CRYPTORCHIDISM: CAUSES AND CONSEQUENCES

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CRYPTORCHIDISM: CAUSES AND CONSEQUENCES

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Cryptorchidism is the failure of one or both testes to descend into the scrotum at birth or shortly thereafter, depending on species, or the ascent of previously descended testes later in life. It is the commonest of all congenital conditions in the human representing between 1 and 9% of all male babies born. It is also common in domestic species such as pigs, dogs and horses. Importantly, cryptorchidism is seen as a sentinel of fetal well-being and is associated with other less common ailments such as testis cancer and hypospadias as part of the testicular dysgenesis syndrome (TDS), as well as being linked to maternal smoking and intrauterine growth restriction. It also likely results from maternal exposure to endocrine disrupting chemicals. Surprisingly, we know relatively little about its immediate causes although deficits in fetal hormonal signaling through INSL3 or testosterone and complex genetic susceptibility seem to be involved. The testes may be affected unilaterally or bilaterally with anatomical arrest anywhere between the abdomen to just above the scrotum. Significantly, we need to distinguish between the consequences of cryptorchidism simply due to retention of the testes within the body cavity at abdominal rather than scrotal temperature, and those sequelae which share a common fetal cause. The former are largely correctable by early orchidopexy.

In this Research Topic we aim to bring together a broad selection of articles (reviews, original research articles, commentaries and hypotheses) from the leading experts in the field, discussing all aspects of this common congenital condition, and throwing light on its genetic and environmental causes including its relationship to TDS and the biological mechanisms involved, its sequelae, and how these relate to optimal treatment.

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Cryptorchidism in Boys With Cerebral Palsy Is Associated With the Severity of Disease and With Co-Occurrence of Other Congenital Anomalies

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Background: Cryptorchidism is reported in 40–50% of small case series of cerebral palsy (CP) and attributed to hypothalamic–pituitary–gonadal axis abnormalities, intellectual disability (ID), or cremaster spasticity. We collected demographic and clinical data to define the frequency of cryptorchidism and clinical comorbidities in a large CP population.

Methods: Electronic health record data were collected for all male patients ≥7 years of age seen in a large, multidisciplinary CP clinic between 2000 and 2016. Variables including age, testicular position, surgical findings, CP severity, birth history, and comorbidities were tested for association using univariable and stepwise backward logistic regression analyses.

Results: Of 839 established patients, testis position was scrotal in 553, undescended in 185 (24%), retractile in 38 (5%), and undocumented in 63 cases. Cryptorchidism were diagnosed at a mean age of 5.8 years, with 20% documented as acquired, and testes were most commonly in the superficial inguinal pouch (41%) and associated with an inguinal hernia (56%). Severity was bilateral in 114/166 (69%) undescended and 24/36 (66%) retractile cases, respectively. Mean birth weight and the frequency of prematurity (55, 58, and 54%) and multiple birth (14, 13, and 9%) were not significantly different among the three groups. We observed a strong ordinal trend in the frequency of comorbidities, including quadriplegia, syndromic features/known genetic disease, intrauterine growth restriction (IUGR), death, brain malformations, seizures, gastrostomy, absent continence, ID and hearing, speech or visual impairment, with the retractile group holding the intermediate position for the majority. The stepwise multivariable analysis showed independent positive associations of cryptorchidism with quadriplegia, syndromic features/known genetic disease, hearing loss, and absent continence, and inverse associations with gestational age and multiple birth.

Conclusion: These data suggest that cryptorchidism is less common than previously reported in CP cases, but most strongly associated with quadriplegia. Delayed diagnosis

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may be related to an acquired condition or to the multiple additional functional deficits that occur in this population. Our data suggest that UDT and CP may both be components of malformation syndromes occurring in singleton births whose clinical features are more likely to include earlier delivery, IUGR, hearing loss, and/or global spasticity.

Keywords: cerebral palsy, cryptorchidism, congenital anomalies, orchidopexy, retractile testes

INTRODUCTION

Cerebral palsy (CP) refers to a broad group of non-progressive motor disorders resulting from multifactorial, pre-, peri-, or postnatal central nervous system maldevelopment or injury. CP manifests as diplegic, hemiplegic, or quadriplegic spasticity, which is often associated with sensory deficits, intellectual disability (ID), and/or seizures (1, 2). CP is attributable to prenatal factors in the majority of cases (1). Risks include pre- or post-term birth, intrauterine growth restriction (IUGR), and twin gestation. Congenital anomalies (CAs) are present in 14–40% of CP cases, about five times higher than seen in the general population (1, 3, 4). Those affected do not have more severe forms of CP, but unlike individuals without CP, the risk of associated CAs is increased in children born full term. These may represent genetic syndromes, consistent with family studies supporting the heritability of CP (5, 6). While genetic association studies have failed to consistently implicate CP-specific loci (7), additional analyses identified potentially pathogenic rare exonic or copy number variants in 10–30% of patients (1, 8). Overall, the available data suggest that the etiology of CP is complex and heterogeneous.

In studies assessing the prevalence of CAs in association with CP, reproductive birth defects are reported rarely (3, 9) or not at all (10, 11). Underascertainment may be the result of underdiagnosis or undertreatment of a relatively minor anomaly in individuals with otherwise significant CAs, and physical or mental disabilities. Nevertheless, almost half of the recognized syndromes identified in CP patients in one series are known to include cryptorchidism as a component (9). Studies that have directly assessed the prevalence of cryptorchidism in males with CP with or without ID have focused on institutionalized individuals. Cryptorchidism was present in 36 of 88 (41%) and 21 of 39 (54%) of severely disabled males with CP in two small studies (12, 13). Similarly, the covariate adjusted multivariable risk ratio for CP was 20.9 in a perinatal cohort that included 385 cryptorchid boys (14). However, to date, no study of the prevalence of cryptorchidism in a large CP population has been reported. To provide potential insight into risk factors for cryptorchidism in subjects with CP, we took advantage of available electronic health record (EHR) data from a large, interdisciplinary clinic to more clearly define the prevalence of clinical characteristics in this subpopulation.

MATERIALS AND METHODS

We analyzed retrospectively retrieved EHR data for all male patients seen at the CP Center at the Alfred I. duPont Hospital for Children (AIDHC) from 2000 to 2016 following approval by the Nemours Office of Human Subjects Protection/Institutional Review Board with waiver of consent requirements. The earliest date coincided with initial EHR implementation at AIDHC. Pediatric subspecialists routinely see and fully examine CP clinic patients, and an extensive template was completed routinely for the majority of visits comprising information regarding full birth history and functional status for each patient. Additional data extracted from the EHR included other primary care visits at AIDHC-associated or other facilities, surgical histories and physicals, and scanned records from other institutions. Boys with a confirmed CP diagnosis who attended at least three clinic visits and were followed until at least age 7 (unless cryptorchidism was diagnosed before that time), were included.

Cerebral palsy cases were categorized as affected (with documentation of cryptorchidism and/or retractile testis at any time point) or unaffected. Subclassification in affected cases included testicular position, side (unilateral or bilateral), surgery (yes or no), and postoperative testicular position. Additional variables collected included race/ethnicity, gestational age, birth weight, birth history, CP severity; presence/absence of a movement disorder (athetoid or dystonic), postnatal injury or death, or at least one other CA (besides cryptorchidism) with or without a known genetic defect; and functional status related to vision and hearing (normal vs. abnormal), feeding (gastrostomy present vs. oral feeding), continence (present vs. absent), seizures (persistent or transient vs. none), speech (normal vs. abnormal or nonverbal), and cognition (characterized as normal/mild, moderate or severe/profound ID). We identified all examinations which documented testicular position, and recorded findings at the time of testicular surgery, when available. We summarized all study variables and analyzed their distribution in each of the case (undescended and retractile testes) and control (descended testes) groups. Categorical data were summarized using frequencies and percentages, and numerical data were summarized using means ± SDs. Two sample *t* tests and the Mann–Whitney *U* tests were used for parametric or non-parametric analysis of continuous variables, respectively, and Chi square tests were used to compare the distribution of categorical variables between affected and unaffected case groups.

We used a univariable logistic regression model to examine the potential association of study variables based on case classification. Odds ratios (ORs) with 95% confidence intervals (CIs) were reported for this purpose. We then used a stepwise backward method of multivariable logistic regression to select the variables significantly associated with the combined case group compared to the control group. All variables were included, and probabilities for entry and removal in this stepwise analysis were set to 0.05 and 0.10, respectively. Adjusted ORs with 95% CIs are reported for variables retained in each model. We used IBM SPSS version 22.0 for these statistical analyses, and all tests were two-tailed with the level of significance defined as <0.05.

RESULTS

Of 2,984 total records of boys identified through initial screening of the EHR data warehouse, 839 established male CP patients met our inclusion criteria. An additional 63 cases for whom available EHR data failed to provide documentation of testicular position during examination and/or of orchidopexy were excluded, leaving a total of 776 cases for analysis. Of these cases, 553 had descended, 185 (24%) had undescended, and 38 (5%) had retractile testes. The age in years at last follow-up was 13.9 ± 4.0 overall, and was slightly but significantly lower for boys with undescended testes (13.0 ± 4.3) as compared to boys with descended testes (14.3 ± 3.8) likely since follow-up to at least age 7 was not required for boys diagnosed with cryptorchidism at a younger age.

The average age at diagnosis of cryptorchidism was 5.8 ± 4.3 years, with 20% of cases documented as acquired, or descended on a prior examination. Retractile testes were first diagnosed at a mean age of 6.0 ± 3.6 years. The affected side was bilateral in 114/166 (69%) undescended and 24/36 (66%) of cryptorchid and retractile cases, respectively, for whom data were available. Of the 185 cryptorchid cases, 145 (78%) had surgery and operative reports were available for 97 (52%). Of these, 8 underwent a scrotal approach, and insufficient data documenting testicular position were available for another 79 cases. Documented testicular position was prescrotal or external ring (37%), superficial inguinal pouch (40%), inguinal or canalicular (15%), and abdominal (8%). An inguinal hernia was documented in 49 of 88 (56%) patients for whom information was available, of which 16 (33%) were repaired prior to the diagnosis of cryptorchidism. The majority (14, 88%) of boys who had an inguinal hernia repair that predated an ipsilateral orchidopexy were born preterm.

The frequency of prematurity (55, 58, and 54%) and multiple birth (14, 13, and 9%), and mean values for birth weight (2,280 ± 1,130, 2,023 ± 1,165, and 2,234 ± 1,141 g) and gestational age (34 ± 6, 33 ± 6, and 34 ± 6 weeks) were not significantly different among descended, retractile, and undescended testis cases, respectively. However, the prevalence of severe spasticity, ID, death, intrauterine growth restriction (IUGR), non-CNS CAs, brain malformations, requirement for gastrostomy, absent continence, seizures, hearing impairment, visual impairment, and abnormal speech were significantly different among the groups (**Table 1**). For the majority of variables, frequencies were intermediate for retractile as compared to descended and undescended testes and significance was enhanced for all variables when the descended and retractile testis groups were combined (**Table 2**). Of note, we identified 93 subjects (12%) who underwent placement of a baclofen pump for uncontrolled spasticity. The frequency of baclofen pump placement did not differ significantly between the undescended or retractile (14.3%) and descended (11%) testis groups ($p = 0.2$). Boys with baclofen pumps were more likely to have quadriplegia (87%; $p < 0.001$) and other severe CP phenotypes. We did not include this variable

TABLE 1 | Frequencies (%) of clinical characteristics by cryptorchidism subtype in boys with cerebral palsy.

Variables (# cases)	Total	Descended	Retractile	UDT	*p*-Value**
Race (776)					
Caucasian	66.8	68.2	60.5	63.7	0.33
African-American	18.8	16.6	26.4	23.8	
Asian	1.8	2.0	2.6	1.1	
Other/unknown	12.6	13.2	10.5	11.4	
Diagnosis (776)					
Quadriplegic	42.3	32.5	47.4	70.3	<0.001
Hemiplegic	37.8	42.1	39.5	24.3	
Diplegic	20.0	25.3	13.2	5.4	
Movement disorder (776)	6.6	6.7	5.3	6.5	0.94
Death (776)	3.2	2.0	5.3	6.3	0.009
Gestational age <37 weeks (764)	54.3	55.4	57.9	53.6	0.85
Multiple birth (764)	12.5	14.0	13.2	8.7	0.18
Intrauterine growth restriction (IUGR) (728)	18.3	15.6	21.6	25.1	0.015
CA[a] and/or genetic defect (764)	11.5	8.3	15.8	20.3	<0.001
Brain malformation (764)	9.7	8.1	10.5	14.3	0.049
Postnatal injury (766)	15.3	14.1	15.8	18.6	0.35
Hydrocephalus (776)	12.5	11.8	13.2	14.6	0.59
Gastrostomy (776)	29.3	21.7	31.6	51.4	<0.001
Absent continence (773)	41.9	33.0	43.2	68.5	<0.001
Seizures (776)	47.7	43.8	50.0	58.9	0.002
Hearing impairment (765)	12.0	8.0	7.9	25.4	<0.001
Visual impairment (771)	34.9	30.7	47.4	45.1	0.001
Abnormal speech (776)	47.8	41.2	50.0	67.0	<0.001
Intellectual disability (776)					
None-mild	43.7	51.5	36.8	21.6	
Moderate	31.6	29.8	31.6	36.8	
Severe/profound	24.7	18.6	31.6	41.6	<0.001

[a]*At least one non-CNS congenital anomaly (CA) in addition to cryptorchidism.*

***Results of chi square analysis.*

TABLE 2 | Frequencies (%) of subtypes and comorbidities in boys with cerebral palsy and descended or retractile vs. undescended testes.

Variables	Descended	UDT or retractile	*p*-Value**
Race			
Caucasian	68.2	63.2	0.1
African-American	16.6	37.0	
Asian	2.0	1.3	
Other/unknown	13.2	11.2	
Diagnosis			
Quadriplegic	32.5	66.4	<0.001
Hemiplegic	42.2	26.9	
Diplegic	25.3	6.7	
Movement disorder	6.7	6.3	0.83
Death	2.0	6.3	0.002
Gestational age <37 weeks	55.4	54.3	0.77
Multiple birth	14.0	9.5	0.09
Intrauterine growth restriction (IUGR)	15.6	24.5	0.004
CA[a] and/or genetic defect	8.3	19.5	<0.001
Brain malformation	8.1	13.6	0.02
Postnatal injury	14.1	18.1	0.17
Hydrocephalus	11.8	14.3	0.32
Gastrostomy	21.7	48.0	<0.001
Absent continence	33.0	64.3	<0.001
Seizures	43.8	57.4	0.001
Hearing impairment	8.0	22.3	<0.001
Visual impairment	30.7	45.5	<0.001
Abnormal speech	41.2	64.1	<0.001
Intellectual disability			
Normal-mild	51.5	24.2	
Moderate	29.8	35.9	
Severe/profound	18.7	39.9	<0.001

[a]*At least one non-CNS congenital anomaly (CA) in addition to cryptorchidism.*
***p-Value: results of chi square analysis.*

in multivariable models because of potential bias related to its elective nature.

In univariable analyses (**Table 3**), estimated ORs for most variables were greater for undescended than for retractile cases. However, the differences were not significant except for quadriplegia, visual impairment, and severe/profound ID, most likely due to the small size of the retractile group. We found that the occurrence of cryptorchidism or retractile testis showed the strongest associations with quadriplegia, postnatal death, presence of at least one non-CNS CA with or without known genetic disease, absent continence, requirement for gastrostomy, hearing impairment, and severe/profound ID. All of these variables, except death, gastrostomy placement, and severe/profound ID, were retained in the multivariable analysis of undescended or retractile testes, which also included independent inverse associations with GA and multiple birth (**Table 4**). The results were similar for a model limited to undescended testes.

In view of these data, we explored the association of other CAs with cryptorchidism and CP in further analyses (**Table 5**). Of 144 CP cases with an associated brain malformation and/or at least one other non-CNS CA (18.8% of the total study group), 61 (42%) also had undescended or retractile testes ($p < 0.001$). We also found significant positive associations between CAs in the setting of CP with postnatal death, IUGR, term delivery, singleton birth, gastrostomy placement, absent continence, seizures, abnormal

TABLE 3 | Univariable logistic regression analysis comparing the prevalence of clinical characteristics/comorbidities based on cryptorchidism subtype.

	UDT or retractile		UDT		Retractile	
	OR	95% CI	OR	95% CI	OR	95% CI
Race						
Caucasian	Reference		Reference		Reference	
African-American	1.6	1.1, 2.3	1.5	1.0, 2.3	1.8	0.8, 3.9
Asian	0.7	0.2, 2.6	0.6	0.1, 2.6	1.5	0.2, 12.0
Other/unknown	0.9	0.6, 1.5	0.9	0.5, 1.6	0.9	0.3, 2.7
Diagnosis						
Diplegic	Reference		Reference		Reference	
Hemiplegic	2.4	1.3, 4.4	2.7	1.3, 5.5	1.8	0.6, 5.1
Quadriplegic	7.7	4.3, 13.6	10.1	5.1, 19.9	2.8	1.0, 7.7
Movement disorder	1.1	0.7, 1.8	1.1	0.7, 1.9	1.0	0.4, 3.1
Death	3.3	1.5, 7.4	3.4	1.5, 7.9	2.7	0.6, 12.8
Gestational age <37 weeks	0.96	0.7, 1.3	0.93	0.7, 1.3	1.1	0.6, 2.1
Multiple birth	0.6	0.4, 1.1	0.6	0.3, 1.0	0.9	0.3, 2.5
Intrauterine growth restriction (IUGR)	1.8	1.2, 2.6	1.9	1.1, 3.2	1.5	0.7, 3.4
CA[a] and/or genetic defect	2.7	1.7, 4.2	2.8	1.8, 4.5	2.1	0.8, 5.2
Brain malformation	1.8	1.1, 2.9	1.9	1.1, 3.2	1.3	0.5, 3.9
Postnatal injury	1.3	0.9, 2.0	1.4	0.9, 2.2	1.1	0.5, 2.8
Hydrocephalus	1.3	0.8, 2.0	1.3	0.8, 2.1	1.1	0.4, 3.0
Gastrostomy	3.3	2.4, 4.6	3.8	2.7, 5.4	1.7	0.8, 3.4
Absent continence	3.6	2.6, 5.1	4.4	3.1, 6.3	1.5	0.8, 3.0
Seizures	1.7	1.3, 2.4	1.8	1.3, 2.6	1.3	0.7, 2.5
Hearing impairment	3.3	2.1, 5.2	3.9	2.5, 6.2	1.0	0.3, 3.3
Visual impairment	1.9	1.4, 2.6	1.8	1.3, 2.6	2.0	1.0, 3.9
Abnormal speech	2.5	1.8, 3.5	2.9	2.0, 4.1	1.4	0.7, 2.7
Intellectual disability						
None/mild defect	Reference		Reference		Reference	
Moderate	2.6	1.7, 3.8	2.9	1.9, 4.5	1.5	0.7, 3.3
Severe/profound	4.6	3.0, 6.8	5.3	3.4, 8.3	2.4	1.1, 5.3

[a]*At least one non-CNS congenital anomaly (CA) in addition to cryptorchidism.*
OR, odds ratio; CI, confidence interval; descended is reference group in these analyses.

TABLE 4 | Stepwise logistic regression models to identify variables substantially associated with cryptorchidism subtype in boys with cerebral palsy.

Variable	Undescended or retractile testes		Undescended testes	
	Odds ratio (OR) (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Diagnosis				
Diplegia	Reference		Reference	
Hemiplegia	1.8 (0.95, 3.4)	0.07	2.0 (0.94, 4.2)	0.07
Quadriplegia	4.0 (2.0, 7.8)	<0.001	4.8 (2.2, 10.5)	<0.001
CA and/or genetic defect	2.5 (1.4, 4.3)	0.001	2.6 (1.5, 4.7)	0.001
Gestational age	0.96 (0.93, 0.99)	0.015	0.96 (0.92, 0.99)	0.023
Hearing impairment	1.9 (1.1, 3.1)	0.016	2.1 (1.3, 3.6)	0.005
Multiple birth	0.53 (0.29–0.96)	0.036	0.46 (0.23, 0.91)	0.025
Absent continence	1.6 (1.0, 2.6)	0.038	1.8 (1.1, 3.1)	0.021
Intrauterine growth restriction (IUGR)	1.5 (0.96, 2.3)	0.079	1.5 (0.94, 2.4)	0.085

Variables included in the model: race, diagnosis, movement disorder, death, birth weight, gestational age, multiple birth, intrauterine growth restriction (IUGR), congenital anomaly (CA; non-CNS in addition to cryptorchidism) and/or genetic defect, postnatal injury, hydrocephalus, gastrostomy, absent continence, seizures, hearing impairment, visual impairment, abnormal speech, and intellectual disability.

TABLE 5 | Frequencies (%) of subtypes and comorbidities in boys with cerebral palsy based on presence or absence of congenital anomalies (CNS and/or non-CNS) other than cryptorchidism.

Variables	Absent $n = 620$	Present $n = 144$ (18.8%)	p-Value*
Race			
Caucasian	67.1	68.1	0.97
African-American	19.0	19.4	
Asian	1.3	1.4	
Other/unknown	12.6	11.1	
Diagnosis			
Quadriplegic	41.5	47.2	0.26
Hemiplegic	37.7	37.5	
Diplegic	20.8	15.3	
Movement disorder	6.5	6.9	0.83
Died	2.6	6.3	0.026
Prematurity	58.8	36.4	<0.001
Multiple birth	13.9	6.3	0.013
Intrauterine growth restriction (IUGR)	16.3	27.0	0.003
Undescended or retractile testis	26.1	42.4	<0.001
Postnatal injury	16.5	10.4	0.07
Hydrocephalus	13.1	11.1	0.53
Gastrostomy	27.6	38.2	0.012
Absent continence	39.0	55.6	<0.001
Seizures	46.0	57.6	0.012
Hearing impairment	11.4	15.6	0.17
Visual impairment	34.2	39.9	0.20
Abnormal speech	44.8	61.8	<0.001
Intellectual disability			
None	47.1	27.1	<0.001
Moderate	30.0	38.9	
Severe/profound	22.9	34.0	

**p-Value: results of Chi square analysis.*

TABLE 6 | Stepwise logistic regression model of comorbidities associated with CNS and/or non-CNS congenital anomalies or genetic defect in boys with CP.

Variable	Odds ratio (95% CI)	p-Value
ID		
None/mild	Reference	
Moderate	2.1 (1.2, 3.4)	0.004
Severe/profound	1.9 (1.3, 3.1)	0.019
Undescended or retractile testis	2.0 (1.3, 3.1)	0.002
Gestational age	1.1 (1.1, 1.2)	<0.001
Postnatal injury	0.4 (0.2, 0.8)	0.006
Intrauterine growth restriction (IUGR)	1.5 (0.93, 2.4)	0.09

Variables included in the stepwise logistic regression model: race, diagnosis, movement disorder, death, birth weight, gestational age, multiple birth, intrauterine growth restriction (IUGR), postnatal injury, hydrocephalus, gastrostomy, absent continence, seizures, hearing impairment, visual impairment, abnormal speech, intellectual disability (ID), and undescended or retractile testis.

speech, and ID (**Table 5**). In a similar multivariable analysis of CAs in CP, we found independent positive associations with ID, undescended, or retractile testes, gestational age and IUGR, and a negative association with postnatal injury (**Table 6**).

DISCUSSION

The present results suggest that common factors contribute to the risk of undescended or retractile testes in the CP population, and that the combined prevalence of 29% for males followed until at least age 7 is lower than identified in previous reports that focused solely on institutionalized, frequently older individuals. Our data also suggest that cryptorchidism is most strongly associated with spastic quadriplegia, with selected functional deficits including abnormal hearing and absent continence, and with non-CNS CAs in males with CP. Although we found associations between cryptorchidism and postnatal death, brain malformations, requirement for gastrostomy placement, seizures, visual impairment, abnormal speech and ID, none of these variables was retained in the multivariable model. This suggests that cryptorchidism in CP is more strongly associated with spasticity than with ID. This contrasts with the results of a prior population-based study showing independent association with both CP and ID, leading the authors to hypothesize that CNS lesions lead to hypothalamic–pituitary–gonadal axis dysfunction and failure of testicular descent (14). However, the total number of affected individuals in that study was small (18 with CP and 53 with ID) and the degree of overlap between the two diagnoses unclear. In the report by Cortada and Kousseff of 148 institutionalized individuals with ID, 44 (30%) had cryptorchidism and the majority of these (82%) also had CP (13). As in the present series, cryptorchidism was bilateral in the majority of cases. The similarity between the frequency distributions of the majority of variables that we analyzed and those reported previously in CP (1, 2, 4, 9–11, 15–22) supports the reliability of our data.

Smith and colleagues suggested a role for muscle spasticity in the etiology of cryptorchidism in boys with CP. In their prospective study, testicular position relative to the pubic tubercle of 50 boys with CP (25 aged <2.5 and 25 aged 5–10 years) was higher than that of aged-matched controls, although no cryptorchidism was reported (23). The authors theorized that progressive cremaster muscle shortening may, therefore, account for cases of acquired cryptorchidism in the CP population. Our documentation of a prior scrotal position for 20% of subsequently cryptorchid testes and the average age at diagnosis (5.8 years) suggest that testicular ascent may be common in the CP population. Failed or delayed diagnosis could also be related to deferral of testicular exams in patients with multiple medical concerns, or potential uncertainty regarding the need for treatment in this population (24, 25). We also observed apparent acquired cryptorchidism in boys who had undergone prior inguinal hernia repair, but biopsy data in this population has suggested that maldescent in these cases is likely primary and not truly iatrogenic (26). In a comparison of the present data and those for acquired cryptorchidism, we found that both sides are more commonly affected (69%) in CP, but the milder phenotype (77% of testes distal to the external ring) and the prevalence of associated inguinal hernia (56%) are similar to those we reported for uncomplicated cases of testicular ascent (79 and 46%, respectively) (27). It remains unclear if cremaster hyperactivity in otherwise normal children and spasticity in boys with CP both predispose to testicular ascent.

The present study, although retrospective, comprises the largest study of testicular position in a CP population to date, providing increased power to detect factors independently associated with cryptorchidism through multivariable statistical analysis. Our results suggest that cryptorchidism is more likely to occur in cases of CP associated with other CAs and with IUGR, a known strong risk factor for both non-syndromic cryptorchidism (28, 29) and for CP (30, 31). Reciprocally, we also identified cryptorchidism

and IUGR as independent variables associated with the occurrence of other CAs in cases of CP. These shared associations are consistent with increasing evidence that CAs and genetic defects are both more common than previously recognized in boys with CP (1, 8, 22). Nevertheless, we did observe differences in the patterns of association of CP with cryptorchidism and with other CAs. While both presentations were more common in singletons, that association was not retained in the final model for CAs (**Table 6**). Cryptorchidism risk in both this study and the general non-CP population (28, 29) is associated with decreased gestational age. In contrast, we found an increased frequency of other CAs in children with CP born near term, as reported previously (1, 11). Similarly, unlike cryptorchidism, we found that the occurrence of other CAs did not correlate with the pattern of spasticity, a finding noted by others who studied the association of non-CNS CAs with CP (11).

Other independent associations that we observed for cryptorchidism in CP include hearing deficit and absence of continence. The reported prevalence of hearing loss in a large cohort of children with CP ($n = 685$) is the same as we found in our population (12%), and its occurrence was associated with quadriplegia, ID, movement disorders, visual impairment, and seizures in univariable analyses (20). However, the causes, which may be genetic or environmental, remain largely unknown. Similarly, a study of children with CP ($n = 79$) and varying degrees of urinary incontinence suggested association with ID, severity of spasticity, movement disorder, and issues related to speech and swallowing in univariable analyses, and with ID and more profound functional impairment in a multivariable model (32).

In conclusion, our data suggest that the prevalence of cryptorchidism in males with CP is approximately 10-fold greater than that of the general population, but lower than previous estimates. We found that its occurrence is most commonly bilateral and associated with measures of increased severity and with the co-occurrence of other CAs. Additional factors that may contribute to a higher risk of cryptorchidism in CP include cremaster spasticity, prematurity, and possibly inguinal hernia repair in infancy. Although testes often descend spontaneously in otherwise normal premature boys, it is possible that comorbidities reduce the likelihood of postnatal descent in boys with CP. Diagnosis in later childhood due to apparent testicular ascent is possible; therefore, serial examination is indicated. The combination of cryptorchidism, other CAs and IUGR may indicate a higher risk of genetic causation in individuals with CP, but further studies are needed. However, the available evidence suggests that the etiologies of both cryptorchidism and CP are complex and multifactorial.

ETHICS STATEMENT

This study was approved by the Institutional Review Board (IRB #262462) of Nemours and requirement for informed consent/parental permission and assent, and authorization for use and disclosure of protected health information was waived based on the retrospective nature of the approved research.

AUTHOR CONTRIBUTIONS

JB designed the study, collected and analyzed data, and wrote the manuscript; AW and KR collected data and approved the manuscript; JH edited and approved the manuscript; MH contributed to study design, analyzed data, edited, and approved the manuscript.

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Amniotic Fluid INSL3 Measured During the Critical Time Window in Human Pregnancy Relates to Cryptorchidism, Hypospadias, and Phthalate Load: A Large Case–Control Study

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The period of the first to second trimester transition in human pregnancy represents a sensitive window for fetal organogenesis, particularly in regard to the development of the male reproductive system. This is a time of relative analytical inaccessibility. We have used a large national biobank of amniotic fluid samples collected at routine amniocentesis to determine the impacts of exogenous endocrine disruptor load on specific fetal biomarkers at this critical time. While adrenal and testicular steroids are highly correlated, they are also mostly positively influenced by increasing phthalate load, represented by the metabolites 7cx-MMeHP and 5cx-MEPP, by perfluorooctane sulfonate (PFOS) exposure, and by smoking, suggesting an adrenal stress response. In contrast, the testis specific biomarkers insulin-like peptide 3 (INSL3) and androstenedione are negatively impacted by the phthalate endocrine disruptors. Using a case–control design, we show that cryptorchidism and hypospadias are both significantly associated with increased amniotic concentration of INSL3 during gestational weeks 13–16, and some, though not all steroid biomarkers. Cases are also linked to a specifically increased variance in the Leydig cell biomarker INSL3 compared to controls, an effect exacerbated by maternal smoking. No influence of phthalate metabolites or PFOS was evident on the distribution of cases and controls. Considering that several animal and human studies have shown a negative impact of phthalate load on fetal and cord blood INSL3, respectively, the present results suggest

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that such endocrine disruptors may rather be altering the relative dynamics of testicular development and consequent hormone production, leading to a desynchronization of tissue organization during fetal development. Being born small for gestational age appears not to impact on the testicular biomarker INSL3 in second trimester amniotic fluid.

Keywords: INSL3, amniotic fluid, fetal steroids, cryptorchidism, hypospadias, phthalate, PFOS, testicular dysgenesis syndrome

INTRODUCTION

Cryptorchidism and hypospadias represent two of the commonest congenital malformations in newborn boys, and both conditions have been linked to a hypothetical TDS (Skakkebaek et al., 2001; Wohlfahrt-Veje et al., 2009) implying a common etiology. This hypothesizes an insult of possible environmental, lifestyle, or pharmacological nature during a critical window of sensitivity in the transition from the first to the second trimester, a time soon after sex determination has occurred and the principal cellular components of the fetal testes are being established. Research in rodents has highlighted a role for Leydig cells and their major androgen products in both conditions, an opinion reinforced by the symptoms induced by some phthalate compounds administered at a comparable gestational time (Foster, 2006; Howdeshell et al., 2008; Wilson et al., 2008; Hu et al., 2009; Veeramachaneni and Klinefelter, 2014; van den Driesche et al., 2017). Recent work using human fetal testis explants and/or xenografts, however, has suggested that the human fetus may be less susceptible to phthalates than rodents (Albert and Jegou, 2014; Habert et al., 2014), and a recent systematic review and meta-analysis could show no epidemiological relationship between endocrine disrupting chemicals and male reproductive disorders (Bonde et al., 2017). Other studies point to a significant role of analgesic compounds, such as paracetamol, taken during pregnancy, in disrupting human fetal Leydig cell function (Kristensen et al., 2012; Mazaud-Guittot et al., 2013; Ben Maamar et al., 2017). In support of an effect of phthalates on the human male phenotype, epidemiological studies have indicated a significant association between maternal phthalate load and the androgen-dependent anogenital distance (Swan et al., 2005; Bornehag et al., 2015).

