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ORIGINAL ARTICLE

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Human and animal fertility studies in cystinosis reveal signs of obstructive azoospermia, an altered blood-testis barrier and a subtherapeutic effect of cysteamine in testis

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Abstract

Cystinosis is an inherited metabolic disorder caused by autosomal recessive mutations in the *CTNS* gene leading to lysosomal cystine accumulation. The disease primarily affects the kidneys followed by extra-renal organ involvement later in life. Azoospermia is one of the unclarified complications which are not improved by cysteamine, which is the only available disease-modifying treatment. We aimed at unraveling the origin of azoospermia in cysteamine-treated cystinosis by confirming or excluding an obstructive factor, and investigating the effect of cysteamine on fertility in the *Ctns*$^{-/-}$ mouse model compared with

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wild type. Azoospermia was present in the vast majority of infantile type cystinosis patients. While spermatogenesis was intact, an enlarged caput epididymis and reduced levels of seminal markers for obstruction neutral α-glucosidase (NAG) and extracellular matrix protein 1 (ECM1) pointed towards an epididymal obstruction. Histopathological examination in human and mouse testis revealed a disturbed blood-testis barrier characterized by an altered zonula occludens-1 (ZO-1) protein expression. Animal studies ruled out a negative effect of cysteamine on fertility, but showed that cystine accumulation in the testis is irresponsive to regular cysteamine treatment. We conclude that the azoospermia in infantile cystinosis is due to an obstruction related to epididymal dysfunction, irrespective of the severity of an evolving primary hypogonadism. Regular cysteamine treatment does not affect fertility but has subtherapeutic effects on cystine accumulation in testis.

KEYWORDS

azoospermia, cysteamine, cystinosis, epididymal obstruction, infertility

1 | INTRODUCTION

Cystinosis is an inherited lysosomal storage disorder caused by recessive mutations in the *CTNS* gene encoding the lysosomal cystine transporter cystinosin.[1] The disease is characterized by lysosomal cystine accumulation in all cells of the body, and initially affects kidneys followed by extra-renal manifestations in several organs. Depending on the severity and age at presentation, three different phenotypes of cystinosis are distinguished: the most severe infantile form (95% of patients), the juvenile (5%), and the rare adult ocular benign form.[2,3] Starting from the early 90s of the last century, cystinosis patients are routinely treated by the cystine-depleting drug cysteamine which delays the progression of kidney disease and postpones or prevents the occurrence of extra-renal complications.[2] The advent of renal replacement therapy and cysteamine treatment significantly improved survival of cystinosis patients who can now reach adulthood. This prolonged life expectancy revealed several adult manifestations of cystinosis, one of which is infertility of male patients.[4,5]

Azoospermia is one of the main causes of male infertility, which is for clinical purposes classified into two subtypes: obstructive (OA) and non-obstructive azoospermia (NOA).[6] OA and NOA are differentiated mainly via testicular volume and secondary sexual characteristics, and biochemically via evaluating the sexual hormonal levels.[7] However, other techniques, such as scrotal ultrasound and evaluation of obstruction markers in the seminal plasma, have recently been put forward as promising novel diagnostic tools.[8-11]

While primary hypogonadism is a well-known extra-renal complication in cystinosis, azoospermia has been identified as the cause of infertility in male cystinosis patients.[5] However, we demonstrated previously that this azoospermia occurs irrespective of kidney function, cysteamine treatment, and sex hormone levels, which leaves the mechanism of azoospermia yet to be revealed.[5] In addition, as cysteamine can act as a direct spermicide or alter fertility at the hormonal level in animals, its potential detrimental effect in male cystinosis patients has been of concern.

In the present study, we aimed to unravel the origin of azoospermia in cystinosis and the role of cysteamine by performing clinical and mechanistic studies in humans and in a mouse model of cystinosis.

2 | MATERIAL AND METHODS

Please see Supporting Information methods for more details.

2.1 | Patients

As cystinosis is a rare condition and given obvious ethical drawbacks related to invasive procedures, in this study we combined data from a retrospective and a prospective cystinosis patient cohorts.

The retrospective cohort consisted of five male patients (infantile phenotype; average age 26 ± 6 years) who already had undergone a testicular biopsy or percutaneous epididymal sperm aspiration (PESA) before, as part of regular clinical care. Three of these patients (patients 2-4) were recruited from our previous study by Besouw et al[5]; however, in this study, we performed additional investigations on their collected tissues. In these five patients, histological sections of testicular tissue (formalin fixed paraffin embedded, FFPE) from three patients, along with the PESA data for the two remaining patients, were retrieved. The corresponding available sex hormone levels including luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and inhibin B, kidney function, genetic background for the *CTNS* mutation, and historical semen analysis results were obtained from the medical records.

The prospective cohort consisted of nine male adult patients (average age 34 ± 7 years; six infantile type, two juvenile type, one ocular type) followed regularly at the University Hospital Leuven (UZ Leuven, Belgium), or the Radboud University Medical Centre Nijmegen (Radboud UMC, The Netherlands), nine patients following vasectomy (average age 42 ± 8 years; average days following vasectomy 56 ± 26), and seven healthy men (controls; average age 33.2 ± 7 years; Table 2, Table S1). A history and clinical examination, scrotal ultrasound, blood sampling for sex hormone levels, and kidney function tests were performed, and a sperm sample produced via masturbation after at least 2 days of abstinence, was analyzed.

2.2 | Scrotal ultrasound

All subjects from the prospective cohort underwent scrotal ultrasound on which the following parameters were assessed: diameter of the caput (craniocaudal), corpus (anteroposterior), and cauda (craniocaudal) epididymis (mm) according to a novel described methodology by Pezzella et al,[8] bilateral testicular volumes (cm^3), and the presence of epididymal cysts and sperm granuloma. At present, this novel methodology by Pezzella et al has not been applied widely in clinical studies and is not being used in regular clinical practice.

For analysis, testicular volumes were calculated as follows: radius long axis × radius short axis a × radius short axis b × 4/3 × π. The average caput epididymis craniocaudal diameter (mm normalized to testicular volume (mm/cm^3) was obtained by the mean of the measurements of right and left, each normalized to the respective testicular volume. Subsequently, the average normalized caput diameter in each group was compared to the other study groups.

