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Testicular function in males with infantile nephropathic cystinosis

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STUDY QUESTION: Do males with the rare lysosomal storage disease infantile nephropathic cystinosis (INC) have a chance of biological fatherhood?

SUMMARY ANSWER: Cryostorage of semen could be an option for approximately 20% of young males with INC, with surgical sperm retrieval from the centre of the testes providing additional opportunities for fatherhood.

WHAT IS KNOWN ALREADY: Biallelic mutations in the cystinosin (*CTNS*) gene in INC cause dysfunction in cystine transport across lysosomal membranes and cystine accumulation throughout the body. Spontaneous paternity in cystinosis has not been described, despite the availability of cysteamine treatment. Azoospermia has been diagnosed in small case series of males with INC. ART using ICSI requires few spermatozoa, either from semen or extracted surgically from the testes of azoospermic men. However, there is limited evidence to suggest this could be successful in INC.

STUDY DESIGN, SIZE, DURATION: In this prospective cohort study performed between 2018 and 2019, we performed a cross-sectional investigation of 18 male patients with INC to delineate endocrine and spermatogenic testicular function.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Serum hormone levels, semen samples (according to World Health Organization 2010 standards), and testicular ultrasound images were analysed in 18 male patients aged 15.4–40.5 years. Surgical sperm extraction was performed in two, and their testicular biopsies were investigated by light and electron microscopy. Past adherence to cysteamine treatment was assessed from medical record information, using a composite scoring system.

MAIN RESULTS AND THE ROLE OF CHANCE: Adherence to cysteamine treatment was high in most patients. Testicular volumes and testosterone levels were in the normal ranges, with the exception of two and three older patients, respectively. Serum LH levels were above the normal range in all subjects aged $\geq$20 years. FSH levels were elevated in all but four males: three with spermatozoa in semen and one adolescent. Inhibin B levels were shown to be lower in older men. Testicular ultrasound revealed signs of obstruction in 67% of patients. Reduced fructose and zinc seminal markers were found in 33%, including two patients with azoospermia who underwent successful surgical sperm retrieval. Histology identified fully preserved spermatogenesis in the centre of their testes, but also tubular atrophy and lysosomal overload in Sertoli and Leydig cells of the testicular periphery.

LIMITATIONS, REASONS FOR CAUTION: Limitations of this study are the small number of assessed patients and the heterogeneity of their dysfunction in cystine transport across lysosomal membranes.

WIDER IMPLICATIONS OF THE FINDINGS: This study suggests that testicular degeneration in cystinosis results from the lysosomal overload of Sertoli and Leydig cells of the testicular periphery, and that this can possibly be delayed, but not prevented, by good adherence to cysteamine treatment. Endocrine testicular function in INC may remain compensated until the fourth decade of life; however, azoospermia may occur during adolescence. Cryostorage of semen could be an option for approximately 20% of young males with INC, with surgical sperm retrieval providing additional opportunities for biological fatherhood.

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Introduction

Cystinosis is a rare lysosomal storage disease caused by bi-allelic mutations in the *CTNS* gene (Town *et al.*, 1998; Gahl *et al.*, 2002). The *CTNS* gene product cystinosin is expressed in the cells of all tissues (Thoene *et al.*, 1999). In patients with cystinosis, dysfunction in cystine transport across lysosomal membranes leads to cystine accumulation and crystal formation in the cells of various organs throughout the body, causing a variety of effects owing to differing susceptibilities of the tissues (Nesterova and Gahl, 2013). The accumulation of cystine in endocrine organs (Abderhalden, 1903), leads to cellular dysfunction and cell death (Schulman *et al.*, 1969; Gahl *et al.*, 1982a,b; Kalatzis *et al.*, 2004; Veys *et al.*, 2017). In individuals affected by the most severe form of cystinosis, i.e., infantile nephropathic cystinosis (INC), the first clinical symptoms generally appear at 6 to 12 months of age, when renal proximal tubular reabsorption become dysfunctional. Urinary losses of water, Na^+, K^+, $HCO3^-$, Ca^{2+}, Mg^{2+}, phosphate, amino acids, glucose and proteins occur (De Toni Debré Fanconi syndrome), leading to polyuria, polydipsia, vomiting and dehydration (Cherqui and Courtoy, 2017). These losses also result in failure to thrive, developmental delay and rickets (Broyer *et al.*, 1981; Veys *et al.*, 2017).

In the past, progressive chronic kidney disease, culminating in end-stage renal failure at approximately 10 years of age, has limited long-term survival of affected children (Kleta *et al*, 2004). Since the advent of oral cysteamine therapy in the 1980s, this has successfully delayed rapid deterioration of renal function and thus end-stage kidney disease (Thoene *et al.*, 1976; Nesterova *et al.*, 2015). Cysteamine prevents lysosomal cystine storage by breaking the disulfide bond in cystine, leading to the formation of cysteine-cysteamine disulphide and cysteine: the former compound exits the lysosome via the lysosomal lysine transporter, bypassing the defective cystinosin pathway (Ariceta *et al.*, 2019). In addition, cysteamine increases the cell's capacity to deal with oxidative stress (Gahl and Tietze, 1985; Jézégou *et al.*, 2012). Timely initiation of cysteamine treatment, with doses every 6 hours around the clock, together with complementary treatment and the availability of kidney transplantation for children, now enables patients with INC to survive into adulthood, with the current life expectancy extending past 50 years (Gahl *et al.*, 2007; Nesterova and Gahl, 2013).

Cystine-depleting therapy has also proven effective in attenuating extra-renal disease manifestations, with endocrine complications such as severe growth retardation (Winkler et al., 1993; Besouw and Levtchenko, 2010), manifestations of diabetes mellitus and development of hypothyroidism being delayed by cysteamine treatment (Greco *et al.*, 2010; Vaisbich and Koch, 2010; Gultekingil Keser *et al.*, 2014; Ariceta *et al.*, 2019). It is unclear whether impairment of gonadal function in cystinosis is positively influenced by cysteamine. The pathophysiologic mechanisms involved in the disturbance of testicular function are also unresolved. Ascertainment of the prevalence and the pathophysiology of gonadal function in cystinosis are limited by the scarcity of published literature. While a few female patients with cystinosis are known to have given birth (Blakey *et al.*,2019), fathering a child through natural conception has not been reported in male patients. In the few male patients with INC assessed for gonadal function, azoospermia was observed (Chik *et al.*, 1993; Besouw *et al.*, 2010). A single report of conception with the help of ART has been published, with sperm from a male patient being retrieved from his epididymis by percutaneous aspiration and used for fertilization via ICSI (Veys *et al.*, 2018). The known detrimental effects of renal failure on spermatogenesis do not fully explain the impairment of testicular function, given that elevated serum FSH levels and decreased sperm motility do not return to normal following kidney transplantation (Lim and Fang, 1975; Akbari *et al.*, 2003; Lundy and Vij, 2019). The age at which spermatogenic or endocrine testicular failure occurs remains unclear. In addition, it is unknown if modern ART can enable biological fatherhood in males with cystinosis.