Besides being major producers of the androgens androstenedione and testosterone during this critical window in human pregnancy (Ivell et al., 2017), fetal Leydig cells are now known to make and secrete another major hormone, INSL3 (Ivell and Anand-Ivell, 2009). Genetic experiments in mice show that ablation of the gene encoding either INSL3 or its unique cognate receptor RXFP2 leads to bilateral cryptorchidism, due to a failure of the gubernacular ligament to expand, and thereby promote the first transabdominal phase of testicular descent (Nef and Parada, 1999; Zimmermann et al., 1999; Kamat et al., 2004). This role is supported by the treatment of mice during mid-gestation with an INSL3 antagonist (Yuan et al., 2010) leading to cryptorchidism. Furthermore, in mice treated gestationally with the estrogen diethylstilbestrol, there is both bilateral cryptorchidism and concomitant down-regulation of *Insl3* gene expression in the fetal testes (Emmen et al., 2000), a finding paralleled by the increased incidence of cryptorchidism amongst boys born of mothers similarly treated with diethylstilbestrol during pregnancy (Palmer et al., 2009). The common consensus is that whereas INSL3 is important for the first phase of testicular descent, fetal androgens are more important for the later inguino-scrotal phase of descent, as well as for the proper formation of the external genitalia (Hutson et al., 2013). However, it should be noted that androgens may be required for the induction of the INSL3 receptor, RXFP2 (Yuan et al., 2010), as well as for the relaxation and involution of the cranial suspensory ligament, which retains the indeterminate gonad in a peri-renal position (Lee and Hutson, 1999), implying that androgens are also required during the first transabdominal phase. Thus it appears that cryptorchidism and hypospadias might have common roots in a disruption of Leydig cell function and the production of INSL3 and androgens during early-mid gestation.

Part of the difficulty in unraveling the etiology of cryptorchidism is its highly heterogeneous manifestation (Hutson et al., 2013). Testes may be arrested in a high abdominal position or at any point before or within the inguinal canal. Cryptorchidism may be bilateral or more commonly unilateral. Moreover, it may also be transient, with a higher incidence of cryptorchidism occurring in newborns compared with 3 month-old infants; there is also a possibility for testes to re-ascend from the scrotum during childhood. Anatomically, particularly the first phase of testicular descent from a peri-renal position to the inguinal ring is complicated by the fact that this occurs with an opposing vector to other abdominal organs. This phase of descent occurs because the thickening of the gubernaculum under INSL3 influence effectively retains the fetal testes in the inguinal region while the remaining organs and tissues, including the kidneys, grow away in an antero-dorsal direction. Thus any factor which would cause a temporal discordance of this fetal growth trajectory could result in a ligament or a tissue being interposed in the normal path of movement of one or other

Abbreviations: 5cx-MEPP, mono(2-ethyl-5carboxypentyl) phthalate; 7cx-MMeHP, mono(4-methyl-7-carboxyheptyl) phthalate; ACTH, adrenocorticotropic hormone; DEHP, di(2-ethylhexyl) phthalate; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; DiNP, diisononyl phthalate; DNPR, Danish National Patient Registry; ED, endocrine disruptor; hCG, human chorionic gonadotropin; HPG, hypothalamo-pituitary-gonadal; INSL3, insulin-like peptide 3; IUGR, intrauterine growth restriction; LC/MS–MS, liquid chromatography with tandem mass spectrometry; LH, luteinising hormone; MoM, multiple of the median; PFOS, perfluorooctane sulfonate; RXFP2, relaxin family peptide receptor 2; SGA, small for gestational age; TDS, testicular dysgenesis syndrome; TRFIA, time resolved fluorescent immunoassay.

testes, leading also to cryptorchidism (mostly unilateral; Hutson et al., 2013).

Regarding hypospadias, evidence points to this being largely a complication involving fetal androgen-deficiency (Kalfa et al., 2009), though it should be noted that androgens are also made in substantial amounts by the fetal adrenals of both sexes as well as by the testes (Wudy et al., 1999; Anand-Ivell et al., 2008; Scott et al., 2009; Ivell et al., 2017). Moreover, it is still not clear whether the human fetal testis is dependent upon an intact and functional HPG axis (and/or fetal hCG), or whether other factors may be involved in determining androgen production (Scott et al., 2009). For example, we know that the mouse fetal testes are largely independent of LH stimulation, rather being driven by ACTH as for the fetal adrenal (O'Shaugnessy et al., 2003). Moreover, one study suggests that human fetal Leydig cells at least up to 18 weeks are also independent of hCG (Word et al., 1989), though a more recent study indicates a clear stimulation of testosterone production by hCG (Hallmark et al., 2007).

One of the major difficulties in understanding the etiology of these two common congenital ailments is the fact that the critical period during human pregnancy which is implicated is also one of the experimentally least accessible. We and others have shown that amniotic fluid collected at routine genetic amniocentesis serves as a useful matrix by which to explore the endocrinology of the growing second trimester fetus (Wudy et al., 1999; Anand-Ivell et al., 2008). Most such samples are collected between gestational weeks 12 and 16, a time at which amniotic fluid still represents very much an exudate of fetal serum combined with other fluid components derived from the amniotic membranes (Underwood et al., 2005; Beall et al., 2007). Later in pregnancy, when the fetal skin has become keratinized, this simple relationship to fetal serum is probably no longer valid (Bacchi-Modena and Fieni, 2004). The present study represents the largest ever (425 controls; 421 cryptorchid; 109 hypospadias) case–control study to assess the role of fetal androgens and INSL3 in subsequent cryptorchidism and hypospadias, using second trimester amniotic fluid samples collected in Denmark as part of a national biobank (Jensen et al., 2012). Preliminary results from this study have already been published (Jensen et al., 2012, 2015; Toft et al., 2016); here we extend these analyses and specifically address those factors influencing Leydig cell function and which may be altered in subsequent cryptorchidism and hypospadias, and focusing on the critical window of sensitivity (weeks 13–16).

MATERIALS AND METHODS

Study Population

Amniotic fluid samples were derived from a Danish biobank maintained at the State Serum Institute (SSI) in Copenhagen. Amniotic fluid samples, which had been collected at routine amniocentesis between 1980 and 1996, were selected from 25,105 samples from live-born singleton male offspring pregnancies which had complete obstetric data in the Danish Medical Birth Registry. Samples were centrifuged, and supernatants stored at −20°C until analysis. Main indications for amniocentesis were age (≥35 years) or increased risk of congenital malformation based on maternal serum analysis. The date of amniocentesis, date of birth, and estimated gestational age at birth were used to calculate the gestational week of amniocentesis (Jensen et al., 2012). Unique person identifiers were used to obtain offspring gender, gestational age at birth, parity, birth weight, and Apgar score (Knudsen and Olsen, 1998; Pedersen et al., 2006). All selected cryptorchid cases (n = 421; 1.64%) had a diagnosis of undescended testis according to the International Classification of Disease, as well as a corrective surgical procedure according to the Surgery and Treatment Classification of the Danish National Board of Health or the Nordic Classification of Surgical Procedures, as listed in the DNPR up to November 2008 (Lynge et al., 2011). To avoid possible inclusion of cases of iatrogenic cryptorchidism, boys with an inguinal hernia repair were excluded. The hypospadias group (N = 109; 0.43%) included all boys with that diagnosis in the DNPR. An equivalent number (n = 425) of controls were randomly selected from the 25,105 boys with complete medical entries in the DNPR. Full details of inclusion criteria are given in Jensen et al. (2015).

This study was approved by the Danish Regional Ethics Committee, the Danish National Board of Health, and the Danish Data Protection Agency.

Analyses and Parameters

Insulin like peptide 3 was measured using a modified TRFIA as recently described (Anand-Ivell et al., 2013; Jensen et al., 2015). This assay used a sample volume of 0.1 ml and had a limit of detection of 10 pg/ml and inter- and intra-plate coefficients of variation of ≤8 and <1%, respectively. Because, it had previously been shown that INSL3 in amniotic fluid indicated a marked dependence on gestational age (Anand-Ivell et al., 2008), values were also converted to MoM to account for this additional variable factor (Lenke et al., 1989). Because of volume restrictions INSL3 could only be measured in 243 controls, 227 cryptorchids (bilateral and unilateral combined), and 73 hypospadias cases. The steroids androstenedione, testosterone, 17OH-progesterone, progesterone, DHEAS, and cortisol were all assayed using LC/MS–MS as previously described (Jensen et al., 2015). Cotinine concentration, as an index for acute maternal exposure to tobacco smoke, was measured as previously (Jensen et al., 2015), with smokers defined as those women with amniotic fluid levels >85 ng/ml, and passive smokers as those with levels 85 > 25 ng/ml (Jauniaux et al., 1999). Non-smokers had <25 ng/ml cotinine. Fetuses were defined as SGA or not using the formula of Marsal et al. (1996). This calculation is based on ultrasound observations of uncomplicated pregnancies from four Scandinavian centers and calculates SGA as >2 standard deviations below the mean corrected birth weight. The two major phthalate metabolites 5cx-MEPP deriving from exposure to DEHP and 7cx-MMeHP, deriving from DiNP, as also PFOS were measured by LC/MS–MS as previously described in detail (Jensen et al., 2012; Toft et al., 2016) with limits of detection at 0.05, 0.02, and 0.02 ng/ml, respectively. Sample numbers available for measuring 5cx-MEPP, 7cx-MMEHP, and PFOS across all gestational ages were 300, 270, and 75 for controls, cryptorchids, and hypospadias cases, respectively, and for weeks 13–16, 190, 146, and 48; for measuring steroids across all gestational ages, N

was 258, 237, and 79 for controls, cryptorchids, and hypospadias cases, respectively, and for weeks 13–16, the corresponding figures were 164, 123, and 51.

Many of the samples measured had been stored for up to 30 years at −20°C prior to analysis. They might thus have suffered evaporation/sublimation and/or analyte degradation in that time. To check this, measured parameters were analyzed against both date of sample collection and total volume of stored sample. No significant trends for any analyte were identified (Jensen et al., 2012). For INSL3 we have shown that serum samples stored at −20°C for at least 5 years remained stable with no significant difference in INSL3 content (data not shown), and previously we have shown that human amniotic fluid samples maintained INSL3 content over several freeze-thaw cycles and when left at room temperature for 24 h without significant change (Anand-Ivell et al., 2008).

Statistical Analysis

Data were analyzed using GraphPad Prism version 7 or the SPSS package (for multiple correlation analysis). For some analyses, data were grouped according to week of amniocentesis, where for example week 16 represented all samples from 16+0 to 16+6. Column statistics were assessed by Levene's test (Levene, 1960), as described in SPSS, to check for equality of variance. Where variance was shown to be unequal, comparisons used the Mann–Whitney non-parametric test; otherwise significance was estimated by ANOVA followed by Newman–Keuls or Student's *t post hoc* tests. Continuous variables were log-transformed prior to statistical analysis. Multiple correlation analysis used untransformed data and was corrected against gestational week of amniotic fluid sample as confounder, since several parameters showed significant trends across gestation (**Supplementary Figure S1**).

RESULTS

Amniotic Fluid INSL3 and Gestational Age at Amniocentesis

Figure 1 shows the distribution of INSL3 concentration in amniotic fluid plotted against gestational age at amniocentesis for controls (red squares), cryptorchid (green triangles), and hypospadias (purple circles) cases. For cryptorchids, unilateral, bilateral and undefined cases were combined, since these showed no significant differences for any parameter measured. All data appear to fit a common distribution with neither cases nor controls appearing to depart from this pattern, which closely follows what we had shown previously for a smaller collective of United States subjects (Anand-Ivell et al., 2008). There is a clear maximum between gestational weeks 13 and 16, with most values declining rapidly after that time. Closer inspection, however, reveals that for all cases compared to controls there appears to be skewness in the data suggesting higher INSL3 values for cases in the earlier weeks of gestation (**Figure 2A**). Since there are no significant differences in INSL3 values within groups between weeks 13 and 16, for which these concentrations represent maximum values, these results can be pooled to provide

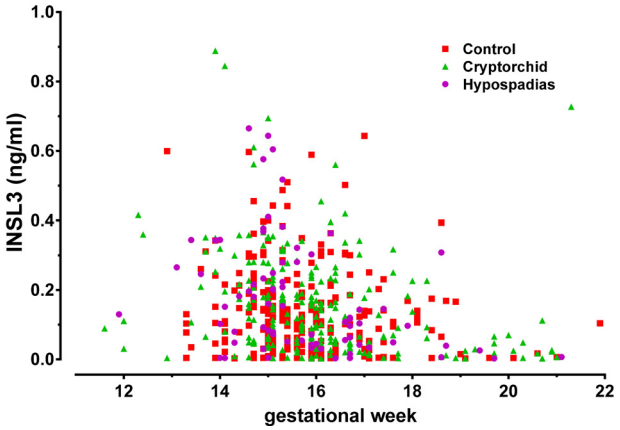

FIGURE 1 | Scatter-plot of human amniotic fluid INSL3 concentration for all available samples from normal controls (red squares), combined cryptorchid cases (green triangles), and hypospadias cases (purple circles), plotted against gestational week of amniocentesis.

the summary **Figure 2B**. This panel shows that in the early second trimester, when amniotic fluid INSL3 is maximal, both cryptorchid and hypospadias cases have significantly elevated INSL3 concentration compared to controls. The mean INSL3 concentration for the control subjects is similar to what had been shown previously for United States male pregnancies at this gestational time (ca. 0.10 ng/ml; Anand-Ivell et al., 2008), as well as for a smaller Danish set collected with a much shorter storage time (ca. 0.12 ng/ml; Bay et al., 2008).

When INSL3 values for weeks 13–16 are corrected for gestational age by converting to MoM, we still observe a significant difference in the mean values for controls versus both cryptorchids and hypospadias cases (**Figure 2C**). There is also a significant difference in the variance between the cryptorchid (unilateral + bilateral) and the control groups for this data-set, as well as between the hypospadias and control groups, assessed using Levene's test.

We also compared the residual INSL3 concentrations in weeks 17–21 for cases and controls (**Figure 2D**). Although the uncorrected mean values are reduced in both cryptorchid and hypospadias cases compared to controls, this does not reach statistical significance, probably due to the large proportion of samples at or close to the assay limit of detection. Converting to MoM does not alter this (not shown).

Amniotic Fluid INSL3 and Maternal Smoking

Comparing amniotic fluid samples for smokers, passive smokers, and non-smokers (cotinine >85, 25–85, and <25 ng/ml, respectively) for INSL3 MoM values for all gestational ages, shows no differences, when all cases and controls are pooled (data not shown). However, when separated out into cases versus controls (**Figure 3**), INSL3 MoMs are significantly greater ($p < 0.003$) in the cryptorchid group for non-smokers versus controls, though just not significant for smokers (passive

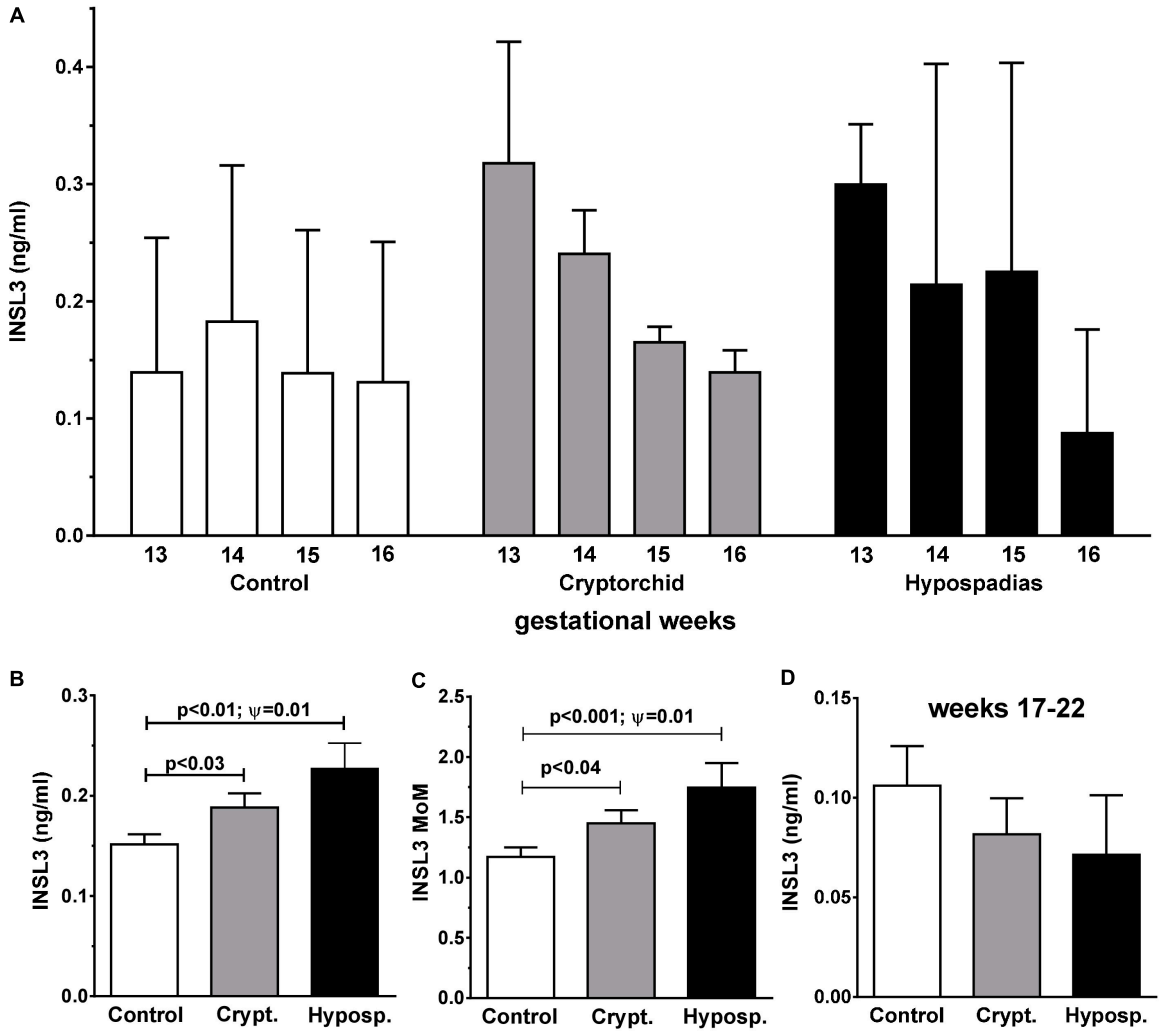

FIGURE 2 | Frequency distributions of amniotic fluid INSL3 concentration (means ± SEM) collected at gestational weeks 13–16 for controls, combined cryptorchids and hypospadias cases **(A)**. Since individual weeks between 13 and 16 were not significantly different from one another for cases and controls, these were combined in **(B)** (expressed as absolute concentration) and **(C)** (expressed age-corrected as MoM). **(D)** As in **(B)** but for gestational weeks 17–22. Differences between means were significant only where indicated. Ψ Represents significance for Levene's test for differences in variance.

smokers are intermediate between smokers and non-smokers and are omitted here for clarity). Both smokers and cases (cryptorchid/hypospadias) indicate a higher overall variance in INSL3 MoM, thus accounting for the failure to achieve significance in the absolute differences. Correlation analysis shows no relationship between individual amniotic cotinine concentration and either INSL3 concentration or INSL3 MoM values in cases or controls or in both combined, nor when data are restricted to weeks 13–16 (**Supplementary Tables S1–S5**).

Amniotic Fluid INSL3 and Fetal Size for Gestational Age

In order to determine whether amniotic fluid INSL3 concentration might be related to intrauterine growth, we applied the formula of Marsal et al. (1996) in order to identify those fetuses born SGA. There was a higher proportion of such SGA pregnancies in both cryptorchid (9.3%) and hypospadias (8.2%) groups, compared to the controls (4.9%). Pregnancies identified as SGA were then compared with non-SGA normal pregnancies in regard to amniotic INSL3 concentration, comparing cases and controls pooled, as well as individually (**Figure 4**). Other than the reported increased variance for cryptorchids, there appeared to be no significant impact on the INSL3 MoM of being born SGA. The same was also true for absolute INSL3 concentrations restricted to weeks 13–16. We also see no relationship between INSL3 expression and gestational age at birth (data not shown).

Amniotic Fluid INSL3 and Steroid Concentrations

For male pregnancies, steroids measured in amniotic fluid derive from the fetal testis, the fetal adrenal gland, and in

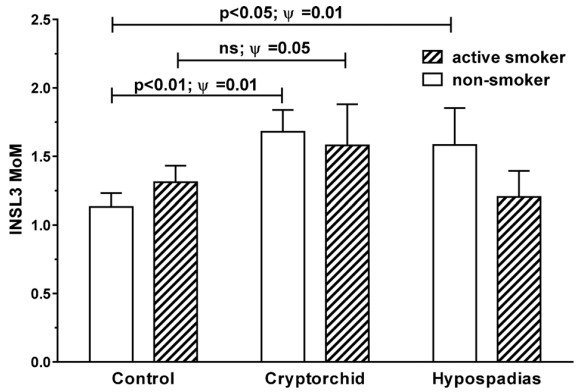

FIGURE 3 | Age-corrected INSL3 MoM values for all gestational weeks combined for cases and controls, separated into active smokers (cotinine > 85 ng/ml) and non-smokers (cotinine < 25 ng/ml). Passive smokers (cotinine 25 < 85 ng/ml) were intermediate and omitted for clarity. Significant differences between means were only evident amongst non-smokers. Ψ Represents significance for Levene's test for differences in variance. ns, not significant.

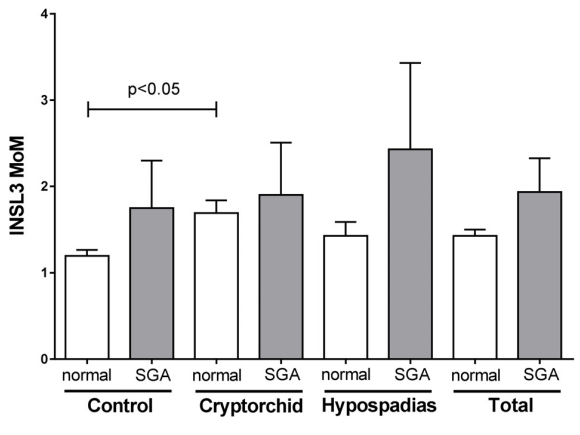

FIGURE 4 | Age-corrected INSL3 MoM values for all gestational weeks combined for cases and controls, separately and together (total), separated into pregnancies calculated to be SGA and normal pregnancies (not SGA). Only the difference between non-SGA controls and cryptorchids was considered significantly different.

small part from the placenta (**Figure 5**; Ivell et al., 2017). Comparisons between amniotic fluid steroids from male and female fetuses (Wudy et al., 1999; Anand-Ivell et al., 2008) indicate that testosterone and androstenedione are predominantly of testicular origin, being enriched for male fetuses. In contrast, progesterone, 17OH-progesterone, DHEAS and cortisol do not differ in relation to fetal gender (Wudy et al., 1999), and are thus predominantly of adrenal and/or placental origin. Not surprisingly, because of their common metabolic pathways, all measured steroids are highly correlated to one another ($p < 0.001$), when measured across gestation for cases and controls combined (**Supplementary Table S1**). Absolute concentrations of the various steroids measured in amniotic fluid show that androstenedione (2–10 ng/ml; 5–95% confidence limits) is 3–4 fold higher than testosterone (0.5–3.0 ng/ml), and comparable in amount to 17OH-progesterone (4–10 ng/ml). Cortisol and DHEAS are greater again (5–100 ng/ml) and illustrate the larger steroidogenic capacity of the adrenal gland at this time of gestation. Progesterone (also of placental/luteal origin and a major steroid precursor in all pathways) is highest at 100–300 ng/ml concentration. Comparing steroid concentrations across gestational weeks, there is no significant effect of gestational age (weeks 11–21) on androstenedione, testosterone, or progesterone. For cortisol, 17OH-progesterone and DHEAS there is a small but significant trend for increasing concentration in amniotic fluid between weeks 11 and 21 (**Supplementary Figure S1**), but only for cortisol is this trend evident between the critical weeks 13–16.

Androstenedione, 17OH-progesterone, DHEAS and cortisol, though not testosterone or progesterone, are positively correlated ($p < 0.02$) with cotinine levels (**Supplementary Table S1**; all cases and controls combined), implying that maternal smoking is promoting increased steroidogenesis.

Comparing all cases and controls for all steroids measured and over all gestational ages, showed small but significant differences for testosterone ($p < 0.05$) and for progesterone ($p < 0.05$) for hypospadias cases versus controls, where for both steroids these were mildly elevated (**Figure 6**). Cryptorchid cases were significantly elevated compared to controls for progesterone and also for androstenedione (**Figure 6**). However, when only weeks 13–16 are considered, then probably because of the reduced sample size only testosterone is significantly different (18% increased; $p < 0.05$) between cases and controls, and then only for hypospadias cases (**Supplementary Figure S2**).

It should be noted that because of lack of sufficient sample availability, it was not possible to measure all parameters in all samples. Thus only where both INSL3 and all measured steroids were available for the same amniotic fluid samples could multiple correlation analysis be carried out. As might be anticipated for major Leydig cell products and comparing samples from all gestational weeks, there is a statistically positive correlation between INSL3 MoM and in order of significance, testosterone ($p < 0.001$), androstenedione ($p = 0.005$), and DHEAS ($p = 0.043$), for cases and controls combined, and corrected for gestational week (**Supplementary Table S1**). For controls only (**Supplementary Table S2**), there is a positive correlation for androstenedione ($p = 0.007$) and testosterone ($p = 0.04$). Amongst cryptorchids (**Supplementary Table S3**), a significant positive correlation with INSL3 MoMs is observed only for testosterone ($p < 0.001$), although INSL3 concentration itself also correlates strongly with androstendione ($p = 0.005$). No correlations were observed for INSL3 amongst the hypospadias cases (**Supplementary Table S4**).

As explained earlier, INSL3 concentrations limited to weeks 13–16 are more informative, representing maximal and physiologically relevant INSL3 expression. When steroids are now compared, we see a significant positive correlation between INSL3 MoMs (as also INSL3 concentration) and both androstenedione ($p = 0.01$) and testosterone ($p < 0.01$; **Supplementary Table S5**).

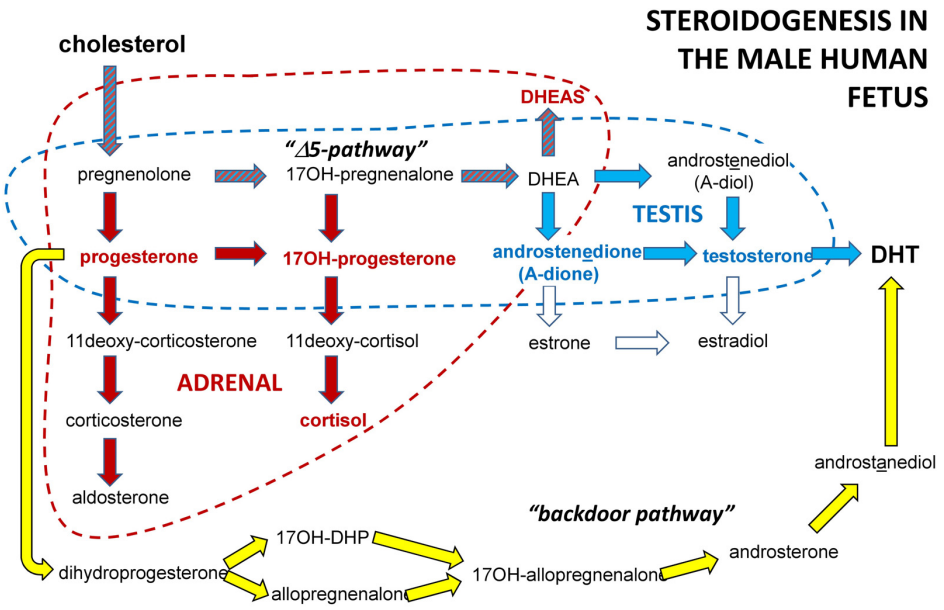

FIGURE 5 | Schematic to illustrate the principal steroidogenic pathways in the male human fetus for both testes and adrenals. In bold type (red or blue) are indicated those steroids used here as biomarkers of fetal steroidogenesis.

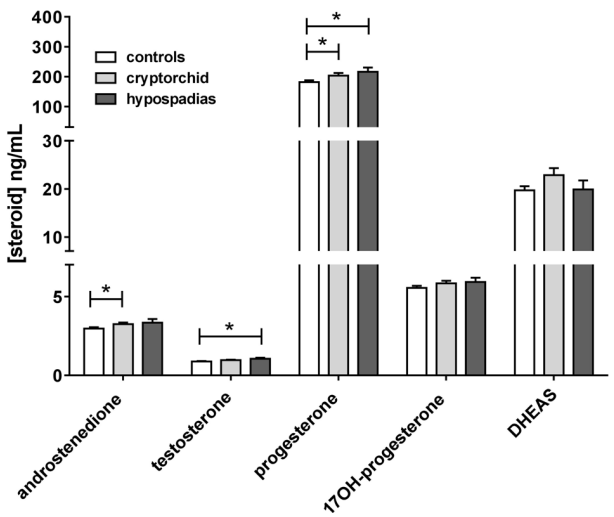

FIGURE 6 | Mean steroid concentrations in amniotic fluid combined across all gestational ages, separated into normal controls, combined cryptorchid and hypospadias cases. *$p < 0.05$.

Influence of Xenobiotic Compounds

The present study has assessed the amniotic fluid concentration of 5cx-MEPP as the chief metabolite of DEHP, and 7cx-MMeHP as the main metabolite of DiNP, as well as the fluorinated compound PFOS. Firstly, both 5cx-MEPP, and 7cx-MMeHP, showed small but significant trends to increase across gestation (weeks 11–22; cases and controls combined; **Supplementary Figure S1**). Only 7cx-MMeHP showed a small but significant ($p < 0.05$) correlation (negative) with cotinine levels (**Supplementary Table S1**; cases and controls combined; **Supplementary Table S2**; controls only). Importantly, both phthalate metabolites were positively correlated amongst each other ($p < 0.001$; cases and controls combined; **Supplementary Table S1**). 5cx-MEPP was also highly positively correlated ($p < 0.5$ to $p < 0.001$; **Supplementary Table S1**) with all steroids measured, except for progesterone (not significant). In contrast, cx7-MMeHP was correlated only with androstenedione ($p < 0.02$; negatively) and DHEAS ($p = 0.002$; positively).

Comparing cases with controls across the gestational range, there was no significant difference in mean or variance for either of the measured phthalate metabolites (not shown). When samples are restricted to the window of sensitivity for testis development in weeks 13–16, there is also no significant difference between cases and controls (**Supplementary Figure S2**).

When we compare INSL3 values for weeks 13–16 as representative of Leydig cell functionality at this critical time, then there is a negative correlation ($p = 0.04$) with 7cx-MMeHP for controls only (not shown), as well as cases and controls combined (**Supplementary Table S5**), and a similar negative correlation ($p < 0.04$) with 5cx-MEPP for cases and controls combined (**Supplementary Table S5**). There is no correlation between INSL3 and any phthalate metabolite within the cryptorchid and hypospadias cases alone at this restricted time window in gestation (not shown).

The environmental pollutant PFOS was significantly positively correlated with all steroids measured (cases and controls combined; all gestational weeks) as well as with the phthalate metabolites 7cxMMEHP (**Supplementary Table S1**). PFOS was not correlated with INSL3 MoM values across gestation (weeks

11–21; **Supplementary Table S1**), nor when data were restricted to gestational weeks 13–16 (**Supplementary Table S5**). As for the phthalate metabolites, also PFOS exposure showed no statistically significant differences between cases and controls within the critical window of weeks 13–16 (**Supplementary Figure S2**).

None of the above significant relationships was altered when multiple correlation analysis was additionally corrected for either cotinine and/or PFOS concentration as possible confounders (not shown).

DISCUSSION

Cryptorchidism and hypospadias are amongst the commonest malformations in newborn infants representing, respectively, 2–9 and 0.2–0.7% (Main et al., 2010) of all male births, and are considered by some to be symptoms of a common TDS (Wohlfahrt-Veje et al., 2009). Such disturbances of sexual development in male human offspring are thought to have their origins relatively early during pregnancy, soon after SRY-driven sex determination and during early testicular organogenesis. This corresponds approximately to the end of the first trimester and the early second trimester of pregnancy, though recent studies using human fetal testis explants suggest weeks 8–10 as those most sensitive in this regard to the analgesic ibuprofen (Ben Maamar et al., 2017). Our understanding of the etiology of these conditions derives largely from animal experimentation as well as from sporadic genetic mutations in the human population. Whilst there is an obvious role here for fetal testicular androgens, we still know relatively little about how these and other factors influence early stages of the relevant organogenesis. Recently and largely based on animal experimentation, there is concern that maternal exposure to certain endocrine disrupting chemicals in the environment may be exacerbating the incidence of such disorders (Diamanti-Kandarakis et al., 2010; Juul et al., 2014), though a recent epidemiological meta-analysis failed to identify such a relationship (Bonde et al., 2017).