TABLE 1 Demographic and clinical characteristics of cystinosis patients included in the retrospective part of the study

Pt	Age (year)	Phenotype	Genotype	Age at initiation cysteamine	KTx	eGFR (mL/min/1.73 m^2)	LH (IU/L)	FSH (IU/L)	Testosterone (nmol/L)	Inhibin B (ng/L)	Testis volume (mL)	Semen analysis	Testicular sperm	Johnsen score	Epididymal sperm
Reference values						> 90	1.7-8.6	1.2-7.7	9-38	105-439					
1	16	INF	57 kb del + 922insG	3	N	39	4.6	5.9	18.0	*nav*	*nav*	*nav*	Y	8-9	*na*
2	25	INF	*nav*	5	Y	> 90	16.5	28.0	21.1	*nav*	*nav*	Azoospermia	*na*	*na*	Y
3	28	INF	Hom 57 kb del	2	Y	50	16.0	19.0	11.4	91	18	Azoospermia	Y	7–8	*na*
4	29	INF	57 kb del + c.141-24 T	4	Y	> 90	12.0	16.0	22.0	127	18	Azoospermia	*na*	*na*	Y
5	33	INF	Hom 57 kb del	18	Y	60	9.6	7.3	22.2	96	18	Azoospermia	Y	8–9	*na*

Abbreviations: eGFR, estimated glomerular filtration rate; FSH, follicle stimulating hormone; INF, infantile; KTx, kidney transplantation status; LH, luteinizing hormone; N, no; *na*, not applicable; *nav*, not available; Pt, patient; Y, yes.

TABLE 2 Demographic and clinical characteristics of cystinosis patients included in the prospective part of the study

Pt	Age (year)	Phenotype	Genotype	Age at initiation cysteamine	KTx	eGFR (mL/min/1.73 m^2)	LH (IU/L)	FSH (IU/L)	Testosterone (ng/dL)	Inhibin B (ng/L)	Testis volume (Le-Ri) (mL)	Days abst	Semen volume (mL)	Semen pH	Sperm concentration ($\times 10^6$/mL)	Spermatocyte morphology (% normal)
Reference values						>90	1.7–8.6	1.2-7.7	5.0-20.0	105–439	≧ 18	≧ 2		≧ 7.2	≧ 20	≧ 4
6	23	INF	Hom 57 kb del	2	N	57	7.4	6.7	7.0	115	12-10	2	0.5	7.8	6.0	1
7	29	INF	Hom 57 kb del	1	Y	28	13.0	9.3	6.0	*na*	17.5-15	2	1.0	7.5	Azoospermia	*na*
8	31	INF	57 kb del + 1015G > A	2	Y	15	41.0	56.0	4.0	53	14-12	7	2.3	7.3	Azoospermia	*na*
9	32	INF	*nav*	1	Y	20	106.0	132.0	4.4	<10	4–4	7	1.5	6.7	Azoospermia	*na*
10	39	INF	Hom 57 kb del	3	Y	31	47.0	140.0	6.0	<10	6–8	2	1.5	8.3	Azoospermia	*na*
11	39	INF	*nav*	2	Y	20	33.2	49.3	7.5	30	6-6	3	0.6	7.0	Azoospermia	*na*
12	29	JUV	Hom c.198_218del	10	N	84	12.0	4.0	8.0	138	15-15	1	0.9	7.8	6.4	5
13	35	JUV	*nav*	12	N	62	8.2	2.6	10.3	234	25-20	3	1.8	7.7	15.9	4
14	48	OC	*nav*	*na*	*na*	90	7.8	2.4	7.1	345	20-20	2	4.0	7.2	71.6	14

Abbreviations: abst, abstinence; eGFR, estimated glomerular filtration rate; FSH, follicle stimulating hormone; INF, infantile; JUV, juvenile; KTx, kidney transplantation status; Le, left; LH, luteinizing hormone; *na*, not applicable; *nav*, not available; OC, ocular; Pt, patient; Ri, right.

2.3 | Semen analysis and collection of seminal plasma in patients

The following parameters were assessed on semen sample within 1 hour after sampling, according to routine laboratory protocols and WHO guidelines: volume, viscosity, pH, concentration, mobility, and morphology. Seminal plasma was isolated from all semen samples by centrifugation (300*g*, 10 minutes at RT), and supernatant was immediately frozen and kept at −80°C until further processing.

2.4 | Evaluation of extracellular matrix protein 1 (ECM1) and neutral α-glucosidase (NAG) activity in seminal plasma in patients

ECM1 and NAG were evaluated in seminal plasma by immunoassay and colorimetric assay, using human ECM1 ELISA kit (SEK10362, Sino Biological Inc.) and EPI-SCREEN PLUS (FertiPro, Belgium), respectively, according to the manufacturer's protocol and as described previously.[11] ECM1 has recently been put forward as a novel seminal marker for non-invasive assessment of vasal obstruction. The samples were run in duplicates in 96-well plates, and values were estimated using standard curves (36.06-2500 pg/mL for ECM1 and 2.32-144 mIU/mL for NAG). The absorbance was measured at 450 nm for ECM1 and at 405 nm for NAG, using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific).

2.5 | Animals and cysteamine treatment

Wild type C57BL/6J male mice, along with wild-type C57BL/6J female mice for the mating experiments, were purchased from Charles River Laboratories, France. Knockout (KO) *Ctns*$^{-/-}$ male mice with C57BL/6J background (C57BL/6J-*Ctns*$^{-/-}$) were obtained as a compliment from Prof. C. Antignac, Laboratory of Hereditary Kidney Disease, Imagine Institute, INSERM U1163, Université de Paris, Paris, France.[12]

2.6 | Animal study design

Sixteen wild type and 16 KO male mice of 2 months of age were randomized to either the treatment group (500 mg/kg/day oral cysteamine, blended with standard diet, Table S2) or the control group (standard diet only), forming four groups; WT control, WT treatment, KO control, and KO treatment, as shown in Figure 4A (4-8 mice per each group). Before the treatment started, the body weight (BW) for all mice was measured. After 4 and 5 months of treatment, the mice were subjected to two mating rounds. After 6 months of treatment, the study was terminated, and the following parameters are measured: BW gain normalized to the baseline BW (%), seminal vesicle weight, and testis weight (weight of two seminal vesicles and weight of one testis were normalized to the total BW for each mouse). In addition, the epididymis was collected and utilized to estimate the epididymal sperm count, while testes were collected and fixed in alcohol formalin (AFA0060AF59001, VWR) overnight at room temperature.

A separate bioavailability study was conducted, where 12 male WT and 12 male *Ctns*$^{-/-}$ KO mice were used at 6 months of age. Both WT and KO mice were randomized into two groups: one group receiving i.p. injections of 1× PBS as a control group, and one group receiving 120 mg/kg/day cysteamine i.p. in 1× PBS vehicle as a treatment group.

2.7 | Cystine and cysteamine levels evaluation

Tissue cystine levels were evaluated in the Laboratory of Metabolic Biochemistry Unit, Department of Pediatric Medicine, Bambino Gesù Children's Hospital, Rome, Italy, with modifications from the method that was published earlier.[13,14] In addition, plasma and tissue cysteamine levels were evaluated in the UCSD Biochemical Genetics and Metabolomics Laboratory, University of California San Diego, as was published earlier.[15]

2.8 | Testis morphological analysis

Testicular tissue biopsies from cystinosis patients were obtained via a clinical standard procedure in which a frontal incision is made over the full length of both testes, and tissue is collected throughout the full length of each of both testes.