In this cross-sectional clinical study, we aimed to delineate endocrine and spermatogenic testicular function from adolescence to adulthood in males with INC, gain additional insight into the pathogenesis of reduced male fertility in INC via the histological evaluation of testicular biopsies, and clarify perspectives of males with INC for biological fatherhood.

Materials and methods

Patients

Eighteen males aged 15.4–40.5 years with confirmed INC, who were referred for assessment of gonadal function between July 2018 and January 2020, were eligible for the study. No patient with INC was excluded.

Cross-sectional investigations were performed at the tertiary Centre of Reproductive Medicine and Andrology, University Hospital Münster, Germany. All males were referred for assessment of gonadal function and potential fertility preservation from a centralized cystinosis care unit, i.e. the Department of Pediatric Nephrology, Children's Hospital RoMed Clinics Rosenheim, and from the Department of Pediatric Kidney, Liver and Metabolic Diseases, Children's Hospital, Hannover Medical School, Hannover, Germany.

Ethics

Ethics committee approval for this study was waived by the ethics committee (Ethikkommission der Bayerischen Landesärztekammer; 11 March 2015), as the planned project of an interdisciplinary cystinosis database does not fall under the consultation obligation according to paragraph 15 of the professional code of conduct for physicians in Bavaria, Germany.

Retrospective evaluation of data

Data taken from medical records included age at diagnosis of INC, molecular genetic data (nucleotide sequence changes in the *CTNS* gene), age at initiation of cysteamine treatment, level of adherence to cysteamine treatment, and kidney function and kidney replacement therapy (dialysis or kidney transplantation). To assess adherence to treatment with cysteamine, a modified composite compliance scoring system (ranging from 0–3) was used, as described previously (Nesterova *et al.*, 2015): a score was assigned to each patient for every year of his life; thereafter, a mean individual score was calculated. Specifically, a score of 0 per year was assigned if the patient's average leucocyte cystine level was $\geq$3 nmol half-cystine/mg protein on cysteamine therapy, or if the patient did not take any cysteamine. A score of 0 was also assigned for every year preceding the diagnosis of INC. A score of 1 was attributed if the average leucocyte cystine level was $\geq$2 and $<$3 nmol, while a score of 2 or 3 was used if this level was $\geq$1 and $<$2 nmol or $<$1 nmol, respectively.

Cross-sectional investigations

Clinical investigations

Clinical investigations included measurements of adult height (with documentation of parental target height), calculation of current BMI, assessment of pubertal Tanner stages, and measurements of testicular volumes by Prader orchiometry.

Analysis of hypothalamic–pituitary–gonadal hormones

Hormones of the hypothalamic–pituitary–gonadal (HPG) axis were analysed in serum from blood samples drawn between 8.00 and 12.00 a.m., including LH, FSH, testosterone and inhibin B. Serum LH and FSH levels were determined using highly specific time-resolved fluoroimmunoassays (Autodelfia, Freiburg, Germany). Serum testosterone was measured by fluorescence photometry (Architect ABBOTT GmbH, Wiesbaden, Germany) and inhibin B by a solid phase sandwich assay (Beckman-Coulter, Krefeld, Germany).

Measurement of serum creatinine and urea

Serum creatinine and urea were measured and estimated glomerular filtration rate (eGFR) was calculated (Stevens and Levin, 2013).

Testicular ultrasound imaging

In each patient, ultrasound was performed by a single experienced physician (JR), using a linear transducer (12 mHz) at the time of fertility assessment.

Analysis of semen

Semen analysis was offered to all males and investigated according to World Health Organization (WHO) 2010 standards, evaluating seminal volumes, sperm concentrations and total sperm counts, sperm motility, and morphology (Cooper *et al.*, 2010).

In addition, the marker concentrations of accessory gland secretory function, that are at the same time indicators for potential obstruction within the male reproductive excretory system (i.e. α-glucosidase for the epididymes, fructose for the vesicular glands, and zinc for the prostate), were determined in the seminal plasma, using commercially available standardized and validated kits (Roche®, FertiPro®, Waco-Chemicals®, respectively), following the instructions of the manufacturers.

Microsurgical testicular sperm extraction

Microsurgical testicular sperm extraction (mTESE) was performed under general anaesthesia in two patients aged 32 and 35 years who wanted to become biological fathers: The tunica albuginea was opened semi-lunarly parallel to the epididymidis. The testicular parenchyma was then scanned from pole to pole using an operating microscope (Zeiss Opmi Vario) fitted with a 1.5–18.2 × objective, to identify expanded tubules. These were found in the central part of both testes. From each testis, eight specimens from the central parts and one of the testicular periphery were excised and transferred into sperm preparation medium (Origio, Måløv, Denmark) maintained at a temperature of 37°C. A small fragment was separated from each specimen and enzymatically digested with 0.8 mg/mL collagenase (type 1A; Sigma, Taufkirchen, Germany) in order to determine whether spermatozoa were present. The remainder of the tissue was placed in a vial containing Sperm Freeze (Ferti Pro, Berneem, Belgium), cryopreserved and stored in liquid nitrogen for future sperm extraction. Specimens from each third segment of the testis were placed in Bouin's fixative, processed, paraffin embedded and cut as per routine procedure for light microscopic evaluation. In preparation of electron microscopy, two specimens from the central and peripheral parts of one testis were transferred and fixed in 2.5% glutaraldehyde with Sorensen's phosphate buffer.

Histologic evaluation

Histologic evaluation of testicular biopsies (taken from the central parts of the testes and the testicular periphery) was performed after haematoxylin and eosin staining, using light microscopy. Crystalline deposits were visualized by birefingence (double refraction) using a polarization microscope.

The samples fixed in 2.5% glutaraldehyde were post-fixed with 1% osmiumtetroxide, then subsequently dehydrated and embedded in Epon. Ultra-thin sections (60–120 nm) were cut using a Leica Ultracut R ultramicrotome (Vienna, Austria), counterstained with 8% uranyl acetate in bi-distilled water and incubated with lead citrate solution, as previously described (Reynolds, 1963). Samples were then inspected on a transmission electron microscope (TEM) EM208S (Philips, Amsterdam, Netherlands).

Statistical analyses

All statistical analyses were performed with Graph Pad Prism, version 5, 2007 (GraphPad Software Inc., San Diego, CA, USA). For all parameters, median values and ranges are reported. Correlations were assessed using the Pearson's *r*-coefficient. A value of $P < 0.05$ was considered statistically significant.

Results

CTNS genotype

In all patients, the diagnosis of INC was made during infancy, based upon clinical symptoms and confirmed biochemically by measurement of leucocyte cystine levels. Molecular genetic analysis was used to confirm the diagnosis in a subset of 13 patients.

Nine of 18 males carried a homozygous 57-kb deletion within the *CTNS* gene, while three were homozygous for the frameshift mutation

Table I. Molecular genetic data, adherence to cysteamine treatment and kidney function data of male patients with cystinosis.

