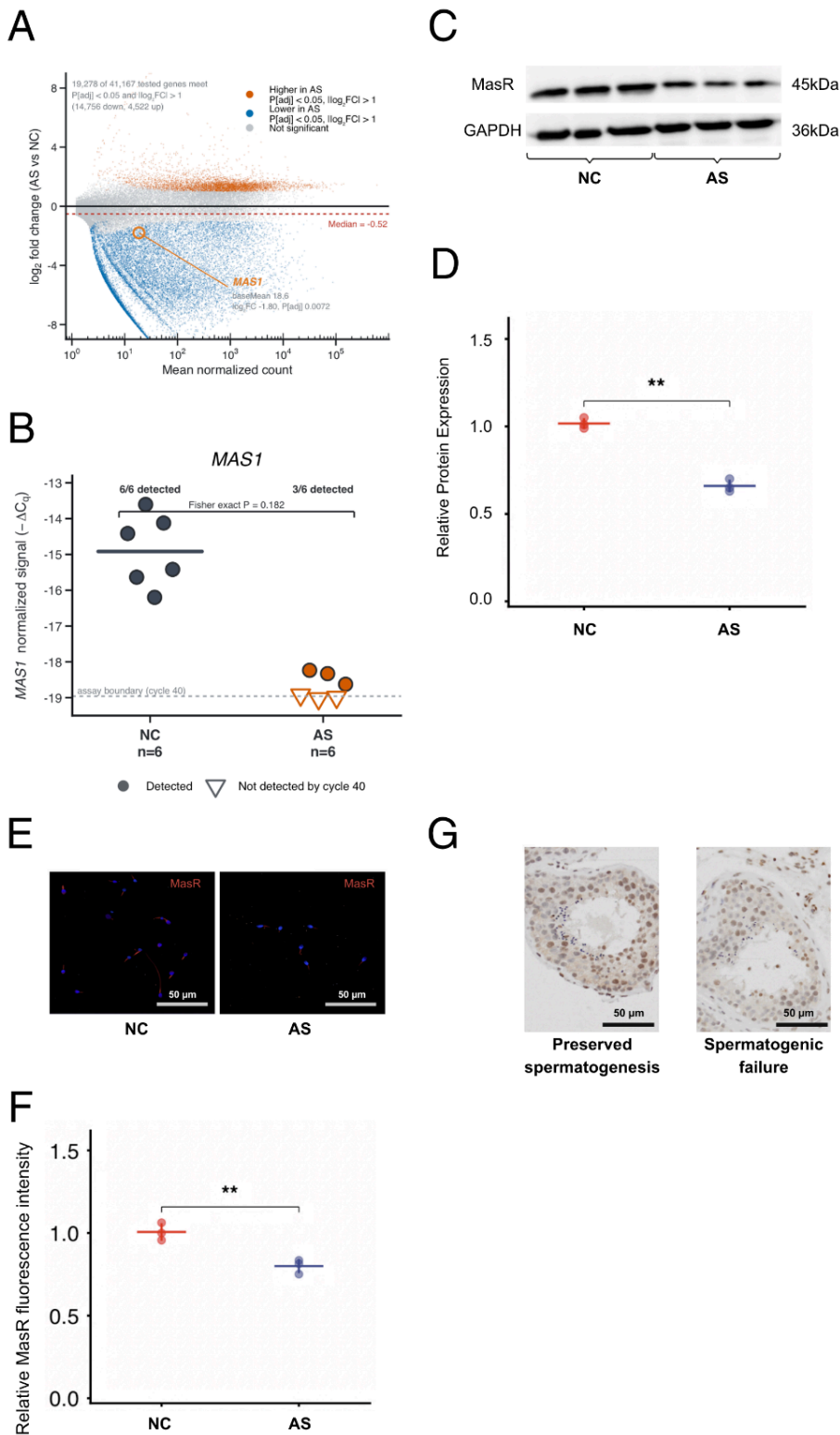


Figure 2

MasR protein signals and exploratory RNA measurements in human spermatozoa

A, MA plot of 41,167 genes in AS versus NC sperm libraries; $n = 3$ donors/group. Orange and blue denote higher and lower relative abundance in AS at adjusted $P < 0.05$ and $|\log_2 \text{fold change}| > 1$; grey denotes other genes. *MAS1*, selected a priori, has baseMean 18.6, $\log_2 \text{fold change} -1.80$ and adjusted $P =$