In patients, FFPE human testicular sections were subjected to periodic acid-Schiff (PAS) staining, and later analyzed under Eclipse CI microscope (Nikon, Japan), while pictures were taken with DS-Fi3 camera (Nikon), and NIS-Elements BR 4.60.00 64-bit software (Nikon).

Johnsen's score was used to evaluate the quality of spermatogenesis in the human samples.[16] Between 195 and 218 tubules were counted for each of the three patients.

In animals, FFPE sections of murine testicular tissues were subjected to PAS staining, and spermatogenic index was calculated as the percentage of tubules at stage VIII to the total number of tubules for each section, as defined by Oakberg.[17] Stage VIII was chosen because it represents the latest developmental stage for the germ cell

inside the tubules. Staining and counting were performed for three sections per sample at different depth (>25 μm in between). Between 58 and 289 tubules were counted for each sample. The average spermatogenic index in each group was calculated and compared between the different groups.

2.9 | Immunofluorescence & immunohistochemistry

FFPE testicular sections from three retrospective infantile cystinosis patients were investigated by immunofluorescence and immunohistochemistry, using antibodies for zonula occludens-1 protein (ZO-1), which is a known marker of blood-testis barrier, CD68 and mast cell tryptase. Meanwhile, murine testicular FFPE sections were investigated by immunofluorescence, using antibodies for ZO-1 (Table S3).[18]

2.10 | Hormonal profile assessment

Sex hormone levels were measured to evaluate gonadal function in patients. Serum levels of testosterone (reflecting Leydig cell function), inhibin B (reflecting Sertoli cell function), and the stimulating hormones LH (for Leydig cells) and FSH (for Sertoli cells) were evaluated in all cystinosis patients of the prospective cohort. The assessment was performed in the clinical laboratories at the participating centers using ELISA according to the routine clinical laboratory protocols being used for regular care.

In all mice (wild type and KO), similar plasma hormone levels were evaluated using commercial ELISA kits for testosterone (EIA-1559, DRG, Germany), LH (CEA441Mu, Cloud-Clone Corp), and FSH (CEA830Mu, Cloud-Clone Corp) according to the manufacturers' protocols, with intra-assay coefficient of variation (CV) <5%, 10%, and 10%, respectively, and inter-assay CV <10%, 12%, and 12%, respectively. The detection ranges were 0.083 to 16 ng/mL for testosterone, 0.149 to 30 ng/mL for LH, and 0.84 to 200 ng/mL for FSH. Absorbance for all kits was measured at 450 nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific).

2.11 | CTNS downregulation in fertile human caput epididymal cell line and RNA sequencing

To further understand the mechanism of epididymal dysfunction, immortalized fertile human caput epididymal (FHCE) cells were cultured as previously described.[19] The cells were transfected with either scrambled siRNA, as a negative control, or *CTNS* siRNA for 48 hours, followed by RNA sequencing (see Supporting Information materials and methods). Sequencing data are available on NCBI.GEO with accession number: GSE148733.

2.12 | Statistical analysis

Graphpad Prism (version 8.1.0 [221] for Mac OS X) (GraphPad Software, La Jolla, California, www.Graphpad.com) was used for the statistical analysis of data from patients. A D'Agostino & Pearson normality test was applied for assessing normal (Gaussian) distribution of the data, followed by parametric or nonparametric tests. In animals, student *t*-test for parametric data and Mann-Whitney test for nonparametric data were applied, using the Sigma Plot software ver.12.0 (Systat Software Inc., Illinois). Data are represented as the mean ± SD for normally distributed data, unless otherwise specified. The means and standard deviations were used in the figures as indicated and each experimental condition was repeated at least three times. A *P*-value <.05 was considered to indicate a significant difference.

3 | RESULTS

3.1 | Epididymal obstruction is the common cause of azoospermia in infantile cystinosis on top of an evolving testicular failure

In order to confirm or reject a possible obstruction as the cause of azoospermia in male cystinosis patients, historical testicular and/or epididymal histological examinations were correlated with semen analyses and sex hormone levels in a retrospective cohort (Table 1), while markers for obstruction were assessed via scrotal ultrasound and on seminal plasma along with semen and sex hormone analysis in a prospective cohort (Table 2). Given the invasive nature of the procedure, and the potential risk for detrimental side effects, testicular or epididymal biopsies were not possible on ethical grounds in the prospective cohort.

First, azoospermia was confirmed in nine out of 10 infantile type cystinosis patients in both cohorts in total (Tables 1 and 2). Remarkably, one infantile cystinosis patient showed oligospermia, similar to both of the two juvenile patients, while the ocular patient showed normozoospermia.