Age at investigation, years	Homozygous or compound heterozygous	Nucleotide change (cDNA) in the CTNS gene (allele one)	Nucleotide change (cDNA) in the CTNS gene (allele two)	Amino acid change	Type of mutation	Gaps in cysteamine treatment 0=<5 1 = 5–10 2 = 10–15 years/ composite compliance score (0–3)	Dialysis, yes/no	Kidney transplant, yes/no	Creatinine, mg/dL (n: 0.8–1.4 mg/dL)	Urea, mg/dL (n: 8–21 mg/dL)	eGFR, mL/min/1.73 m^2
15.4	Homozygous	57-kb del	57-kb del		10 exon del	0/2.60	Yes	No	9.4	66	7.4
15.5	Homozygous	57-kb del	57-kb del		10 exon del	0/3.00	No	No	1.2	12	89.3
16.3	n/a	n/a	n/a		n/a	0/2.00	No	No	7.1	38	10.4
16.9	n/a	n/a	n/a		n/a	0/2.40	No	No	2.6	19	37.4
17.5	Homozygous	57-kb del	57-kb del		10 exon del	0/2.80	No	No	4.4	54	18.3
18.5	Comp heteroz	c.926 dup G	400-400 + 2delGGT	p.Ser310Gln fs	splice site	0/2.80	No	No	1.1	11	>90
18.7	Homozygous	57-kb del	57-kb del		10 exon del	0/2.80	No	No	1.3	12	80
20.6	n/a	n/a	n/a		n/a	0/2.70	No	No	2.4	25	37
23.5	Homozygous	57-kb del	57-kb del		10 exon del	0/2.70	No	No	2.2	37	41
24.8	Homozygous	c.18_21 del GACT	c.18_21 del GACT	p.Thr7 Phe fs	frameshift	0/2.90	No	No	2.2	22	40
25.4	n/a	n/a	n/a		n/a	0/2.60	No	No	3.9	41	20
27.5	n/a	n/a	n/a		n/a	1/2.30	Yes	No	4.1	11	19
27.5	Homozygous	57-kb-del	57-kb del		10 exon del	0/2.70	No	Yes	1.7	25	54
28.4	Homozygous	c.18_21 del GACT	c.18_21 del GACT	p.Thr7 Phe fs	frameshift	0/2.30	Previously	Yes	1.0	18	>90
32.3	Homozygous	57-kb del	57-kb del		10 exon del	1/2.30	No	Yes	1.9	30	46
33.5	Homozygous	57-kb del	57-kb del		10 exon del	0/2.60	No	Yes	1.2	21	79
34.7	Homozygous	57-kb del	57-kb del		10 exon del	0/2.90	No	Yes	1.2	18	78
40.5	Homozygous	c.18_21 del GACT	c.18_21 del GACT	p.Thr7 Phe fs	frameshift	2/1.3	No	Yes	3.9	44	18

CTNS: cystinosin; eGFR: estimated glomerular filtration rate.
Patients with sperm in semen are highlighted in dark grey, while patients with sperm extracted via microsurgical testicular sperm extraction (mTESE) are highlighted in light grey.

c.18_21 del CACT. One male was compound heterozygous for a splice site mutation (c.926 dup G and 400-400 + 2delGGT). In five males, no results of mutation analysis were available (Table I).

Adherence to cysteamine treatment

The length of cumulative cysteamine treatment gaps during infancy, childhood, and adolescence, as well as the individual composite compliance scores (median: 2.65; range: <1.3 (low adherence) – 3.0 (high adherence)) are listed in Table I. In all but three males, cumulative treatment gaps were shorter than 5 years. Two males (aged 27 and 32 years) had discontinued cysteamine treatment for 5 –10 years. The oldest male (aged 40 years) had not been treated for more than 10 years, as cysteamine treatment was not available at the time of diagnosis. In these patients with large treatment gaps, the composite compliance scores were 1.3, 2.3, and 2.3, respectively. Only two other males had relatively low compliance scores (2.0 and 2.3) owing to irregularities of medication intake.

Renal function

Ten males had stage 2–5 chronic kidney disease (median eGFR 39 mL/min/1.73 m^2; range 10–>90); two were receiving maintenance dialysis and six had previously received a kidney transplant (median eGFR 66 mL/min/1.73 m^2; range 18–90; Table I).

Anthropometric data

All but the youngest male had reached adult height. Median final adult height of these 17 males was 173.6 cm (range: 164–191), i.e. 7.7 cm below the median for parental target height (181.3 cm; range: 173–190). All but five males had a normal BMI; four males were underweight, while one was slightly overweight (median BMI of all males: 19.05 kg/m^2; range: 16.6–25.6).

Tanner stages, testicular sizes, endocrine and spermatogenic function

All patients had spontaneously reached a late pubertal Tanner stage G4–5, P4–5 A2, with a normal adult penile length; the youngest male reached a Tanner stage G4, P4, A2. Body hair was sparse and voice was muted, but high-pitched, in all males. Testicular volumes (summated median: 35 mL; range: 12–80) were in the normal adult range in all males, except for two 'older' men (aged 33.5 and 40.5 years) who had testicular volumes below the adult reference range (i.e. ≤ 24 mL) (Fig. 1, Table II).Testicular volumes negatively correlated with age: Pearson's r: −0.532; $P = 0.02$.

Serum LH concentrations above the upper limit of the normal range (>10 U/L) were reported in all patients above the age of 20 years, indicating hypergonadotropic hypogonadism (median: 12.6 U/L; range: 2–105). However, all but three patients had normal serum testosterone concentrations (median: 20.9 nmol/L; range: 6.1–30.4) (Fig. 1, Table II). A 15-year-old adolescent male had mid-pubescent testosterone levels because of delayed puberty, so his levels were deemed to be appropriate for his pubertal Tanner stage (4–5). The two previously mentioned 'older' men with testicular atrophy had decompensated hypogonadism, i.e. low serum testosterone, despite elevated serum LH.

None of the patients had received testosterone supplementation or complained of symptoms of androgen deficiency.

Spermatozoa were present in semen from 3/15 (20%) males aged 18, 24 and 28 years, respectively. The youngest of them had normal semen quality according to WHO 2010 criteria (sperm concentration: 55.7 mill/mL), although he was carrying a homozygous 57-kb deletion within the *CTNS* gene. The other two patients (brothers) had oligozoospermia (sperm concentration of 12.7 mill/mL and 0.7 mill/mL, respectively); both were carriers of a homozygous frameshift mutation (c.18_21delGACT).

The remaining 12 males had azoospermia (Fig. 1, Table II). Of those, six had a homozygous 57-kb deletion within the *CTNS* gene, one had a homozygous c.18_21 del GACT mutation, and one was compound heterozygous with a c.926 dup G and a 400-400 + 2delGGT mutation (Table II).

In three younger males aged 15–17 years, semen samples could not be obtained for analysis for psychological reasons.

Serum FSH concentrations were elevated in all patients, except for the youngest male (aged 15 years with Tanner G4, P4, A2) and the three men with sperm in their semen, who had FSH levels within the reference range (median: 14.7 U/L; range 2.7–99.8; normal: <7 U/L) (Fig. 1, Table II).

Serum inhibin B concentrations (median 140 pg/mL; range: 8–336) were lower in older patients than in the younger ones. Concentrations below the normal adult range were observed in all males older than 23 years (Fig. 1, Table II); Pearson's r for inhibin B over age: −0.585; $P = 0.01$.