0.0072. The dashed line marks the genome-wide median $\log_2$ fold change of -0.52 . B, *MAS1* assay signals relative to *ACTB*, expressed as $-\Delta Cq$; $n = 6$ donors/group. Filled circles indicate detection by cycle 40; open triangles indicate non-detection at donor-specific assay boundaries, *ACTB* Cq minus 40, rather than measured expression values. The dashed line marks the mean assay boundary. Detection frequencies were 6/6 NC and 3/6 AS; two-sided Fisher exact $P = 0.182$. C,D, Western blot and GAPDH-normalised MasR abundance; $n = 3$ donors/group, one lane/point per donor. MasR and GAPDH regions were cropped from the same source image and separated by white space. Both strips were horizontally flipped to display NC before AS, corresponding to original source lanes 6–1. No individual lanes were independently rearranged or images from different sources combined. The complete source image in its original orientation and lane annotations are provided in the Supplementary Information. The approximately 45-kDa band was not identified by deglycosylation or mass spectrometry. E,F, MasR immunofluorescence and donor-level intensity; $n = 3$ /group, with ≥ 10 fields averaged per donor. G, Illustrative testicular MasR immunohistochemistry from one preserved-spermatogenesis case and one spermatogenic-failure case; no between-case test was performed. Scale bars: 50 μm in E and G. D,F show mean $\pm$ SD; two-sided Welch t-tests. In D,F, $**P < 0.01$ for the bracketed comparisons; the Fisher test in B applies only to detection frequency. Protein/fluorescence comparisons are exploratory.