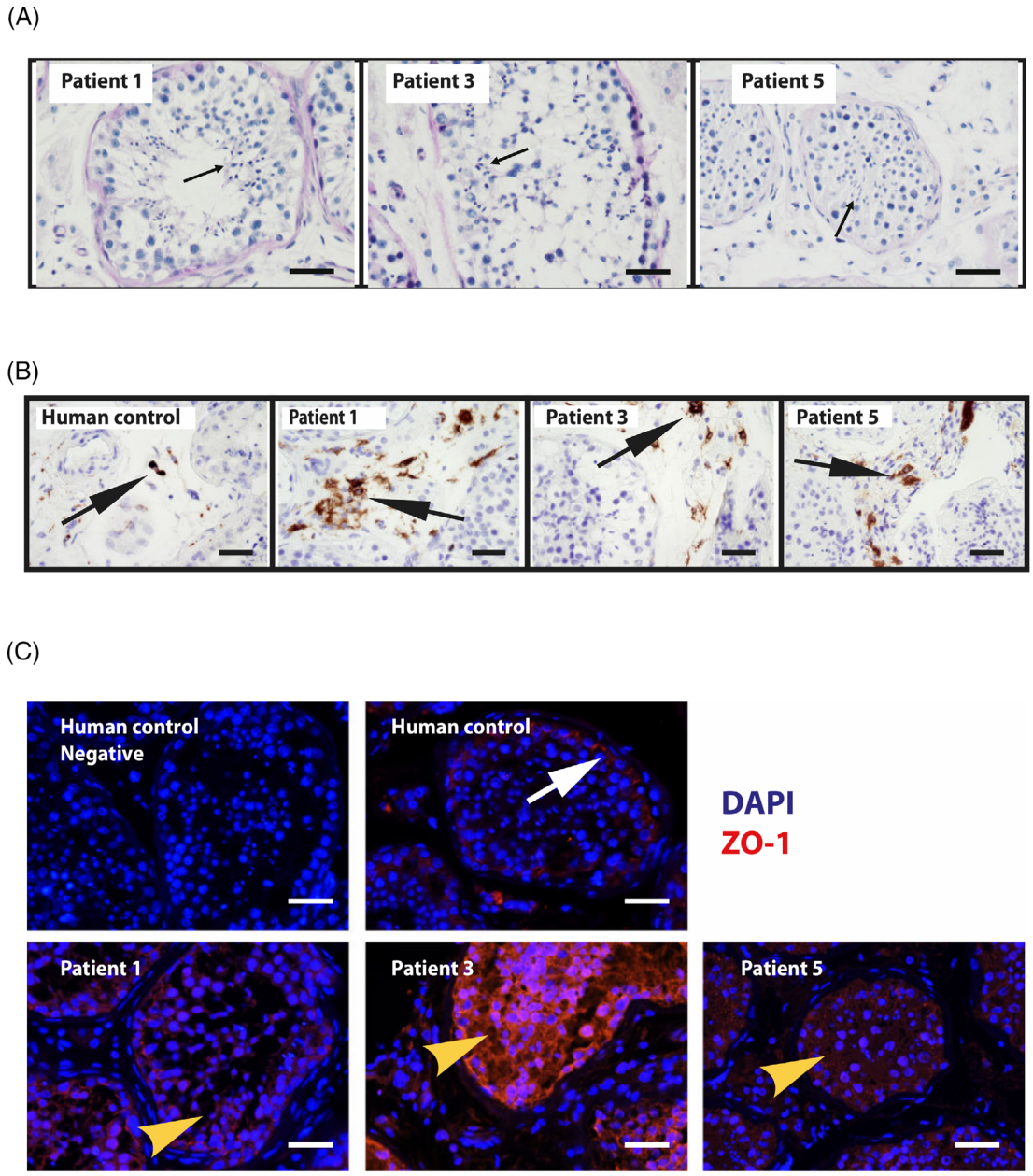

FIGURE 1 Testicular tissue in azoospermic infantile cystinosis patients shows macrophage infiltration and alterations of the BTB while spermatogenesis is ongoing. Panel (A): PAS staining for formalin-fixed paraffin-embedded (FFPE) testicular sections (5 μm thickness) from three infantile cystinosis male patients (patients 1, 3, and 5). Black arrows indicate the presence of post-meiotic elongated spermatids. Panel (B): Immunohistochemistry staining for CD68 (macrophage marker) in FFPE testicular sections (5 μm thickness) from one human male control (control subject n°1) and three infantile cystinosis male patients (patients 1, 3, and 5). The black arrows indicate the CD68 positive cells. Panel (C): Immunofluorescence staining for zonula occludens-1 (ZO-1) in FFPE testicular sections (5 μm thickness) from one human male control (control subject n°1) and three infantile cystinosis male patients (patients 1, 3, and 5). ZO-1 is shown in red and nucleus in blue (DAPI). The upper left square of the panel shows the negative control for the staining, performed by omitting the primary antibody. The white arrow indicates the intact blood-testis barrier (BTB), shown by the positive ZO-1 red staining lining the inner side of the seminiferous tubules, while the yellow arrowheads indicate the disturbed diffuse tubular positive ZO-1 red staining. Scale bars in all panels (A, B, and C) represent 50 μm

In all azoospermic patients, sex hormone levels and testicular volumes revealed a pattern compatible with primary hypogonadism, which is in line with previous observations and confirms primary testicular failure as a contributing factor to azoospermia.[4,5,20] However, similarly to our previous report,[5] while the sex hormone levels showed a wide range of inter-patient variation and testicular volumes were mostly reduced with increasing

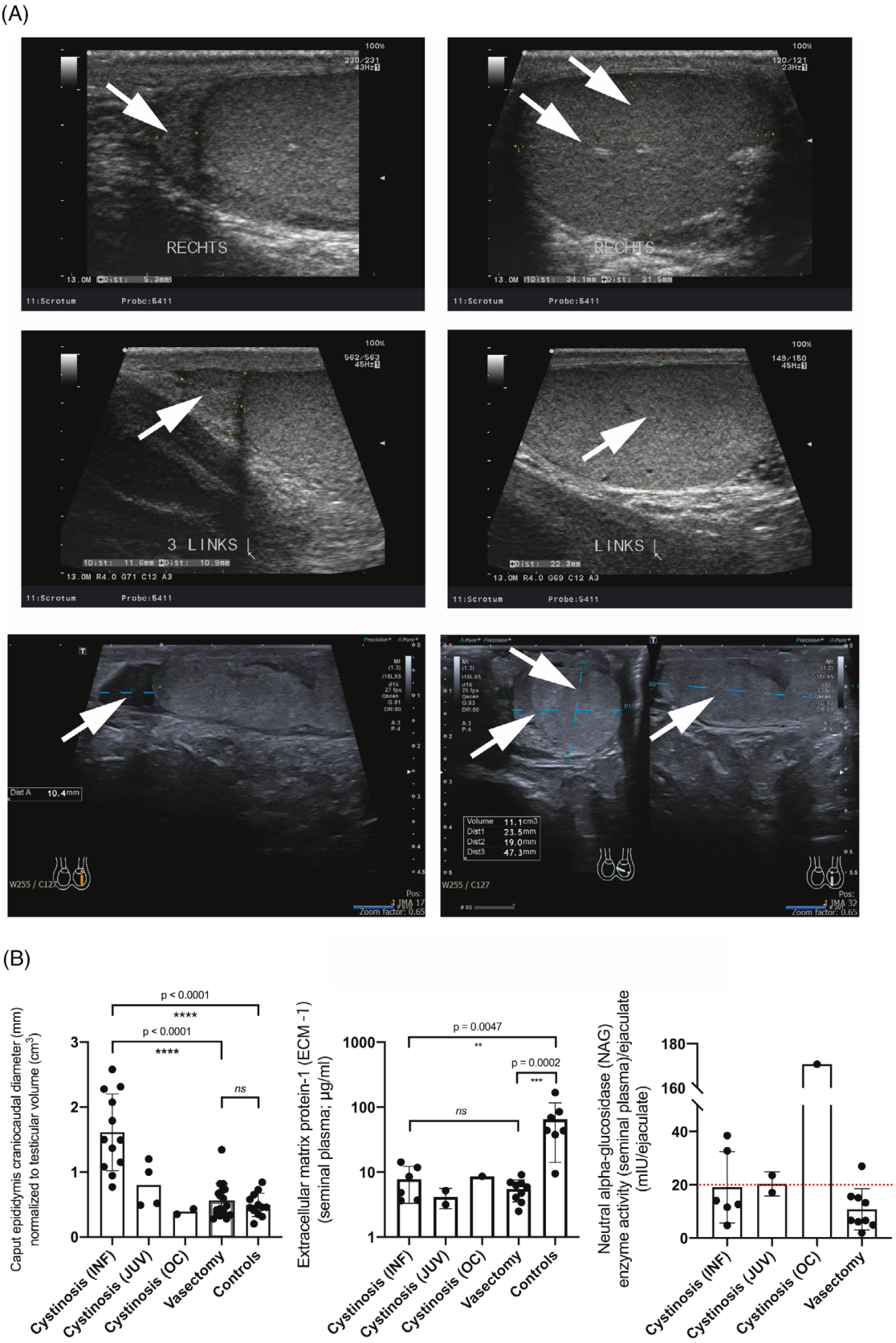

FIGURE 2 Legend on next page.