Unfortunately, the highlighted critical period of early pregnancy is also a time in the human which is analytically relatively inaccessible, with assessment of pollutants and/or end-point physiology relying heavily on indirect measurement, e.g., maternal urine, breast milk, cord blood hormones, perinatal, juvenile, or even adult phenotype. Amniocentesis is a procedure whereby a sample of amniotic fluid and contained fetal cells is removed by abdominal needle puncture. It is routinely offered to older women, or where a genetic risk is suspected, during weeks 12–20 of pregnancy for fetal genetic analysis. The resulting amniotic fluid supernatants thus represent a unique opportunity to explore human fetal metabolism at an otherwise relatively inaccessible period of pregnancy. In the present study we have taken advantage of the largest known biobank of preserved amniotic fluid samples to assess known markers of fetal testis physiology within the sensitive window of development and compare these in a case–control format, together with known sentinel ED substances, to determine how these might be related at this critical period during human gestation.

Of the biomarkers assessed, only INSL3 is absolutely specific for the steroid-producing Leydig cells of the fetal testis (Ivell and Anand-Ivell, 2009). Because of this specificity of expression at a time when many organ systems are actively developing, INSL3 has been considered an important biomarker for more general fetal organogenesis at this time (Anand-Ivell and Ivell, 2014). Amongst the steroids, both androstenedione and testosterone are relatively specific for the fetal testis, though are also produced by the fetal adrenal gland which is very active at this time in pregnancy. Of the other steroids measured, these are largely products of the fetal adrenal gland (Ivell et al., 2017). INSL3 is responsible for the first transabdominal phase of testicular descent and is thus directly implicated in cryptorchidism. Activated androgen receptors are involved both in cryptorchidism (early and late stages) as well as in hypospadias and associated malformations. In this context it is important to note that whilst fetal testosterone is a very important androgen, androstenedione also acts as a weak androgen (Chen et al., 2004), and can additionally activate estrogen receptors alpha and beta (Miller et al., 2013), which may also be involved in gonadogenesis (Cederroth et al., 2007). However, testosterone itself is less important than its metabolite DHT, which is produced locally in target tissues. Recently, it has been shown also for the human fetus, that significant amounts of DHT can be generated in the fetal testis by the so-called 'back-door' pathway, which avoids testosterone (Fukami et al., 2013; **Figure 5**). Thus whilst the biomarkers selected in this study are likely to reflect well aspects of testicular (in particular Leydig cell) physiology, they may not be fully informative about factors responsible for the etiology of cryptorchidism and hypospadias.

Reflecting its high cell-type and fetal specificity, amniotic INSL3 concentration is most informative as a biomarker of Leydig cell function. However, a factor of concern is the high number of samples with low or undetectable INSL3 concentration. Similar observations were made previously (Anand-Ivell et al., 2008). There is no evidence for any loss of INSL3 during storage, or repeated freeze-thaw cycles, and the TRFIA assay itself is very robust and reliable. The INSL3 concentration may be reflecting the dynamic nature of amniotic fluid at this time in early pregnancy when its volume relationship to fetal serum is changing dramatically, and its source is probably also changing (Anand-Ivell and Ivell, 2014). Studies in the cow, pig, and rat suggest that amniotic fluid samples in mid-late gestation represent between 1-3rd and 1-10th of the INSL3 concentration in fetal serum at that time (Anand-Ivell et al., 2011; Anand-Ivell and Ivell, 2014; Vernunft et al., 2016). Thus while maximal INSL3 levels, as are evident between weeks 13 and 16, will depend on Leydig cell numbers and differentiation status, the subsequent decline in INSL3 concentration may be less due to a fall in production as to the changing fluid dynamics of the amniotic compartment. It should also be noted that amniotic fluid volume itself changes markedly across gestation from approximately 10 ml at gestational weeks 8–9, to about 100 ml at week 12, increasing to nearly 400 ml by week 18 (Brace and Wolf, 1989; Falcon et al., 2005; Rolo et al., 2010; Sandlin et al., 2014). More importantly, the individual variation in amniotic fluid volume at any one time point is also large (up to 10-fold

between the 25th and 75th percentile; Sandlin et al., 2014). Thus a change in amniotic hormone concentration can be as much due to a specific change in hormone production as to a change in amniotic fluid volume. It should be noted that significantly reduced amniotic fluid volume (oligohydramnios) is associated with utero-placental insufficiency and fetuses born SGA (Morris et al., 2014). Unfortunately, no information regarding amniotic fluid volume was available in the present retrospective study. In a previous study (Anand-Ivell et al., 2008) ultrasound estimates of amniotic fluid volume did not appear to vary in normal pregnancies.

Comparing the cryptorchid and hypospadias cases with controls, particularly within the physiologically informative weeks 13–16, indicates firstly, a small mean increase of INSL3 concentration in both cryptorchid and hypospadias cases. This had not been noted in the earlier analysis which made use of a logistic regression analysis using tertiles from all gestational ages (Jensen et al., 2015). Secondly, there appears to be a significantly increased variance within these populations in cases compared to controls, particularly noticeable in non-smokers. Thirdly, there is an altered distribution, with the higher INSL3 values amongst the cases occurring at an earlier gestational time-point. Interestingly, looking at the later time-points in gestation (weeks 17–21) suggests (though not significantly) that later in gestation INSL3 concentration becomes reduced in cases versus controls, thus lending support to the recent studies indicating a reduced INSL3 concentration in the cord blood of infants with cryptorchidism (Bay et al., 2007; Fenichel et al., 2015). Taken together, these data imply an altered Leydig cell functionality in the cases compared to controls, these cells possibly responding earlier and more vigorously to the conditions which eventually lead to the pathologies. There is no evidence for a reduced Leydig cell function in these cases compared to controls. All subjects can be considered as representative of the general population; this differs therefore from the more extreme pharmacological situations in humans or rodents, for example, where high diethylstilbestrol or phthalate concentrations can lead to a high incidence of cryptorchidism with accompanying INSL3 depletion (Emmen et al., 2000; McKinnell et al., 2005; Palmer et al., 2009). A similar significant increase in INSL3 expression was also noted in the previous, smaller study of United States amniotic fluid samples (Anand-Ivell et al., 2008) in relation to preeclampsia. Moreover, a study of INSL3 in rats during puberty suggested that one of the effects of maternal treatment with dibutyl phthalate or diethylstilbestrol was to cause a precocious shift in the dynamics of INSL3 expression, with the pubertal increase in hormone expression being advanced upon maternal ED treatment (Ivell et al., 2013). This appeared to be due to altered relative rates of Leydig cell proliferation and differentiation.

There is no evidence for an influence of phthalate metabolites or PFOS within the normal ranges of human exposure on the incidence of cryptorchidism or hypospadias, even when analysis is restricted to the sensitive window between weeks 13 and 16.

When looking at data from all available weeks of gestation, both testosterone and progesterone also appear to be elevated compared to controls in hypospadias samples both as mean, but also in their variance (Levene's test). Similarly, androstenedione and progesterone are both slightly elevated in cryptorchid cases. This effect is not apparent when assessing steroids only within weeks 13–16 of pregnancy; here only testosterone is significantly elevated, and then only in the hypospadias cases compared to controls. This is an important finding, since it would tend to refute the alternative explanation that any increases in the concentration of amniotic hormones in cases compared to controls might be due to a significantly reduced amniotic fluid volume. These findings reinforce the notion that Leydig cell function may indeed be physiologically different in cases compared to controls, possibly over-responding to some external 'stress' factors. The increased variance is particularly interesting since it implies that in this large population there are an increased number of discrepant testes, some of which may be under-producing, many of which may be over-producing, compared to controls. Altogether the results indicate a larger 'at risk' population, more likely to be impacted by other confounding factors, such as maternal smoking. The fact that INSL3 and androstenedione (both Leydig cell biomarkers) are closely correlated in the weeks 13–16 samples (cases and controls combined) and that also androstenedione shows an increased variance (Levene's test), if not mean, in the hypospadias cases reinforces this notion, that there is a larger 'at risk' population amongst the cases compared to controls. None of the adrenal steroids show similar patterns of significantly altered mean or variance between cases and controls. The notion of increased variance (as opposed to or in addition to increased mean) for a parameter linked to pathology is not new. It is similar in a way to the concept of "superfertility" that is reported to be associated with at-risk pregnancies (Teklenburg et al., 2010) or in the positive fertility effects of being a "quiet" embryo (Leese et al., 2007).

Of possible extrinsic factors that might be involved in altered Leydig cell function, maternal smoking and ED load are likely to be relevant. It should be noted that of the ED substances tested, both PFOS and the DEHP metabolite 5cx-MEPP indicate a positive correlation with almost all steroids, including the androgens, measuring all samples across gestation. Interestingly, only the DiNP metabolite cx7-MMeHP, as well as the DEHP metabolite 5cxMEPP showed significant negative correlations, but only for INSL3 and androstenedione in this sample population (**Supplementary Tables S1–S5**), whereas PFOS and cotinine showed no specific interactions with INSL3. In other words, only the specific Leydig cell biomarkers INSL3 and androstenedione were negatively impacted by putative phthalate metabolites. Otherwise, these, like PFOS and cotinine, had rather a positive impact on steroidogenesis, most of which occurs in the fetal adrenal gland, suggesting an increased fetal stress response due to the increased ED load. It is to be noted that the results presented here have mostly used partial correlation analysis corrected for gestational age, whereas an earlier analysis of the same data, which showed a significant negative effect of PFOS on INSL3 concentration, had corrected for additional confounders and transformed the PFOS and hormone data prior to logistic regression analysis using tertiles (Jensen et al., 2015; Toft et al., 2016). Here, our analysis also indicated a weak negative relationship between PFOS and INSL3 for weeks

13–16 (**Supplementary Table S5**), though this failed to achieve significance.

Cotinine, as an indicator of maternal smoking was generally positively correlated with most steroids, when cases and controls were combined across gestation. Similar findings have been shown in the adult human, where smoking is linked to increased circulating testosterone, though to decreased INSL3 (Atlantis et al., 2009). In the present study there is no direct association of any kind between cotinine levels and INSL3. However, cotinine, being a relatively short-lived metabolite, is representative only of acute rather than chronic smoking and hence is less likely to correlate with differentiation-dependent parameters.

Finally, we assessed the possible impact of fetal growth on the various biomarkers, using a calculated estimate of SGA. Altogether, 7.2% of all pregnancies could be identified as SGA. Amongst these SGA pregnancies, and as suggested by others (Main et al., 2006), there appeared to be an increased risk of hypospadias and cryptorchidism (together 9% of all cases compared to 5% of control pregnancies), although none of the measured biomarkers, including INSL3, showed any association (negative or positive) with SGA. This thus appears to differ from an earlier, smaller study of INSL3 in amniotic fluid, where INSL3 values were shown to be significantly associated with age-corrected birth weight (Anand-Ivell et al., 2008).

Taken together, the results from this first large case–control study of amniotic fluid biomarkers, collected during the sensitive gestational window for testicular organogenesis, shows that particularly Leydig cell biomarkers appear to be increased, rather than decreased as might have been expected, in cryptorchidism and hypospadias. Significantly also, the variance about these biomarkers is also increased, strongly suggesting that at a population level Leydig cell functionality is more likely to be impaired or at risk to other confounding factors in the cases compared to controls. Moreover, when attempting to identify possible factors with a negative impact on Leydig cell functionality, the phthalate metabolites 5cx-MEPP or 7cx-MMeHP indicated the requisite negative correlation with INSL3 and/or androstenedione, without, however, implicating these compounds directly in the etiology of cryptorchidism or hypospadias. This agrees with a recent quantitative study on the impact of INSL3 and testosterone reduction on phenotype in rats, wherein it is suggested that >40% reduction in either hormone (or its mRNA) is required before there is evidence of any phenotypic impact (Gray et al., 2015). Recently, several studies have shown that INSL3 measured in cord blood, i.e., substantially later than its expression maximum in the fetus, is significantly reduced in cryptorchid cases compared to controls (Bay et al., 2007; Fenichel et al., 2015). Taken together, these data support the notion that cryptorchidism and/or hypospadias appear to be associated with a shift in the dynamics of INSL3 expression in the fetus, with precocious hormone expression and presumably Leydig cell differentiation in cases compared to controls. Thus INSL3, possibly as a result of exposure to EDs, such as phthalates, would increase earlier and decline sooner, and thus be out of synchrony with other ongoing morphological processes, thereby encouraging cryptorchidism and/or hypospadias.

AUTHOR CONTRIBUTIONS

RA-I, RI, BN-P, ML, and J-PB conceptualized and funded this study. BN-P, DH, and AC sorted and retrieved amniotic fluid samples from the biobank and performed the steroid assays. BJ and CL carried out the assays for environmental contaminants. RA-I and RI were responsible for the conception and writing of this article, for the measurement of INSL3, and for the statistical analysis presented here. CL, GT, and J-PB performed data management and reported to the major financial contributors. All authors critically revised previous versions of this manuscript, and approved the final version for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00406/full#supplementary-material

FIGURE S1 | Regression analyses across gestational age for amniotic fluid levels of **(A)** DHEAS, **(B)** cortisol, and **(C)** 17OH-progesterone. All other steroids measured showed no significant age-related change. **(C–E)** Represent regression analyses for PFOS, and for the phthalate metabolites 5cx-MEPP and 7cx-MMeHP, respectively. The outlined boxes indicate those samples lying within the testicular window of sensitivity, weeks 13–16.

FIGURE S2 | Mean steroid and environmental xenobiotic concentrations in amniotic fluid for weeks 13–16, separated into normal controls, combined cryptorchid and hypospadias cases. $^{*}p < 0.05$.

TABLE S1 | Cases and controls combined (weeks 11–22; corrected for gestational week).

TABLE S2 | Controls only (weeks 11–22; corrected for gestational week).

TABLE S3 | Cryptorchid only (weeks 11–22; corrected for gestational week).

TABLE S4 | Hypospadias only (weeks 11–22; corrected for gestational week).

TABLE S5 | Cases and controls combined (weeks 13–16; corrected for gestational week).

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Anti-Müllerian Hormone and Testicular Function in Prepubertal Boys With Cryptorchidism

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Introduction: The functional capacity of the testes in prepubertal boys with cryptorchidism before treatment has received very little attention. The assessment of testicular function at diagnosis could be helpful in the understanding of the pathophysiology of cryptorchidism and in the evaluation of the effect of treatment. Anti-Müllerian hormone is a well-accepted Sertoli cell biomarker to evaluate testicular function during childhood without the need for stimulation tests.

Objective: The aim of the study was to assess testicular function in prepubertal children with cryptorchidism before orchiopexy, by determining serum anti-Müllerian hormone (AMH). We also evaluated serum gonadotropins and testosterone and looked for associations between testicular function and the clinical characteristics of cryptorchidism.

Materials and methods: We performed a retrospective, cross-sectional, analytical study at a tertiary pediatric public hospital. All clinical charts of patients admitted at the outpatient clinic, and recorded in our database with the diagnosis of cryptorchidism, were eligible. The main outcome measure of the study was the serum concentration of AMH. Secondary outcome measures were serum LH, FSH, and testosterone. For comparison, serum hormone levels from a normal population of 179 apparently normal prepubertal boys were used.

Results: Out of 1,557 patients eligible in our database, 186 with bilateral and 124 with unilateral cryptorchidism were selected using a randomization software. Median AMH standard deviation score was below 0 in both the bilaterally and the unilaterally cryptorchid groups, indicating that testicular function was overall decreased in patients with cryptorchidism. Serum AMH was significantly lower in boys with bilateral cryptorchidism as compared with controls and unilaterally cryptorchid patients between 6 months and 1.9 years and between 2 and 8.9 years of age. Serum AMH below the normal range reflected testicular dysfunction in 9.5–36.5% of patients according to the age group in bilaterally cryptorchid boys and 6.3–16.7% in unilaterally cryptorchid boys. FSH was elevated in 8.1% and LH in 9.1% of boys with bilateral cryptorchidism, most of whom were anorchid. In patients with present testes, gonadotropins were only mildly elevated in less than 5% of the cases. Basal testosterone was mildly decreased in patients younger than 6 months old, and uninformative during childhood.

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Conclusion: Prepubertal boys with cryptorchidism, especially those with bilaterally undescended gonads, have decreased AMH production. Although serum AMH may fall within the normal range, there is a considerable prevalence of testicular dysfunction during childhood in this frequent condition.

Keywords: anti-Müllerian hormone, gonadotropins, hypogonadism, Sertoli cells, testosterone, undescended testes

INTRODUCTION

Cryptorchidism is one of the most frequent congenital malformations in the male, with a prevalence at birth ranging from 1.6 to 5.7% (1–4), and up to 9% in Denmark (5). In patients with a history of cryptorchidism, the risk for infertility (6–8) and testicular cancer (7, 9) is highly increased.

Cryptorchidism is the consequence of the lack or insufficiency of the process of testicular descent taking place during fetal life. The testes are initially formed near the kidneys and descend following a two-phase process (10); in the first phase, between the 8th and 15th fetal weeks, the testes are anchored to the internal inguinal ring by the gubernaculum. A Leydig cell factor named insulin-like 3 (INSL3) and its receptor RXFP2 are major regulators of gubernacular development (11). In addition, the androgen-regulated regression of the testicular cranial suspensory ligament also seems to play a role (12). Anti-Müllerian hormone (AMH) has also been suggested to participate, although this remains controversial (13). In the second phase, the testes migrate from the internal inguinal ring to the scrotum, mainly driven by the effect of androgens and intra-abdominal pressure (14). This phase is usually completed in humans by the time of birth; therefore, prematurity is associated with a higher incidence of cryptorchidism (5). Spontaneous descent may still occur in the first months of postnatal life (4, 5), and re-ascent can occur later in life probably associated with the development of the cremasteric reflex and the existence of a remnant of the processus vaginalis preventing normal elongation of the spermatic cord, leading to the concept of acquired cryptorchidism (4, 15).

While a large bibliography exists on the controversies regarding the most adequate treatment for cryptorchidism and its timeliness (16, 17), it is surprising that less attention has been given to the functional capacity of the prepubertal testes before treatment. It is clear that cryptorchidism may be a sign of several conditions with different underlying pathogeneses (7). In the vast majority of the cases, the sign is treated—i.e., the abnormal position is repaired—without knowing the degree of affectation of the gonadal axis. Assessing testicular function at the time of diagnosis may help in the understanding of the pathophysiology of cryptorchidism in each patient and in the ensuing evaluation of the effect of treatment.

Because the hypothalamic–pituitary–gonadal axis undergoes a relative quiescence after the age of 6 months and during childhood (18, 19), classical markers like testosterone and gonadotropins are of little use in the assessment of testicular function in the prepubertal boy. Conversely, Sertoli cells maintain an active secretory activity and serum levels of Sertoli cell biomarkers like AMH (20–22) and inhibin B (21, 23, 24), can readily inform about testicular function without the need for stimulation tests. Indeed, undetectable serum AMH is clearly more robust than testosterone post-hCG to diagnose or rule out anorchidism in boys with non-palpable gonads (25). AMH is secreted exclusively by the Sertoli cells of the testis in males, from early fetal life (26) where it is involved in male sex differentiation by inducing the regression of the Müllerian ducts, i.e., the anlagen of the uterus and Fallopian tubes. Although this process is completed in the first trimester of the fetal period, Sertoli cells continue to secrete high amounts during infancy and childhood. Testicular AMH production varies with age; therefore, the normal reference ranges of serum AMH levels change during postnatal development (27–29).

The aim of this study was to assess testicular function in prepubertal children with cryptorchidism before orchiopexy, by determining the serum concentration of AMH. Secondarily, we evaluated the serum concentrations of gonadotropins and testosterone and looked for associations between testicular function and the clinical characteristics of cryptorchidism.

SUBJECTS AND METHODS

Study Design and Setting

We performed a retrospective, cross-sectional, analytical study at the Division of Endocrinology of the Ricardo Gutiérrez Children's Hospital, a tertiary pediatric public hospital in Buenos Aires, Argentina.

The same pediatric endocrinologist performed a careful review of clinical charts. Clinical description of cryptorchidism as unilateral and bilateral, testicular volume measured by comparison to Prader's orchidometer, position of the gonads, pubic hair, and genital development according to Marshall and Tanner (30), and of the presence of hernia or micropenis were extracted from the history chart. Patients' personal history data, including gestational age and birth weight, history of hCG treatments, and orchiopexy were registered. Hormonal values were extracted from the history chart and the laboratory Cobas® Infinity system (Roche).

Patients

Patients With Cryptorchidism

All clinical charts of subjects admitted at the outpatient clinic of the Division of Endocrinology of the Ricardo Gutiérrez Children's Hospital between 2000 and 2017, and recorded in our database with the diagnosis of cryptorchidism, were eligible. Cryptorchidism was defined by the absence of one or both testes in the scrotum. Inclusion criteria were the presence of cryptorchidism, normal virilization (urethral orifice at the end of the penis and complete fusion of the scrotum) and the availability of

a result of serum AMH determination performed before orchiopexy at a prepubertal stage, defined by testicular volume ≤ 3 ml as compared with Prader's orchidometer and according to Tanner stages. The following exclusion criteria applied: history chart absent or incomplete, a diagnosis of disorders of sex development or genetic syndromes known to affect testicular function, genital, or abdominal–pelvic surgeries susceptible of affecting the gonadal vessels performed before the first hormonal evaluation, radiotherapy, or chemotherapy.

Healthy Controls

For comparison, we used serum levels of AMH, testosterone, FSH, and LH from a sample of 179 apparently normal prepubertal boys, which have been published previously (29). This cohort fulfilled the following criteria: (i) written informed consent was given by the participant's parents, (ii) a blood sample was being drawn for routine clinical evaluation independently of the research study, (iii) anamnesis ruled out cryptorchidism or other genital or urologic malformations, endocrine diseases, and chronic or acute general pathologies that could affect endocrine function, and (iv) a clinical examination was performed to assess Tanner pubertal stage and to determine testicular position and volume by comparison with Prader's orchidometer.

Outcome Measures and Definitions

The main outcome measure of the study was the serum concentration of AMH. Secondary outcome measures were serum concentrations of LH, FSH, and testosterone. Circulating levels of reproductive hormones were compared between patients with cryptorchidism and normal boys. Serum AMH and FSH were, respectively, used as a direct and an indirect biomarker of the functional mass of prepubertal Sertoli cells (21), whereas serum testosterone and LH were, respectively, used as a direct and an indirect biomarker of Leydig cells. All data were obtained at first referral, before any hormonal or surgical treatment was attempted. In a small subset of patients, a second assessment was available after orchiopexy in these cases, a longitudinal comparison (at referral vs after surgery) was made.

For the primary analysis, patients with cryptorchidism and controls were grouped by age intervals (all prepubertal). Subsequent stratification for subgroup analysis was done according to the clinical characteristics of the cryptorchidism, such as palpability and position of the testes. According to the AMH values, patients were classified as functionally anorchid when serum AMH was non-detectable, hypogonadal when serum AMH was detectable but below the normal reference level (below the 3rd percentile for age), and eugonadal when serum AMH was within the normal reference level.

Other potentially associated variables considered in this study were as follows: gestational age at birth, birth weight, penile size, presence of inguinal hernia, and hCG treatment for cryptorchidism. Gestational age was a dichotomic variable (preterm/full-term), considering preterm birth when gestational age was <37 weeks. Birth weight was analyzed as a continuous variable. Weight for gestational age was a dichotomic variable (small for gestational age/adequate for gestational age), considering small for gestational age when weight was <3rd percentile for gestational age according to local references (31). Penile size was dichotomized (micropenis/normal), and micropenis was defined by penile size < −2 SD according to the Argentine references for age (32). Presence of inguinal hernia was dichotomized (yes/no), according to the attending physician's description in the clinical chart. Treatment with hCG followed a standardized protocol used at the Division of Endocrinology, consisting of 1,000 IU of hCG administered IM once weekly for 5 weeks, and assessment within 1 month following the last injection. Treatment was considered successful if the gonad was in the scrotum at physical examination, and unsuccessful if it was not in scrotal position.

Study Size

The sample size was calculated for the main outcome measure, i.e., the prevalence of hypogonadism (patients with serum AMH below the normal reference range) in prepubertal patients with cryptorchidism. The estimated study size required 181 patients with bilateral cryptorchidism and 116 patients with unilateral cryptorchidism, to detect a prevalence of hypogonadism of 36 and 18%, respectively, based on previously unpublished own studies, with an accuracy of 7% and a confidence level of 95%.

Hormone Assays

Anti-Müllerian Hormone

Results of serum AMH determinations were all obtained with an enzyme-linked immunoassay specific for human AMH (EIA AMH/MIS®, Beckman-Coulter Co., Marseilles, France), as previously validated by our group (29, 33). Intra- and inter-assay coefficients of variation were, respectively, 10.5 and 9.4%, for a serum AMH concentration of 700 pmol/L, and 11.1 and 12.8% for a serum AMH concentration of 7 pmol/L. When serum AMH levels were undetectable, the value of the limit of quantification (functional sensitivity = 2.5 pmol/L) was attributed.

Gonadotropins

LH and FSH were determined using electrochemiluminescent immunoassays (ECLIAs, Roche Diagnostics GmbH, Mannheim, Germany) as described (33). The limits of quantification of both LH and FSH assays were 0.10 IU/L, according to the 2nd NIBSC IS 80/552 for LH and the 2nd WHO IRP 78/549 for FSH. Intra- and inter-assay coefficients of variation were 1.1 and 1.8% for LH, respectively, for a mean LH concentration of 2.8 IU/L and 1.4 and 1.5% for a mean LH concentration of 16.9 IU/L. Intra- and inter-assay coefficients of variation were 1.0 and 4.2% for FSH, respectively, for a mean FSH concentration of 14.8 IU/L and 1.1 and 4.1% for a mean FSH concentration of 23.4 IU/L. When serum LH or FSH levels were undetectable, the value of the limit of quantification (functional sensitivity) was attributed.

Testosterone

Testosterone was determined in serum using an ECLIA (Roche Diagnostics GmbH, Mannheim, Germany) as described (29). Intra- and inter-assay coefficients of variation were 2.4 and 2.6%, respectively, for a mean testosterone concentration of 176 ng/dL (6.10 nmol/L) and 1.2 and 2.3% for a mean testosterone concentration of 455 ng/dL (15.78 nmol/L). When serum testosterone

levels were undetectable, the value of the limit of quantification (functional sensitivity = 10 ng/dL) was attributed.

Statistical Analyses

Because serum AMH varies with age in normal boys during infancy and childhood, values were analyzed using the standard deviation score (SDS) for age in the overall evaluation. In the age subgroup analyses, absolute serum levels and percentiles were used. Data distribution was assessed for normality using the Shapiro–Wilk test. Results are expressed as median and range. Because non-Gaussian distribution was found in most cases, non-parametric tests were used for comparisons. The Wilcoxon Signed Rank Test was used to compare median SDS with the theoretical value of 0 SDS. Mann–Whitney test was used to compare serum hormone levels between two independent groups. Kruskal–Wallis test with Dunn's multiple comparison posttest was used when more than two groups were compared. Fisher's exact test was used to compare categorical variables. Logistic regression was performed to analyze potential risk factors associated with decreased AMH, considered as a categorical variable (serum AMH below the 3rd percentile for age). The level of significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 13 (StataCorp LLC, College Station, TX, USA).

RESULTS

Characteristics of the Study Population

Our database contained 1,557 patients with "cryptorchidism" as a diagnosis (524 bilateral and 1,033 unilateral cryptorchidism). Filtering the database for lack of clinical chart number, history of genital or abdominal surgery and for other exclusion diagnoses (see Subjects and Methods), a total of 178 cases were excluded, leaving 393 cases of bilateral and 986 cases of unilateral cryptorchidism as eligible for our study. Using a randomization software, we selected and reviewed 390 clinical charts (240 with bilateral and 150 with unilateral cryptorchidism). Eighty (54 with bilateral and 26 with unilateral cryptorchidism) were discarded because the history chart was incomplete, the diagnosis was not cryptorchidism (database recording error), there was a history of genital or abdominal surgery or of chemotherapy or radiotherapy reported in the chart, a result of a serum AMH measurement was not available in the prepubertal period, or other exclusion diagnoses were found in the chart. Finally, 310 patients with cryptorchidism, 186 with bilateral cryptorchidism, and 124 with unilateral (50% right cryptorchidism) were analyzed in the study (**Figure 1**).

Median age at first hormonal evaluation was approximately 3 years, with a wide range (**Table 1**). The proportion of patients born preterm, with micropenis or hernia, or having received hCG treatment for cryptorchidism varied according to the age groups. Only two preterm patients were born at <28 weeks.

Anti-Müllerian Hormone

Median AMH SDS was below 0 in both the bilaterally (Wilcoxon signed rank test, $P < 0.0001$) and the unilaterally ($P = 0.0052$) cryptorchid groups (**Figure 2**), indicating that testicular function is overall decreased in patients with cryptorchidism. Testicular function was more affected in the bilaterally than in the unilaterally cryptorchid group (Mann–Whitney test, $P < 0.0001$). In the bilaterally cryptorchid boys, serum AMH was below 0 SDS in 80.1% of the cases; furthermore, it was below −1 SDS in 39.8% of the cases. In the unilateral cryptorchidism group, the impairment was milder, with only 62.9% of the patients with serum AMH below 0 SDS.

To assess testicular function specifically in patients with unilateral or bilateral cryptorchidism according to age, we analyzed absolute serum AMH levels and the percentile distribution. In patients with bilateral cryptorchidism, serum AMH was undetectable—thus indicative of anorchidism—in nine cases (**Figure 3A**). This represents 4.8% of all patients with bilateral cryptorchidism and 26.5% of those with non-palpable gonads. Patients with anorchidism were excluded from all the subsequent comparisons of testicular function between groups. Serum AMH concentration of boys with bilateral cryptorchidism was significantly lower than in the control and the unilaterally cryptorchid groups between 6 months and 1.9 years and between 2 and 8.9 years of age (**Table 2**).

The prevalence of serum AMH below the normal range (<3rd percentile) indicated an increased proportion of patients with hypogonadism in all age groups, even in those groups with a normal median serum AMH (**Table 2**). The prevalence of AMH below the normal range was greater in patients with bilateral cryptorchidism than in boys with unilateral cryptorchidism between 6 months and 1.9 years (Fisher's exact test, $P = 0.006$) and in boys between 2 and 8.9 years (Fisher's exact test, $P = 0.043$). In fact, AMH was below the normal range in 16.6% of the whole group of patients with cryptorchidism and present gonads, 22.6% of boys with bilateral cryptorchidism and 8.1% of boys with unilateral cryptorchidism. These results showed only minor changes when patients with central hypogonadism were excluded (Table S1 in Supplementary Material).

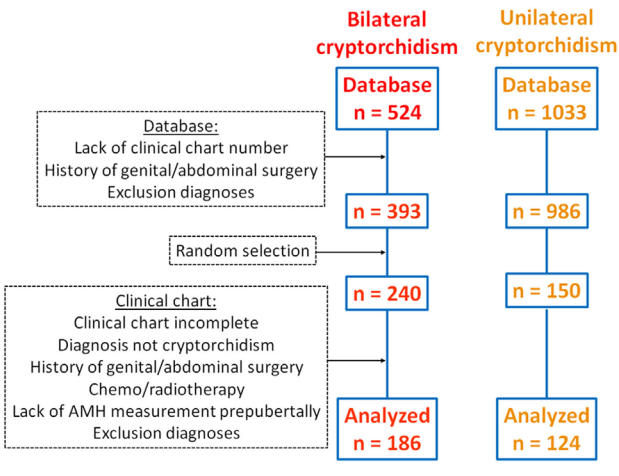

FIGURE 1 | Flowchart of patient inclusion in the study.

TABLE 1 | Clinical characteristics of patients with unilateral or bilateral cryptorchidism included in this study.