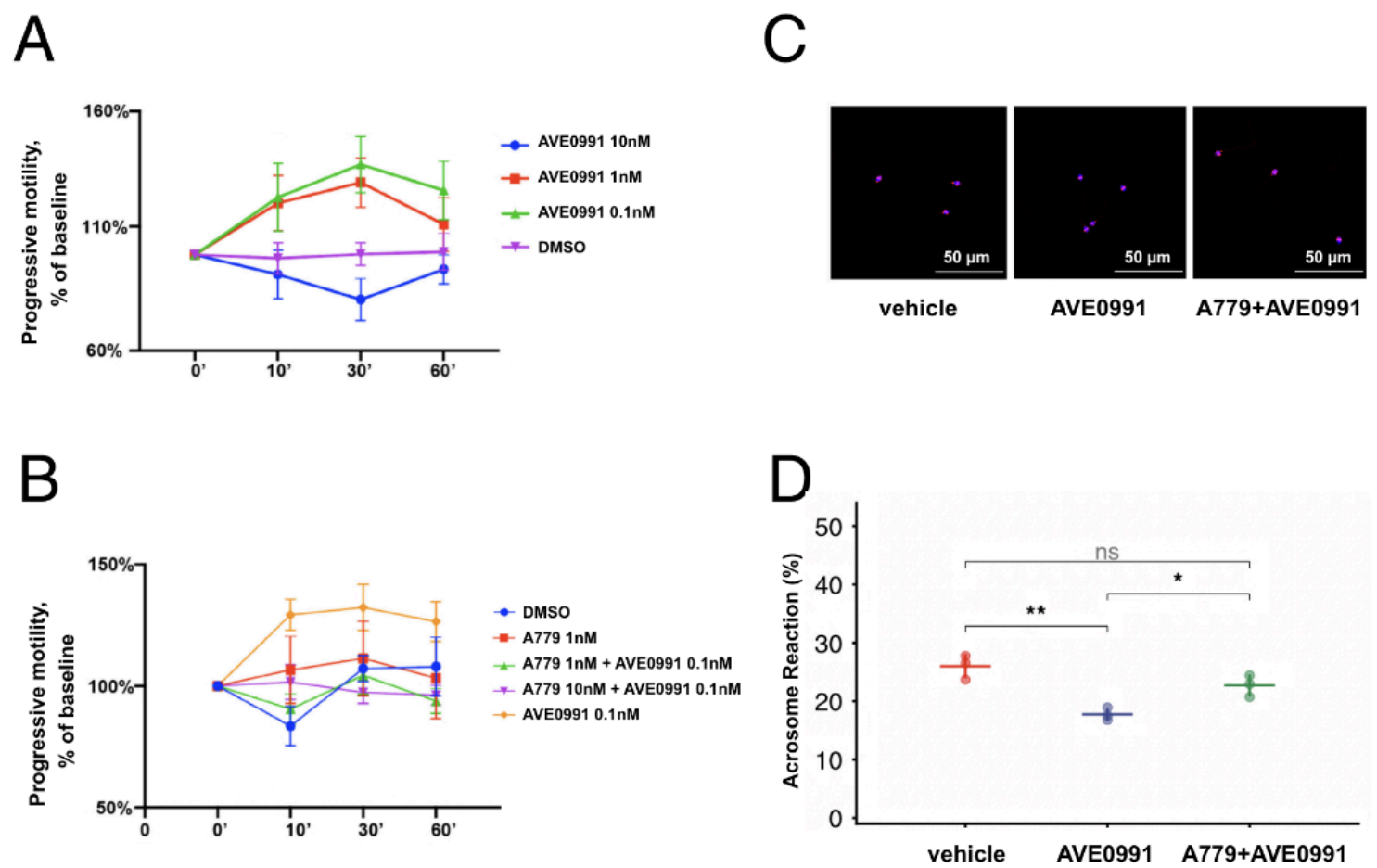


Figure 3

Ex vivo effects of AVE0991 on human sperm motility and acrosomal status

A, Progressive motility after DMSO vehicle or AVE0991 at 0.1, 1 or 10 nM. B, A779 at 1 or 10 nM for 15 min before AVE0991 at 0.1 nM, with vehicle, AVE0991-alone and A779-alone at 1 nM as comparators. CASA measurements were obtained at 0, 10, 30 and 60 min in five independent donors per comparison. Values are percentages of each donor's baseline, set to 100%, rather than absolute percentages of motile cells. A,B show mean $\pm$ SEM. Time courses are descriptive. C,D, Representative acrosomal staining and percentages of acrosome-reacted spermatozoa after 60 min of vehicle, AVE0991 at 0.1 nM, or A779 pretreatment at 1 nM followed by AVE0991 at 0.1 nM. At least 200 spermatozoa across at least ten fields were scored per donor in three donors. Points are donor-level percentages; spontaneous reaction was not subtracted. Scale bars: 50 μ m in C. Cells and fields are nested observations. D shows mean $\pm$ SD; one-way repeated-measures ANOVA with Tukey-adjusted comparisons across donor-matched conditions. For bracketed comparisons in D, *P <0.05, **P <0.01 and ns, P $\geq$ 0.05. Acrosome comparisons are exploratory.