age, no correlation was observed between the occurrence of azoospermia, and sex hormone levels, kidney function, or age of initiation of cysteamine. All azoospermic patients (n = 9) had a kidney transplant, and only one patient had chronic kidney disease (CKD stage 5); hence, the fertility status cannot be solely explained by kidney dysfunction.[21-24]

Moreover, morphological examination of testicular biopsies displayed the presence of post-meiotic germ cells (elongated spermatids) with Johnsen's score between 7 and 9, indicating an intact spermatogenesis (Table 1, patients 1, 3, and 5; Figure 1A).[16] In addition, data on epididymal sperm extraction from the cauda epididymis of two patients demonstrated the presence of viable epididymal sperm (Table 1, patients 2 & 4). Taken together, azoospermia in the presence of an intact spermatogenesis at the testicular and epididymal level highly suggests obstruction to be involved in its pathogenesis.

Therefore, non-invasive investigations including scrotal ultrasound (US) and the assessment of markers for obstruction in seminal plasma were applied to confirm or rule out an obstructive cause for azoospermia.

In the infantile cystinosis patients, a significantly enlarged diameter of the caput epididymis normalized to the ipsilateral testicular volume was demonstrated, compared with healthy controls. The two juvenile cystinosis patients who were oligospermic, and the ocular cystinosis patient showing normospermia, had a normal caput diameter similar to the control group (Figure 2A,B).

Further evaluation of the markers of vasal obstruction in seminal plasma, revealed that infantile cystinosis patients, similar to vasectomy patients, had significantly reduced ECM1 levels compared to control subjects. In addition, evaluation of seminal NAG activity showed that infantile cystinosis patients, similar to vasectomy patients, had median levels below the lower limit of normal (Figure 2B).

Taken together, these ultrasonographic and seminal plasma data are in support for an obstructive cause of azoospermia in the epididymis, on top of an evolving primary hypogonadism, and suggest epididymal dysfunction.

3.2 | Transcriptomic analysis in *CTNS KD* human epidydimal cell line hints to inflammation and loss of epithelial polarization as mechanisms of epididymal dysfunction

In order to underpin and further unravel a potential epididymal dysfunction in cystinosis, transcriptomic analysis was performed on an immortalized fertile human epididymal cell line 48 h following *CTNS KD* via *CTNS* siRNA transfection. Quantitative real-time PCR results confirmed that *CTNS* was downregulated by 94 ± 3% (Figure S1A). There were no obvious morphological differences between scrambled and *CTNS* siRNA transfected cells (Figure S1B). RNA sequencing revealed 55 genes that were significantly upregulated and 84 genes that were significantly downregulated by more than twofold upon *CTNS* downregulation (Figure 3A,B, and Table S4). While principle component analysis showed no clear clustering of gene expression (Figure S1C), a gene set enrichment analysis showed altered biological processes,

FIGURE 2 Scrotal ultrasound (US) of the caput epididymis and testicular volume, and analysis of markers for obstruction in seminal plasma of cystinosis patients, vasectomy patients and healthy control subjects. Panel (A): Representative scrotal US images illustrating the assessment of the caput epididymis. Upper left: measurement of the right caput epididymis craniocaudal diameter of a healthy control (control subject n°17), measured in green dots (5.3 mm) which is indicated by the white arrow. Upper right: measurement of testicular volume via two dimensions measured in green dots (34.1 and 21.5 mm) which is indicated by the white arrows. Middle left: measurement of left caput epididymis craniocaudal diameter of a vasectomized patient (control subject n° 36), measured in yellow dots (11.6 mm) which is indicated by the white arrow. Middle right: measurement of the testicular volume of the ipsilateral testis of the vasectomized patient (control subject n° 36) with one dimension measured in green dots (22.3 mm) which is indicated by the white arrow. Lower left: measurement of the left caput epididymis craniocaudal diameter of an infantile cystinosis patient (patient n°10), measured in green dots (10.4 mm) which is indicated by the white arrow. Lower right: measurement for the ipsilateral (same left-sided) testis of the same infantile cystinosis patient, with three dimensions measured in green dots (23.5, 19.0, and 47.3 mm, respectively) which is indicated by the white arrows. Panel (B): Scrotal US and seminal plasma markers indicate an obstructive cause for azoospermia. Left: Caput epididymis craniocaudal diameter (mm) normalized to the testicular volume for the ipsilateral testis (cm^3) in cystinosis (infantile (INF), juvenile (JUV), and ocular (OC)) and vasectomy patients, and healthy control subjects. Each dot represents a single-sided measurement of a single patient. The craniocaudal diameter of the caput epididymis is significantly increased in infantile cystinosis patients compared to patients following vasectomy or healthy controls ($P < .0001$). Middle: ECM1 levels in seminal plasma (μg/mL) of infantile cystinosis male patients is comparable to vasectomy patients, and significantly lower compared to healthy control subjects ($P = .0047$) which parallels the significant lower level of ECM1 in seminal plasma of vasectomy patients compared to healthy control subjects ($P = .0002$). Right: Neutral alpha-glucosidase (NAG) seminal plasma activity per one ejaculate (mIU/ejaculate) falls below the WHO lower limit of normal NAG activity (20 mIU/ejaculate; red dotted line) and is comparable to vasectomy patients

comprising response to fluid shear stress, interleukin-6 production, actin cytoskeleton reorganization, and modified amino acids and sulfur compounds transport (Figure 3C). Taken together, these data hint to inflammation and loss of polarity as potential mechanisms involved in epididymal malfunction.