Males with spermatozoa in their semen had a median inhibin B concentration of 198 pg/mL (range: 162–287), while the median level in those with azoospermia was only 90 pg/mL (range: 8–336; $P = 0.129$; n.s.).

Influence of cysteamine therapy on testicular impairment

Among the three males with cumulative treatment gaps longer than 5 years, aged 27.5, 32.3 and 40.5 years, and with the lowest composite compliance scores (2.3, 2.3 and 1.3 respectively), two men had testicular atrophy (bi-testicular volumes of 24 and 12 mL) and decompensated hypogonadism, i.e. low serum testosterone (10.5 and 7.3 nmol/L, respectively), despite elevated serum LH (36.5 and 105.5 U/L, respectively). The youngest of these three males had a serum testosterone at the lower limit of normal (13 nmol/L), resulting from increased stimulation by LH (44.5 U/L). Of the three other males with lower composite compliance scores (2.0, 2.4 and 2.3), two were still young with adequate endocrine function. The oldest of these three had an only moderately elevated LH (11.7 U/L) with normal testosterone (21 nmol/L).

Among the INC males with proven azoospermia, the composite compliance score ranged from 1.3 to 2.8.

Among males with sperm in semen, two with higher sperm concentrations had a high cysteamine composite compliance score of 2.8 and 2.9, respectively, indicating good long-term adherence to treatment and one with lower sperm concentrations had a score of 2.3, indicating moderate adherence.

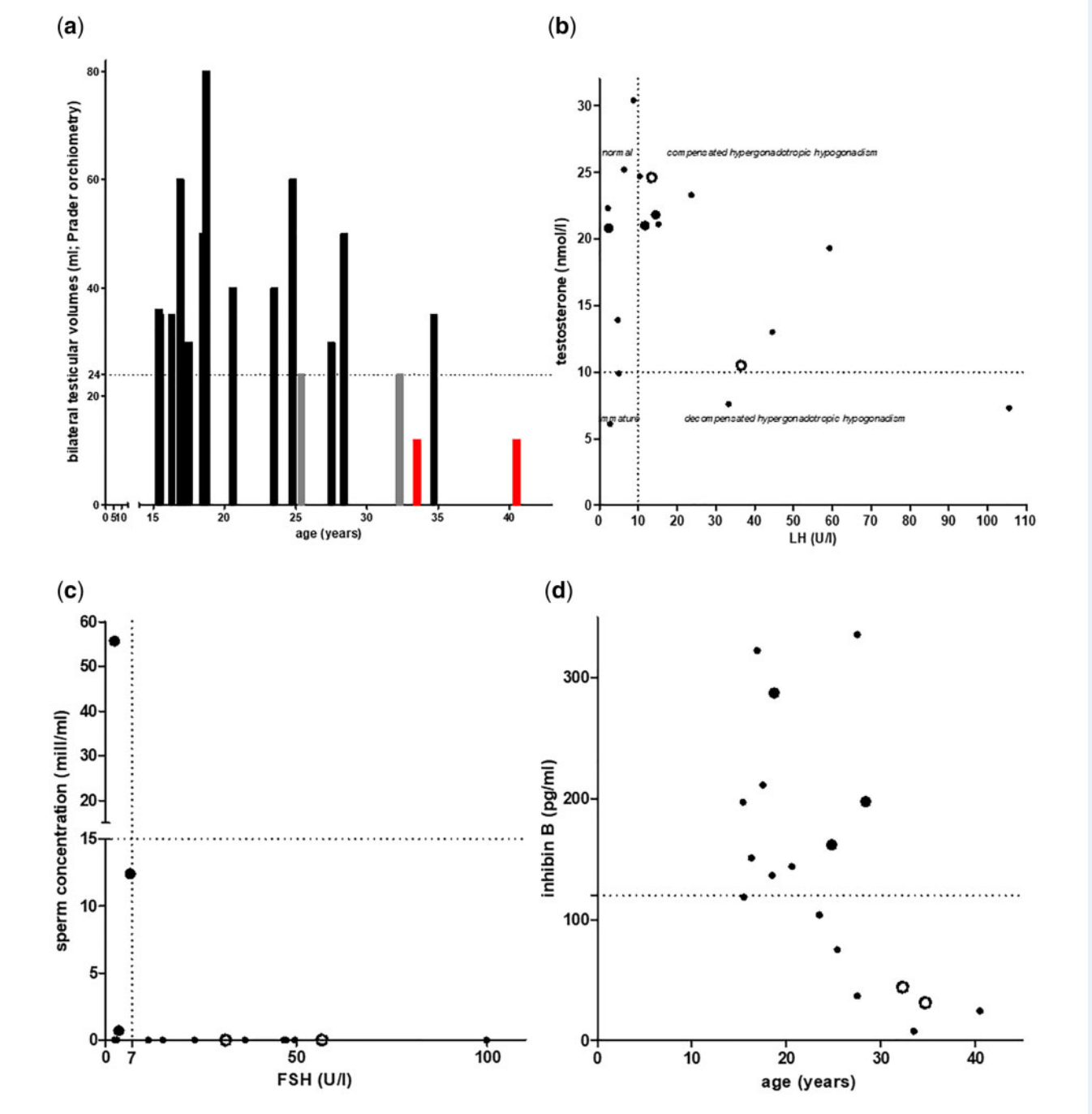

Figure 1. Testicular size, and endocrine and spermatogenic testicular function of 18 males with infantile nephropathic cystinosis. a) Bilateral testicular volumes (measured by Prader orchiometry) against age (years). The grey bars indicate bilateral testicular volumes at the lower limit of the normal adult range; the red bars refer to bilateral testicular volumes below the lower limit of the normal adult range. **b)** Serum testosterone concentrations (nmol/L) against serum LH (U/L). **c)** Sperm concentrations (mill/mL) against serum FSH (U/L). **d)** Serum inhibin B (pg/mL) against age (years). The large dark circles indicate that these patients had sperm in their semen, while the large open circles indicate those patients with successful surgical sperm retrieval. The mottled/dotted horizontal and vertical lines represent limits of the reference ranges.

Testicular ultrasound imaging and seminal markers of obstruction

Ultrasound revealed signs of obstruction (with a dilated rete testis) in the testes of 12/18 males (67%). Figure 2 illustrates the testicular images of selected patients. Of those males with sperm in their semen, only one with normal sperm counts had no signs of obstruction while two with oligozoospermia had signs of obstruction, with dilation of the rete testis. The ejaculate volume was decreased (<1.5 mL) in 5/15 patients.

Table II. Semen parameters, testicular volumes and hypothalamo–pituitary–gonadal axis hormones of males with cystinosis.