	All ages		1–5.9 months		6 months–1.9 years		2–8.9 years		≥9 years	
	Unilateral	Bilateral	Unilateral	Bilateral	Unilateral	Bilateral	Unilateral	Bilateral	Unilateral	Bilateral
n	124	186	12	9	28	54	68	100	16	23
Age, median (range)	3.06 (0.03–11.10)	3.33 (0.06–13.62)	0.39 (0.03–0.47)	0.31 (0.06–0.49)	1.26 (0.55–1.97)	1.21 (0.54–1.94)	4.01 (2.00–8.98)	4.65 (2.02–8.67)	10.18 (9.06–11.12)	10.47 (9.04–13.62)
Preterm, *n* (%)	11 (8.9)	25 (13.4)	1 (8.3)	0	2 (7.1)	8 (14.8)	5 (7.4)	13 (13.0)	3 (18.8)	4 (17.4)
Micropenis, *n* (%)	3 (2.4)	14 (7.5)	1 (8.3)	2 (22.2)	0	5 (9.3)	1 (1.5)	7 (7.0)	1 (6.3)	0
Hernia, *n* (%)	32 (25.8)	31 (16.7)	3 (2,500)	2 (22.2)	9 (32.1)	8 (14.8)	18 (26.5)	17 (17.0)	2 (12.5)	3 (13.0)
hCG treatment, *n* (%)	43 (34.7)	89 (47.9)	2 (16.7)	2 (22.2)	7 (25.0)	19 (35.2)	27 (39.7)	55 (55.0)	7 (43.8)	13 (56.5)

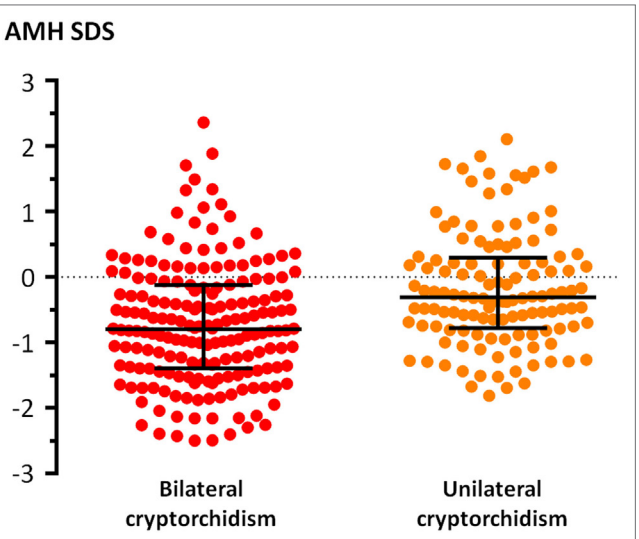

FIGURE 2 | Serum levels of anti-Müllerian hormone (AMH), expressed as standard deviation score (SDS) for age, in patients with unilateral or bilateral cryptorchidism. Bars indicate medians and interquartile ranges.

Testicular function was not worse in patients with bilaterally non-palpable gonads, as compared with patients with bilateral cryptorchidism and at least one palpable gonad (Fisher's exact test, $P = 0.45$). Median serum AMH was not significantly different in any of the age groups (**Table 3**).

To identify risk factors for hypogonadism (AMH levels < 3rd percentile) in cryptorchid boys, we performed a logistic regression (**Table 4**). The factors that were significantly associated with AMH levels < 3rd percentile were bilateral cryptorchidism, as compared with unilateral cryptorchidism, and the presence of micropenis. Sixteen out of 17 boys (94.2%) with micropenis had AMH levels below the normal range (**Figure 3A**; **Table 5**). One patient was anorchid (undetectable AMH) and five of them were diagnosed with central hypogonadism (data obtained from clinical charts reporting testosterone treatment at age ≥14 years). There were no significant differences in the prevalence of hypogonadism (AMH < 3rd percentile) between preterm and full-term patients, either in the total group or in patients with unilateral or bilateral cryptorchidism (**Table 6**). Likewise, serum AMH did not differ significantly between preterm and full-term cryptorchid patients (Mann–Whitney test, $P = 0.275$; **Figure 4A**). We hypothesized that, in patients with hernia, testicular maldescent would be the result of an anatomical hindrance rather than a testicular dysfunction. However, the prevalence of hypogonadism was not significantly lower than that observed in patients with cryptorchid gonads not associated with an inguinal hernia (**Table 6**). Also, no difference was found in serum AMH between cryptorchid patients with or without inguinal hernia (Mann–Whitney test, $P = 0.288$; **Figure 4B**).

Treatment with hCG for cryptorchidism was performed in 132 of 310 (42.6%) patients according to local standard procedures. Treatment was performed in 43 of the 124 (34.7%) patients with unilateral cryptorchidism, with a success rate of

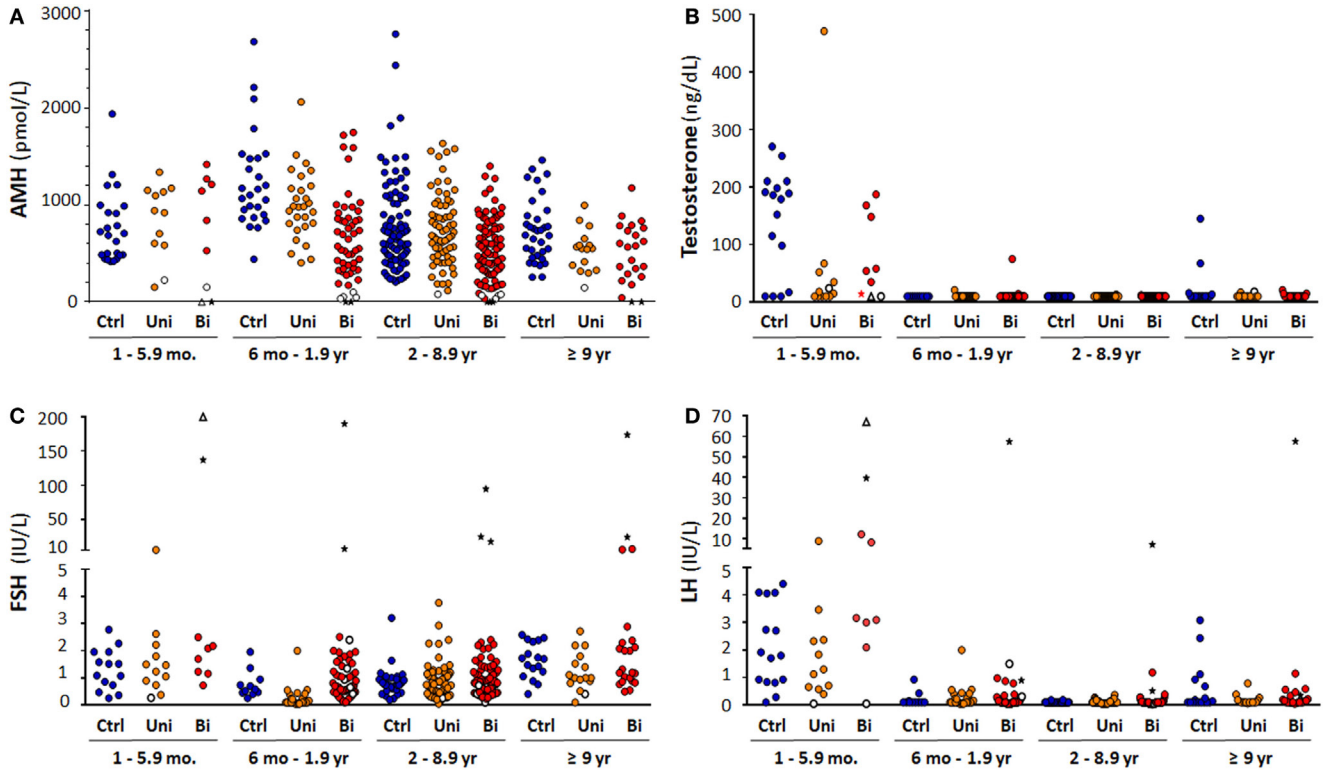

FIGURE 3 | Hormone serum levels in cryptorchid boys: **(A)** anti-Müllerian hormone (AMH), **(B)** testosterone, **(C)** FSH, and **(D)** LH. Empty circles indicate cryptorchid boys with micropenis, stars indicate anorchid boys, and triangles indicate anorchid boys with micropenis. Abbreviations: Ctrl, normal controls; Uni, unilateral cryptorchidism; BI, bilateral cryptorchidism.

20.9%. AMH levels did not differ significantly between patients in whom hCG treatment was successful or unsuccessful (Mann–Whitney test, $P = 0.581$; **Figure 5A**). In the group of bilaterally cryptorchid boys, 89 of 186 (47.9%) received hCG with a success rate of 28.1% for the descent of both testes and 15.7% for one testis. AMH levels of bilaterally cryptorchid boys who showed a successful response of both testes to hCG were higher than those of boys with no response (Kruskal–Wallis test followed by Dunn's Multiple Comparison Test, $P = 0.0001$; **Figure 5B**).

Orchiopexy was performed in 151 patients. Serum AMH levels were available in 76 patients at referral, i.e., before any treatment was attempted, and at least 1 month after surgery. A statistically significant increase was observed in AMH levels after orchiopexy (Paired T test, $P = 0.0030$, **Figure 6**).

Testosterone

In patients aged 1–5.9 months, lower testosterone levels were observed in the unilaterally cryptorchid group as compared with controls (Kruskal–Wallis test followed by Dunn's Multiple Comparison Test, $P = 0.029$; **Figure 3B**). During the rest of childhood, testosterone levels are usually very low or undetectable in normal boys. Accordingly, serum testosterone was below the limit of detection of the assay (10 ng/dL) in 265 of 276 cryptorchid patients aged >6 months; in the remaining 11 patients, serum testosterone ranged between 11 and 75 ng/dL. Therefore, no statistical comparisons were made between groups.

Gonadotropins

Serum gonadotropin levels were within the normal range in the vast majority of patients with unilateral or bilateral cryptorchidism independent of age (**Figures 3C,D**). Only 4 of 124 (3.2%) boys with unilateral cryptorchidism had elevated FSH (>97th percentile for age), between 2.7 and 6.2 IU/L. In boys with bilateral cryptorchidism, FSH was elevated in 15 of 186 (8.1%) cases. Eight of them proved to be anorchid, with FSH levels ranging from 7 to 200 IU/L. In the remaining seven cases, FSH was mildly elevated, between 2 and 7.3 IU/L.

Serum LH was elevated (>97th percentile for age), between 0.2 and 8.9 IU/L, in 9 of 124 (7.3%) boys with unilateral cryptorchidism, and in 17 of 186 (9.1%) boys with bilateral cryptorchidism (**Figure 3**). Seven of the latter were anorchid, with LH levels ranging from 0.25 to 67.1 IU/L. In the remaining 10 cases, LH was mildly elevated, between 0.2 and 12.2 IU/L.

When the subgroup of patients with abnormally low AMH (<3rd percentile for age) was analyzed, 13 of 59 (22.0%) had elevated FSH for age, 8 of whom were anorchid. In the 51 cryptorchid patients with bilaterally present gonads, elevated FSH was

TABLE 2 | Serum hormone levels in normal controls and in patients with unilateral or bilateral cryptorchidism.

	1–5.9 months			6 months–1.9 years			2–8.9 years			≥9 years		
	Controls	Unilateral	Bilateral	Controls	Unilateral	Bilateral	Controls	Unilateral	Bilateral	Controls	Unilateral	Bilateral
AMH[a]												
n	24	12	7	26	28	52	95	68	97	34	16	21
Median (pmol/L)	697	931	1,145	1,132	981	563	684	682	525	713	551	580
Range (pmol/L)	418–1,939	153–1,337	153–1,417	441–2,682	405–2,061	34–1,747	207–2,761	80–1,635	31–1,400	256–1,462	147–966	43–1,175
<3rd percentile, *n* (%)		2 (16.7)	1 (14.3)		2 (7.1)	19 (36.5)		5 (7.2)	18 (18.6)		1 (6.3)	2 (9.5)
Kruskal–Wallis + Dunn[b]	a	a	a	a	a	b	a	a	b	a	a	a
FSH												
n	14	12	7	11	28	52	33	66	90	17	16	21
Median (IU/L)	1.30	1.36	1.70	0.59	0.10	0.71	0.75	0.84	0.83	1.70	1.0	1.20
Range (IU/L)	0.26–2.78	0.27–6.16	0.73–2.5	0.26–1.96	0.05–2.00	0.10–2.51	0.20–3.21	0.05–3.77	0.10–2.40	0.41–2.59	0.10–2.72	0.50–7.33
>97th percentile, *n* (%)		1 (8.3)	0		1 (3.6)	3 (5.8)		1 (1.5)	0		1 (6.3)	4 (19.1)
Kruskal–Wallis + Dunn[b]	a	a	a	a	a	a	a	a	a	a	a	a
LH												
n	15	12	7	11	28	52	34	67	90	17	16	21
Median (IU/L)	1.8	1.215	3.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Range (IU/L)	0.10–4.41	0.10–8.90	0.10–12.20	0.10–0.93	0.10–2.00	0.10–1.50	0.10–0.19	0.10–0.37	0.10–1.19	0.10–3.09	0.10–0.79	0.10–1.15
>97th percentile, *n* (%)		1 (8.3)	2 (22.2)		1 (3.6)	3 (5.8)		7 (10.6)	6 (6.7)		0	0
Kruskal–Wallis + Dunn[b]	a	a	a	a	a	a	a	a	a	a	a	a

[a]*To obtain serum anti-Müllerian hormone (AMH) in ng/mL, divide by 7.14.*

[b]*Kruskal–Wallis test followed by Dunn's multiple comparison test (Controls vs Unilateral vs Bilateral). In each age subgroup, a different letter indicates that there is a significant difference when comparing Controls vs Unilateral vs Bilateral ($P < 0.05$).*

observed in only 5 cases (9.8%). Serum LH was elevated in 9 of the 59 patients (15.3%), and in only 2 (3.9%) of the boys with present testes.

DISCUSSION

Controversial results on whether testicular AMH production is decreased in prepubertal boys with cryptorchidism have been reported in a few studies with small number of patients of a large range of ages and including cases of both unilateral and bilateral cryptorchidism (25, 34–38) (**Table 7**). Our study including 310 prepubertal patients randomly selected from a cohort of more than 1,500 boys with cryptorchidism shows that, as a group, cryptorchid patients have lower AMH production than normal boys. Even though most of cryptorchid patients have serum AMH levels within the reference range, there is a considerable prevalence of cases with abnormally decreased serum AMH, indicating an affected testicular function during childhood. The prevalence of testicular dysfunction did not increase with age. As could be expected, low serum AMH concentration was found more often in patients with bilateral than unilateral cryptorchidism.

With the aim of evaluating the pituitary–testicular axis in patients with cryptorchidism, serum levels of another Sertoli cell marker—inhibin B—and of Leydig cell markers—testosterone and INSL3—have also been assessed in different studies. INSL3, but not the other markers, has consistently been found low in cord blood from cryptorchid newborns (47, 48). Basal testosterone and INSL3 are no longer informative in childhood after postnatal activation wanes at 3–6 months of age, since their circulating levels are very low or undetectable. Like AMH, inhibin B also shows controversial results in boys with cryptorchidism. However, most studies indicate that there is an increased prevalence of patients with low basal inhibin B or inhibin B/FSH ratio in older infants and children with cryptorchidism (38, 44, 49–51). Altogether, these results and our present data using basal AMH as a Sertoli cell marker suggest that there is an increased risk of seminiferous tubule dysfunction already in childhood even though the gonadotropin axis is relatively quiescent. Furthermore, lower levels of inhibin B have been shown to correlate with decreased number of spermatogonia in infancy (50, 52), a predictor of infertility in males with a history cryptorchidism (53–56).

In our large cohort of cryptorchid boys, the median SDS for serum AMH was below 0 both in the unilaterally and bilaterally cryptorchid groups, which indicates that the testicular Sertoli cell compartment is overall affected. Serum AMH levels found in patients with bilateral cryptorchidism were clustered in the lowest ranges: indeed, 39.8% of the values were below 1 SDS, as compared with 15.9% expected according to a Gaussian distribution. Furthermore, more than one-fifth of patients with bilateral cryptorchidism had overtly abnormal serum AMH, i.e., levels below the 3rd percentile for age, thus validating the prevalence of childhood hypogonadism previously reported in a smaller series of cryptorchid boys (38). Other studies report mean serum AMH levels but do not discriminate the percentage of patients with abnormally low AMH production (34, 35,

TABLE 3 | Serum anti-Müllerian hormone (AMH) in bilaterally cryptorchid boys with non-palpable gonads and boys with at least one palpable gonad.

	1–5.9 months		6 months–1.9 years		2–8.9 years		≥9 years	
	Non-palpable gonads	At least one palpable gonad	Non-palpable gonads	At least one palpable gonad	Non-palpable gonads	At least one palpable gonad	Non-palpable gonads	At least one palpable gonad
n	1	6	9	43	12	85	3	18
Median (pmol/L)	1,269	993	530	574	498	535	260	593
Range (pmol/L)	N.A.	153–1,417	277–1,003	34–1,747	35–938	31–1,400	43–835	178–1,175
Mann–Whitney test	N.A.		a	a	a	a	a	a

N.A., not applicable.
To obtain serum AMH in ng/mL, divide by 7.14.
Mann–Whitney test ("Non-palpable gonads" vs "At least one palpable gonad"): same letter indicates lack of significant difference (P > 0.05).

TABLE 4 | Logistic regression performed to identify potential risk factors for hypogonadism (AMH levels < 3rd percentile) in boys with cryptorchidism (unilateral and bilateral considered together).

	Odds ratio	95% CI	*P*
Bilateral cryptorchidism	3.63	1.52–8.66	0.004
Micropenis	91.70	10.96–767.05	<0.001
Hernia	1.41	0.60–3.3327	0.430
Preterm	0.81	0.27–2.41	0.705
Birth weight	0.99	0.99–1.00	0.389
SGA	0.54	0.15–1.91	0.339
Age at evaluation	1.02	0.91–1.14	0.748

CI, confidence interval; AMH, anti-Müllerian hormone; SGA, small for gestational age.

37, 44). When analyzed by age groups, we found a prevalence of hypogonadism—as indicated by AMH < 3rd percentile—in 36.5% of the patients between 6 months and 2 years, an age at which most cryptorchid patients are referred to the pediatric endocrinologist. In our series, recruited between 2000 and 2017, we have noticed a high frequency of relatively late referral, with a predominance after 2 years of age, which raises the concern of a potential progressive testicular damage until the time of treatment (57–59). However, the proportion of bilaterally cryptorchid patients with impaired Sertoli cell function was not higher in the 2- to 9-year-old group than in the younger group. Furthermore, no influence of age at first hormonal evaluation (always performed before treatment in our series) was found on the prevalence of impaired Sertoli cell function as determined by serum AMH < 3rd percentile, in agreement with other observational studies indicating no progressive damage associated with delayed orchiopexy (57, 60–62). The latter studies clearly identified that there are at least two conditions in patients with cryptorchidism: in one, testes are already affected at early infancy, showing absence of spermatogonia type Ad, and in the other, testes have Ad spermatogonia. After long-term follow-up until adulthood, patients who had Ad spermatogonia at biopsy during orchiopexy were fertile with normal spermiograms in contrast to those with absence of Ad spermatogonia, who showed abnormal spermiograms, regardless of the age at orchiopexy (57). Nonetheless, it should be emphasized that our study was not designed to assess the effect of early vs late treatment on testicular function, and definitive results of long-term prospective clinical trials will certainly shed light on this concern (58, 63). Another limitation of our study is linked with the relatively small number of patients of the two extreme age groups, which may result in low power to detect decreased AMH or increased prevalence of Sertoli cell dysfunction with enough statistical significance.

In the unilateral cryptorchidism group, the impairment was milder, with only 62.9% of the patients with serum AMH below 0 SDS and less than 10% of patients with AMH below the 3rd percentile. These results confirm results of a recent study comparing serum AMH between unilaterally cryptorchid patients and normal boys (40), and are in line with the observation in a large scale, long-term follow-up study, that unsuccessful paternity was 10.3% in patients with a history of unilateral cryptorchidism and 6.8% in controls (64).

With the aim of identifying risk factors associated with Sertoli cell dysfunction in patients with cryptorchidism, in addition to the previously discussed bilaterality, logistic regression analysis detected micropenis with a very high odds ratio. A limitation of our study, related to its retrospective design, is that certain recently detected genetic, maternal, and environmental risk factors (65) were not screened by the attending clinician. The occurrence of micropenis at birth is indicative of insufficient androgen exposure during the second half of fetal life (66), which can be due to fetal testicular regression syndrome resulting in congenital anorchidism. This was the case in one patient with non-palpable gonads of our cohort, in whom the finding of undetectable AMH in serum lead to early diagnosis of anorchidism. More frequently, congenital micropenis is associated with central (hypogonadotropic) hypogonadism. In these cases, the low levels of AMH are the consequence of insufficient FSH in intrauterine life resulting in decreased number of Sertoli cells and impaired AMH gene expression in each Sertoli cell (67–69).

Excluding anorchid patients, the majority of whom showed very high gonadotropin levels, only a very low proportion of prepubertal cryptorchid boys had a mild elevation of serum gonadotropins, even in the case of patients with manifest primary hypogonadism as revealed by abnormally low serum AMH levels, thus emphasizing that primary hypogonadism is rarely hypergonadotropic in prepubertal patients (33, 70, 71).

The pathogenesis of cryptorchidism may involve disorders of the hypothalamic–pituitary–gonadal axis or anatomical defects with no primary endocrine deficiency. In central or primary

TABLE 5 | Serum hormone levels in cryptorchid boys with micropenis.

	1–5.9 months	6 months–1.9 years	2–8.9 years	≥9 years
AMH[a]				
n	3	5	8	1
Median (pmol/L)	153	53	79	147
Range (pmol/L)	N.D.–226	34–321	31–525	N.A.
<3rd percentile, *n* (%)	3 (100.0)	5 (100.0)	7 (87.5)	1 (100.0)
Anorchia	1	0	0	0
Bilateral/unilateral	2/1	5/0	8/0	0/1
FSH				
n	3	5	8	1
Median (IU/L)	0.73	0.94	0.45	0.41
Range (IU/L)	0.27–200	0.42–2.4	0.10–0.74	N.A.
>97th percentile, *n* (%)	1 (33.3)	1 (20.0)	0	0
LH				
n	3	5	8	1
Median (IU/L)	0.1	0.1	0.1	0.1
Range (IU/L)	0.10–67.09	0.1–1.5	0.10–0.10	N.A.
>97th percentile, *n* (%)	1 (33.3)	1 (20.0)	0	0

N.A., not applicable.

[a]*To obtain serum AMH in ng/mL, divide by 7.14.*

TABLE 6 | Prevalence of patients with hypogonadism (anti-Müllerian hormone levels < 3rd percentile) according to prematurity and the presence of hernia in cryptorchid boys.

	Preterm	Term	*P*[a]	Hernia	No hernia	*P*[a]
All, *n* (%)	10 (27.8)	39 (15.4)	0.093	11 (17.5)	48 (19.4)	0.858
Unilateral cryptorchidism, *n* (%)	2 (18.2)	8 (7.3)	0.303	2 (6.3)	8 (8.7)	1.000
Bilateral cryptorchidism, *n* (%)	8 (32.0)	31 (22.0)	0.229	9 (28.0)	40 (26.0)	0.826

[a]*Fisher's exact test, "Preterm" vs "Term" or "Hernia" vs "No hernia."*

hypogonadism, low testosterone and/or INSL3 are responsible for the testicular maldescent. In anatomical defects, like an inguinal hernia, the obstruction of the inguinal canal may preclude the normal gonad from completing its descent to the scrotum. Therefore, we hypothesized that AMH would not be affected in cryptorchid patients with inguinal hernia. However, when comparing between patients with and patients without inguinal hernia, we did not find a significant difference in serum AMH levels, or in the prevalence of patients with evident Sertoli cell dysfunction (AMH < 3rd percentile). A possible explanation is that the abnormal testicular position *per se* would affect the Sertoli cell population, independent of the pathogenic mechanism underlying cryptorchidism.

The overtly low AMH levels detected in a subset of patients with cryptorchidism could be associated with one or more possibilities. One is that fetal testicular development is primarily defective, and low AMH behaves as a biomarker. Evidence for a primary testicular defect arises from large association studies showing that reproductive conditions observed at birth, like cryptorchidism and hypospadias, and in adults, e.g., low sperm counts and testicular cancer, are increasing in incidence concomitantly, and are signs of the so-called testicular dysgenesis syndrome (72). Furthermore, the risk of testicular cancer in patients with a history of unilateral cryptorchidism is increased in both testes, indicating that in addition to the ectopic position of the testis, there are other preexisting factors involved in the mechanism underlying the association between cryptorchidism and testicular cancer (73). Another possibility is that the abnormal position of the testes impairs Sertoli cell development. Normally, Sertoli cells proliferate during the postnatal activation of the gonadal axis (74) resulting in a mild but significant increase in testicular volume (75, 76) and serum AMH (27–29). Conversely, in cryptorchid boys the increase in Sertoli cell number (58, 77) and testicular volume (58) is impaired. The increase in serum AMH levels after orchiopexy observed in our study suggests that testicular damage might be at least partially reversible. Finally, decreased AMH has been postulated as one etiologic factor for cryptorchidism.

In fact, in addition to testosterone and INSL3, AMH has been proposed to be involved in testicular descent on the basis of observations made in patients with persistent Müllerian duct syndrome (PMDS) due to mutations in the genes coding for either AMH or its specific receptor AMHR2, although an experimental proof-of-concept is still lacking (13). Because the testes remain in ovarian position in approximately 40% of PMDS boys (78), AMH was initially thought to be involved in the first (transabdominal) phase of testicular descent as a candidate to stimulate the swelling reaction in the gubernaculum (79), until INSL3 was discovered as the factor controlling gubernaculum shortening (80). Furthermore, normal testicular descent was observed in mice with an experimental knockout of the *Amh* (81) or the *Amhr2* (82) gene and in male pups of a female rabbit with high levels of blocking anti-AMH antibodies (83). Altogether, these observations favored the hypothesis that cryptorchidism in PMDS patients is related to the anatomical attachment of

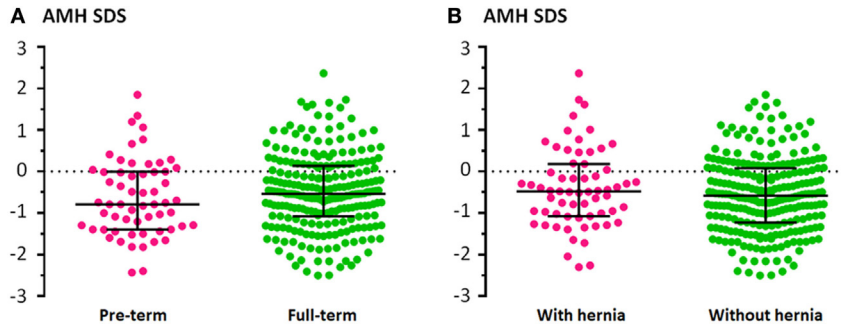

FIGURE 4 | Serum levels of anti-Müllerian hormone (AMH), expressed as standard deviation score (SDS) for age, in preterm or full-term patients with unilateral or bilateral cryptorchidism **(A)** and in unilaterally and bilaterally cryptorchid patients with or without hernia **(B)**. Bars indicate medians and interquartile ranges.

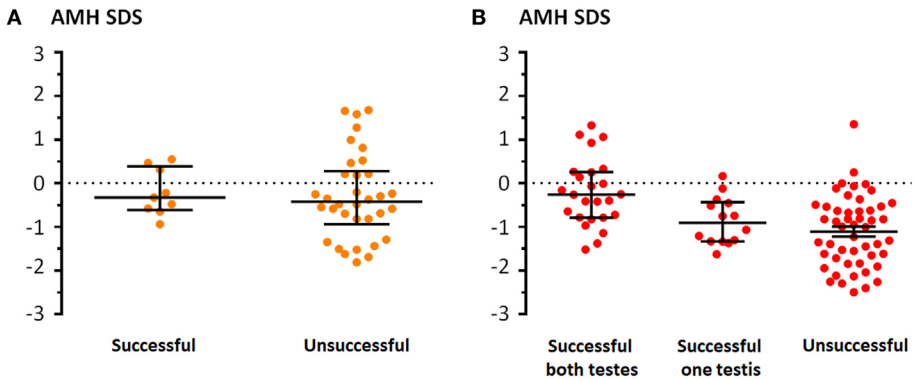

FIGURE 5 | Serum levels of anti-Müllerian hormone (AMH), expressed as standard deviation score (SDS) for age, in patients who received hCG treatment for cryptorchidism. **(A)** Unilateral cryptorchidism. **(B)** Bilateral cryptorchidism. Successful indicates that the testis was in scrotal position at physical examination in the visit following hCG treatment.

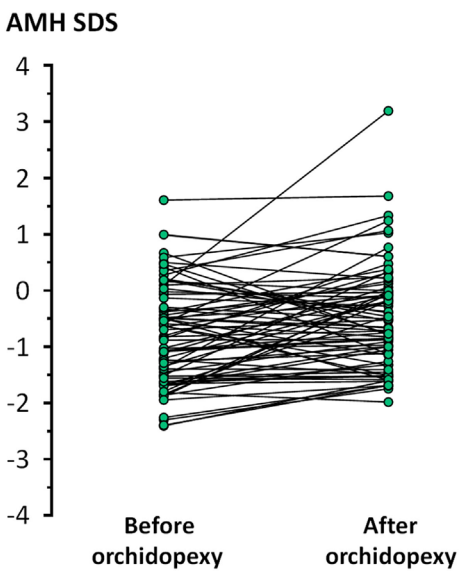

FIGURE 6 | Serum levels of anti-Müllerian hormone (AMH), expressed as standard deviation score (SDS) for age, at referral and at least 1 month after orchidopexy in patients with cryptorchidism (unilateral or bilateral).

the testes to the broad ligaments of the persistent uterus (78). However, there are anatomical differences that exist between humans and mammalian species used as experimental models (13), and two anatomical forms responsible for about 60% of PMDS cases, presenting either with unilateral cryptorchidism where either one testis in inguinal position along with its attached tube and uterus (known as *hernia uteri inguinalis*) or with both testes and Müllerian derivatives herniated on the same side (known as transverse testicular ectopia), show an abnormally long gubernaculum. These observations, consistent with a role for AMH gubernacular shrinkage, have led the hypothesis that AMH may increase INSL3 action on the gubernaculum by stimulating the shortening of the gubernacular cord (13). This hypothesis needs experimental evidence. Our observational study design was not conceived to address the potential implication of AMH in testicular descent in the largely most frequent cases of cryptorchidism, i.e., non-PMDS patients.

The choice between surgical and hormonal treatment for cryptorchidism has raised controversies in the last decade (84, 85), that go beyond the scope of this work. A meta-analysis demonstrated that the lower the original position of the testis, the better the effect of the hormonal treatment, thus suggesting that LHRH or hCG can be tried in the treatment of

TABLE 7 | Comparison between this work and previously published articles reporting on AMH serum or expression levels in boys with cryptorchidism.

Reference	Sample size	Population	Age	Unilateral/bilateral cryptorchidism	AMH serum or testicular expression levels
Present work	310	Argentine	0.03–13.6 years (prepubertal)	124 unilateral and 186 bilateral	Decreased in 22.6% of boys with bilateral and 8.1% of boys with unilateral cryptorchidism
(39)	156	USA	1 day–20 years	16 unilateral and 140 bilateral	Undetectable AMH in 30%, low in 21%, and normal in 49%
(40)	105	Polish	1–4 years	Unilateral	Mean AMH not statistically different from controls Gene polymorphisms in cryptorchid boys, no association with AMH levels
(41)	104	Australian	0–18 years	76 unilateral and 28 bilateral	Lower mean AMH in bilateral cryptorchid as compared with unilateral and controls
(38)	94	Danish	0.5–13.1 years (prepubertal)	53 unilateral and 41 bilateral	Decreased in 5% (no distinction made between unilateral and bilateral)
(25)	65	USA	2 days–11 years	Bilateral non-palpable	Undetectable AMH in anorchid, low AMH in 14 cryptorchid with histological damage, normal AMH in 34 with histologically normal testes
(42)	50	Polish	1–4 years	Unilateral	Mean AMH not statistically different before and after orchiopexy
(37)	50	Polish	1–4 years	Unilateral	Lower mean AMH in cryptorchid as compared with controls
(36)	43	Various	85 ± 31 days	N.S.	Mean AMH not statistically different from controls
(43)	31	Brazilian	0.75–9 years	24 unilateral and 7 bilateral	Mean AMH within normal range
(44)	27	French	14–32 months	17 unilateral and 10 bilateral	Lower mean AMH in bilateral but not unilateral cryptorchid as compared with controls
(35)	20	Turkish	12 months	20 unilateral	Lower mean AMH in cryptorchid as compared with controls
(45)	18	N.S.	0.6–6.1 years	N.S.	No differential AMH mRNA levels reported between patients with high risk and those with low risk for azoospermia
(34)	15	French	1 day–10 years	Unilateral and bilateral	Decreased in 75% (no distinction made between unilateral and bilateral)
(46)	15	N.S.	7–55 months	7 unilateral and 8 bilateral	No changes reported in AMH mRNA levels in testicular biopsy

N.S., not specified; AMH, anti-Müllerian hormone.

inguinal or high scrotal position (86). In our series of patients with bilateral cryptorchidism, serum AMH concentration was higher in those with a bilaterally successful testicular descent in response to a 5-week hCG treatment protocol than in those with no response. These results are in line with the concordance observed between serum AMH and testosterone concentrations during hCG stimulation in boys undergoing gonadal function assessment (87), and suggest that basal AMH determination may serve as predictive marker of response to hCG treatment, in addition to testicular position.