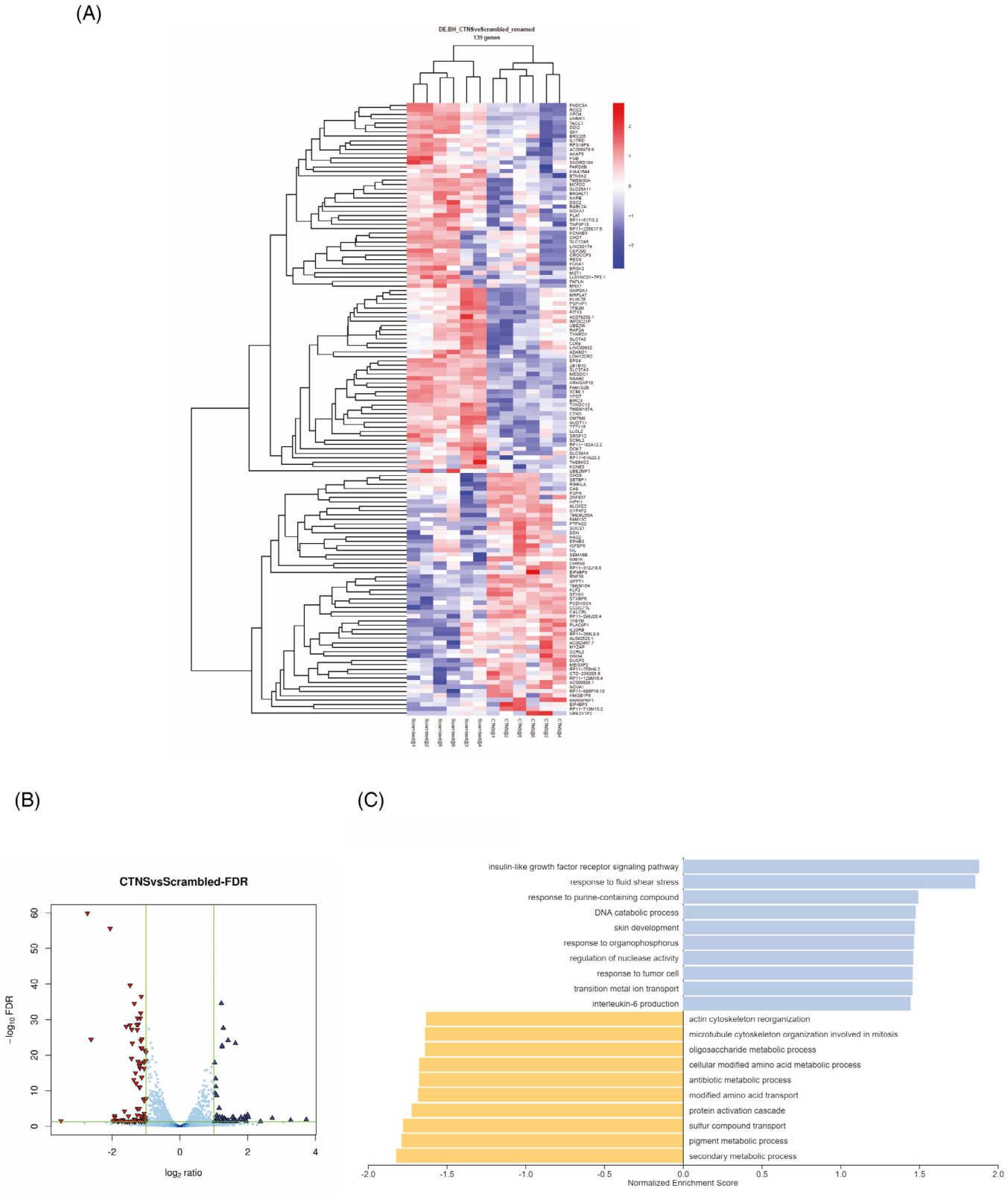

FIGURE 3 Legend on next page.

3.3 | A perturbed BTB and enhanced macrophage infiltration characterizes testicular tissue in azoospermic infantile cystinosis

As tissue cystine accumulation is accompanied by an inflammatory response, and in order to explore whether inflammation could also be involved in the development of the primary hypogonadism in cystinosis, we performed immunohistochemistry for CD68 (a marker of macrophages) in the cystinosis patients of whom testicular tissue was available.[25] This showed an enhanced infiltration of macrophages into the testicular interstitium (Figure 1B, and Figure S2). Immunofluorescence for ZO-1 showed a more diffuse cytoplasmic ZO-1 staining as compared with the control, indicating a profoundly perturbed BTB (Figure 1C). Taken together, our data indicate that inflammation related cystine accumulation might be a common factor in the pathogenesis of primary hypogonadism and epididymal dysfunction leading to obstruction.

3.4 | Cysteamine does not affect fertility status of the C57BL/6J-*Ctns*$^{-/-}$ mouse model

In order to study the potential detrimental effect of cysteamine on fertility, animal studies in the C57BL/6J-*Ctns*$^{-/-}$ mouse model were undertaken.

We demonstrated that cysteamine had no significant effect on epididymal sperm count, number of litters per mouse, plasma LH, FSH, and testosterone (Figure S3A-C). In addition, there were no significant effects on BW gain relative to the baseline BW (%) at the start of the experiment, and the seminal vesicle weight normalized to the BW.

Also, cysteamine had no effect on the spermatogenic index of testes in mice (Figure 4B). As was observed in cystinosis patients, the BTB was significantly perturbed in KO mice, which could not be restored by cysteamine treatment. In WT animals, cysteamine had no effect on the immunolocalization of ZO-1 at the plasma membrane level of sertoli cells (Figure 4C).

3.5 | Bio-availability of cysteamine in the C57BL/6J-*Ctns*$^{-/-}$ mouse model

Remarkably, cystinosis KO mice showed an excessive cystine accumulation in the testis as compared to WT mice (36.1 ± 14.5 μmol/g protein for KO vs 0.1 ± 0.2 μmol/g protein for WT) which was ~15 times higher than in the kidney of KO animals (57.7 ± 27.2 μmol/g protein for testis vs 4.4 ± 5.0 μmol/g protein for kidney; Figure 4D). Cysteamine could not significantly reduce testicular cystine levels in KO mice following 6 months of oral cysteamine. Therefore, a separate cysteamine bioavailability study was performed, which showed that in plasma, cysteamine reached therapeutic levels in both WT and KO mice, with no difference between both groups and cysteamine reached similar levels in testis and kidney (Figure 4D), Nevertheless, similar to oral treatment, i.p. cysteamine did not significantly reduce testicular cystine accumulation in the KO mice.

4 | DISCUSSION

In this study, we aimed to further unravel the origin of azoospermia in male cystinosis patients. While juvenile patients displayed oligospermia and the ocular patient normospermia, azoospermia was confined to the vast majority of the infantile type cystinosis patients. In our study, nine out of 10 KTx infantile cystinosis patients were azoospermic. While in the majority of these patients, hormone levels and testicular volumes displayed a pattern compatible with primary hypogonadism, primary testicular failure cannot fully explain the

FIGURE 3 Transcriptomic analysis of *CTNS* KD on immortalized human epididymal epithelial cells. Panel (A): Heat map for differentially expressed genes (DEGs) for scrambled siRNA (n = 6) vs *CTNS* siRNA (n = 6) treated immortalized human epididymal epithelial cells. The heat map was created by computing Spearman-correlation between all samples using the normalized counts as expression values. Panel (B): Volcano plot for the DEGs for scrambled siRNA vs *CTNS* siRNA treated immortalized human epididymal epithelial cells. The Y-axis shows ($-\text{Log}_{10}$) of the Benjamini-Hochberg corrected *P*-values to control the false discovery rate (FDR), while the X-axis shows the fold change in expression by the (Log_2) ratio. The horizontal line defines significance with FDR values less than .05 indicating significant dysregulation, while the vertical lines indicate genes that had higher than twofold change in expression; downregulated on the left and upregulated on the right. Panel (C): Gene ontology (biological processes) by gene set enrichment analysis (GSEA) for the differentially expressed genes after transfection of the immortalized human epididymal epithelial cells with either scrambled siRNA or *CTNS* siRNA. To obtain the gene ontology, a list of the differentially expressed genes with their scores (based on the significance and the fold expression) were loaded to WEB-based GEne SeT AnaLysis Toolkit (WEBGESTALT) online. The blue bars demonstrate the top 10 upregulated enriched gene sets, while the yellow bars demonstrate the top 10 downregulated enriched gene sets. The normalized enrichment scores are shown on the X-axis