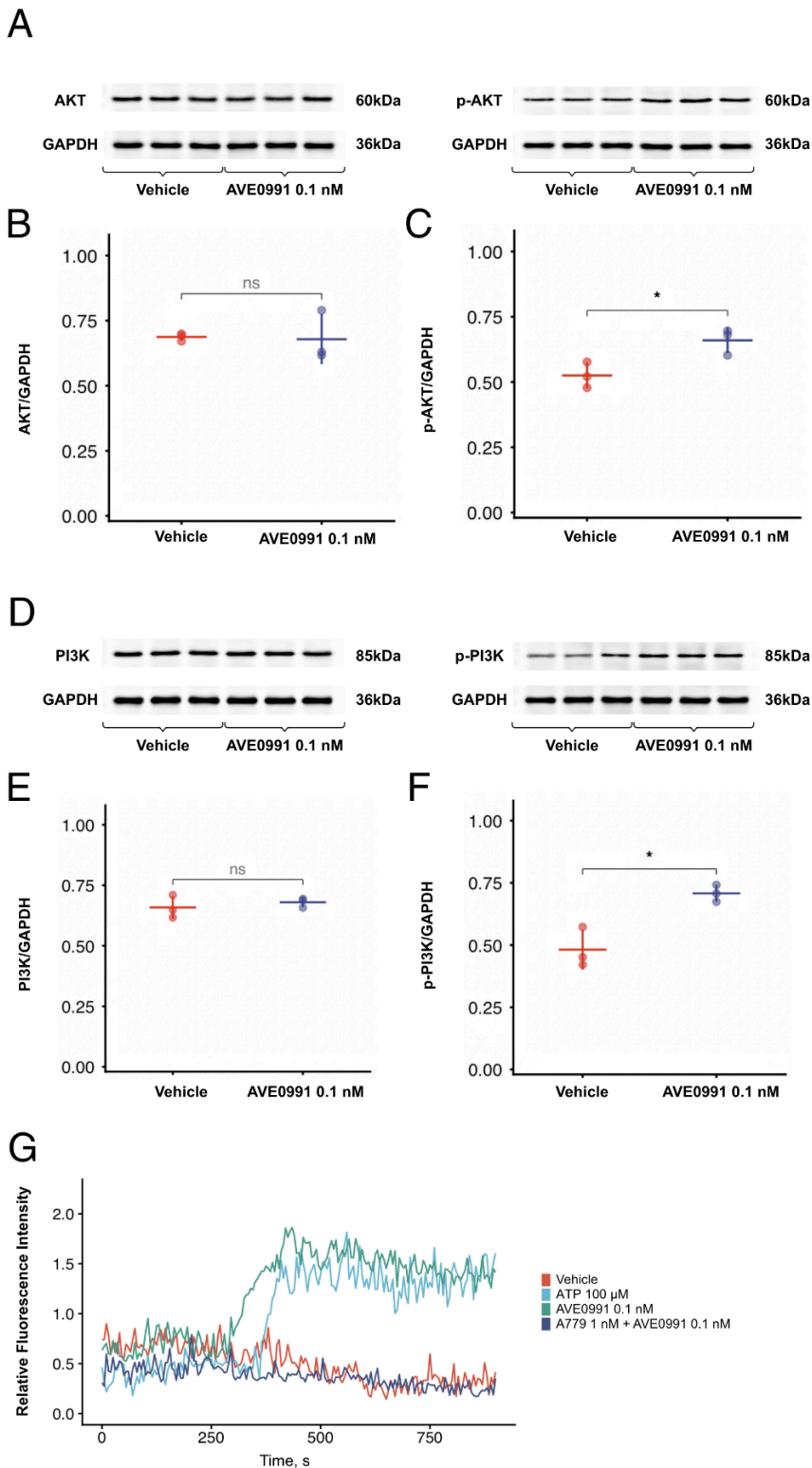


Figure 4

Protein and calcium responses to AVE0991 in human spermatozoa

Spermatozoa received vehicle or AVE0991 at 0.1 nM for 30 min before protein analysis. A, Western blots for total AKT and phospho-AKT Ser473, with corresponding GAPDH controls. B,C, GAPDH-normalised total and phospho-AKT signals. D, Total and phosphorylated PI3K p85 blots with GAPDH controls. E,F,

Corresponding GAPDH-normalised PI3K signals. Each condition contains three independent donors. Total and phosphorylated proteins were measured on parallel membranes with matched lysate aliquots; values are separate GAPDH-normalised signals, not phospho/total ratios. B,C,E,F show mean $\pm$ SD; two-sided paired t-tests with Holm adjustment across the four protein endpoints. G, Relative calcium-fluorescence trajectories with vehicle, ATP at 100 μ M, AVE0991 at 0.1 nM, or A779 at 1 nM for 15 min before AVE0991 at 0.1 nM. Thirty spermatozoa per donor were examined in three donors; cells are not independent biological replicates. The horizontal axis shows recording time in seconds. Traces are descriptive. Protein panels contain no A779 arm. For bracketed protein comparisons, *P < 0.05 and ns, P $\geq$ 0.05. All experiments are exploratory.