Age at investigation, years	Semen parameters (WHO 2010)					mTESE result	Testicular volumes summated Prader, mL	HPG axis hormones			
	n: ≥1.5 mL Ejaculate volume, mL	n: ≥15 mill/mL Sperm concentration, mill/mL	n: ≥39 mill Total sperm counts, mill	n: ≥32% Motility, %	n: ≥4% Morphology, %			n: ≥12 nmol/L Testosterone, nmol/L	n: 1–7 U/L LH, U/L	n: 2–10 U/L FSH, U/L	n: >125 pg/mL Inhibin B, pg/mL
15.4	0.5	0	0				36	6.1	2.7	3.0	197.1
15.5	n/a	n/a	n/a	n/a	n/a		35	25.2	6.4	14.4	118.8
16.3	n/a	n/a	n/a	n/a	n/a		35	13.9	4.7	8.3	151.1
16.9	1.1	0	0				60	22.3	2.2	2.4	322.3
17.5	n/a	n/a	n/a	n/a	n/a		30	9.9	5.0	6.6	211.3
18.5	0.3	0	0				50	30.4	8.8	15.0	136.6
18.7	3.0	55.7	167.1	47	5		80	20.8	2.4	2.4	287.3
20.6	1.6	0	0				40	24.7	10.4	11.2	144,0
23.5	2.9	0	0				40	19.3	59.3	47.3	104.0
24.8	1.5	12.4	18.6	42	6		60	21.8	14.5	6.5	161.9
25.4	2.1	0	0				24	21.1	15.2	23.4	75.2
27.5	0.8	0	0				30	13.0	44.5	36.6	335.6
27.5	3.9	0	0				30	23.3	23.7	46.7	37,1
28.4	1.6	0.7	1.1	36	n/a		50	21.0	11.7	3.5	197.6
32.3	1.5	0	0			Positive	24	10.5	36.5	56.7	44.4
33.5	2.2	0	0				12	7.6	33.3	49.6	8
34.7	4.0	0	0			Positive	35	24.6	13.5	31.5	31.5
40.5	0.3	0	0				12	7.3	105.5	99.8	24.6

HPG: hypothalamo–pituitary–gonadal; WHO: Word Health Organization; n: normal range. Patients with sperm in semen are highlighted in dark grey, patients with sperm extracted via mTESE are highlighted in light grey.

Regarding seminal markers, both fructose (median: 15.9 µU/ejaculate; range: 2.8–63; normal: >13), a marker for seminal vesicle secretions, and zinc (median: 3.5 µU/ejaculate; range: 0.7–9.1; normal: >2.4), a marker for prostate secretions, were below the lower limit of normal in 5/15 (33%) patients. In an additional two men, markers could not be determined because of extremely reduced semen volumes (both 0.3 mL). Alpha-glucosidase (median: 40 mU/ejaculate; range: below detection in one male; 15.6–140 in the others; normal: >20), a marker for epididymal secretions, was below the reference value in 1/13 patients.

Surgical sperm extraction and microscopic exploration of testicular biopsies

mTESE was performed successfully in the two males who underwent surgery, which allowed for cryobanking of testicular spermatozoa. Both patients were homozygous for the *CTNS* 57-kb deletion. While the 32-year-old male had not taken cysteamine for a cumulative period of approximately 7 years, the 35-year-old male had strictly adhered to treatment. Both had received a kidney transplant after end-stage renal failure. The macroscopic and microscopic intraoperative view of the gonads (of the 32-year-old male) after incision of the tunica albuginea showed a white-coloured and thickened testicular 'area underneath the albuginea' (Supplementary Fig. S1). In both patients, spermatozoa could be obtained from all ($2 \times 8 = 16$) testicular samples that were taken from the deeper (central) parts of both testicles.

Histology

Light microscopy and TEM of the testicular biopsies revealed preserved spermatogenesis in the central parts of the testicles. By contrast, the biopsies taken from testicular peripheries displayed tubular shadows and seminiferous tubules with spermatogonial arrest, surrounded by crystalline deposits and lymphocytic infiltrates in the interstitium (Fig. 3).

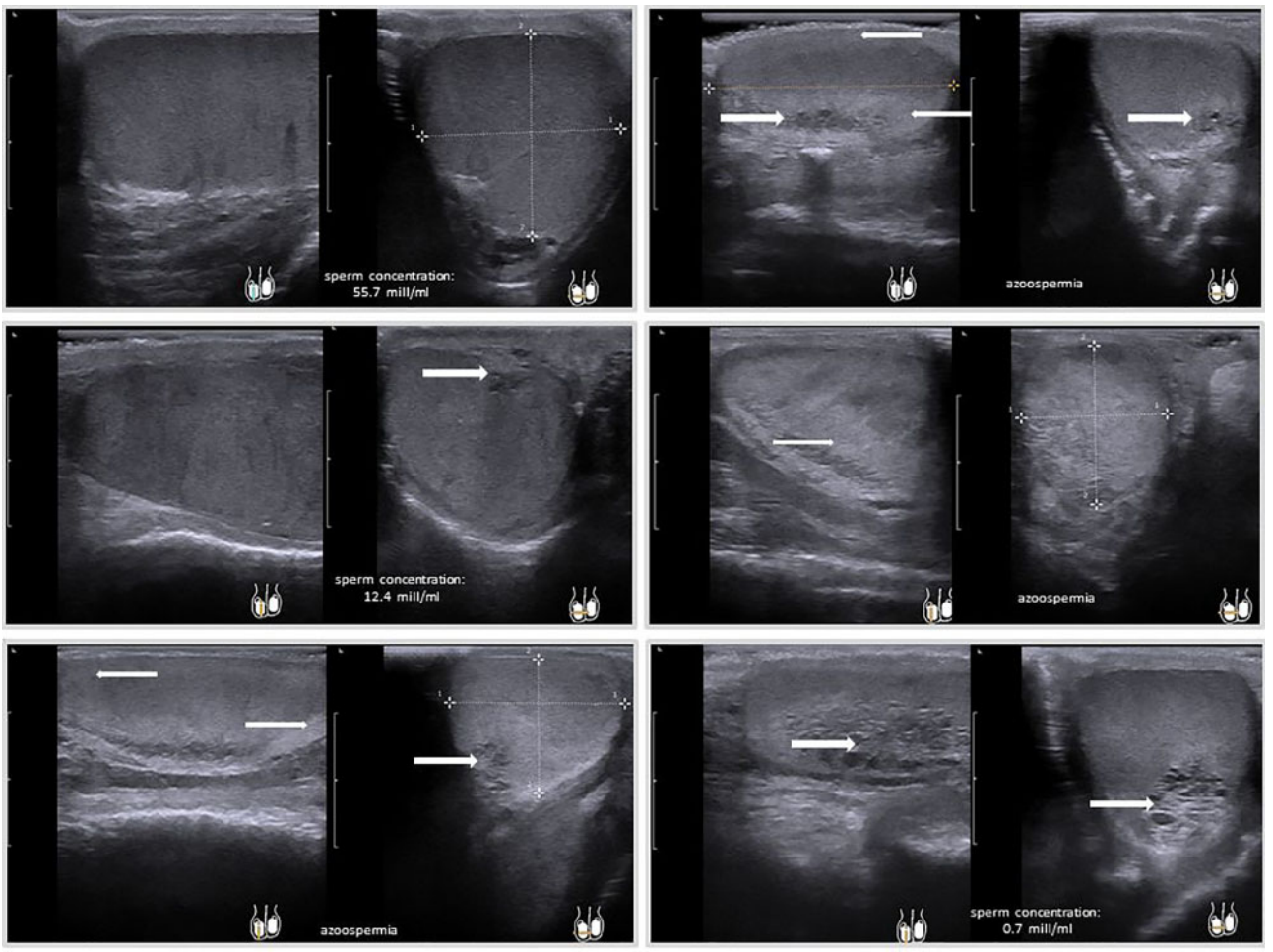

Figure 2. Testicular ultrasound imaging of selected males with infantile nephropathic cystinosis, variably showing dilation of the rete testis (thick arrows). A hyperechogenic rim in the peripheral regions of testicular parenchyma (thin arrows) is seen in patients without sperm in their semen.