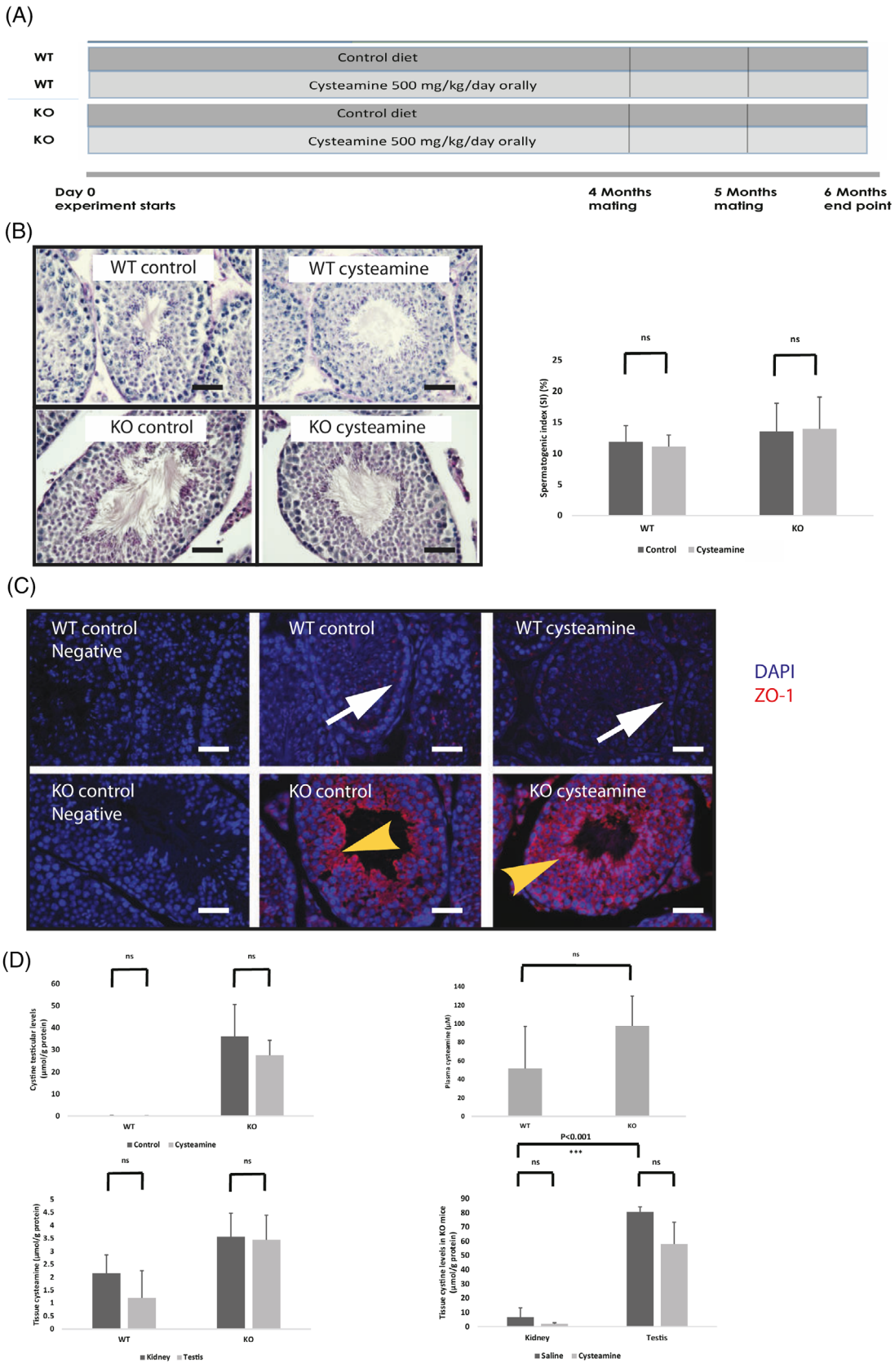

FIGURE 4 Legend on next page.

azoospermia observed in all patients, given the variability in the severity of the hypogonadism, and corresponding testicular or epididymal histopathological findings. Indeed, in azoospermic patients, we have found either intact spermatogenesis at the testicular level or viable sperm in the epididymis, which suggests that obstruction was implicated in its pathogenesis, apart from a primary hypogonadism. In addition, while all azoospermic patients had a kidney graft, only one patient was in end-stage kidney disease, thus the fertility status could not be attributed to kidney failure. The increased diameter of the caput epididymis normalized to testicular volume as measured via scrotal ultrasound, and the significant lower levels of ECM1 and NAG activity in seminal plasma in these azoospermic patients, support our hypothesis and point toward an obstruction leading to azoospermia, irrespective of the level of primary gonadal failure. While ECM1 in seminal plasma is a recently identified marker of obstruction,[11] NAG activity is an established seminal marker, with a recommended WHO lower cut-off level of 20 mIU/ejaculate.[26,27] ECM1 is highly expressed by the epididymis,[11] while NAG is a specific marker for epididymal secretion.[26] Hence, reduced ECM1 levels and NAG activity in seminal plasma also suggest that the obstruction is occurring in the epididymis and may be related to epididymal dysfunction. In accordance with our findings, it has been recently reported that 67% of male infantile cystinosis patients show signs of obstructive azoospermia around adolescence, while testicular function shows gradual impairment with age, reaching gonadal failure in the fourth decade of life.[20]

In order to gain better mechanistic insights into a possible epididymal dysfunction, we performed *CTNS* downregulation in an immortalized FHCE epididymal cell line, using siRNA technology. Our data showed that downregulation of *CTNS* altered multiple cellular pathways, including the upregulation of pathways related to fluid shear stress and inflammation, and downregulation of pathways involved in actin cytoskeleton reorganization and amino acid- and sulfur compounds transport. This suggests that *CTNS* downregulation results in a pro-inflammatory effect and a possible loss of cellular polarization. While it is plausible that inflammation related to cystine accumulation is involved in the development of epididymal dysfunction and obstruction, further studies are required to understand the role of cystinosin on the epididymis in general.

Likewise, little is known about the mechanism leading to primary hypogonadism in cystinosis.