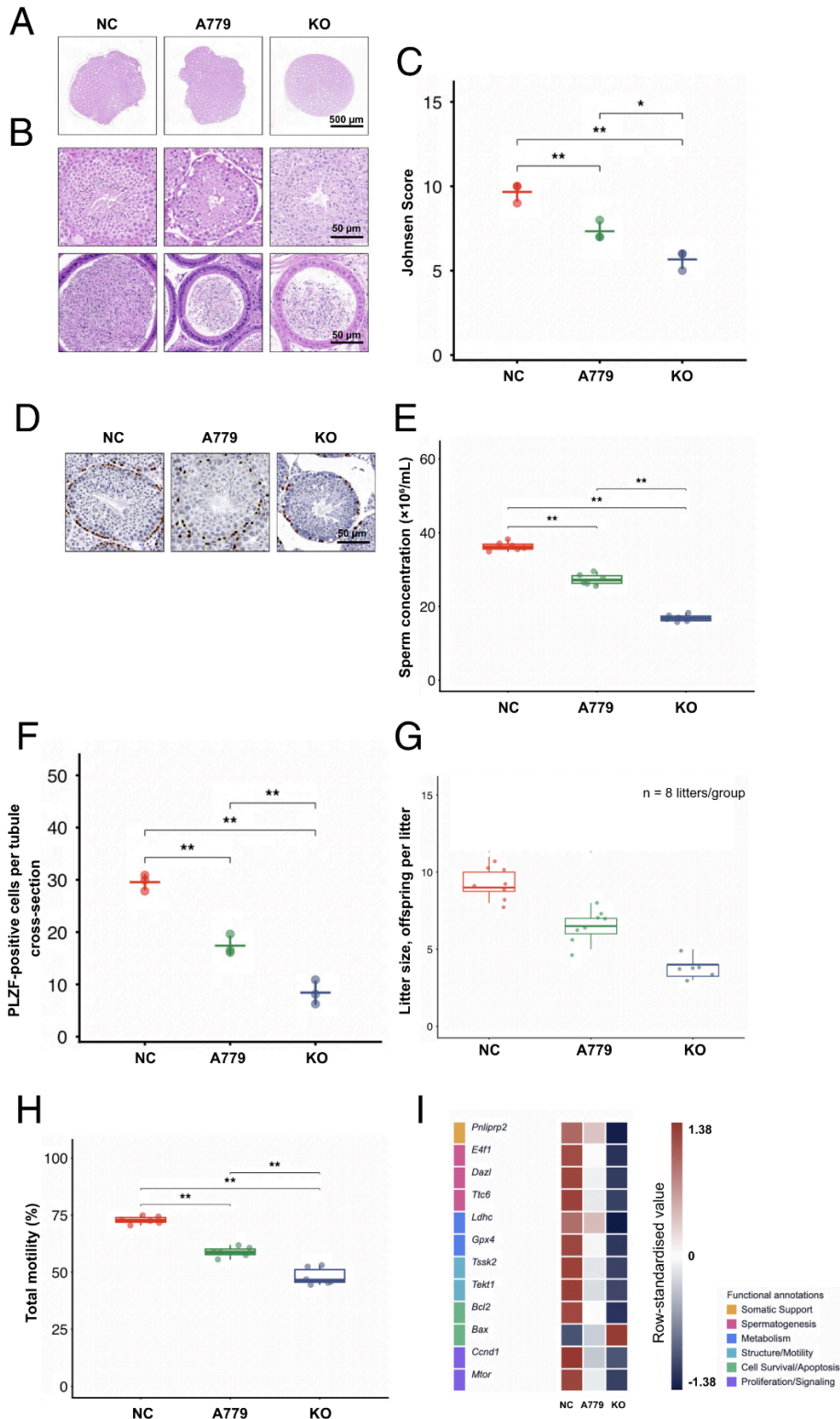


Figure 5

Testicular and sperm phenotypes after *Mas1* deletion or chronic A779 treatment

Groups are wild-type vehicle controls, NC; wild-type mice receiving A779 at 0.05 mg/kg/day for six weeks; and non-diabetic *Mas1*-null mice receiving vehicle, KO. A,B, Low- and high-magnification haematoxylin and eosin sections. C, Modified Johnsen score: each point is the animal-level mean across

≥50 tubule cross-sections from three non-consecutive sections, averaged across two blinded pathologists; n = 3 animals/group. D, Promyelocytic leukaemia zinc finger protein, PLZF, immunohistochemistry. E, Cauda epididymal sperm concentration; n = 6/group. F, PLZF-positive cells per tubule cross-section, averaged across ≥30 round cross-sections per animal. Each point represents one animal; n = 3/group. G, Descriptive offspring counts per delivered litter; n = 8 litters/group. Sire-to-litter linkage was unavailable; points are not assumed to represent independent sires. H, Total cauda epididymal sperm motility; n = 6/group. I, Targeted 12-gene testicular RT-qPCR panel, shown as row-standardised group summaries; n = 3/group. Functional annotations are descriptive, not enrichment results. Colours are scaled within each gene and separately from Figure 6K. Scale bars: 500 µm in A; 50 µm in B and D. C,F show mean ± SD. E,G,H show the median, interquartile range and minimum–maximum whiskers, with individual observations. C,E,F,H were analysed using one-way ANOVA with Tukey-adjusted comparisons. In C,E,F,H, *P <0.05 and **P <0.01 for bracketed comparisons. No inferential comparison is reported for G. Tissue endpoints are exploratory.