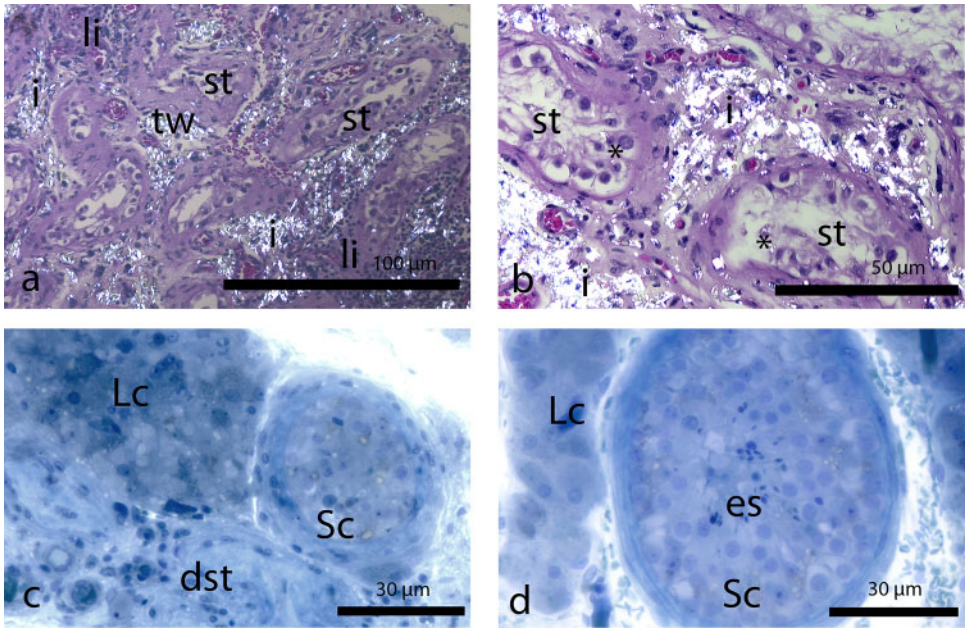

Figure 3. Histology of peripheral and central testicular tissues from a patient with cystinosis. a) Polarisation microscopy of peripheral testicular tissue after haematoxylin and eosin (HE) staining shows huge clusters of "shining" crystalline material in the testicular interstitium (i) and infiltration of this compartment with lymphocytes (li). The crystals surround degenerated seminiferous tubules (st) with thickened and hyalinised tubular walls (tw) containing Sertoli cells (Sc), but no germ cells. **b)** Crystalline structures (*) are rarely present within the seminiferous tubules (st). **c)** Semi-thin sections (methylene blue-stained) of peripheral testicular tissue show degenerated tubules (dst), containing Sertoli cells (Sc) with brownish content. The tubules are surrounded by Leydig cell (Lc) clusters, containing a high number of vesicles with brownish content. **d)** Semi-thin section of central testicular tissue shows a seminiferous tubule with full spermatogenic progression up to the stage of elongated spermatids (es), with Sertoli cells (Sc) of normal morphology. Interstitial Leydig cells (Lc) also appear normal.

On TEM, Sertoli cells with clusters of lysosomes of increased size and variable content were visualized in the peripheral testicular biopsies. In the Leydig cells of these biopsies, multiple enlarged lysosomes were present. By contrast, in Sertoli cells from biopsies of the central region of the testes, fewer clusters of smaller lysosomes were observed. In the central biopsies, spermatogonia were of normal appearance, and germ cell differentiation progressed normally to elongated spermatids. The number and morphology of lysosoma in Leydig cells also appeared normal (Fig. 4).

Discussion

The present study sheds some light on both endocrine and spermatogenic testicular function of a relatively large series of males of different ages with INC. One important result is that testicular endocrine function in males with INC may remain compensated until the fourth decade of life, while impairment of semen quality, culminating in azoospermia, apparently occurs during adolescence. The observed decline in testicular volumes and inhibin B serum concentrations in our older male INC patients, together with the results of our analysis of adherence to cysteamine treatment, suggests that the degenerative process of the testicles may be delayed, but cannot be fully prevented by treatment with cysteamine.

Pathogenesis of male fertility impairment in INC

Evidence from ultrasound imaging data, hormone and semen analysis, and testicular histology indicates that two major factors are involved in male fertility impairment in INC.

Firstly, progressive testicular degeneration, primarily occurring in the testicular periphery, appears to alter semen quality. This area underneath the tunica albuginea corresponds to the supply area of the terminal vessels of testicular perfusion, as testicular arteries go from the periphery to the testicular centre, then turn again from the centre to the periphery and end underneath the tunica albuginea.

Atrophy appears to be associated with lysosomal cystine overload of both somatic Sertoli cells within seminiferous tubules and Leydig cells in the testicular interstitium. While Sertoli cell lysosomal overload obviously results in tubular hyalinization or spermatogonial arrest, ballooning of lysosomes in Leydig cells seems to contribute to endocrine testicular dysfunction, thereby altering spermatogenesis, specifically as intratesticular testosterone (in concert with FSH) is important for the differentiation of type A spermatogonia to type B spermatogonia (Meachem *et al.*, 1998; Matthiesson *et al.*, 2005).