Hence, we examined testes of cystinosis patients in greater detail, showing a disruption of the BTB, accompanied by macrophage infiltration. While cystinosis mice were fertile despite high testicular cystine levels, which is in line with our previous study by Besouw *et al.*,[28] these also displayed a disrupted BTB and cysteamine could neither sufficiently reduce cystine accumulation, nor could it restore the perturbed BTB. These data suggest a sub-therapeutic effect of cysteamine treatment at the testicular level. Interestingly, our observations are in line with the previously suggested role of inflammation in the pathogenesis of cystinotic kidney disease, in which similar to infertility, the phenotype cannot be cured by cysteamine.[27,29-31]Macrophages are known to secrete

FIGURE 4 Effect of oral cysteamine on fertility in wild type and *Ctns* knockout male mice. Panel (A): Overview of the pre-clinical animal study design. Wild type (WT) and *Ctns* knockout (KO) male C57BL/6J mice at 2 months of age were randomized to either the treatment group or the control group for 6 months, forming four groups; WT control, WT treatment, KO control, and KO treatment (n = 4 to 8 mice per each group). After 4 and 5 months of treatment, the mice were subjected to two mating rounds. After 6 months of treatment, the study was terminated. Panel (B): Effect of oral cysteamine for 6 months on spermatogenesis in WT and KO male mice. Left: PAS staining for formalin-fixed paraffin-embedded (FFPE) testicular sections (5 μm thickness) of mice from the four groups after 6 months of treatment, showing seminiferous tubules at stage VIII of the spermatogenic cycle. Scale bar is 50 μm. Right: spermatogenic index (SI). In the different study groups ($P = .51$ for WT and .89 for the KO; n = 4 to 8 mice per group; 58 to 289 seminiferous tubules were counted for each mouse). Panel (C): IF staining for zonula occludens-1 (ZO-1) in FFPE testicular sections (5 μm thickness) of mice from the four different study groups after 6 months of oral cysteamine treatment. Left: negative controls. Middle & right: The white arrow indicates the intact blood-testis barrier (BTB), shown by the positive ZO-1 red staining lining the inner side of the seminiferous tubules, while the yellow arrowheads indicate the disturbed diffuse tubular positive ZO-1 red staining. Scale bar is 50 μm. Panel (D): Cystine levels in tissues and cysteamine levels in plasma and tissues (in WT and KO mice). Upper left: testicular cystine levels after 6 months with or without oral cysteamine treatment in WT and KO male mice ($P = .45$ for the WT groups and .30 for the KO groups; n = 4 to 8 mice per group). Upper right: plasma cysteamine levels after 2 days of intra-peritoneal (i.p.) cysteamine injection (120 mg/kg/day divided in two doses) in WT and KO male mice at 6 months of age ($P = .23$; n = three mice per group). Lower left: tissue (testis and kidney) cysteamine levels after 2 days of intra-peritoneal (i.p.) cysteamine injection in WT and KO male mice at 6 months of age ($P = .26$ for the WT groups, and .88 for the KO groups; n = three mice per group). Lower right: tissue (testis and kidney) cystine levels in KO male mice at 6 months of age treated with either saline or cysteamine i.p. injection ($P = .07$ for treated to untreated testis comparison, $P = .29$ for treated to untreated kidney comparison, and $P < .001$ for untreated testis to untreated kidney comparison; n = three mice per group). Student's *t*-test was performed for the statistical comparison between the different groups, using the Sigma Plot software ver.12.0, and values are represented as mean ± SD

several inflammatory cytokines which have been shown to disturb the tight junctions of the BTB.[31] This may explain why the localization of tight junction proteins, such as ZO-1, displays a more cytoplasmic localization as the assembly of the tight junctions may be compromised.[28,29,32] These observations hint to a potential important role for inflammation in the pathogenesis of hypogonadism which will require further studies.

There has been a genuine concern that cysteamine treatment may be detrimental for male fertility in cystinosis patients. In sheep, oral administration of cysteamine resulted in reduced sperm count, sperm motility, and testosterone levels, as well as alterations in the BTB.[33] Other studies suggested that cysteamine can act as a spermicide.[34] In contrast to those data, our report does not indicate any negative effect of cysteamine on male fertility in either WT or cystinosis mice. On the other hand, cysteamine was unable to significantly lower testicular cystine accumulation, nor was it able to restore alterations in the BTB. Following this observation, we hypothesized that the bioavailability of cysteamine in the testis could be limited. Our bioavailability study showed that cysteamine levels in the testis are comparable with those observed in kidney, in both WT and KO mice. Similar levels of cysteamine in the kidney were achieved following intraduodenal administration in rats. Likewise, plasma levels were similar to those observed in mice injected intraperitoneally.[15] This suggests that the cysteamine dose used in this study is subtherapeutic for the testes which is likely to be the case in cystinosis patients, which could, in turn, explain the lack of effect of cysteamine on the development of gonadal failure.

In conclusion, male infertility in infantile cystinosis is caused by an obstructive azoospermia due to epididymal dysfunction, on top of an evolving non-obstructive process of primary hypogonadism. While cystine accumulation in testis is associated with inflammation, disruption of the BTB and primary hypogonadism, obstruction in the epididymis is linked to inflammation and the final determining cause for the observed azoospermia. As cysteamine treatment cannot reverse these pathological processes, novel therapies targeting these mechanisms are required.

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CONFLICT OF INTEREST

Elena Levtchenko performed consultancy for Recordati, Chiesi, Advicenne, and Kyowa Kirin. All other coauthors have no relevant disclosures.

AUTHOR CONTRIBUTIONS

Koenraad Veys, Ahmed Reda, Maarten Albersen, Carl Spiessens, Leo Monnens, Lambertus van den Heuvel, Ellen Goossens, and Elena Levtchenko: Responsible for study conception and design. **Koenraad Veys, Lambertus van den Heuvel, and Elena Levtchenko**: Responsible for funding acquisition. **Ahmed Reda, Koenraad Veys, Prashant Kadam, Anna Taranta, Laura Rita Rega, Bianca M. Goffredo, Chelsea Camps, Martine Besouw, Daniel Cyr, Liesbeth de Wever, Robert Hamer, Mirian C.H. Janssen, Kathleen D'Hauwers, and Alex Wetzels**: Responsible for data acquisition and interpretation. **Ahmed Reda and Koenraad Veys**: Responsible for the statistical analysis. **Ahmed Reda**: Wrote the first draft of the article. All authors contributed to the final article. **Koenraad Veys and Elena Levtchenko**: revised the final version of the article.

DATA AVAILABILITY STATEMENT

All raw data supporting the results can be provided upon request.

ETHICS STATEMENT

Approval from the institutional ethical board of UZ/KU Leuven (Ethische Commissie Onderzoek UZ/KU Leuven) and Radboud UMC Nijmegen (Centrum Mensgebonden

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The use and handling of animals were approved by the ethics committee for experimental laboratory animals at VUB (ethical permit number 16-216-4) and by the ethics committee of Italian Ministry of Health for experimental laboratory animals at Plaisant (ethical permit number 66/2018-PR).

PATIENT CONSENT STATEMENT

All study participants signed a written informed consent form prior to the inclusion in the study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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