The large sample size of cryptorchid patients included in this study and the randomized method used to select cases from a database of more than 1,500 potentially eligible patients is one major strength of this work. We acknowledge the existence of the possibility of a selection bias related to the fact that our study was performed in the endocrinology service of a tertiary hospital. Cryptorchidism is a condition traditionally treated by surgeons. However, the rate of referral of cryptorchid patients to the endocrine unit is high in our hospital, due to the multidisciplinary approach that has been implemented in the last two decades and deems endocrine assessment as essential in the management of patients with cryptorchidism. The relatively low number of patients in the 1–5.9 and >9 years precluded certain analyses with the desired power, yet reflect that a low number of patients are referred in the first months of life, awaiting a potential spontaneous descent (1, 4, 5, 88) but that most are referred before pubertal onset, as expected.

Our study included patients over a period of 18 years (years 2000 through 2017) but had a cross-sectional design, which precluded us from performing longitudinal analyses. Another concern that may arise from such a long study whose main outcome measure derives essentially from AMH determination is related to changes in the hormone assay methodology. Because we were involved in the development of the commercial AMH/MIS Beckman-Immunotech assay [usually referred to as IOT (89, 90)], we have been able to use the same assay all through this study. The applicability of our results is assured, even though the IOT assay is no longer available, because results of serum AMH in the male range are comparable with those obtained with the newly developed AMH assays (91).

In conclusion, the population of prepubertal boys with cryptorchidism have lower AMH production than normal boys, especially those with bilaterally undescended gonads. This could be the result of a decreased number of Sertoli cells in the cryptorchid gonads and/or to an impaired AMH secretion by each Sertoli cell. Although cryptorchid boys may have serum AMH within the normal range, our results in a large cohort, together with those previously reported in smaller studies, indicate that there is a considerable prevalence of testicular dysfunction during childhood in this frequent condition. The proportions of unilaterally or bilaterally cryptorchid patients with decreased AMH in our study are consistent with the risk of azoospermia in adulthood (45). However, to know whether Sertoli cell dysfunction early in life underlies germ cell failure in adulthood will need longitudinal follow-up for many years.

ETHICS STATEMENT

The study protocol was approved by the Institutional Review Board (Comité de Docencia e Investigación) and Ethics Committee (Comité de Ética en Investigación) of the Buenos Aires Children's Hospital (Hospital de Niños Ricardo Gutiérrez de Buenos Aires). Because the study of patients with cryptorchidism was based on a retrospective clinical chart review with

descriptive purposes and no anticipated effect on prognosis or therapeutic management of the patients whose charts were included, the need for a written informed consent was waived. For the control group, written informed consent was given by the participant's parents, and assent was given by the participants over 7 years of age.

AUTHOR CONTRIBUTIONS

RG and RR conceived the study design and drafted the manuscript; all the authors collected clinical and laboratory data and approved the final version; RG analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00182/full#supplementary-material.

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The Expression of Markers for Intratubular Germ Cell Neoplasia in Normal Infantile Testes

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Background: Positive immunohistochemical expression of testicular cancer markers is often reported beyond 12 months of age in cryptorchid testes, which is assumed to indicate delayed maturation of the fetal germ cells, or neoplastic changes. These findings allowed for questions as to the extent of positive reaction in normal testes. The aim of the study was to clarify the expression of these markers in a normal material up to 2 years.

Methods: Testicular material from 69 boys aged 1–690 days, who died of causes with no association of testicular pathology. Histology sections were incubated with primary antibodies including anti-placental-like alkaline phosphatase (PLAP), anti-C-Kit, anti-D2–40, and anti-Oct3/4. The mean germ cell number per tubular transverse section (G/T) was calculated based on the G/T of both testes of every boy.

Results: The mean G/T declined through the 690 days. PLAP appeared stably expressed throughout the ages studied. The likelihood of a positive reaction for C-Kit waned with increasing age within the study period. Positive staining for D2–40 and Oct3/4 was demonstrated up to 6 and 9 months respectively.

Conclusion: Up to 1 or 2 years of age, normal infantile testes contain germ cells positive for the immunohistochemical markers commonly utilized to aid in the detection of testicular cancer. This finding supports the concept of germ cells undergoing a continuous maturational process in a heterogeneous fashion, and that this process is not complete by 2 years of age.

Keywords: testis maturation, testis, immunohistochemistry, germ cells, testicular neoplasms

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INTRODUCTION

Intratubular germ cell neoplasia (ITGCN) is a recognized precursor of invasive testicular cancer (1). In adults it has a well-described histological appearance with atypical germ cells lined adjacent to a thickened basement membrane inside the seminiferous tubuli. The cells are large, have large nuclei with a hyperchromatic, coarse chromatin pattern, large prominent nucleoli, and abundant pale cytoplasm. In prepubertal boys the cells are morphologically similar, but located both centrally and peripherally, and the basal membrane is not thickened (2).

Commonly, the detection of ITGCN in adults is aided by the application of immunohistochemistry with antibodies toward the receptors for placental-like alkaline phosphatase (PLAP), C-Kit, D2–40, and Oct3/4. The sensitivity and specificity of detecting ITGCN in adults, using these markers, is high (3).

The observation that fetal germ cells express these same markers funneled the hypothesis of ITGCN originating from fetal germ cells failing to mature properly (4).

Cryptorchidism—when one, or both, testis has failed to descend into the scrotum—is a congenital condition, where there is evidence of disruption in spermatogenesis (2). It is among the more common congenital defects, for which 2–3% of boys in the western world are operated. Boys with cryptorchidism have an associated relative risk of developing testis cancer ranging from 3.7 to 7.5 times higher than in normal boys (5). Cryptorchidism also confers a risk of infertility ranging from 10% in unilateral cases to 70% in those bilaterally afflicted (2, 6, 7).

We recently published our findings of positive immunostaining of spermatogonia in biopsies from 404 cryptorchid boys with PLAP, C-Kit, D2–40, and Oct3/4, aged up to 15 years without histological features of concomitant cancer or ITGCN, except for two boys. One boy 13 months old was diagnosed with Prader–Willi syndrome and had been treated with growth hormone; the other was 44 months old with 45X/46XY, penoscrotal hypospadia, persistent vaginal, and uterine remnants, and both testes located intra-abdominally (8, 9). This is consistent with ITGCN in a previously published cohort of cryptorchid boys of around 0.5% (7/1403) (10). Conversely, in a cohort study from our region, the incidence of testicular cancer in adults treated for cryptorchidism in childhood was 1.2% (6 out of 506 subjects), and in general around 5% of testicular cancers are statistically referred to cryptorchidism (5, 11).

Thus in relation to cryptorchidism, it does not immediately follow, that a subsequent development of ITGCN or testicular cancer resides within dormant, dysmature spermatogonia. Moreover, screening by way of immunohistochemistry, can not stand alone in cryptorchid boys.

These findings naturally allowed for questions as to the extent of positive reaction in normal testes, and after reviewing the literature, we found very few studies regarding human tissue beyond the neonatal period. Most studies have been done on tissue from aborted fetuses and/or stillborn children and from adults (4, 12–20). From these it would appear that in normal testes the germ cells become negative for these immunohistochemical markers after the first year of age. Therefore, our previous findings of a positive reaction to the same markers in undescended testes beyond this age support the prevailing concept of delayed maturation of spermatogonia within cryptorchid testes (8).

Thus, this study aims to substantiate the hypothesis: that the pool of spermatogonia is heterogeneous and continues to transform and differentiate after birth; that this is demonstrable through the varying expression of different immunohistochemical markers in neonatal and infantile testes; and that we hereby can strengthen the concept of delayed maturation in the cryptorchid testis.

This process would also follow an evaluation of the timing of the disappearance of a positive reaction to the aforementioned panel of antibodies in normal infantile testes.

MATERIALS AND METHODS

From the Department of Forensic Medicine was acquired testicular material from the testes of 69 boys, aged 1–690 days (mean and median 360 days), who died of causes with no known association of testicular pathology.

The tissue had been fixed in 4% formalin. The exact ischemia time was unknown, but estimated to be between 1 and 5 days.

Due to scarcity of tissue, tissue microarrays (TMA) of the paraffin-embedded biopsies were performed, with two cores from each biopsy. The resulting block was cut in 2 µm sections and mounted on coated slides (Dako Flex IHC microscope slides) for immunohistochemical analysis, which was performed on Ventana Benchmark Ultra Stainer.

One hematoxylin-eosin (H + E) slide was produced.

For immunohistochemistry sections were dewaxed with EZ-prep from Ventana. Antigen-retrieval slides were pretreated with Ventana CDI (cell conditioning pH 8.5) for 64 min and incubated with antibodies for 32 min at 36°C following the manufacturer's instructions. The reaction was visualized using Ventana Ultra View DAB-kit. Counterstaining was with Ventana hematoxylin for 8 min.

Histology sections were incubated with primary antibodies, including anti-PLAP (1:200, PL8-F6, Biogenex), anti-C-Kit receptor (1:50, C-Kit, Dako, Glostrup, Denmark), D2–40 (1:25, M3619, Dako, Glostrup, Denmark), and anti-Oct3/4 (RTU, MRG, Cellmarque, CA, USA).

The number of germ cells per tubular transverse section was measured from at least 30 tubular transverse sections (G/T) from testes and the mean G/T was calculated for each boy.

For identification of germ cells with positive reaction to antibody, all seminiferous tubuli were examined by two observers. In cases where positive signals from germ cells were seen in some seminiferous tubules only, this focal expression was classified as a positive result.

Both the counting and identification was done in blinded fashion, and only when the results were established, was the identity of the boy revealed.

We evaluated the timing of the disappearance of a positive reaction to the aforementioned panel of antibodies in these normal infantile testes.

Statistical analysis was performed using SAS University Edition.

The study was conducted according to the Helsinki II declaration and approved by the ethics committee of Copenhagen (Protocol #H-3-2010-074).

RESULTS

From the records, we extracted age, weight, and cause of death. The birth weight was only available in less than one-third of cases, and gestational age not at all. The causes of death were infection, congenital heart disease, suffocation, homicide, trauma, and unknown.

In the H + E stained sections it was possible to discriminate between Sertoli cells and germ cells, but not to positively discern fetal spermatogonia from adult dark (Ad) and adult pale (Ap)

spermatogonia, or between the latter two, due to edema and clustering of the cells centrally within the tubuli, rendering it also impossible to discern whether the germ cells were centrally or peripherally located.

The mean G/T of the testes from every boy is shown in **Figure 1**.

The results of the immunostaining are summarized in **Table 1**. Regarding the positive markers there was concordance between the two testes of the same boy.

Placental-like alkaline phosphatase appeared stably expressed throughout the ages studied. The likelihood of a positive reaction for C-Kit waned with increasing age, but remained positive until 2 years of age. Statistical analysis with regression analysis on the percentage of positive staining confirmed this. Positive staining for D2–40 and Oct3/4 was demonstrated up to 6 and 9 months, respectively. In the groups 1–3, 3–6, and 6–9 months all D2–40 positive testes were also positive for the other three markers and those who were D2–40 negative but Oct3/4 positive were also C-Kit and PLAP positive (**Table 1**). For all the other groups the results regarding positive staining were unambiguous (**Table 1**).

There was a sense, between the observers, that the intensity of staining was also inversely related to age. However, as the biopsies were obtained from autopsies and processed by way of TMA, the general staining pattern was quite heterogeneous and no attempt was made to quantify the intensity.

Figure 2 shows positive staining for the immunohistochemical markers in testicular biopsies.

DISCUSSION

Our results are somewhat surprising. To our knowledge this is the first time a positive staining by PLAP and C-Kit has been reported beyond 12 months of age in normal testes.

We had expected to confirm, what others had found before, namely that there would be no positive reaction beyond 12 months of age (4, 12, 21). However, when the maturation of the primordial germ cells (PGC's) through sequential steps into adult spermatogonia is viewed as a continuum, our results are not in conflict. They merely suggest that the maturation is neither uniform nor complete at 2 years of age.

Previous studies with normal fetuses and neonates have evaluated the expression of several different immunohistochemical markers of differentiation and stem cell-ness. Some of these are also used to detect ITGCN (13, 20), and hence have supported the concept of ITGCN originating in fetal life.

Jørgensen and colleagues in 1993 and 1995 reported that in normal infantile testis the expression of PLAP was not detected beyond 12 months of age (4, 12). This was later confirmed and also applicable for C-Kit and Oct3/4 by Vigueras-Villasenor et al. in 2015 (21). They reported that C-Kit and Oct3/4 positive germ cells were not seen after 4 months of age and PLAP positive germ cells were seen in newborns until 1 year of age (21). Others have found similarly diminished to absent expression of the other markers of the panel in normal boys (13, 20). Rajpert-De Meyts et al. (13) found that the oldest specimen in their series with a few nuclei weakly positive for OCT3/4 was from a 4 months old infant. Thereafter, all prepubertal, peripubertal, and adult testicular samples in their series of normal males were consistently negative. However most authors have only included gonads from fetuses and neonatal boys (14–17, 20).

The fact, that we find much stronger expression of particularly PLAP and C-Kit in the present normal testes, as previously shown in cryptorchid testes (8), could be ascribed to enhanced staining due to technical refinement both with regards to staining procedure and antibody production.

Taken together, the studies of the literature suggest that once the PGC's have arrived from the allantois to the gonadal ridge by week 6 of gestation and become enclosed in the newly formed seminiferous tubuli they continue to differentiate and proliferate, but not in unison (22). Rather they make up a heterogeneous pool of gonocytes at different differential stages, gradually maturing

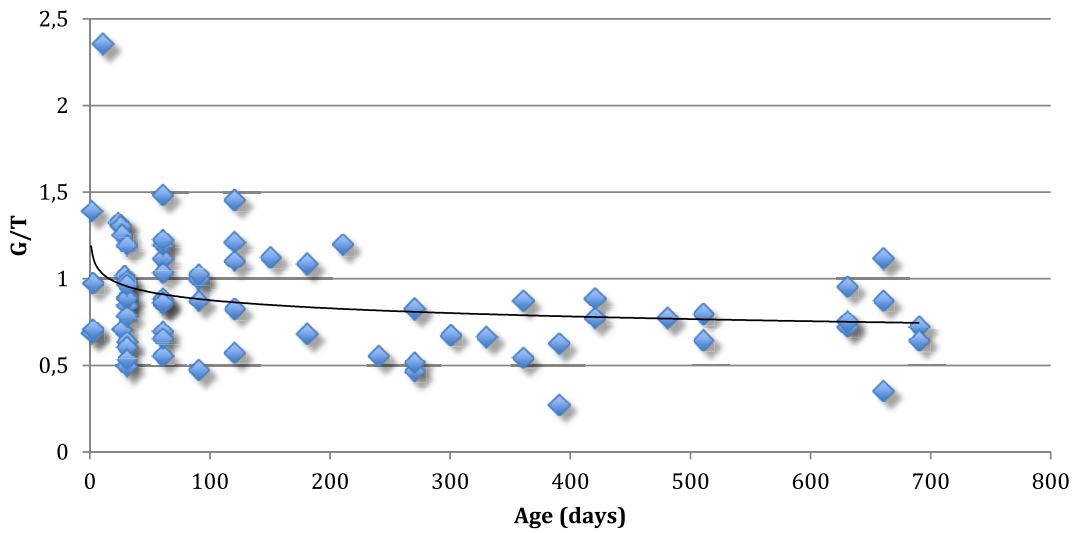

FIGURE 1 | Mean germ cell number per tubular cross-section (G/T) of the testes from 69 normal boys up to 2 years of age. Every boy is represented with one value. The line represents median G/T for every age.

into fetal spermatogonia. This is evidenced by their spatial expression of the varying immunohistochemical markers (4, 12–18, 20), their morphology (16, 17) and localization (14, 16, 17) within the tubuli as showed in **Table 2**.

The D2–40 antibody recognizes the M2A antigen, a marker for adult ITGCN and seminoma. Downregulation of D2–40 expression coincides with the movement of gonocytes toward the basement membrane as they lose embryonic stem-cell-like phenotype and differentiate into spermatogonia according to Sonne et al. (23). Those D2–40 positive germ cells were the first to disappear in our material and are, therefore, likely gonocytes. Oct3/4 positive germ cells in this age group represent other early stages of fetal germ cell maturation (possibly gonocytes at other differential stages and pre-spermatogonia) which normally proceeds into the neonatal age and early infancy (8, 20).

TABLE 1 | Number of boys with a positive reaction to each the immunohistochemical markers grouped by age in months.

Age	*n*(boys)	Placental-like alkaline phosphatase	Oct3/4	C-Kit	D2–40
0–1 month	10	10/10 (100%)	10/10 (100%)	10/10 (100%)	8/10 (80%)
1–3 months	22	22/22 (100%)	15/22 (68%)	18/22 (82%)	12/22 (55%)
3–6 months	10	10/10 (100%)	6/10 (60%)	8/10 (80%)	4/10 (40%)
6–9 months	4	4/4 (100%)	1/4 (25%)	3/4 (75%)	0
9–12 months	6	6/6 (100%)	0	4/6 (67%)	0
12–15 months	6	6/6 (100%)	0	3/6 (50%)	0
15–18 months	3	3/3 (100%)	0	2/3 (67%)	0
18–21 months	0	NA	NA	NA	NA
21–24 months	8	8/8 (100%)	0	3/8 (38%)	0

NA, no boys within this age group.

The process continues, and gradually more Ad and Ap spermatogonia appear according to the literature (2, 6). Of these Ad spermatogonia has been regarded as the "reserve" stem cell and Ap spermatogonia as the "active" stem cell, dividing once every epithelial cycle and eventually giving rise to type B spermatogonia (24). The PLAP and C-Kit positive staining in our material may likely represent persisting germinative stem cell properties.

Particularly the period of mini-puberty—a term coined for the phase of transient rise in gonadotropins and androgens that sets in between 2 and 4 months of life—seems to be crucial in driving the maturation of the germ cells (7, 25). In some cryptorchid boys, this hormonal activation may be a stunted, as evidenced by a transient hypothalamus–pituitary–gonadal hypo-function seen at surgery for the undescended testis (26). Because of the forensic medicine character of the material we had no possibility

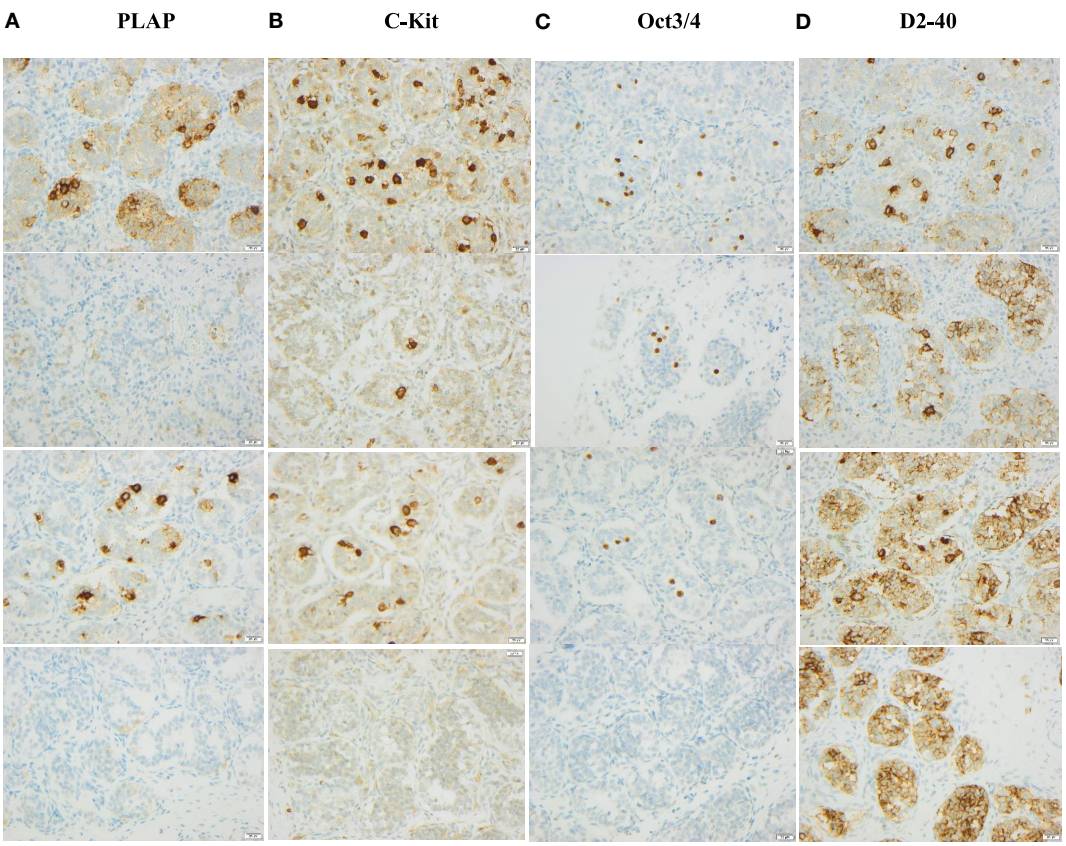

FIGURE 2 | Examples of staining for immunohistochemical markers in testicular biopsies of boys 10, 30, 60, and 660 days old, respectively. Column A shows PLAP staining in age order starting with the youngest age in upper row. Column B shows C-Kit staining in the same age order. Column C shows Oct3/4 staining in the same age order. Column D shows D2–40 staining in the same age order.

TABLE 2 | Previously reported immunohistochemical staining of primordial germ cells (PGC's) and germ cells in fetuses and neonates.

	PGC	1st trimester	2nd trimester	3rd trimester	Neonatal
Placental-like alkaline phosphatase	+	+++	++	+	+/−
OCT3/4	+	+++	++	+	+/−
C-Kit	+	+++	++	+	+/−
D2–40	?	+	++	++	+
AP-2γ	+	++	++	+	+/−
MAGE-A4	−	+	++	+++	++
VASA	+/−	+	++	+++	++
TSPY	?	+	++	+++	+++
Ki-67	?	?	+++	++	++

+, positive reaction; ++, intermediate positive reaction; +++, strong positive reaction; −, negative reaction; ?, unclassified. Data compiled from Jørgensen et al. (4, 12), Hoei-Hansen et al. (18), Robinson et al. (15), Gaskell et al. (16), Rajpert-de Meyts et al. (13), Pauls et al. (17), Honecker et al. (20), and Franke et al. (14).

TABLE 3 | Percentage of testes with a positive reaction to each of the immunohistochemical markers grouped by age in years.

Age	*n* (boys)	*n* (testes)	Placental-like alkaline phosphatase (PLAP) pos (%)	Oct3/4 pos (%)	C-Kit pos (%)	D2–40 pos (%)
Testes from normal boys. Present forensic medicine material						
0–<1/2 years	42	84	100	73	86	57
1/2–<1 years	10	20	100	10	70	0
1–<2 years	17	34	100	0	47	0
Testes from boys with cryptorchidism. Materials from Thorup et al. (30)						
0–<1/2 years	33	43	98	91	93	54
1/2–<1 years	189	220	95	59	70	23
1–<2 years	249	324	89	12	33	4

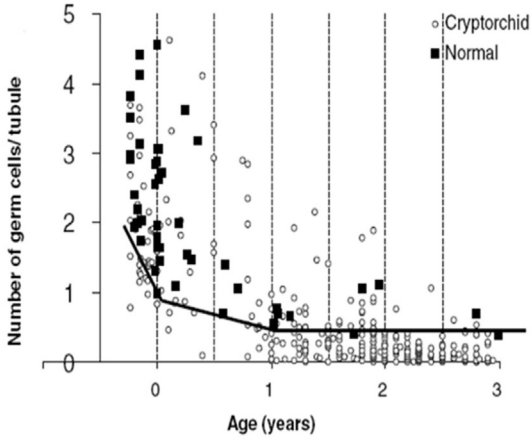

FIGURE 3 | The number of germ cells (spermatogonia and gonocytes if any) per tubular cross-section (G/T) in human cryptorchid fetuses, and in boys who underwent surgery for cryptorchidism <3 years of age. Furthermore, G/T in normal human fetuses and normal boys who died <3 years of age. The solid line indicates the lowest normal G/T. Data with permission from Cortes (31) and Cortes et al. 1995 (32).

to determine serum levels of reproductive hormones (gonadotropins, androgens, and inhibin-B). It would have been interesting to relate the results of such blood samples to the immunohistochemical staining pattern of germ cells, especially during the period of mini-puberty.

In cryptorchid testes a diminished number of spermatogonia—both Ad and Ap—are particularly described, and we have previously reported positive reaction to D2–40, and Oct3/4 upto 19 months of age (6–8).

These findings—diminished number of spermatogonia, stunted mini-puberty, and positive reaction to the above panel of markers—have supported the prevailing concept of delayed maturation of germ cells in cryptorchid testes.

Our findings of the aforementioned positive immunohistochemical reactions in normal testis upto 2 years of age, suggest that there is both a maturational delay in cryptorchid testis and an accelerated loss of spermatogonia (27–29). Indeed, D2–40 and Oct3/4 positive germ cells persist in cryptorchid testes into the second year of life and PLAP and C-Kit positive germ cells persist with almost the same frequency as in our material of normal testes (30) (**Table 3**). The decline in mean G/T with age in this study is in accordance with previous findings reported (2, 31) (**Figures 1** and **3**). However, when compared to our previously published normal material a significant loss of germ cells in cryptorchid testes is seen already 1 year of age (31, 32) (**Figure 3**).

This loss could be attributed to increased apoptosis, which in turn could be ascribed to decreased hormonal values and the increased temperature, the retained testis is subjected to Ref. (27–29). Several studies have described the deleterious effect of increased temperature on the testes and the displacement of a testis to the abdomen in experimental settings often result in a Sertoli-cell-only pattern. In intra-abdominally located cryptorchid testis biopsied at the time of surgery, less than 10% contain germ cells after the age of 3 years (33, 34).

We do not know if any of the boys in our material would eventually have developed testicular cancer. However, our findings do question the hypothesis that such cancers in general develop from dormant fetal germ cells, as it raises the age at which PLAP and C-Kit positive germ cells with stem cell properties should no longer be present. In this study, PLAP and C-Kit positive germ cells are only reported up to 2 years of age. But this information may be added to the findings that PLAP-positive cells were seen in 57–82% and c-Kit-positive in 5–21% of cryptorchid testes between 4 and 13 years, who unlikely would develop cancer (30). Also, in contrast, among 11 men with testicular cancer 24–37 years old previously operated for non-syndromic cryptorchidism in childhood only one had PLAP positive germ cells in the prepubertal biopsy. In all the others all staining were negative except for one case that had Oct3/4 and D2–40 positive cells in the prepubertal biopsy and developed a teratocarcinoma 27 years old. But a teratoma anlage is known to be congenital precursor to cancer (10).

One weakness of this study is the poor histological preservation of the tissue, rendering finer observations impossible, as mentioned above. Another weakness, when making comparison to the other studies, is that we used TMA, making quantification

of the immunohistochemical reactions unreliable; being heterogeneous in a section of a biopsy of a biopsy. Finally, but not least, we are unable to say when the maturation of germ cells is complete. Or at which age a positive reaction to the above panel of markers is pathological, as we only studied testes in boys up to 2 years of age. Alas, this age was chosen, as we had expected to confirm their disappearance at 12 months.

CONCLUSION

We showed that normal infantile testes contain germ cells positive for PLAP a C-Kit commonly utilized to aid in the detection of ITGCN upto 2 years of age. Positive staining for D2–40 and Oct3/4 was demonstrated up to 6 and 9 months, respectively. Therefore, a positive reaction to these antibodies is not diagnostic of neoplastic changes in this age group, but reflects that the normal germ cell maturational process is not completed by the age of 2 years.

Compared to our previous findings of a positive reaction to the same immunohistochemical markers in undescended testes beyond this age, our new findings confirm the hypothesis of delayed maturation of spermatogonia within cryptorchid testes.

ETHICS STATEMENT

The study was conducted according to the Helsinki II declaration and approved by the ethics committee of Copenhagen (Protocol #H-3-2010-074). As the individuals from where the biopsies were taken have passed away and were anonymous and taken for forensic medicine legal causes the ethical committee accepted that no consent was needed.

AUTHOR CONTRIBUTIONS

KK did germ cell evaluation and wrote first draft of manuscript. EC-L stained biopsies, evaluated germ cells, supervised cell counting and manuscript. OL wrote protocol of study, applied for ethical consent. SH took all biopsies of the material, supervised manuscript. DC planned the study and revised manuscript. JT planned the study and revised manuscript All authors accepted the final manuscript. None of the authors have any disclosures.

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Selecting Infants With Cryptorchidism and High Risk of Infertility for Optional Adjuvant Hormonal Therapy and Cryopreservation of Germ Cells: Experience From a Pilot Study

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Introduction: Orchiopexy for congenital cryptorchid testes is recommended between ½ and 1 year of age to preserve testicular germ cell maturation. Early operation is not enough to preserve fertility in 22 and 36% of cases. Aim of this study was to set up a protocol for optional adjuvant hormonal therapy after orchiopexy and thereafter cryopreservation of testicular biopsies from infants with bilateral cryptorchidism and high infertility risk.

Materials and methods: We included 17 boys with bilateral cryptorchidism, normal FSH, and impaired germ cell number per tubular transverse section (G/T) in testicular biopsies at orchiopexy, 7 months to 3½ years old. Postoperatively, optional adjuvant LHRH (kryptocur®) 0.2 mg/0.1 mL 2× every second day in 16 weeks were offered. Ten boys were applicable for age matching according to parent's choice of treatment regime and G/T. Five of them had kryptocur®, and five were controls. Repeat bilateral testicular biopsy evaluation and cryopreservation were offered to all boys 12 months after primary orchiopexy. For cryopreservation, tissue pieces were incubated with a cryoprotectant with a slow program freezing.

Results: Two out of five kryptorcur®-treated boys normalized both the average G/T and the number of adult dark spermatogonia (Ad-S). Another kryptocur®-treated boy with initial low G/T and no Ad-S increased the G/T and achieved normal number of Ad-S at time of cryopreservation. In the control group, two patients reached only normal lower range regarding the G/T and the number of Ad-S. None of boys with less than average 0.2 G/T improved significantly, whether they were kryptocur®-treated or not.

Conclusion: Based on literature and the present results, we recommend adjuvant LHRH treatment to boys with cryptorchidism and insufficient genuine gonadotropin stimulation at time of surgery, as these patients have high infertility risk. Cryopreservation should

be an option in case of treatment failure of adjuvant LHRH. However, to avoid repeat surgery with biopsy, some parents may choose biopsy for cryopreservation at time of the initial bilateral orchiopexy, well informed that the procedure may only be truly indicated in 22 and 36% of the cases.

Keywords: cryptorchidism, germ cells, LHRH, cryopreservation of cells and tissues, orchiopexy, fertility

INTRODUCTION

Today, orchiopexy for congenital cryptorchid testes is recommended within the first ½–1 year of life to preserve testicular germ cell maturation (1–4). In accordance with the strategy of early operation follow-up studies on adult men operated for cryptorchidism in childhood have shown significant improvement of fertility even in bilateral cases (5–7). However, these studies have not conclusively proven that early surgery will protect from azoospermia development.

For a subgroup of boys with cryptorchidism, early operation is not enough to preserve fertility. If, there is an underlying endocrinopathy causing inadequate maturation of the testis, merely putting the testis into the scrotum may not correct that endocrinopathy (4, 8). However, some of the reported endocrinopathies may be temporary and part of a maturational process. Specifically, there is incomplete evidence whether an appropriately time orchiopexy increases adult dark spermatogonia (Ad-S) numbers later similar to what LHRH may aid earlier on. One author showed that early and successful orchiopexy (before 9 months of age) could not prevent infertility development in 36% of cryptorchid males (1, 7, 9). Cortes et al. (10) found similarly that germ cell hypoplasia, which is germ cells per tubular cross section value below the lowest normal value for age, was present in 22% of the testes in 0- to 1-year-old boys with cryptorchidism. From about 15 months of age, germ cells may even start to lack and this process will progress (10, 11). Germ cell hypoplasia in both testes of prepubertal boys with cryptorchidism at time of orchiopexy generally leads to infertility in adulthood (12).

For more than a decade, Hadziselimovic and Hoecht have advocated that a group of boys with cryptorchidism and high risk of infertility could be identified in infancy according to histopathological evaluation of testicular biopsies showing low germ cell number and lack of Ad-S in the germinative epithelium (1, 2). Recently, Thorup et al. (13) proposed to combine the results of blood samples (gonadotropins and inhibin B) with histopathology (bilateral testicular biopsies during orchidopexy) to identify the group of patients at high risk of infertility. They reported that boys with normal gonadotropin levels and normal germ cell number have a good fertility prognosis and boys with increased gonadotropin levels may have testicular dysgenesis, and some of these boys may benefit from early surgery alone. However, boys with normal gonadotropin levels and a decreased total number of germ cells and decreased number of Ad-S have transient hypothalamus hypofunction and a poor fertility prognosis, as there was no gonadotropin response to the impaired histological state of seminiferous tubules as reflected either directly with the mean germ cell count per tubular transverse section or indirectly by the serum levels of inhibin B. The reason could be a significant prepubertal transient hypothalamus–pituitary–gonadal hypofunction (14). Impaired transformation of the neonatal gonocytes into type Ad-S during the first 12 months of age and subsequent apoptosis of germ cells may be a pathogenic factor. This transformation may be impaired if gonadotropins are insufficient and when the testis is undescended this lead to germ cell deterioration. Adjuvant hormonal treatment has been used to improve the fertility potential in such cases (15, 16).