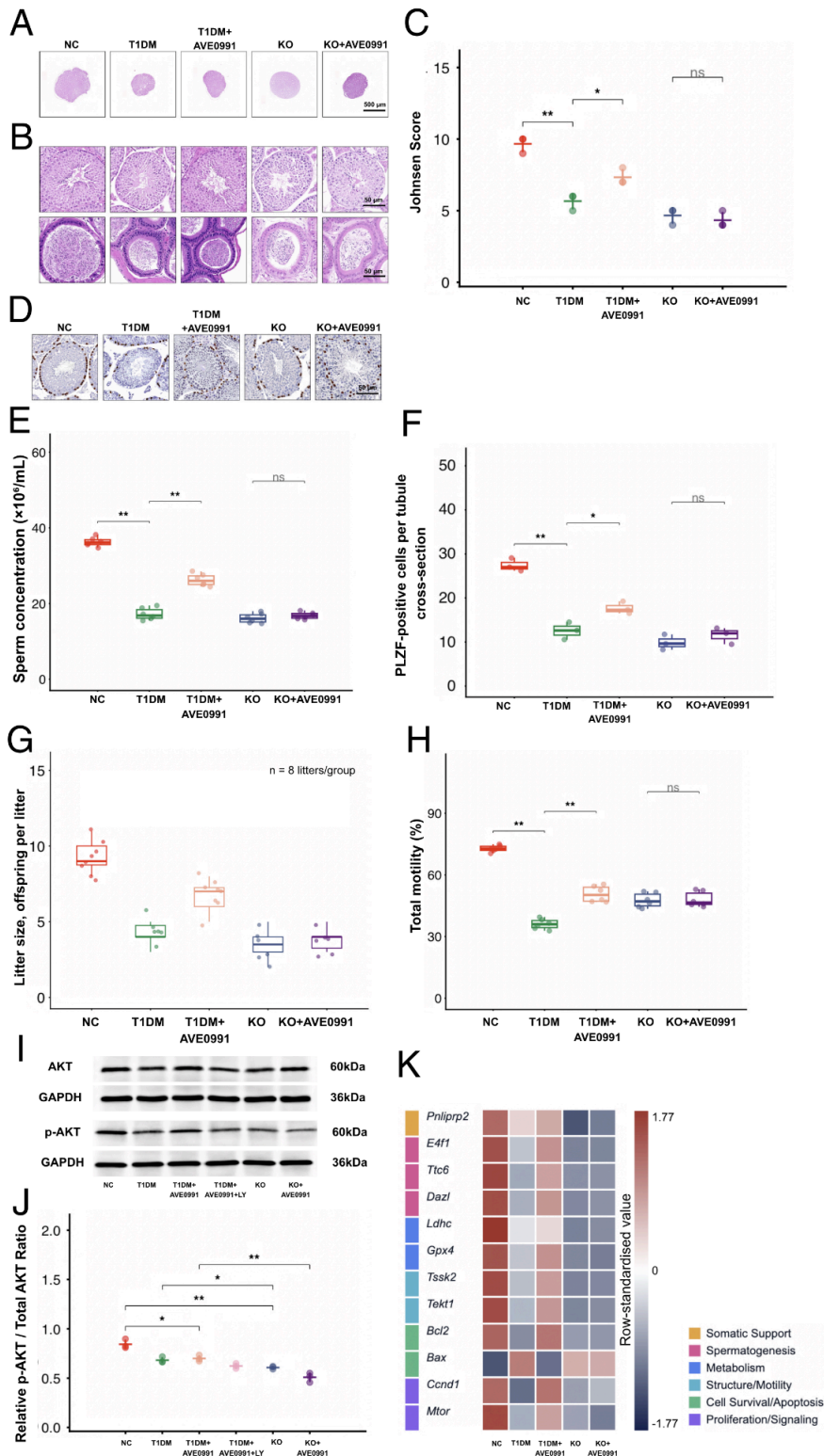


Figure 6

AVE0991 responses in diabetic wild-type mice and non-diabetic *Mas1*-null mice

Main groups are wild-type vehicle controls, NC; diabetic vehicle-treated mice, T1DM; T1DM + AVE0991 at 0.6 mg/kg/day for six weeks; non-diabetic KO; and non-diabetic KO + AVE0991. A,B, Haematoxylin and eosin sections. C, Modified Johnsen score. D, PLZF immunohistochemistry. E, Cauda epididymal sperm

concentration. F, PLZF-positive cells per tubule cross-section. C,F use three animals/group after averaging nested observations within each animal. G, Descriptive offspring counts per delivered litter; n = 8 litters/group. Sire-to-litter linkage was unavailable, and points are not assumed to represent independent sires. H, Total cauda epididymal sperm motility. E,H use six animals/group. I,J, Testicular Western blots and p-AKT/total AKT ratios; n = 3/group. Molecular group order is NC, T1DM, T1DM + AVE0991, T1DM + AVE0991 + LY294002, KO, and KO + AVE0991. The LY294002 subgroup contributed molecular endpoints only. K, Targeted 12-gene panel, shown as row-standardised group summaries; n = 3/group. Functional annotations are descriptive; colour intensities must not be quantitatively compared between figures. The KO arms do not constitute a diabetic knockout rescue experiment. Scale bars: 500 μ m in A; 50 μ m in B and D. C,J show mean $\pm$ SD. E,F,G,H show the median, interquartile range and minimum–maximum whiskers, with individual observations. C,E,F,H,J were analysed using one-way ANOVA with Tukey-adjusted comparisons. In C,E,F,H,J, *P <0.05, **P <0.01 and ns, P $\geq$ 0.05, for bracketed comparisons. No inferential comparison is reported for G. Tissue/molecular endpoints are exploratory.

Supplementary Files

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- [WBSupplementaryInformationCombined.pdf](#)