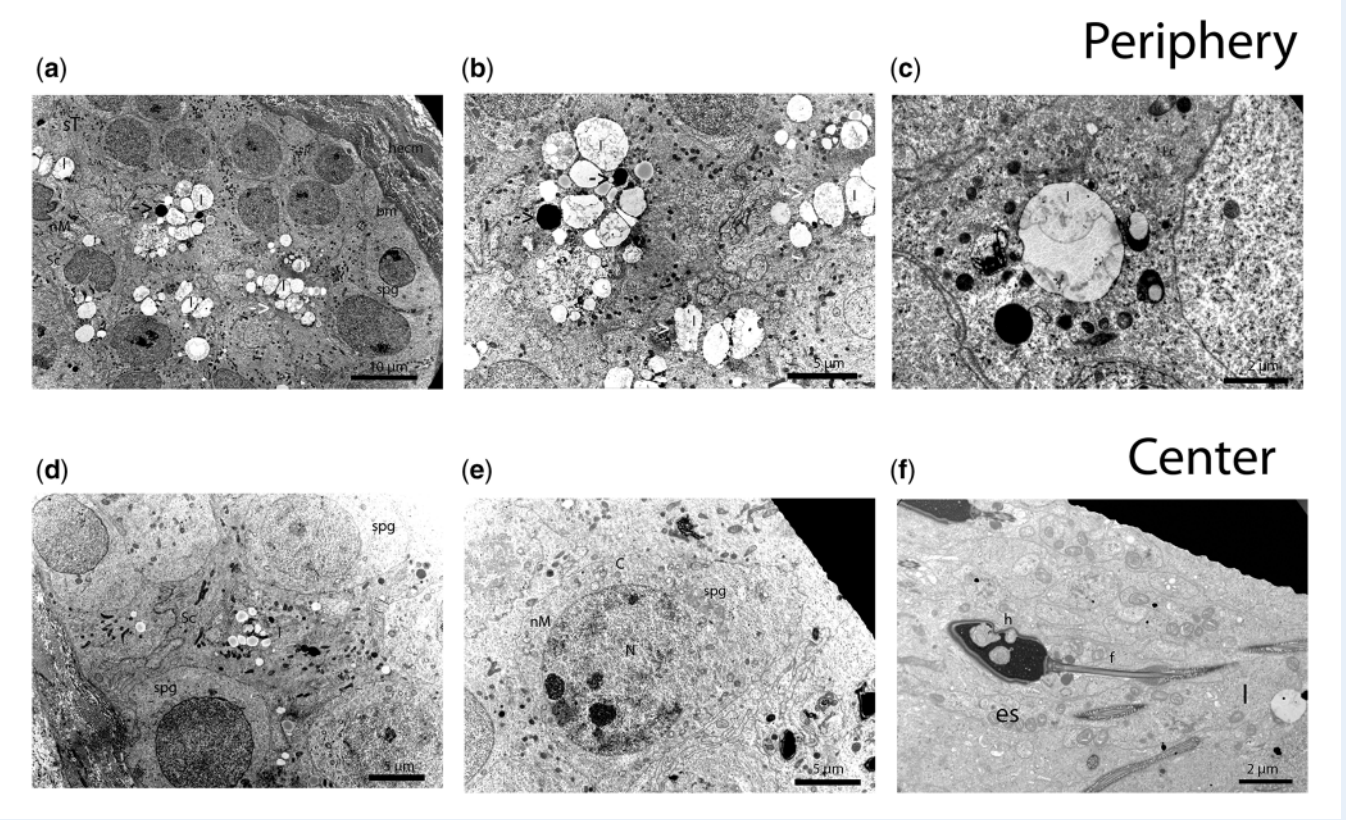

Figure 4. Transmission electron microscopy of testicular tissues from a patient with cystinosis. Panels a–c) show peripheral tissue and d–f central tissue. **a)** Section of peripheral testicular tissue showing a degenerating seminiferous tubule (sT). The seminiferous epithelium consists of Sertoli cells (Sc) and spermatogonia (spg). Clusters of electron dense (black arrow) and electronlight (white arrow) lysosomes (l) are present within Sertoli cells (Sc) identified by their folded nuclear membrane (nM). The basal membrane (bm) of the seminiferous tubule (sT) is surrounded by hyalinized extracellular matrix (hecm). **b)** Lysosomes within Sertoli cells are of heterogenous appearance, either electron dense (black arrow) or light (white arrow), due to varying content. **c)** Lysosomes (l) within Leydig cells (Lc) in the testicular interstitium are much bigger than those of Sertoli cells. **d)** Section of a seminiferous tubule of the central part of testicular tissue. In the Sertoli cell cytoplasm (Sc) between the spermatogonia (spg), lysosomes (l) smaller in number and size than those of peripheral parts of the testes are observed. **e)** Normal spermatogonia (spg) (as identified by the relation of nucleus (N) and cytoplasm (C), the roundish nuclear shape and a dark nuclear membrane (nM)) are present in the central part of testicular tissue. **f)** Germ cell differentiation fully progresses up to the stage of elongated spermatids (es) with morphologically normal head (h) and flagellum(f) formation.

Enhanced FSH serum concentrations in our cohort reflect the degenerative changes that affect spermatogenesis in the testes

One hypothesis of other investigators regarding potential mechanisms involved in the disturbance of tissue function in INC has related the amount of intracellular cystine content to the severity of the phenotype. However, an association between cysteine overload and renal tubular dysfunction was not observed in *in-vitro* studies of renal epithelial tubular cells (Park *et al.*, 2002). Aberrant energy production and/or apoptosis are other mechanisms that have been suggested to explain functional impairment of tissues in INC patients (Park *et al.*, 2005).

In the present study, we observed extensive crystal formation and consequent lymphocytic infiltration of the testicular interstitium in the testicular peripheries; this could contribute to progressive testicular involution. Our findings are in accordance with previous observations that crystals are preferentially present in interstitial macrophages, which can lead to the production of proinflammatory substances (Prencipe *et al.*, 2014).

Secondly, obstruction in some parts of the male excretory system appears to contribute to impaired semen quality in males with INC: we observed a dilation of the testicular rete testis on ultrasound imaging of the testes in two-thirds of patients. We also observed reduced seminal volumes, along with reduced fructose and zinc seminal markers in one-third of patients. Dilation of the rete could result from stenosis within the efferent ductal system, i.e. the connection between the rete testis and epididymis, while alterations of biochemical secretions from accessory glands (epididymis, seminal vesicles and prostate) in the seminal plasma could either be caused by a dysfunction of the respective secretory gland and/or a stenosis within the male reproductive excretory system, distal of it. The observed reduced fructose (the energy source for spermatozoa, and important for the alkaline nature of semen) levels in the seminal plasma in 5/15 males with INC

could point to an obstruction at the level of the excretory ducts of the seminal vesicles (vesicular glands), before their convergence with the respective ampullae of the vasa deferentia. This hypothesis would also explain our observation of reduced seminal volume in these males, as the secretions of the seminal glands contribute around 70% of the fluid volume of semen. The reduced zinc levels in semen of 5/15 of our patients would point to an obstruction of the small intraprostatic ducts that transport prostate secretions to the ejaculatory ducts, crossing the prostate. As we observed normal α-glucosidase in the seminal fluids in all (but one) patients, this indicates that epididymal secretory capacity is not altered and that the flux of epididymal secretions through the vas deferens, and further through the ejaculatory ducts into the urethra, is preserved. Therefore, stenosis of the ejaculatory ducts, that join the urethra within the prostate, does not seem probable.

Taking all our observations into consideration, we speculate that crystal formation and the consequent fibrotic changes, occurring after inflammation, specifically within those parts of the male excretory system where the ducts have a very small diameter, could be causative of stenosis and explain the observed biochemical signs of obstruction.

Endocrine testicular function in INC

Our study illustrates that hypogonadism in males with INC remains compensated for a long time, without the need for testosterone supplementation. However, elevated LH serum levels found in males from approximately 20 years of age onwards indicate that Leydig cell function becomes compromised. LH elevation in our cohort was accompanied by normal adult serum testosterone levels, indicating compensated hypogonadism. Only two older patients had decompensated hypogonadism; suprisingly these men did not complain of symptoms of androgen deficiency.