For prepubertal infant boys with cryptorchidism, when full spermatogenesis is not yet ongoing, cryopreservation of immature testicular tissue might be an option to try to preserve their fertility while germ cells are still present in the cryptorchid testes, although the number of germ cells, as previously mentioned, is impaired (17, 18). Cryopreservation of testicular tissue has the advantage of preservation of integrated tissue. This allows to maintain cell-to-cell contact between spermatogonia and the neighboring cells, especially Sertoli cells, which are important for subsequent maturation of spermatogonia. We and another team have reported the freezing protocols of testicular tissue in prepubertal boys, both yielding good structural integrity (17, 19). Several centers have now established testicular tissue banking for prepubertal males at risk of infertility (20, 21). Young boys, before starting gonadotoxic cancer treatment (20, 21) or undergoing bilateral orchiopexy (21), are offered the option of testicular biopsy and cryopreservation under the same general anesthesia for either port-a-cath-insertion (for chemotherapy) or orchiopexy surgery. A biopsy of about 0.35 mm^3, either 5% of testicular volume, is considered as a sufficient amount of tissue for culture and transplantation. Although testicular tissue preservation offers the prospect of realistic applications, fertility restoration after cryopreservation has not yet been successful in humans. Nevertheless, promising results of restoration of fertility from donor stem cells have been achieved in animals (22).

The aim of this study was to set up and evaluate a useful protocol for optional adjuvant hormonal therapy and cryopreservation of testicular biopsies from infant boys with cryptorchidism and high risk of infertility. We hypothesize that this strategy is relevant, because within a 20-year period the technique of restoration of fertility from autologous donor stem cells has been achieved in humans.

MATERIALS AND METHODS

Testicular Biopsies

Testicular biopsies at time of orchiopexy were taken and evaluated according to a previous described method (10). All tissue specimens were fixed in Stieve's solution, embedded in paraffin, and 2-μm sections were stained with hematoxylin-eosin, CD99

(MIC-2) and PLAP. In blinded fashion, the total number of germ cells (S/T), including gonocytes and Ad-S, per tubular transverse section was measured from at least 100 tubular transverse sections. For every patient, the mean adult dark spermatogonia per tubule (Ad/T) and mean-S/T were found. The mean-S/T was considered normal when the value was at least 1.0 at birth, 0.65 at 6 months and 0.38 in 1- to 4-year-old boys, based on our previously published normal material (10). The total number of Ad/T for each patient was stratified into present (greater than 0.02) or abnormal (0.02 or less).

Hormonal Assays

Blood samples were obtained by venipuncture between 8:00 and 11:00 a.m. Serum samples were separated from the clot by 10 min of centrifugation at 2,000 × *g*. Serum was stored at −80°C until analysis. Serum inhibin B levels were measured using a commercial available inhibin B ELISA kit (Serotec Ltd., Oxford, UK) with research kit as recommended by the manufactory instructions. The lower detection limit was 5 pg/mL, and the measurements were made in duplicate.

LH and FSH were measured by sandwich electrochemiluminescence immunoassay. The lowest value of FSH to be measured was 0.05 IU/L. Normal median: 0.5 IU/L and range: 0.1–1.4 IU/L. The lowest value of LH to be measured was also 0.05 IU/L. Normal median: 0.1 IU/L and range: 0.05–0.3 IU/L.

FSH more than 1.4 IU/L was considered as a high serum value for this age group.

Patients and Setup

We offered boys age 7 months to 3½ years old with bilateral cryptorchidism to join the study if they met the inclusion criteria.

In order for patients to be qualified for inclusion, the average germ cell count per tubular transverse section should be between 0.05 and 0.40. PLAP positive germ cells should be present in the biopsy. The serum FSH level should not be increased.

After 3 months postoperatively, when testes should be in scrotum and the hormone profile and testicular histology was evaluated, the patients were offered adjuvant hormonal treatment with LHRH (kryptocur®) 0.2 mg/0.1 mL 2× every second day in 16 weeks (dosing according to consensus conference, Cortes and Hadziselimovic; Liestal 2009). Patients, with matched germ cell number in testicular biopsies, who did not choose hormonal treatment were used for controls. Repeat bilateral testicular biopsy for histology and cryopreservation were offered to all the patients 12 months after the primary orchiopexy.

Cryopreservation was performed according to the following protocol: tissue pieces were incubated with a cryoprotectant (5% DMSO) with a slow program freezing.

Statistics

Non-parametric statistics were used for analyses: Mann–Whitney test was used to asses statistical significance, and two-sited *p* values less than 0.05 were considered significant.

Ethics

The study was conducted according to the Helsinki II declaration, and informed consent was obtained from the parents of the patients. The study received approval from the ethics committee of Copenhagen (H-2-2012-060.anm.37655) and Danish Medicines Agency (SST jr. nr. LMST-2012083184).

RESULTS

Seventeen boys with bilateral cryptorchidism had testicular biopsies taken for cryopreservation. Ten boys were applicable for age matching according to parent's choice of treatment regime and histopathological evaluation of germ cell status. These boys were included in the study. Five of them had kryptocur® treatment and five were controls. None had any associated anomalies.

At inclusion, there were no differences between the two groups in respect of age ($p = 0.92$), inhibin-B level ($p = 0.69$), and the average germ cell count per tubular transverse section ($p = 0.40$) (**Figure 1**).

Two out of five kryptorcur®-treated boys normalized completely as well the average germ cell count per tubular transverse section as the number of Ad-S. One other kryptocur®-treated boy with an initial germ cell count per tubular transverse section of 0.06 and no Ad-S increased the germ cell count and achieved normal number of Ad-S at time of cryopreservation. In the control group, two patients reached normal lower range regarding the average germ cell count per tubular transverse section and the number of Ad-S (**Figure 1**). None of the boys with less than average 0.2 germ cell number per tubular transverse section improved significantly, whether they were kryptocur® treated or not (**Figure 1**). There were no surgical complications after the re-biopsy procedure. All specimens for cryopreservation were prepared, frozen, and stored according to the protocol without complications. The median and (range) hormonal serum levels at time of orchiopexy and re-biopsy for cryopreservation were FSH: 0.7 (0.6–1.4) and 0.4 (0.1–1.7) IU/L, respectively; LH: 0.1 (0.05–0.4) and 0.1 (0.05–0.4) IU/L, respectively; inhibin B: 114 (17–300) and 55 (28–132 pg/mL), respectively.

DISCUSSION

This is the first study on cryopreservation of testicular tissue from infant boys with documented high risk of infertility after treatment for cryptorchidism. Although the study sample is very modest, we demonstrate important results, which are useful for further investigations. Since 2/5 (and one partial) of LHRH-treated patients responded, adjuvant LHRH treatment to cryptorchid boys with insufficient genuine gonadotropin stimulation or in patients with low germ cell count or number of Ad-S by testicular biopsy should be instituted at time of surgery. However, since 2/5 control patients had almost similar improvements in germ cell counts, one could also argue that the study does not show any significant difference between treated and control patients. Thus, the conclusion should be that a larger sample size is needed to fully discern whether the germ cell count improvement is truly only seen in LHRH-treated patients. Our findings are evidently in agreement with the research by Hadziselimovic, who states that if germ cell

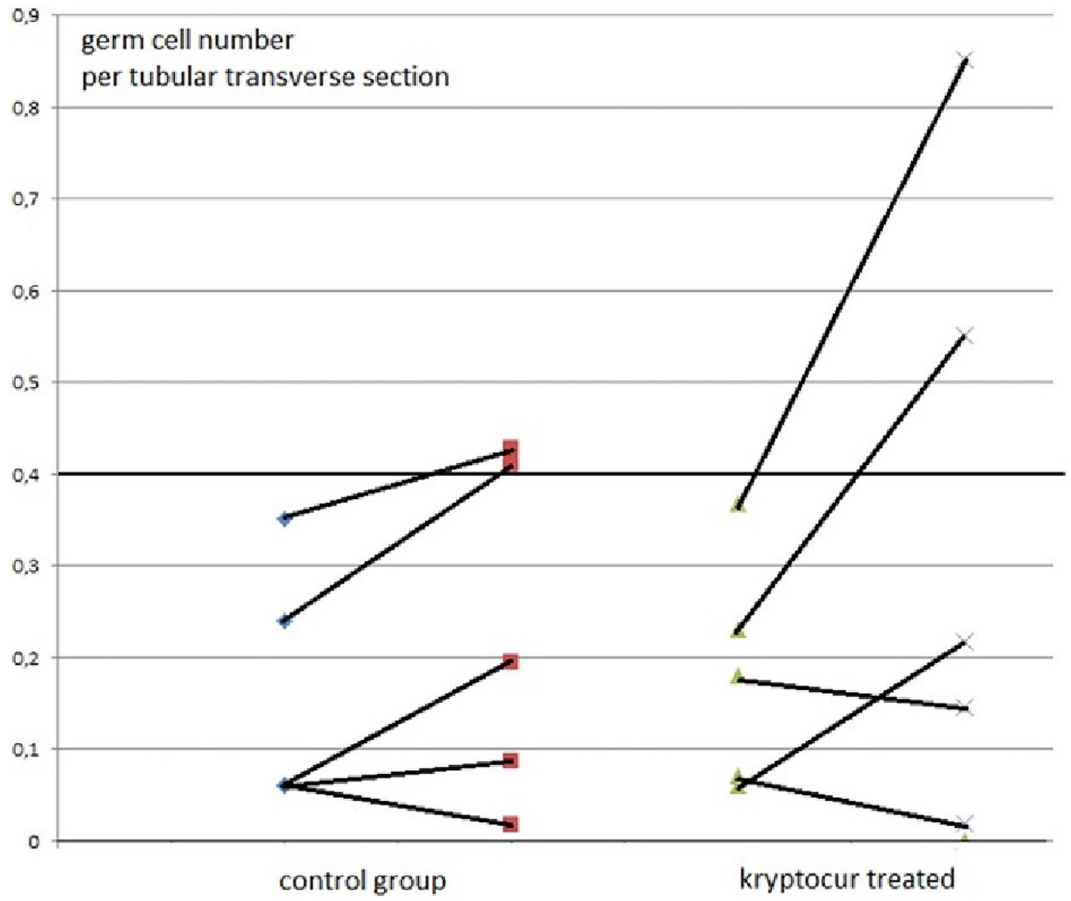

FIGURE 1 | The average number of germ cells per tubular transverse section from 10 boys with bilateral cryptorchidism at time of orchiopexy and 1 year after, at time of cryopreservation. The individual results of primary and follow-up biopsies from five kryptorcur® treated and five bilateral controls without hormonal treatment aged 10 months to 3 years are connected with lines [there was no age difference between groups ($p = 0.92$)].

count per tubular transverse section is below 0.2, a majority of patients will develop infertility irrespective of whether they had only surgery or hormonal treatment before orchiopexy (9). So, the only realistic way to achieve biological paternity for these patients may be through cryopreservation of testicular tissue. Great challenges in this respect are obvious. We have previously clearly shown that infant testicular biopsies tolerate the freezing procedure (19). In that study the morphology of the fresh and frozen–thawed samples was similar, with well-preserved seminiferous tubules and interstitial cells. A similar picture appeared after 2 weeks of culture, but a few of the cultured biopsies contained small necrotic areas. The presence of spermatogonia was verified by c-kit-positive immunostaining. Production of testosterone and inhibin B (ng/mm^3 testis tissue) in the frozen–thawed pieces were on average similar to that of the fresh samples (19). However, although PLAP positive germ cells were present in our present selected material, possibly representing germ cells with stem cell properties and with preserved ability for germ cell transformation (23, 24), presence of Ad-S was not identified in all biopsies for cryopreservation. Cultivation of a spermatogonia cell population is a prerequisite for later restoration of fertility from an autologous donor. So, development in our laboratory of a technique for testicular autograft enzymatically dispersed leading to single spermatogonia stem cell suspension is needed and is now in process.

Although there are too few patients in our study to show any statistical significance our data may support the studies in a recent review implicating that LHRH treatment increases the germ cell number in cryptorchid testes (16): "Hadziselimovic and Herzog reported in 1997 that the luteinizing hormone-releasing hormone analog, Buserelin®, administered intranasally every other day for 6 months following successful orchidopexy, appeared to have a long-lasting, positive effect on germ cells (15). In another study, Huff et al. (25) reported that GnRH treatment after orchidopexy improved total germ cell counts in 75% of the patients. These results have partly been confirmed by other groups. Schwentner et al. (26) gave intranasal GnRH 1.2 mg/day for 4 weeks preoperatively in 21 cases versus orchidopexy only in 21 controls. The mean number of spermatogonia per tubule was 1.11 in unilateral and 0.96 in bilateral cases versus 0.47 and 0.56, respectively, in controls. Jallouli et al. (27) prospectively assigned a total

of 24 boys, 12–123 months old (median 34.5), with 24 UDT into two groups during a 24-month period. The patients were randomized to receive either orchidopexy alone ($n = 12$) or orchidopexy combined with neoadjuvant GnRH therapy (kryptocur®) ($n = 12$) as a nasal spray for 4 weeks at 1.2 mg/day. In both groups, testicular biopsies were performed at orchidopexy, and the number of germ cells per tubule was determined. The mean number of germ cells per tubule in the group treated with GnRH before surgery was significantly greater (0.88 ± 0.31) than in the group without hormonal stimulation (0.49 ± 0.52; $p = 0.02$). Zivkovic et al. (28) also found that hormonal therapy (Buserelin®) improved the histopathology of the abnormal contralateral descended testis in unilateral cryptorchidism without harming the germ cells." Vincel et al. (29) presented recently a randomized study of 10 boys 8 months to 5 years old. Boys with high infertility risk (no Ad-S in the testicular biopsy) were randomly divided into two groups. First group underwent just the second orchidopexy without any hormonal treatment. The second group received intranasal LHRH (Buserelin®) therapy for the period of 6 months followed by second orchidopexy. Biopsy was taken from both groups during the second surgery. Five boys in each arm were included. There was no difference in the mean number of germ cells per tubule at the first surgery between the first and the second groups. Only the second group showed statistically significant increase in the number of germ cells per tubule: medians from 0.11 to 0.42 ($p = 0.04$). There were no Ad spermatogonia in both groups in the first biopsy. They were detected only in patients from the second group, who had adjuvant hormonal therapy, in the second biopsy ($p = 0.008$). These findings are very similar to our present results (**Figures 1** and **2**).

So, adjuvant hormonal therapy stimulating gonadotrophins may probably have a positive effect on germ cell maturation (16). Due to our previous findings that LHRH in recommended doze may harm the germ cells in young infancy (30), we have chosen a lower doze and a longer treatment period in the present study. Further pharmaceutical safety studies and randomized placebo controlled efficiency studies are needed to find the optimal adjuvant treatment regime. However, hormonal treatment should be restricted to boys with genuine insufficient gonadotropin stimulation, which may also include unilateral cryptorchidism with bilateral disease. The hormonal changes from orchiopexy to time of re-biopsy are in accordance with the expected changes related physiological decline of serum values seen after the minipuberty (31). The weakness of the study is related to the modest sample size and the lack of a randomized design. Furthermore, there is no guaranty that the development of technique for restoration of fertility from autologous donor stem cells will be successful within 15–20 years, although very promising rodent studies have recently been published (32).

In 1994, a first report showed that spermatogonia stem cell transplantation restored spermatogenesis, resulted in functional sperm and subsequently gave rise to normal offspring in mice (33, 34). Till now, spermatogonia stem cell transplantation has been successful in various species such as pig, bovine, and monkey (35–37). In addition, it has been shown that spermatogonia stem cells from humans and other species could settle in their niche on the basal membrane of testis tubules after transplantation into immunodeficient mice but currently there is no experience from transplanting spermatogonia stem cells to humans (38–41).

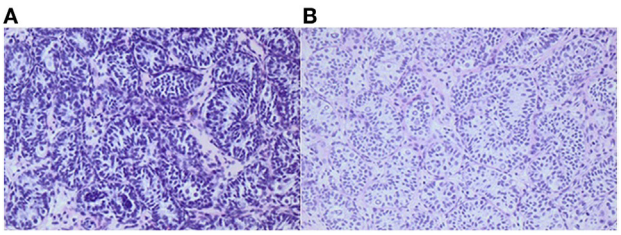

FIGURE 2 | Histology of testicular biopsies from a boy when he was 1¼ and 2¼ years old, respectively. After bilateral orchiopexy for cryptorchidism, 4 months of adjuvant LHRH was given. Average germ cell number per tubular transverse section increased from 0.27 **(A)** to 0.55 **(B)**. Adult dark spermatogonia (Ad-S) are noted in the re-biopsy **(B)**. The Ad-S are characterized as large gem cells with pale cytoplasm. In the nuclei, a vacuole is observed which appears in the sections as a circular light area. Informed consent to show the pictures was given by the parents.

CONCLUSION

Based on the literature and the present results we recommend adjuvant LHRH treatment to boys with cryptorchidism and insufficient genuine gonadotropin stimulation at time of surgery, as these patients have high risk of infertility. But a larger study sample size is needed to fully discern whether the germ cell count improvement is truly only seen in LHRH-treated patients. Cryopreservation should be an option in case of treatment failure of adjuvant LHRH. However, to avoid repeat surgery with biopsy, it may be more attractive for some parents to choose biopsy for cryopreservation at time of the initial bilateral orchiopexy, well informed that the procedure may only be truly indicated in 22 and 36% of the cases.

ETHICS STATEMENT

The study was conducted according to the Helsinki II declaration, and informed consent was obtained from the parents of the patients. The study received approval from the ethics committee of Copenhagen (H-2-2012-060.anm.37655) and Danish Medicines Agency (SST jr. nr. LMST-2012083184).

AUTHOR CONTRIBUTIONS

JT designed study, recruited and treated patients, wrote first manuscript draft, and discussed and revised manuscript. EC-L evaluated histology and discussed and revised manuscript. LD and SK did cryopreservation an discussed and revised manuscript. SH and CA discussed and revised manuscript. DC counted germ cells and discussed and revised manuscript.

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PERSPECTIVE
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Perspective: A Neuro-Hormonal Systems Approach to Understanding the Complexity of Cryptorchidism Susceptibility

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Nonsyndromic cryptorchidism is a common multifactorial, condition with long-term risks of subfertility and testicular cancer. Revealing the causes of cryptorchidism will likely improve prediction and prevention of adverse outcomes. Herein we provide our current perspective of cryptorchidism complexity in a synthesis of cumulative clinical and translational data generated by ourselves and others. From our recent comparison of genome-wide association study (GWAS) data of cryptorchidism with or without testicular germ cell tumor, we identified RBFOX family genes as candidate susceptibility loci. Notably, RBFOX proteins regulate production of calcitonin gene-related peptide (CGRP), a sensory neuropeptide linked to testicular descent in animal models. We also re-analyzed existing fetal testis transcriptome data from a rat model of inherited cryptorchidism (the LE/orl strain) for enrichment of Leydig cell progenitor genes. The majority are coordinately downregulated, consistent with known reduced testicular testosterone levels in the LE/orl fetus, and similarly suppressed in the gubernaculum. Using qRT-PCR, we found dysregulation of dorsal root ganglia (DRG) sensory transcripts ipsilateral to undescended testes. These data suggest that LE/orl cryptorchidism is associated with altered signaling in possibly related cell types in the testis and gubernaculum as well as DRG. Complementary rat and human studies thus lead us to propose a multi-level, integrated neuro-hormonal model of testicular descent. Variants in genes encoding RBFOX family proteins and/or their transcriptional targets combined with environmental exposures may disrupt this complex pathway to enhance cryptorchidism susceptibility. We believe that a systems approach is necessary to provide further insight into the causes and consequences of cryptorchidism.

Keywords: cryptorchidism, testicular descent, gubernaculum, androgen, insulin-like 3, systems biology

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The undescended testis has been the object of continued interest over many years. In April 2018, a search for "cryptorchidism" in PubMed (https://www.ncbi.nlm.nih.gov/pubmed) yielded nearly 10,000 articles spanning almost 100 years. Cryptorchidism has been an area of interest because of its inheritance patterns in domesticated mammals, its high prevalence in man (2–9% of all newborn boys) and its co-morbidities, including subfertility and testicular cancer. Despite sustained and focused attention, the pathogenesis of cryptorchidism and its associated conditions remain poorly

understood. Indeed, the more we study the condition, the more complex it seems to become. It stands to reason that better knowledge of the global mechanisms of testicular descent would provide important insight into the causes of cryptorchidism, and would allow us better to predict and prevent this common birth defect and its consequences.

Through our work over the years studying testicular descent and cryptorchidism in animal models and in man, we now propose a consolidated model of testicular descent comprising multilevel integration of neuro-hormonal signaling, and that cryptorchidism results when a combination of genetic and environmental factors target this integrated pathway. Below, we present relevant published and unpublished evidence supporting this perspective.

LEYDIG CELL HORMONES WORK SEPARATELY AND TOGETHER TO BRING ABOUT TESTICULAR DESCENT

Enlargement and regression/migration of the gubernacular ligament (gubernaculum) connecting the ventral pole of the testis/epididymis to the body wall in the inguinal region are indispensable for testicular descent (1, 2). Based on Hutson's hormonal regulation model (3), transabdominal (Phase 1) and transinguinal (Phase 2) descent are distinct, and largely regulated by a non-androgenic hormone, now known to be insulin-like 3 (INSL3), and androgens, respectively. At the same time in most mammals a second ligament, the cranial suspensory ligament (CSL), linking the dorsal pole of the testis to the dorsal surface of the body cavity close to the embryonic kidney, needs to dissolve. Studies of androgen receptor knockout (ARKO) and *tfm* (testicular feminization) male mice, androgen-exposed female mice, and anti-androgen-exposed rats clearly show that the CSL is regulated by androgens and that its persistence leads to cryptorchidism (4–9). Yet CSL regression is thought to facilitate transabdominal rather than transinguinal, descent. Other inconsistencies exist, complicating efforts to show that distinct hormones regulate distinct phases of descent across species. Remodeling of the CSL may not truly occur, or may be less relevant in human fetuses (10, 11). Some human subjects with complete androgen insensitivity syndrome have testes located close to ovarian position (12). Studies in rodents suggest that INSL3 overexpression leads to partial ovarian descent and transinguinal migration of the processus vaginalis, leading to hernia (13), which could be interpreted as an evolutionary relic of a primitive mode of testis excorporation. Similarly, RXFP2, the INSL3 receptor, appears to augment the role of androgens in transinguinal descent (14) and together with AMH may influence gubernacular cell proliferation in culture (15).

Knockout experiments in mice clearly show independent requirements for INSL3/RXFP2 and androgens in testicular descent (16, 17); nevertheless causative mutations in *INSL3, RXFP2, AR* (androgen receptor) or the Leydig cell regulator *NR5A1* (steroidogenic factor-1), are rare in cases of cryptorchidism (18, 19). WNT signaling appears to be a downstream target of both INSL3 and androgen in the fetal rat gubernaculum (20, 21), and cryptorchidism and/or gubernacular maldevelopment occur in mice with transgenic deletion of WNT pathway genes, such as *Sfrp1/Sfrp2, Wnt5a, Ctnnb,* or *Vangl2* (16, 22–25). Yet none of these genes has been implicated in human cryptorchidism. If INSL3 and androgen are each indispensable for testicular descent, fetal Leydig cell function must play a central role in cryptorchidism susceptibility. This is strongly echoed by studies on the effects of maternal exposure to phthalates in rats where the fetal Leydig cells are seen as primary targets for this endocrine disruptor, leading to a reduction in both INSL3 and testosterone production as well as cryptorchidism (26). Yet the effects of phthalates appear to be species-specific, with humans and mice seemingly more resistant to these inhibitory effects on testicular hormone production (27, 28). While detailed studies of Leydig cell function during the prolonged process of testicular descent in human fetuses are unavailable, it is reasonable to assume that genetic and/or environmental factors that alter this function may contribute to cryptorchidism.

THE SENSORY NEUROPEPTIDE CALCITONIN GENE-RELATED PEPTIDE (CGRP) PLAYS A ROLE IN TESTICULAR DESCENT

A role for CGRP in testicular descent and cryptorchidism is supported by *in vitro* and *in vivo* rat studies [reviewed in (29)]. Experiments in newborn rodents showed that transection of the genitofemoral nerve (GFN; which innervates the gubernaculum) causes cryptorchidism, and that CGRP release by the sensory limb of the GFN regulates proliferation and motility of the gubernaculum. Hutson and colleagues found evidence for interaction between CGRP and androgens in rodent models (30, 31), and in the absence of clear AR expression in the fetal gubernaculum (32, 33) they theorized that androgens indirectly modulate CGRP via effects on surrounding AR+ mammary tissue. However, other data suggest that the fetal gubernaculum does express its own functional AR (17, 21, 34–36). Clinical data have not shown an association of genetic variants in the CGRP pathway with cryptorchidism (37). However, we recently found a potential role for RBFOX proteins, which regulate production of CGRP, in genetic association analyses of cryptorchidism (see below), which may provide evidence supporting a role for CGRP in humans.

HERITABLE CRYPTORCHIDISM IN THE LE/ORL RAT IS ASSOCIATED WITH MULTI-LEVEL DYSREGULATION OF TESTICULAR DESCENT, AND MULTILOCUS INHERITANCE OF CRYPTORCHIDISM

The Long Evans-derived LE/orl rat exhibits incompletely penetrant cryptorchidism that is associated with variants in

at least 2 genes, *Syne2* and *Ncoa4,* which encode AR-interacting proteins (ARIPs) (38, 39). As frequently observed in cryptorchid rats exposed prenatally to anti-androgens (40–42) and cryptorchid boys, affected LE/orl testes are located in the superficial inguinal pouch, suggesting that this strain is a good model for a common form of clinical cryptorchidism. Testosterone levels and DHT-responsive transcript expression are reduced in LE/orl males, suggesting altered AR signaling (43). Testosterone deficiency alone is likely not sufficient to cause cryptorchidism in this strain, since other work suggests that a more marked reduction in Leydig cell hormone production is required to elicit this effect (44). Interestingly, in a recent re-analysis of transcriptome data (45) based on new information (46), we found that 110 of 315 (35%) differentially expressed LE/orl fetal testis transcripts map to genes whose expression is enriched in Leydig cell progenitors ($n = 62$; $p = 2 \times 10^{-24}$) or fetal Leydig cells ($n = 48$; $p = 4 \times 10^{-11}$; Fisher's exact test using Ingenuity Pathway Analysis/IPA®). The majority (59 of 62, 95%) of Leydig cell progenitor-enriched genes are downregulated at E17 in LE/orl as compared to the parent outbred strain (LE/wt). In addition, 40 of these transcripts are also differentially expressed in fetal gubernaculum, of which 37 (92%) are similarly downregulated. This evident coordinate gene regulation is lost by E19 (**Figure 1A**). These data are consistent with work published by the Agoulnik lab, which has shown that a retinoic acid receptor β type 2 Cre transgene is expressed in both Leydig cell progenitors and gubernaculum, and that conditional deletion of *Ar* in these cells impairs both testicular descent and fetal Leydig cell survival (17, 47). Others have confirmed that Leydig cell progenitors express AR (48), raising the possibility that the mesenchymal progenitors in the testicular interstitium and the gubernaculum may have a common origin, making AR important for both testicular hormone production and response.

LE/orl rats also carry a homozygous insertion within the *Prrxl1* (*Drg11, Drgx)* gene that is inherited together with the *Ncoa4* variant. *Prrxl1* regulates development of sensory neural circuitry (49) and transgenic deletion in mice leads to loss of CGRP-expressing neurons through apoptosis (50). Using qRT-PCR as described previously (51), we found that *Prrxl1* and other sensory transcript levels are altered in the L1-L2

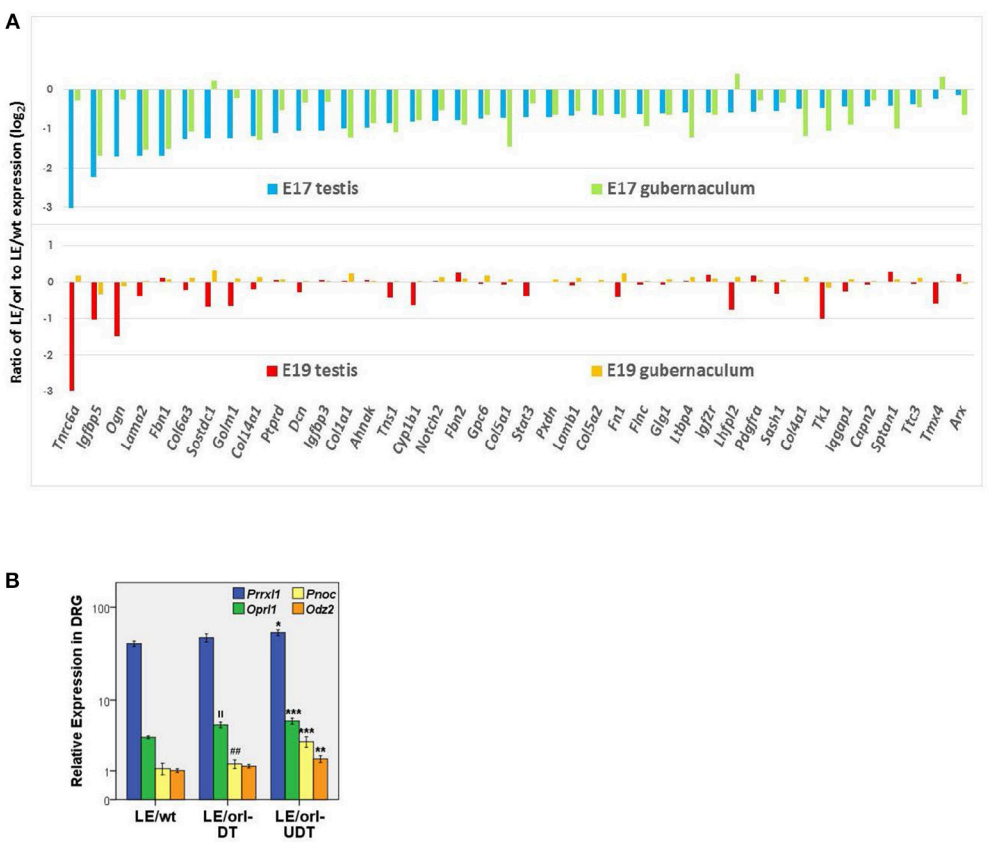

FIGURE 1 | (A) Of 315 differentially expressed genes in LE/orl testis (45), 62 (20%) are Leydig cell progenitor-specific (46) and expression was reduced in 59 (95%) of these at E17. Forty of 62 (shown here) are also differentially expressed in LE/orl gubernaculum, and 37 of these (92%) are also downregulated. By E19, coordinate expression of these genes in these tissues is lost. **(B)** Expression levels of the sensory transcripts *Prrxl1* (paired related homeobox protein-like 1, also known as *Drg11* or *Drgx*), *Pnoc* (prepronociceptin) and its receptor, *Oprl1* (opioid receptor-like 1), and *Odz2* (odd oz/ten-m homolog 2, also known as *Tenm2*) measured by qRT-PCR (logarithmic mean ± SEM) in LE/wt and LE/orl L1-L2 dorsal root ganglia (DRG) ipsilateral to descended (DT) or undescended (UDT) testes. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. LE/wt; $^{II}p < 0.01$ vs. LE/wt; $^{\#\#}p < 0.01$ vs. LE/orl-UDT by ANOVA; $n = 5$–10 per group, postnatal day 3 DRGs.

dorsal root ganglia (DRG) of postnatal LE/orl rats, particularly ipsilateral to cryptorchid testes (**Figure 1B**). These data suggest that altered development of an integrated system comprising Leydig cells, sensory nerves and the gubernaculum together augment the risk of cryptorchidism in LE/orl rat fetuses. Yet even with apparent defects at multiple levels, at least half of all LE/orl testes descend normally. Moreover, we must be cautious when dealing with the potential complexities of gene-environment interaction, and the anatomical and contextual differences between humans and other mammals. The levels of endocrine disrupting chemicals (EDC) required to cause cryptorchidism in experimental animals are much higher than the typical range of human exposures; yet genetic heterogeneity and the combined effects of multiple environmental influences may put some boys at increased risk. The complexity of the spontaneous LE/orl rat model of cryptorchidism may provide insight into the complexity of cryptorchidism in humans.