According to consented guidelines, treatment with testosterone is indicated only for symptomatic men with androgen deficiency, to induce and maintain secondary sex characteristics and to improve their sexual function, sense of well-being, muscle mass and strength, and bone mineral density (Bhasin *et al.*, 2006).

Spermatogenic testicular function in INC (including hormonal markers)

In the present study, serum FSH concentrations were in the normal range in males with INC who had sperm in their semen, but were elevated in all azoospermic males. Thus, FSH levels in the normal range could indicate that there is a chance of finding spermatozoa in semen for potential cryostorage. However, young patients may have only recently entered puberty and thus have normal FSH serum levels because of immaturity. Inhibin B was not helpful regarding whether or not sperm could be found in semen. However, we observed a progressive decline of serum inhibin B over age. Levels of inhibin B could be decreased not only because of Sertoli cell dysfunction caused by lysosomal overload, but also because of hampered testicular blood flow in atrophic testicles.

Testicular histology in two subjects with INC

On light-microscopic imaging, seminiferous tubules were replaced by tubular shadows in the peripheral parts of the testes. (This phenomenon was also visualized by ultrasound, showing a hyperechogenic 'rim' in the testicular periphery, surrounding a relatively hypoechogenic testicular centre). By contrast, in the central parts of the testicles, full spermatogenesis was observed.

TEM imaging of the peripheral areas of testicular parenchyma showed that in both somatic Sertoli cells, known to 'nurture' spermatogenesis, and in Leydig cells, necessary for supporting meiotic divisions of spermatogonia, the number and size of lysosomes was increased. Restricted oxygen and nutrient supply in the terminal areas of testicular perfusion could contribute to altered tissue pH, which could favour crystal formation and consequent hyalinization of seminiferous tubules (Nesterova and Gahl, 2013).

CTNS genotype in our INC cohort

Mutations in the *CTNS* gene have been categorized into three classes, according to whether they abolish, severely inhibit (<30% of wild-type) or do not alter cystine transport (Attard *et al.*, 1999; Kalatzis *et al.*, 2004).

In our cohort, a relatively large proportion of patients ($n = 9$) were homozygous for the 57-kb deletion within the *CTNS* gene, known to cause a severe cystinosis phenotype, i.e. the infantile nephropathic form, owing to a total loss of function of the lysosomal cystinosin transporter. This founder mutation is present in the homozygous state in around 50% of patients with cystinosis, who are of northern European descent (Emma *et al.*, 2014; Levtchenko *et al.*, 2014; Shotelersuk *et al.*, 1998). The two men undergoing successful mTESE had a 57-kb deletion.

However, over 121 other pathogenic *CTNS* mutations reported in cystinosis patients worldwide are known to cause the infantile phenotype (David *et al.*, 2019). Of those, only three mutations were found in our cohort: the 57-kb deletion, the compound heterozygous splice site mutation c.926 dup G/400-400 + 2delGGT (in one male) and the homozygous frameshift mutation c.18_21 del GACT (in three other males). In three of these four men without a 57-kb deletion, sperm were present in semen.

Measures to enable biologic fatherhood for males with INC

Our findings of sperm in semen in some of the males with INC and of preserved spermatogenesis in the centres of the testicles of two azoospermic males provide hope for patients with INC who may want to produce their own biological offspring, with the help of ART.

We found that cryostorage of semen is an option for around 20% of males with INC, up to the third decade of life. In azoospermic subjects, sperm retrieval by surgical means appears to be possible even in males carrying severe *CTNS* mutations, with a kidney graft, and at a relatively advanced age, despite prolonged gaps in cysteamine treatment. Nevertheless, in view of the gonadal degeneration occurring over their lifetime, the recommendation of an early 'search for sperm' approach in young males with INC is warranted. ICSI is a successful method for the fertilization of oocytes *in vitro*, independent of whether freshly ejaculated or cryopreserved spermatozoa are used.

As cystinosis follows an autosomal recessive mode of transmission, the chances of producing a healthy child are high, provided that the

partner does not carry a pathogenic mutation in the *CTNS* gene. In this case, PGD or prenatal molecular genetic testing via chorionic villus biopsy or amniocentesis would be an option to clarify whether the offspring could be affected by cystinosis.

Considerations regarding endocrine care of males with INC

Our observations indicate that regular evaluation of serum testosterone levels, along with serum LH and FSH concentrations from puberty onwards, is reasonable. Whether testosterone treatment of latent hypogonadism, i.e. normal testosterone but elevated LH, would provide benefit to males with INC regarding voice, bone mineralization and muscle strength, remains to be explored in future studies.

Comparison of our findings with the results of previous studies

The results of the present study confirm some findings from previous case studies, but are also partially in contrast to earlier publications, when cysteamine was not used systematically. An early retrospective study of 30 male and female patients with cystinosis reported that the onset of puberty was delayed and that gonadotropin levels rose above the normal adult range during puberty (Winkler *et al.*, 1993); testosterone levels remained in the low-normal range in the male patients.

Another earlier report of 10 males aged 15–28 years with INC and kidney allografts observed pubertal arrest at Tanner G4 in all patients, low testosterone levels in three patients and elevated LH and FSH in seven patients (Chik *et al.*, 1993).

In a study of seven males aged 19–43 years with INC (five with renal allografts), six had pubertal arrest at a Tanner stage G3 or G4, and only one reached Tanner G5 (Besouw *et al.*, 2010); all had normal mean testicular volumes (18 mL; range: 10–18), three had low testosterone levels, five had elevated LH and FSH, and all five of those investigated had azoospermia.

The improved characteristics of our patient cohort, who largely adhered to a regimen of oral cysteamine therapy, may indicate that early and regular treatment with cysteamine is effective in delaying, although not fully preventing, endocrine and spermatogenic gonadal dysfunction.

It is important to highlight the limitations of this study. While this study comprises data from a relatively large cohort of male patients with INC, the actual number of patients with this rare disease remains small. In addition, selection bias with INC patients attending our clinic for fertility assessment who were less seriously affected cannot be fully excluded. Finally, these patients show heterogeneity of dysfunction in cystine transport across lysosomal membranes, and the heterogeneity in past adherence to cysteamine treatment may not always be satisfactorily reflected by our composite compliance score.

In summary, endocrine testicular function in males with INC potentially remains compensated until the fourth decade of life, but azoospermia occurs during late adolescence in the majority of patients. Obstruction of efferent tubules, tubular atrophy and spermatogonial arrest in the testicular peripheries, presumably as a consequence of lysosomal cystine overload in Sertoli and Leydig cells, contribute to impaired semen quality. Cryostorage of semen is an option for around 20% of young males with INC, while for azoospermic patients surgical sperm retrieval provides additional opportunities for biological fatherhood.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

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Authors' roles

JR and KH designed the study; JR performed all cross-sectional clinical investigations and wrote the manuscript. KH provided long-term patient care, contributed retrospective clinical data, evaluated adherence to cysteamine treatment and edited the manuscript. DH and DW provided patient care and contributed to retrospective clinical data collection. JFC and SK performed mTESE and edited the manuscript, SH and JW performed histology of testicular samples and edited the manuscript.

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Conflict of interest

The authors have no competing interests to declare.

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