THE ETIOLOGY OF HUMAN CRYPTORCHIDISM IS COMPLEX, AND LIKELY ASSOCIATED WITH GENETIC AND ENVIRONMENTAL FACTORS

Subtle Leydig cell dysfunction, characterized by increased variance in INSL3 levels (52, 53) and hence increased risk for susceptibility to other factors, and reduced testosterone/luteinizing hormone (T/LH) ratio (54–56), may occur in boys with cryptorchidism. It is unclear if these defects are primary or secondary, genetic or environmental. Our genome-wide association study (GWAS) of cryptorchidism identified many suggestive signals, but none surpassed the genome-wide significance threshold (57–59), typical of a polygenic disorder. Pathway analysis of suggestive intragenic signals showed enrichment of genes encoding proteins involved in cytoskeletal functions, including known or predicted ARIPs. Thus, complementary human and rat data suggest that cryptorchidism susceptibility is heterogeneous, multilocus and potentially multifactorial.

RBFOX PROTEINS MAY FUNCTION AS MAJOR REGULATORS OF NEURO-HORMONAL SIGNALING IN TESTICULAR DESCENT AND CONTRIBUTE TO CRYPTORCHIDISM SUSCEPTIBILITY

Recently, we compared GWAS data from non-syndromic cryptorchidism cases vs. controls (57) and from men with TGCT with or without a history of cryptorchidism vs. controls, and discovered suggestive signals in 19 genes, including *RBFOX1* and *RBFOX3*, paralogs that encode RNA-binding proteins (RBPs) (60). We found that predicted RBFOX targets are strongly enriched among developmental or differentially expressed Leydig cell- and gubernaculum-specific transcripts.

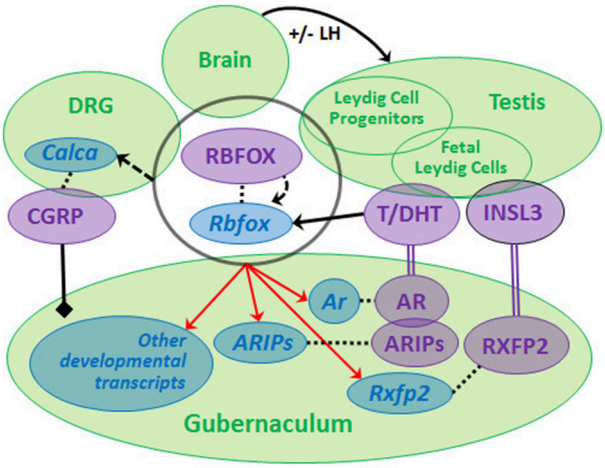

FIGURE 2 | We propose that a neuro-hormonal signaling network regulates testicular descent via direct and indirect interactions among multiple target tissues (green circles). Development of the gubernaculum requires androgen (T, DHT) and insulin-like 3 (INSL3) binding, respectively, to RXFP2 and AR (together with AR-interacting proteins, ARIPs), and regulation by calcitonin gene-related peptide (CGRP). LH regulates Leydig cells during later phases of gestation. Rbfox/RBFOX transcripts/proteins are expressed (overlapping black circle) at all levels of the system with evidence for autoregulation (curved dashed arrow) and for regulation (solid straight arrow) by androgens [data from (46, 60), Barthold et al., unpublished]. RBFOXs regulate alternative splicing of *Calca* (dashed straight arrow) in dorsal root ganglia (DRG) to generate CGRP, which is released by sensory nerves innervating the gubernaculum (solid black line/diamond). *Ar, Rxfp2* and other developmental transcripts are predicted experimental and/or computational RBFOX targets (as denoted by red arrows; http://lulab.life.tsinghua.edu.cn/postar/). Androgens and INSL3 (20, 21) and possibly CGRP regulate other developmental transcripts in the fetal gubernaculum (not shown). Transcripts (blue italic) and proteins (purple, capital letters) are linked by dotted lines.

The RBFOX proteins have relevant functions that include sex determination (61) and alternative splicing of *Calca* to produce CGRP (62). *Rbfox1* and *Rbfox2* are expressed in the rat fetal gubernaculum and L1-L2 DRGs, which produce the CGRP needed for gubernacular development, and *Rbfox2* expression is increased by DHT (data not shown). Based on these observations, we hypothesize that a neuro-hormonal RBFOX-AR-INSL3-CGRP signaling network regulating testicular descent may exist (**Figure 2**). We base this model on the neuro-hormonal data from rodent models, and these novel human genetic data suggesting a role for RBFOX genes in cryptorchidism susceptibility. Together, the human and rat data suggest that RBFOX family genes expressed in gubernaculum, testis and DRG (**Figure 2**) may regulate themselves and each other, playing complex roles in post-transcriptional regulation of CGRP, hormone receptor and/or other developmental molecules. The RBFOX family may therefore connect the hormonal and neural components of this complex network. Genetic variation impacting this network may interact (locally and/or systemically) with adverse effects of environmental endocrine disrupting chemicals (EDCs), augmenting susceptibility to cryptorchidism.

CONCLUDING SYNTHESIS

A feature of cryptorchidism which we need to take into account is that its etiology is primarily occurring in the fetus at a time shortly after sex determination when hormonal regulation is largely via local diffusion-based processes, and not by systemic circulation-borne events (52). This probably accounts for the preponderance of unilateral, as opposed to bilateral cryptorchidism and the prevalence of ipsilateral rather than general associations between factors. Localized regulatory networks such as we describe here, which may become differentially susceptible through increased variance (statistical "noise") to a range of environmental or possibly epigenetic effects, are unlikely to reveal causality in single elements (genes or hormones) especially when using insensitive and systemic methodological approaches. Moreover, such localized and complex networks are linked to a highly dynamic and irreversible pathway of events, making them increasingly prone to stochastic/serendipitous localized influences, or dosage effects.

The complexity of such pathways (**Figure 2**) could explain the general failure to identify specific genes or EDCs associated with clinical cryptorchidism. Such data inform our perspective and underscore the need for a broader approach, utilizing systems biology and predictive modeling, to increase the likelihood of identifying both the causes and consequences of cryptorchidism.

ETHICS STATEMENT

This study was carried out in accordance in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The protocol was approved by the Nemours Animal Care and Use Committee (ACUC).

AUTHOR CONTRIBUTIONS

JB collected and analyzed original data, and JB and RI wrote the manuscript.

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Molecular Mechanisms of Syndromic Cryptorchidism: Data Synthesis of 50 Studies and Visualization of Gene-Disease Network

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Background: Cryptorchidism is one of the most frequent congenital birth defects in male children and is present in 2–4% of full-term male births. It has several possible health effects including reduced fertility, increased risk for testicular neoplasia, testicular torsion, and psychological consequences. Cryptorchidism is often diagnosed as comorbid; copresent with other diseases. It is also present in clinical picture of several syndromes. However, this field has not been systematically studied. The aim of the present study was to catalog published cases of syndromes which include cryptorchidism in the clinical picture and associated genomic information.

Methods: The literature was extracted from Public/Publisher MEDLINE and Web of Science databases, using the keywords including: syndrome, cryptorchidism, undescended testes, loci, and gene. The obtained data was organized in a table according to the previously proposed standardized data format. The results of the study were visually represented using Gephi and karyotype view.

Results: Fifty publications had sufficient data for analysis. Literature analysis resulted in 60 genomic loci, associated with 44 syndromes that have cryptorchidism in clinical picture. Genomic loci included 38 protein-coding genes and 22 structural variations containing microdeletions and microduplications. Loci, associated with syndromic cryptorchidism are located on 16 chromosomes. Visualization of retrieved data is presented in a gene-disease network.

Conclusions: The study is ongoing and further studies will be needed to develop a complete catalog with the data from upcoming publications. Additional studies will also be needed for revealing of molecular mechanisms associated with syndromic cryptorchidism and revealing complete diseasome network.

Keywords: candidate genes, comorbidity, cryptorchidism, diseasome, syndrome, undescended testes, systems biology, biological network

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INTRODUCTION

Cryptorchidism or undescended testes is characterized as the failure of one (unilateral) or both (bilateral) testes and associated structures to descend from retroperitoneal abdomen to their normal position in scrotal sac during fetal development (1). It is one of the most frequent congenital birth defects in male children and is present in 2–4% of full-term male births (2). It has also been reported

that cryptorchidism may develop after infancy, in some cases as late as young adulthood, but that is exceptional and was observed in only about 1–3% of male children (3). Around four out of five cases of cryptorchid testes descend by the first year of life (the majority within first 3 months) on their own (4).

Men with a history of cryptorchidism have an increased risk of infertility. Outcomes related to infertility include the paternity rate, semen analysis, measurement of serum luteinizing hormone (LH), follicle-stimulating hormone and inhibin B, and testicular size (5). Paternity rates in formerly bilaterally criptorchid men who have attempted to father a child (65.3%) are significantly lower than formerly unilaterally criptorchid men (89.7%) and control men (93.2%). Sperm density and inhibin B levels are lower in the bilateral group whereas follicle-stimulating hormone and luteinizing hormone levels are higher, compared to unilateral and control groups (6).

Hormone treatment with androgens, human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone (LHRH), intended to sufficiently bring testes to a scrotal position, has so far proved to have limited success (7). However, it has been observed that in combination with orchidopexy (a surgical procedure to move undescended testes to scrotum), hormonal treatment significantly increased testosterone levels and the number of adult spermatogonia compared to orchidopexy alone (8). In all cases, orchidopexy in early stages of life is universally recommended since it prevents degeneration of spermatogenic tissue and decrease of spermatogonia which occurs if the issue is not resolved (9).

The second common effect of cryptorchidism is increased risk for testicular neoplasia, specifically seminoma and testicular torsion. About 2–3% of males born with unilateral or bilateral cryptorchidism develop testicular cancer, the risk factor is therefore ∼4–40 times increased (1). Testes descent is a complex, multistage series of events requiring the interaction of several anatomical factors, including normal gubernaculum development, nervous system, and hormonal factors. Testes descent is described in three distinct phases: (1) the abdominal translocation; (2) followed by transinguinal migration; and (3) migration to the bottom of the scrotum. The developmental process, during which testes descend, was subdivided into three phases due to three general locations where non-scrotal testes were found (9).

Abbreviations: KDVS, Koolen de Vries syndrome; DWS, Dandy-Walker syndrome; OKS, Opitz-Kaveggia syndrome (FC syndrome); DDS, Denys-Drash syndrome; AAS, Aarskog-Scott syndrome; TKCR, Torticollis, keloids, cryptorchidism, and renal dysplasia; OCRL, Lowe oculocerebrorenal syndrome; TDS, Testicular dysgenesis syndrome; MEND syndrome, Male EBP disorder with neurologic defects; HJCYS, Hajdu Cheney syndrome; PMDS, Persistant mullerian duct syndrome; BRESEK/BRESHECK, Brain anomalies, retardation, hirschsprung disease, ear/eye anomalies, cleft palate/criptorchidism, and kidney dysplasia/hypoplasia; PPS, Popliteal pterygium syndrome; BWS, Beckwith-Wiedemann syndrome; LEOPARD syndrome, multiple Lentigines, ECG conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormal genitalia, Retardation of growth, and sensorineural Deafness; PBS, prune belly syndrome; MDP syndrome, Mandibular hypoplasia, deafness, progeroid features, lipodystrophy syndrome; OA/TOF, Oesophageal atresia/tracheoesophageal fistula and anal atresia.

In most cases, causes of cryptorchidism usually cannot be determined. It can occur as an isolated disorder in males with no other genital or birth defects, however, it can also appear as co-occurring disorder due to the presence of other congenital anomalies. It is viewed as a complex disorder which according to a high number of studies and research work in the recent years is believed to be direct consequence of combination of several factors such as: (1) environmental factors such as maternal health and chemicals acting as endocrine disruptors; (2) premature birth; (3) genetics causes such as polymorphisms in different genes and chromosomal aberrations; (4) comorbidity due to other abnormalities (2, 9).

Among the factors critically responsible for testicular descent are insulin-like factor 3 (INSL3) and hormones, in particular androgens such as testosterone. Mutations in genes encoding for these hormones or their receptors often lead to the occurrence of cryptorchidism. Insulin-like 3 is a peptide hormone produced by the Leydig cells and its expression is upregulated in the fetal testes and downregulated after birth. Deficiency of this hormone during the development of fetus can cause undescended testes. The role of insulin-like factor 3 is related to its effect on gubernaculum differentiation and development during the transabdominal phase (10). For androgens, it is generally believed that they act on both the cranial suspensory ligament and gubernaculum, normal functionality of the two is needed for regular descent of the testes since they are major mediators of the inguinoscrotal phase. During the transabdominal phase androgens also contribute to and aid the regression of cranial suspensory ligament. Mutations in androgen receptor (AR) gene are a frequent cause of cryptorchidism development. It has been observed that during development from fetus sex differentiation to puberty Sertoli cells produce anti-Müllerian hormone which prevents the development of the Müllerian ducts into the uterus and other Müllerian structures. It also enables normal development of Wolffian ducts, which eventually progress into male reproductive organs. However, the research in case of this particular hormone is still incomplete and inconclusive, therefore its role in cryptorchidism is still controversial (1).

Various genetic loci have been associated with cryptorchidism development, including protein-coding genes, chromosomal mutations, copy number variations and microRNAs (11). Genomic loci are dispersed through an entire genome, therefore a genome wide screening for identification of smallest regions of overlaps in cryptorchidism has been performed. These narrowed regions present candidate regions for further identification of stronger candidates and biomarker development (12).

Testicular maldescent can occur as an isolated event, or as part of a variety of syndromes (syndromic cryptorchidism) and other non-syndromic diseases (non-syndromic cryptorchidism) (13–15). Some syndromes with cryptorchidism in the clinical picture are extremely rare, with only few cases reported worldwide [for example 2p14p15 microdeletion syndrome (16)], while others are quite frequent [for example Beckwith-Wiedemann syndrome (1/13,000)] (1).

Molecular mechanisms for co-presence of symptoms in a syndrome could be explained using systems biology approach.

For example, in our previous study molecular mechanisms underlying co-occurrence of cryptorchidism and cardiovascular diseases in RASopathies have been proposed (17). Foresta et al. reviewed syndromes that include cryptorchidism in clinical picture from OMIM database (1). Cryptorchidism gene database included 32 syndromes with cryptorchidism in clinical picture (11). However, the study field has not yet been systematically studied.

The aim of the present study was therefore to catalog the genetic loci associated with syndromic cryptorchidism. Genomic locations of candidate genes linked to development of syndromic cryptorchidism were visualized on a karyotype view to identify possible genomic hotspots associated with the development of cryptorchidism. The visualization of network consisting of syndromes connected to microdeletions or genes which caused them was also interpreted in systems biology manner as a contribution to growing diseasome map. The aim of the study is also to contribute to the standardization of reporting of congenital disorders and comorbidity defects together with corresponding hereditary causes.

MATERIALS AND METHODS

Candidate genes associated with syndromic cryptorchidism were extracted from published literature as described previously (11). Various keywords were used for identification of syndromic cryptorchidism loci: syndrome, cryptorchidism, undescended testes, loci, microdeletions, and genes. Data from publications were obtained up to March 2018 and manually analyzed. Gene names were updated according to Ensembl genomic browser, release 92 (18). The network revealing connections between candidate genes and disorders was visualized using Gephi (19). Visualization of chromosomes and corresponding abnormalities associated with the development of cryptorchidism was performed using a figure of human karyotype downloaded from Ensembl genomic browser release 92 (18) and javascript programming language. Genomic location (base pairs) of breakpoints of chromosomal regions involved in chromosomal mutations were obtained from UCSC database (https://genome.ucsc.edu/cgi-bin/hgTables).

RESULTS

We retrieved data from PubMed and WoS. After manual elimination there were 50 studies left, from which we harvested data for current study. The catalog of genetic loci of syndromic cryptorchidism consists of 60 genetic loci associated with 44 syndromes that include cryptorchidism in the clinical picture. Among those 60 loci 38 were protein-coding genes and 22 of those loci were structural variations including microdeletions and microduplications. Among candidate genes residing within chromosomal locations the genes which were discussed or functionally analyzed to be associated with the development of a syndrome (or cryptorchid phenotype, if possible) in reviewed publications were cataloged and visualized in an interaction network.

Catalog of Genetic Loci Associated With Syndromic Cryptorchidism

Data were retrieved from PubMed and WoS. Study types of obtained publications including relevant genomic information were performed using different study approaches including: case reports, association, and functional studies, genome-wide and single locus studies, and different omics types. Syndromes that include cryptorchidism in clinical picture were reported to be associated with protein-coding genes and chromosomal mutations. **Table 1** includes protein coding genes associated with syndromic cryptorchidism, which are alphabetically ordered by their locus names. Proteins, encoded by the genes listed include enzymes (BRCC3), hormones (AMH), transcription inhibitors (ANKRD11), inhibitors of enzymes (CDKN1C), transmembrane receptors (RET), and many other types and subtypes of regulatory proteins. **Table 2** includes chromosomal mutations associated with syndromic cryptorchidism. Chromosomal mutations include microdeletions or microduplications, of various size ranging from 3.5 to 43.7 Mb. In some cases chromosomal mutations were associated with candidate genes, in total 8 possibly responsible for cryptorchidism phenotype. Each row in the catalog represents a genetic origin of a syndrome, containing gene or cytogenetic location of mutation, deletion or duplication, name of a syndrome, DOID (Disease ontology ID if available), reference of a publication in which the connection to cryptorchidism was proposed and PMID (PubMed ID) or OMIM ID. Chromosomal mutations are ordered by the chromosome number. The systematic approach enables future researchers to use this manually checked data in further studies more efficiently.

Genetic Distribution of Loci Associated With Syndromic Cryptorchidism

Genomic location of loci linked to syndromes are presented in the karyogram view in **Figure 1** which shows microdeletions and microduplications with blue lines and genes with red dots. Loci, associated with syndromic cryptorchidism are located on 16 chromosomes. Number of loci, associated with syndromic cryptorchidism per chromosome are shown in **Supplementary Table 1**. The highest number of loci, associated with syndromic cryptorchidism are located on chromosome X ($n = 16$) (for example Lenz dysplasia, X-linked Kallmann syndrome, and Aarskog-Scott syndrome) and chromosome 1 ($n = 8$) (for example Noonan syndrome, TDS syndrome, and Meier-Gorlin syndrome. Visualization of loci on a karyotype view revealed regions of overlaps, where multiple microdeletions coincide on one chromosome to produce single phenotype output, or causative genes located within chromosomal mutation. Analysis revealed that candidate gene *BRCC3* coincides with genetic loci recognized as a causative for TKCR syndrome. *BRCC3* gene is also considered causative for 2p15p16.1 microdeletion syndrome's cryptorchidism as there was a male case studied which, apart from microdeletion on chromosome 2, also had deletion on X chromosome in q28 region (55). Furthermore, the gene

TABLE 1 | Protein-coding genes, associated with syndromes with cryptorchidism in clinical picture.

Locus name	Chromosome	Name of the syndrome	Disease ontology ID (DOID) for the syndrome	References	PMID (or OMIM ID)
AMH	19	Persistent Müllerian duct syndrome (PMDS)	0050791	(20)	25026127
				(21)	28742509
ANKRD11	16	KBG syndrome	14780	(22)	21782149
ANOS1	X	Kallmann syndrome	3614	(23)	18160472
ATRX	X	Smith-Fineman-Myers syndrome	NA	(24)	10751095
BMP7	20	TDS syndrome	NA	(25)	22140272
BRAF	7	Cardiofaciocutaneous syndrome	60233	(26)	22190897
CDC6	17	Meier-Gorlin syndrome	0060306	(27)	22333897
CDKN1C	11	Beckwith-Wiedemann Syndrome	5572	(28)	20503313
CDT1	16	Meier-Gorlin syndrome	0060306	(27)	22333897
CHD7	8	CHARGE syndrome	0050834	(29)	17661815
				(30)	25606431
CHD7	8	Kallmann syndrome	3614	(31)	19021638
CHRM3	1	Prune Belly syndrome	0060889	(32)	22077972
EBP	X	MEND syndrome	NA	(33)	20949533
FGD1	X	Aarskog– Scott syndrome (ASS)	6683	(34)	23443263
GFRA1, RET	10	CAKUT	NA	(35)	22729463
HRAS	11	Costello syndrome	50469	(26)	22190897
				(36)	19213030
ICR1	11	Beckwith-Wiederman syndrome	5572	(37)	21863054
IRF6	1	Popliteal pterygium syndrome	0060055	OMIM (38)	119500
					12219090
KRAS	12	Noonan syndrome	3490	(39)	16474405
MAP2K2	19	Cardiofaciocutaneous syndrome	0060233	(40)	23885229
MBTPS2	X	BRESEK/BRESHECK	NA	(41)	22105905
MED12	X	Opitz-Kaveggia syndrome (OKS) (FC syndrome)	14711	(42)	17334363
MEGF8	19	Carpenter syndrome	0060234	(43)	23063620
NOTCH2	1	Hajdu-Cheney syndrome(HJCYS)	2736	(44)	25696021
NSD1	5	Sotos syndrome	14748	(45)	15942875
OCRL	X	Lowe oculocerebrorenal syndrome (OCRL)	1056	(46)	24778696
ORC1	1	Meier-Gorlin syndrome	0060306	(27)	22333897
ORC6	16	Meier-Gorlin syndrome	0060306	(27)	22333897
POLD1	19	Mandibular hypoplasia, deafness, progeroid features, lipodystrophy syndrome (MDP syndrome)	NA	(47)	23770608
PROK2	3	Kallmann syndrome	3614	(48)	18559922
PROKR2	20	Kallmann syndrome	3614	(48)	18559922
PTPN11	12	Noonan syndrome	3490	(26)	22190897
PTPN11	12	LEOPARD syndrome	14291	(49)	18505544
RAB23	6	Carpenter syndrome	0060234	(50)	20358613
RAB3GAP2	1	Martsolf syndrome	NA	(51)	16532399
RAF1	3	Noonan syndrome	3490	(40)	23885229
RAF1	3	LEOPARD syndrome	14291	(49)	18505544
RIT1	1	Noonan syndrome	3490	(52)	25124994
SEMA3A	7	Kallmann syndrome	3614	(53)	22416012
SOS1	2	Noonan syndrome	3490	(26)	22190897
TGFBR3	1	TDS syndrome	NA	(25)	22140272

NA, not available.

is also recognized as causative for MOYAMOYA disease which has wide range of testicular abnormalities in clinical picture (74).

As can be observed in **Table 1**, there are many different genetic causes associated with one syndrome. In Carpenter syndrome, for instance, there are two genes, *MEGF8* located

TABLE 2 | Chromosomal regions, associated with syndromes with cryptorchidism in clinical picture.

Chromosome	Locus name; chromosomal region and proposed candidate genes	Name of the syndrome	Disease ontology ID (DOID) for the syndrome	References	PMID (or OMIM ID)
1; 10	NOTCH2; 46,XY,del(10)(q25.3-q26.13)	Persistent Müllerian duct syndrome (PMDS) with distal monosomy 10q	0050791	(54)	25820398
1	1p22–p21	Zellweger syndrome	905	(2)	15136137
2	del(2p14p15)	2p14p15 microdeletion syndrome	NA	(16)	23266801
2; X	46,XY,del(2)(p15p16.1); del(X)(q28), BRCC3***	2p15p16.1 microdeletion syndrome	0060415	(55)	22406401
3	3p22–p26	Fanconi anemia	13636	(2)	15136137
7	46,XY,dup(7p21)	7p22.1 microduplication syndrome	NA	(56)	21998864
				(57)	25124455
				(58)	25893121
8	46,XY,del(8)(q23q24)pat	Langer-Giedion syndrome (LGS) or Trichorhinophalangeal syndrome (TRPS) type II	4998	(59)	26269715
10	del(10q25.3q26.3), WDR11*,PLPP4*(PPAPDC1A)	10q deletion syndrome	NA	(60)	14598339
				(61)	18661548
				(62)	19253379
				(63)	19558528
				(64)	26114870
11	11p13	Denys–Drash syndrome (DDS)	3764	(2)	15136137
11	11p13	Genitourinary dysplasia- component of WAGR	NA	(2)	15136137
15	del(15q25.2), RPS17	15q25 deletion syndrome	0060396	(65)	20921022
15	15q11q13	Prader–Willi syndrome	11983	(2)	15136137
16	16p13	Rubinstein–Taybi syndrome	1933	(2)	15136137
17	del(17q21.31), KANSL1**	Koolen de Vries syndrome (KDVS)	0050880	(66)	18628315
				(67)	26306646
17	dup(17p12)	Oesophageal atresia/tracheoesophageal fistula and anal atresia	0080171	(68)	24239950
X	Xp21.1	Aarskog– Scott syndrome (ASS)	6683	(2)	15136137
X	Xq25–q27	Dandy–Walker syndrome (DWS)	2785	(2)	15136137
X	Xq28	Frontometaphyseal dysplasia	NA	(2)	15136137
X	(Xq27q28),ANOP1	Lenz dysplasia	NA	(2)	15136137
X	(Xp11.4p21.2), BCOR(ANOP2)	Lenz dysplasia	NA	(2)	15136137
X	Xq26.1	Lowe oculocerebrorenal syndrome (OCRL)	1056	(2)	15136137
X	Xq12–q21.31	Opitz-Kaveggia syndrome (OKS) (FG syndrome)	14711	(2)	15136137
X	Xq28	Torticollis, keloids, renal dysplasia, cryptorchidism (TKCR)	NA	(2)	15136137

NA, not available.

**genes were determined via shortest region of overlap.*

***a SNP in KANSL1 or del(17q21.31) is sufficient to cause KDVS.*

****the only gene on del(X)(q28) with an intragenic loss (4 exons).*

on chromosome 19 (43), and *RAB23* located on chromosome 6 (50), malfunctions of each of them was associated with cryptorchidism. Interestingly, for Kallmann syndrome, five different genes were found to cause cryptorchidism, each of which is located on separate chromosomes: *PROK2* on chromosome 3 (48), *SEMA3A* on chromosome 7 (53), *CHD7* on chromosome 8 (31), *PROKR2* on chromosome 20 (48), and *ANOS1* on X chromosome (23). Such diversity is difficult to notice when data are scattered across different references, but come to attention when summarized in one analysis.

Visualization of a Gene-Disease Network View

Cryptorchidism often appears together with several other diseases and disorders or it is present in clinical picture of syndromes. Syndromes associated with cryptorchidism are

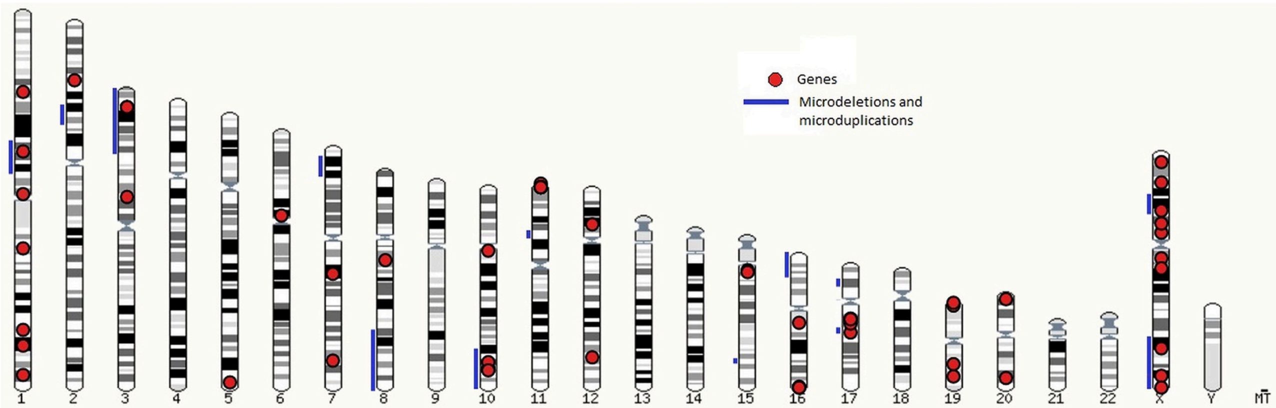

FIGURE 1 | A karyotype view with loci associated with syndromic cryptorchidism in human.

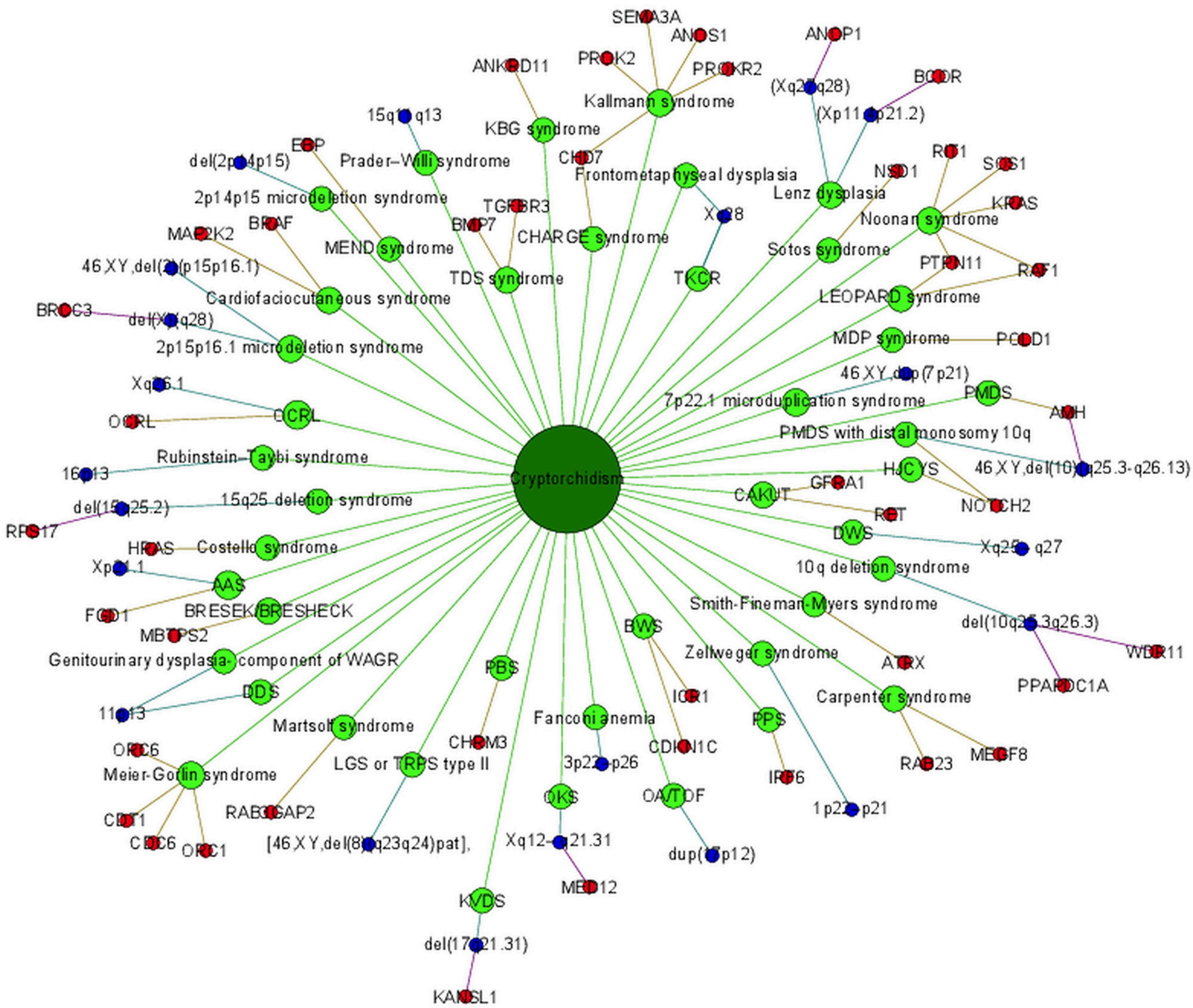

FIGURE 2 | Portrayal of cryptorchidism associated syndromes and genetic loci.

presented in **Figure 2**. **Figure 2** presents all 44 syndromes associated with cryptorchidism connected to 60 candidate genetic loci which include 38 genes and 22 chromosomal mutations. Some studies reporting chromosomal mutations associated with syndromic cryptorchidism additionally performed functional analysis or discussed one or more candidate genes potentially causing the syndrome. From those studies we extracted 8 genes which were proposed to contribute to development of cryptorchidism. The **Figure 2** therefore includes 22 chromosomal mutations and 46 (38 + 8) genes associated with syndromes. Visualization presents the presence of cryptorchidism in several syndromes such as KBG syndrome, Kallmann syndrome, Noonan syndrome and Lenz dysplasia. Altogether there are 11 syndromes which are connected to at least one other syndrome via eight different genetic locations. For example, LEOPARD syndrome and Noonan syndrome, belonging to RASopathies, share several phenotypic features: characteristic facies, congenital heart defects, delayed development, and cryptorchidism for which the molecular causes are heterozygous mutations in various exons of *RAF1* and *PTPN11* (26, 40, 49). Likewise, as reported in a study by (31) features of Kallmann syndrome, hypogonadotrophic hypogonadism and anosmia or hyposmia are often present in patients with CHARGE syndrome for which the molecular cause is a mutation in *CHD7* as can be seen in **Figure 2**. Figure also revealed a great heterogeneity of associations in the molecular syndromology field. For example, a locus can be associated with: (1) a syndrome with cryptorchidism in the clinical picture, (2) can be a genetic cause for the development of a syndrome, but the patient does not have cryptorchidism in the clinical picture, or (3) a locus can be associated with another disease, which is not a syndrome. Examples of such heterogeneity of genotype-phenotype are presented in **Table 3** (2, 23, 31, 48, 53).

TABLE 3 | Example of heterogeneity of genotype-phenotype relations in association with cryptorchidism.

Locus	Disease	Cryptorchidism present	References	PMID
SEMA3A	Kallmann syndrome	yes	(53)	22416012
ANOS1	Kallmann syndrome	yes	(23)	18160472
CHD7	Kallmann syndrome	yes	(31)	19021638
CHD7	Kallmann syndrome	no	(31)	19021638
CHD7	CHARGE syndrome	yes	(31)	25606431
PROK2	Kallmann syndrome	yes	(48)	18559922
PROKR2	Kallmann syndrome	yes	(48)	18559922
Xp22.31	Kallmann syndrome	yes	(2)	15136137

DISCUSSION

The etiology of cryptorchidism is complex and has yet to be fully determined. Current evidence indicates that there are several factors that cause the disease, among which the most important ones are genetic and environmental factors (9, 17). If these elements significantly deviate from the ones seen in unaffected males, there is a high probability that cryptorchidism will appear. The results of research and literature mining have led us to conclusion that there is a vast quantity of seemingly unrelated syndromes co-occurring with cryptorchidism which are just being discovered and that specialized databases for syndromic cryptorchidism, do not exist yet.

Retrieving information of relevant genes from chromosomal mutations regarding cryptorchidism is a challenging task as propositions of candidate genes are presented in various ways. Some publications only listed a chromosomal location [for example 11p13 in Denys-Drash syndrome (2). Choucair et al. (64)] determined candidate *WDR11* and *PLPP4* (previous symbol *PPAPDC1A*) genes for genital anomalies development in patients with 10q deletion syndrome by smallest regions of overlap. The largest chromosomal mutation is present in Fanconi anemia, which extends over 43.7 Mb (2) and according to the latest version of the genomic browser Ensembl it includes 213 protein-coding genes. A study by Wat et al. (65) considered a role of *RPS17* (ribosomal protein S17) in 15q25.2 deletion syndrome. According to Ensembl genomic browser, this region extends is 3.5 Mb and includes 26 protein-coding genes. Within some microdeletion syndromes where candidate genes were not discussed causative genes for the occurrence of the syndrome can be found in OMIM database. For example, Prader-Willi syndrome in a study by Klonisch et al. (2) did not include candidate genes. Further investigation of OMIM database revealed that *NDN* and *SNRPN* are listed as candidate genes for Prader-Willi syndrome and that cryptorchidism is present frequently in the clinical picture of this syndrome. However, our study only includes cases in which genetic loci were associated with a syndrome and a patient also had diagnosed cryptorchidism. Such scattered information makes it very challenging to perform effective scientific research and the vast heterogeneity of genotype-phenotype relation which is why a standardized database of gene-disease interactions is necessary.

Investigation of comorbidity of diseases and genetic associations, as well as developing networks of disorders such as the Diseasome (69), allows elucidation of genotype-phenotype associations between several diseases that may have common genetic origin. The relevance of protein-protein interactions was examined in a study by Cannistraci et al. (17), where such interactions revealed the relation between co-presence of cardiomyopathy and cryptorchidism; two symptoms of the RASopathies. The study by Pevec et al. (70) also provided a baseline for future studies of associations between interactome and phenome in RASopathies, additionally presenting data at genome, interactome, and phenome levels and as an integrated network of all three data types.

Malfunctioning genes may result in seemingly unconnected phenotypes in different tissues which could hide the actual molecular background of such disease. Clustering on the basis of phenotypic similarities represents true biological relationships of the genes involved—we may then use such similarities to prioritize potential disease genes and make predictions

of a certain phenotype (71). The visualizations presented in the current study could be seen as a tool for better and more perceivable understanding of connection between genotype and phenotype. Some complex interaction between genotype and phenotype have been published, for example, the TKCR syndrome (Torticollis, keloids, cryptorchidism, and renal dysplasia), which is caused by deletion of Xq28 (72) coincides with genomic coordinates of a *BRCC3* gene. Protein product of this gene is a part of a holoenzyme complex BRCC, which has a role in DNA repair mechanisms (73). This gene has been also mutated in a patient which had two diagnosed chromosomal mutations; 2p15p16.1 and Xq28 (55). Furthermore, *BRCC3* gene is also connected to MOYAMOYA disease (74) which also has similar symptoms including short stature, hypergonadotropic hypogonadism, facial dysmorphism, and in some cases decreased testicular volume and azoospermia (75). There are few candidates, for instance NOTCH2 and KANSL1, which play an important role in regulatory pathways, expressed in testicular cells. NOTCH2 is a membrane receptor protein with extracellular domain and an intracellular domain, which both play an important role in controlling cell fate decision pathways (76). Similarly, KANSL1 is a nuclear protein which has an important role in chromatin modifications as it is a part of a multiprotein complex that works as a histone acetylase (77). Both appear to be essential for proper cell differentiation during testicular development, although there has been no reported case of cryptorchidism caused just by mutation in *NOTCH2* gene, but it's contribution to reported, more complex cases, cannot be excluded (54). Additionally, *RPS17* encodes a protein subunit of ribosome, which is recognized as causative for Diamond-Blackfan anemia, but it has frequently coincided with syndromic cryptorchidism as well (65). A visualization tool simplifies search for causing genes and contributes to our molecular understanding of microdeletion syndromes. Our approach leads the integration of syndromic phenotypical abnormalities in the field of systems biology.

There are other syndromes which, according to a study by Foresta et al. (1) and OMIM database have cryptorchidism in the clinical picture, and also loci, where causative genes are not yet identified. That is why there is still possibility for further investigation that might lead us to understanding of novel signaling pathways involved in human development. As data is limited to few specific cases it is crucial that one extrapolates and includes new knowledge in systems of known data and therefore slowly unveil full picture behind complex genetic complications in human diseases. Various clinical subtypes of cryptorchidism exist, including unilateral, bilateral, syndromic, non-syndromic, comorbid, thus further studies are required to additionally clarify the role each particular gene contributes to different cryptorchid phenotypes. We must also not neglect the contribution of post transcriptional modification and epigenetic modification such as methylation and acetylation to development of different types of cryptorchidism.

As shown in **Table 2** based on the collected data there are genomic hotspots on chromosomes X and 1 which have the highest number of genes associated with syndromic cryptorchidism. With knowledge of causative genes and other genetic loci one might develop a genetic therapy or hormonal treatment that would counteract the malfunctioning gene (78). Since the majority of genes are positioned on the X chromosome, genetic tests could be developed to consider applying treatment in advance of pregnancy and therefore counteract potential development of monogenic syndrome or at least minimize the symptoms.

The results of the present study contribute to the development of molecular syndromology field and understanding of associations between genome and phenome—Diseasome. The developed database and results of this study also contribute to better understanding of the genetic causes of cryptorchidism and associated comorbid syndromes. The developed protocol could now be also applied to other multifactorial traits and diseases. Further studies of the linkage between genetic variations and phenotype could contribute to the development of specific markers, which would in turn enable the diagnosis of the disease early during fetal development. Thus, better understanding of the causes for the disorder could also help to elucidate the complete mechanism that occurs during the development of testicular tissue—a process that is currently not completely understood.

EXECUTIVE SUMMARY

- Cryptorchidism is one of the most common genital defects in men which causes infertility, testicular neoplasia, psychological damage, and other health defects.
- Cryptorchidism is often comorbid with other symptoms and syndromes associated with chromosomal mutations and genetic variations.
- We have extracted data from WoS and PubMed and cataloged 60 genetic variants reported to be associated with syndromic cryptorchidism.
- Variants associated with syndromic cryptorchidism are heterogeneous and include 38 protein-coding genes and 22 structural variants (deletions, microdeletions, and microduplications). Using systems biology approach we visualized a biological network of genes connected to syndromes which present a baseline for further identification of novel candidate genes for cryptorchidism.
- Research methods and network visualization used in present study could be further extended to develop a complete diseasome and lead to systemic understanding of gene-phenotype relationship.
- The progress of the project was hampered by heterogeneous data presentation in published reports, therefore we call for standardization of gene and phenotype terminology in future publications. Independent validation in a larger number of cases will also be needed.

AUTHOR CONTRIBUTIONS

KU, MH, ŽK and LS gathered data, conducted data analysis, and drafted the manuscript, MH and ŽK conducted graphical

representation. TK designed and coordinated the study, PD and TK revised the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00425/full#supplementary-material

Supplementary Table 1 | The number of genetic loci associated with syndromic cryptorchidism according to chromosome location.

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Regional Variation in Androgen Receptor Expression and Biomechanical Properties May Contribute to Cryptorchidism Susceptibility in the LE/orl Rat

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Background: The process of testicular descent requires androgen and insulin-like 3, hormones secreted by fetal Leydig cells. Knowledge concerning distinct and common functions of these hormones in regulating development of the fetal gubernaculum remains limited and/or conflicting. The current studies were designed to better define characteristics of androgen receptor (AR) expression, function and regulation, as well as the biomechanical properties of normal and cryptorchid gubernaculum during fetal development.

Methods: We studied fetal gubernacula from Long Evans outbred (LE/wt) rats and an inbred (LE/orl) strain with an inherited form of cryptorchidism associated with an AR signaling defect. Gubernacular cells or whole organs obtained from LE/wt and LE/orl fetal gubernacula underwent AR immunostaining and quantitative image analysis. The effects of dihydrotestosterone (DHT) on AR expression, muscle fiber morphology, hyaluronan (HA) levels and glycosaminoglycan (GAG) content were measured in LE/wt gubernacula. Finally, the spatial mechanics of freshly harvested LE/wt and LE/orl fetal gubernacula were compared using micropipette aspiration.

Results: AR is expressed in the nucleus of mesenchymal core, tip and cord cells of the embryonic (E) day 17 and 21 fetal gubernaculum, and is enhanced by DHT in primary cultures of gubernacular mesenchymal cells. Enhanced AR expression at the tip was observed in LE/wt but not LE/orl gubernacula. In *in vitro* studies of whole mount fetal gubernaculum, DHT did not alter muscle fiber morphology, HA content or GAG production. Progressive swelling with reduced cellular density of the LE/wt gubernaculum at E19–21 was associated with increased central stiffness in LE/wt but not in LE/orl fetuses.

Conclusions: These data confirm nuclear AR expression in gubernacular mesenchyme with distal enhancement at the tip/cord region in LE/wt but not LE/orl rat fetuses. DHT enhanced cellular AR expression but had no major effects on muscle morphology or matrix composition in the rat fetal gubernaculum *in vitro.* Regional increased stiffness and decreased cell density between E19 and E21 were observed in LE/wt but not LE/orl fetal gubernacula. Developmental differences in cell-specific AR expression in LE/orl fetal gubernacula may contribute to the dysmorphism and aberrant function that underlies cryptorchidism susceptibility in this strain.

Keywords: gubernaculum, cryptorchidism, androgens, glycosaminoglycans, hyaluronan, myogenesis, microaspiration, biomechanics

INTRODUCTION

Cryptorchidism or undescended testis, is the most common reproductive congenital anomaly (1, 2). Testicular descent normally occurs prior to birth in humans and requires developmental programming of the fetal gubernaculum, an organ comprising a central core of mesenchyme infiltrated and surrounded by cremaster muscle. Studies in rodents indicate that development and function of the fetal gubernaculum are regulated by the Leydig cell hormones insulin-like 3 (INSL3) and androgens via their receptors, relaxin/insulin-like family peptide receptor 2 (RXFP2) and the androgen receptor (AR).

Despite a wide range of studies in mammalian models, not all aspects of the hormonal control of testicular descent are fully understood. A prevailing view, first proposed by Hutson over 30 years ago (3), defines descent as a process that occurs in two separate stages, Phase 1/transabdominal, and Phase 2/transinguinal descent, each regulated by distinct hormones. Transgenic studies confirm that cryptorchidism is present in mice with conditional ablation of either *Insl3, Rxfp2* or *Ar* (the GU-ARKO mouse) in the gubernaculum (4, 5). However, the effects of knockdown or deletion of *Insl3* or *Rxfp2* are more profound than those of *Ar* deletion on gubernacular swelling and testicular descent. With partial knockdown of *Rxfp2,* the murine fetal gubernaculum is disorganized, with aberrant muscle cells in the mesenchymal core, while *Rxfp2* or *Insl3* deletion results in an atrophic and disorganized fetal gubernaculum (4, 6–9). In contrast, cryptorchidism in the GU-ARKO mouse is associated with more subtle gubernacular defects (5) that include abnormal cremaster muscle development, which was also reported in the *Tfm* mouse, a strain with an *Ar*-inactivating mutation (10).

The role of androgens in gubernacular development and the target(s) of androgen action in testicular descent remain controversial. Hutson et al. reported absence of AR expression within the fetal gubernaculum, and hypothesized that prenatal androgens act indirectly via masculinization of the genitofemoral nerve (GFN) (11). Most recently they have proposed that androgens exert indirect effects on tissues extrinsic to the gubernaculum, including stimulation of AR-expressing cells in the inguinoscrotal fat pad and repression of the mammary branch of the GFN (12, 13). In contrast, other investigators have observed AR expression in fetal rodent gubernacular mesenchyme (14–16).

The functional role(s) of AR in testicular descent, in addition to cellular proliferation (17, 18), have been hypothesized to include myogenesis and regulation of extracellular matrix (ECM) composition, although there is little direct evidence in support (4, 5). ECM production is likely important in determining the size and biomechanical properties of the gubernaculum. Additionally, there is supportive evidence of a role for the neuropeptide calcitonin gene-related peptide (CGRP) release by gubernacular sensory nerves, providing non-hormonal stimulation of cellular proliferation and motility in the gubernaculum (11). Studies of rat gubernacula exposed to antiandrogens and of *Tfm* mouse gubernacula suggest a role for neuro-hormonal interaction between androgens and CGRP in the process of testicular descent (19, 20).

To more clearly define the role of AR in gubernacular development, we studied the effects of dihydrotestosterone (DHT) stimulation on AR expression, muscle fiber morphology, and ECM composition, and we compared the pattern of AR expression and the biomechanical properties of the gubernaculum in outbred and LE/orl fetal rats with evidence for impaired AR signaling (21, 22). Prior work has shown the inbred LE/orl strain is susceptible to cryptorchidism, with only 45% of LE/orl fetuses exhibiting bilateral descent (23). Our data suggest that nuclear AR is expressed in a subset of gubernacular mesenchymal cells, particularly within the tip/cord region, and that androgen regulates expression but not muscle size or ECM production in the fetal rat gubernaculum. Although our data suggest that ECM composition is not altered in the LE/orl fetal gubernaculum, we found regional differences in AR expression, cellular density and organ stiffness that are consistent with previous evidence of dysmorphology and may contribute to gubernacular malfunction in this strain.

MATERIALS AND METHODS

Animals and Tissue Samples

We obtained fetal gubernacula from timed-pregnant Long-Evans wild-type (LE/wt) and LE/orl (an LE substrain with inherited cryptorchidism) males by microdissection during Phase 1 (E17 and E19) and Phase 2 (E21) of testicular descent, at the onset of gubernacular inversion. Samples obtained from $\geq$3 fetuses and $\geq$2 separate litters were immediately processed for each

experiment. All work was conducted under a protocol approved by the Nemours Institutional Animal Care and Use Committee (#NBR-2016-002).

AR Expression in Fetal Gubernaculum

For tissue clearing we used the benzyl alcohol:benzyl benzoate (BABB) protocol (24) prior to confocal imaging, as described previously (21). Immediately after harvesting, fetal gubernacula were fixed in freshly prepared 4% paraformaldehyde/phosphate buffered saline (PBS) and incubated overnight at 4°C. The gubernacula where then placed in 100% methanol for 1 h on ice followed by storage at −20°C. Samples were then treated with Dents bleach, blocked and incubated overnight with a 1:10 dilution of A4.1025, a myosin-specific mouse monoclonal antibody (Developmental Studies Hybridoma Bank) and 1:200 dilution of a rabbit anti-AR antibody (Abcam cat# ab133273) which we have validated by Western blot to recognize the full length AR protein (data not shown). Gubernacula were then incubated overnight at 4°C, washed 4X with PBS Tween (PBST) and then incubated overnight with donkey anti-rabbit Alexaflour 555 (ThermoFisher Cat#A31572) and DRAQ5 (Themofisher Cat#62254) as a counterstain for nuclei. Gubernacula were then washed 3X for 20 min in PBST and then exposed to 100% methanol on ice and stored at 4°C prior to imaging. Just before imaging, gubernacula were cleared by incubation in BABB (1:2). The LSM880 confocal microscope with 25X oil objective was used to create an image z-stack, with images viewed sequentially to visualize 3-dimensional AR expression within the gubernaculum. Representative single images were in the central sagittal plane were qualitatively reviewed to define developmental AR distribution.

Quantitative Image Analysis

We quantified AR expression in E17 and E21 LE/wt and LE/orl immunostained gubernacula ($n = 3$–9 per group) using a custom analysis algorithm formulated in MATLAB (MATLAB 2018a; Mathworks, Natick, MA). Each confocal volume was analyzed in full. Segmentation of the gubernacula was accomplished through manual elimination of any remaining the abdominal wall tissue and enhancement of the DRAQ5 channel. Briefly, DRAQ5 signal was median filtered with a 3.3 × 3.3 × 4.3 μm kernel, plane by plane rolling ball filtering (with a 66.4 μm radius) and morphological closing (with a 20 μm radius) to fill in gaps between the cells. The gubernaculum was finally segmented using hysteresis thresholding. Identification of the "tip" region was performed manually by a masked observer, and was defined as the region between the cord (arrow in **Figure 1A**) and the beginning of the dense muscular layer. The mesenchymal core comprised the region deep to the muscle (dotted yellow line in **Figure 1A**). Average AR intensity for the tip region and the rest of the gubernaculum was accomplished by taking the mean intensity value of the raw stain over the segmented volumes.

Androgen Stimulation of Gubernaculum Cells

E19 gubernaculum pairs were dissociated overnight at 4°C in 5 mg/ml collagenase with 2.5 mM CaCl. Samples were triturated and then plated on collagen or poly-L lysine (PLL)/laminin-coated 6-well tissue culture plates. Cells were then incubated for a total of 6 days in DME/F12 containing 18% fetal calf serum (FCS), rat basic fibroblast growth factor (bFGF; 2.5 ng/ml), rat epidermal growth factor (EGF; 10 ng/ml), dexamethasone (0.4 ug/ml), and penicillin-streptomycin supplemented with 0, 0.1, 1.0, or 10.0 nM DHT. Media was changed on each of the 6 days of culture. Immunofluorescent staining of AR in gub cells was carried out using 1:200 dilution of the rabbit anti-AR antibody (Abcam cat# ab133273) in PBS at 4°C overnight. Cells were washed 3X with PBS and then incubated with donkey anti-rabbit Alexaflour 555 and Dapi (Fisher cat#EN62248) to visualize nuclei. On day 6, 5 random areas were captured from each treatment protocol using an Olympus BX-60 florescence microscope with an Evolution QEi 12-bit digital camera (Media Cybernectics) and a 20X objective. AR+ cells were counted manually using Image J software (National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/).

Membrane Organ Culture and Muscle Development Assay

Individual gubernacula were dissected and placed on Millipore membranes in a six-well dish containing 2 ml of DME/F12 media with 2% stripped FCS, ITS and pen-strep as described previously (25). Media with or without 10 nM DHT was added, and gubernacula were harvested 24 h after incubation in PBS. Conditioned media (200 μl) from individual gubernacula grown on Millipore membranes with and without DHT stimulation was removed at 24, 48, 72, and 96 h with replacement of an equivalent volume of media to the appropriate wells.

Membrane culture was also used to assay muscle development by embedding E17 gubernacula in Matrigel® for 6 days, allowing gubernaculum myoblasts to migrate peripherally and form striated muscle fibers as described previously (25). We used myosin IF to visualize individual fibers with 1:10 dilution of A4.1025, a myosin-specific monoclonal antibody (Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa). We measured muscle fibers using the Image-Pro histogram generated masking (v.6.3, Media Cybernetics). These measurements included area, feret minimum and maximum diameter (length) and aspect, as defined by the Image-Pro program. In order to reduce background, non-fiber counts cutoffs were established for area and aspect. Every data point was tabulated in ascending order, and aspect and area measurements <5 and 500, respectively, were deleted.

Hyaluronan (HA) Analysis

DHT-treated gubernaculum pairs were homogenized in cell lysis buffer using a Rotor stator. The extracts were then spun at 17,800 rpm for 5 min. Supernatants were removed and stored at 4°C and the pellets were frozen at −20°C. After completion of sample collection, we measured HA levels concurrently in all available samples using R&D Biosystems Hyaluronan Quantikine ELISA Kit (cat# DHYAL0) in accordance with the manufacturer's instructions.

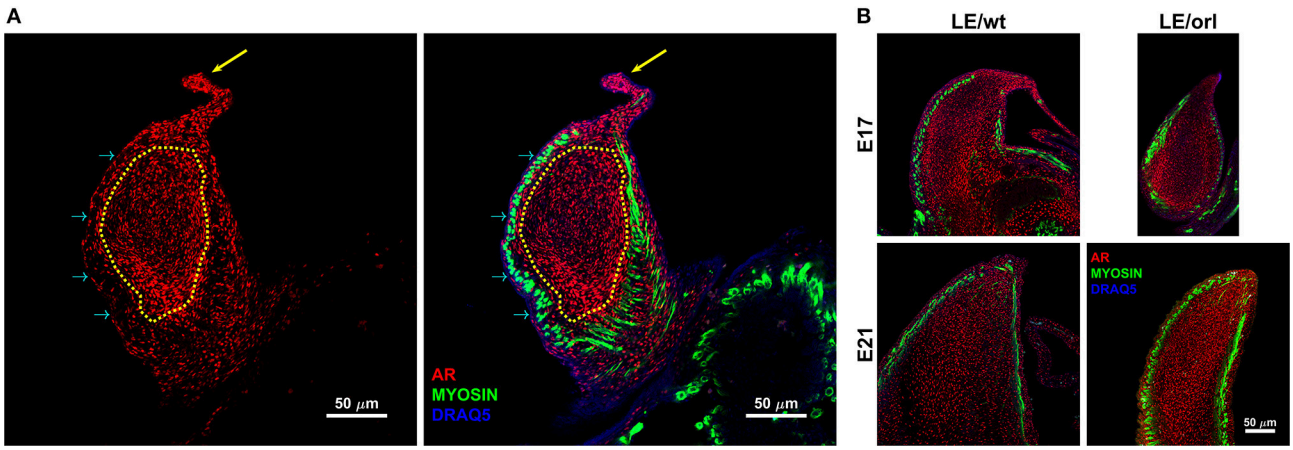

FIGURE 1 | Confocal images of the sagittal midportion of LE/wt gubernacula show androgen receptor (AR) and muscle expression defined by anti-AR (red) and anti-myosin heavy chain (green) immunofluorescent staining. Images are oriented in the longitudinal plane with the cord oriented superiorly. **(A)** Representative image of E17 LE/wt gubernaculum with localization of AR visible throughout the inner mesenchymal core (traced in yellow) and the gubernacular cord (yellow arrow). As noted in the merged image (second panel), nuclear localization (magenta) is strongly evident in the tip region and cord. However, myosin positive cells are negative for AR, several prominent examples highlighted by cyan arrows. **(B)** In E17 and E21 gubernacula isolated from LE/wt and LE/orl fetal rats, AR + cells are visible within the central mesenchyme and near the tip, which contains a previously described growth center. By E21, as the gubernaculum is starting to invert, areas of clearing with reduced AR + cell density appear proximally.

Glycosaminoglycan (GAG) Analysis

Rat gubernacula were digested with 0.3 ml of 4 mg/ml Pronase (Roche, 11459643001) in PBS for 1.5 h at 37°C. Collagenase (Gibco, 17101015) 0.1 ml, 2 mg/ml in PBS, was added to digestion samples, and samples were incubated at 37°C overnight. After centrifugation at 14,000 rpm for 10 min, the enzyme solution was removed. 150 μl of 50 mM Tris-HCl (pH = 7.0) was added to the pellets, and the sample solution was sonicated on ice for 15 s, 2 times. The sample solutions were stored at −20°C before pretreatment for GAG analysis. Further, preparation of gubernaculum and conditioned media samples for GAG analysis and quantification of GAG levels by liquid chromatography tandem mass spectrometry (LC-MS/MS) was previously described (26). In brief, 40 μl of sample solution was added into a 96 well Omega 10 K filter plate (PN 8034) (Pall Co, MI) with 60 μl of 50 mM Tris-HCl (pH = 7.0) with a 96 well receiver plate at the bottom. After centrifugation for 15 min at 2,500 g, a cocktail mixture with 60 μl of 50 mM Tris-HCl (pH = 7.0), 10 μl of 200 mU/ml Heparatinase, 10 μl of 200 mU/ml keratanase II (Seikagaku Co., Japan), 10 μl of 50 mU/ml Chondroitinase B, and 10 μl of 5 μlg/ml IS (chondrosine) were added to the filter plate, and incubated overnight at 37°C to digest oligosaccharides into disaccharides. All enzymes were provided by Seikagaku Co. (Tokyo, Japan). After centrifugation for 15 min at 2,500 g, filtrated pretreatment sample was stored at −20°C before GAG analysis by LC-MS/MS. A sample aliquot of 5 μl was injected into LC-MS/MS consisting of a 1,290 Infinity LC system with a 6,460 triple quad mass spectrometer (Agilent Technologies, Palo Alto, CA). After separation of disaccharides on a Hypercarb column (2.0 mm i.d. 50 mm length; 5 μm particles; Thermo Scientific, USA), specific precursor ion and product ion were detected to quantify each disaccharide, respectively, (HS-0S 378.3; 175.1, HS-NS 416, 138; mono-sulfated KS 462, 97; di-sulfated KS 542, 462; IS 354.3, 193.1). GAG levels in rat gubernaculum was normalized by protein concentrations. The protein assay was performed using a bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Prod#23227).

Apparent Elastic Modulus of the Fetal Gubernaculum

Isolated gubernacula were incubated in RS-I media (Aqix Ltd, London, United Kingdom) for no more than 6 h before measurement. Mechanical characterization of each intact gubernaculum was performed using micropipette aspiration on a stereoscope stage (Discovery.V8; Carl Zeiss Inc, Thornwood, NY), similar to previous descriptions (27–33). Two micromanipulators were used to position the tissue samples and the aspiration pipette. Aspiration was conducted with micropipettes freshly pulled from 1 mm thin wall capillaries (WPI, Sarasota, FL) using Flaming/Brown Micropipette Puller (P-97; Sutter Instruments, Novato, CA). Micropipettes were trimmed to diameters of approximately 80 μm using a ceramic cutting square (Sutter). For measurement, gubernacula were equilibrated in ice-cold PBS for 15 min. Suction was applied to the micropipette through a vacuum regulator at −70 kPa. Three positions were aspirated along the outer curvature of each gubernaculum: the *base* (defined as the circumferential bottom 25% of height adjacent to the pelvic floor), *midportion* (between 40 and 60% of the height), and *tip* (top 25% of the height) of the organ. Each aspiration was allowed to stabilize for 3 min to allow the tissue deformation to reach steady state and imaged with a camera mounted on the stereoscope (Canon EOS EOS T5 DSLR;

Canon, Melville, NY). Apparent elastic modulus (stiffness) is defined by Equation 1 (27):

$$E_{app} = \frac{3a\Delta p}{2\pi L}\Phi \quad (1)$$

Where a is the micropipette radius, Δp is the aspiration pressure, L is the length of aspirated tissue, and Φ is the wall function, a parameter to account for differences in pipette geometry, given by Equation 2 (27), below, where w is the pipette wall thickness:

$$\Phi \cong \frac{1}{2}\frac{1+\frac{w}{a}}{1+\frac{w}{2a}}\ln\frac{8a}{w} \quad (2)$$

Statistical Analysis

We used ANOVA, UNIANOVA or independent sample t tests (Mann-Whitney) in SPSS® v25 (IBM Corporation) to test for differences between groups. Parametric or non-parametric p values < 0.05 were considered significant.

RESULTS

Full-Length AR Has a Distinct Expression Pattern in Fetal Gubernacular Mesenchyme

Using a specific anti-AR antibody that recognizes the full-length form of the receptor and confocal imaging, we found that nuclear AR is expressed widely in mesenchymal cells of the fetal rat gubernaculum (**Figure 1A**, yellow dashed region), and is excluded from cremaster muscle cells (**Figure 1A**; cyan arrows). At E17 in LE/wt fetuses (**Figure 1B**), we observed increased AR staining intensity, which could indicate either increased cell density or increased expression levels. While we observed qualitative differences in size of the gubernacula (with LE/orl tissues being thinner), we did not observe any qualitative differences in cell density. In aggregate, the results indicate enhanced AR expression in nuclei of mesenchymal cells in the tip region and adjacent to developing cremaster muscle. AR immunofluorescence was less strongly nuclear within cells defined as a distal growth zone in studies of the postnatal rat gubernaculum (34, 35). By E21 (**Figure 1B**), we noted areas of decreased cellular density beneath the cremaster muscle layers. The pattern of AR expression was similar in LE/orl fetuses at E17 and E21, although a narrower organ at E21 was associated with increased density of AR+ cells (**Figure 1B**). A higher power view of the central mesenchyme (**Figure 2**) confirms variable AR expression between cells and nuclear localization of AR in this region. Quantitative analysis suggested enhanced AR expression in the tip region as compared to the mesenchymal core in LE/wt but not LE/orl gubernacula (**Figure 3**).

DHT Stimulates AR Expression in Primary Gubernaculum Cells

In the process of developing fetal gubernaculum cell lines (25), we observed loss of AR expression over time with repeated passaging of cultured E17 gubernaculum cells, both by immunofluorescent staining and Western blotting of protein extracts. In the current experiments, we confirmed that AR+ cells in culture show varying degrees of nuclear expression of the receptor, consistent with *in vivo* distribution as shown in images of the intact mesenchymal core (**Figure 2**) and AR expression is enhanced in a dose-dependent fashion by DHT stimulation (**Figure 4**). This effect was highly significant ($p < 0.001$ by ANOVA) for cells grown on either collagen type I or PLL/laminin substrata, but the response was more robust for PLL/laminin.

DHT Does Not Affect the Morphology of Gubernacular Muscle Cells in a Developmental Assay

We previously reported a fetal gubernaculum muscle development organ culture model, which utilizes intact E17 gubernacula embedded in Matrigel on PLL-coated plates (25). In this model, muscle precursors migrate away from the organ to form striated muscle fibers. Using this assay, we found no significant effect of DHT on muscle morphometry following 6 days of LE/wt gubernaculum culture, nor any significant differences in muscle fiber size between LE/wt and LE/orl samples (**Figure 5**). Although the assay could not provide accurate quantitation of developing muscle fibers, we found no

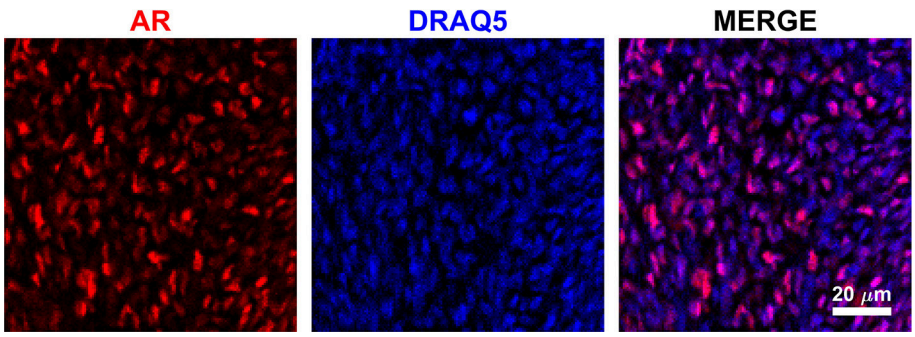

FIGURE 2 | Image of gubernacular mesenchymal core in culture shows variable AR expression among cells, and predominantly nuclear localization of the receptor (red = AR, blue = DRAQ5 nuclear stain). Image shown of E17 LE/wt, localization and staining patter qualitatively conserved at E19 and E21 in both LE/wt and LE/orl.

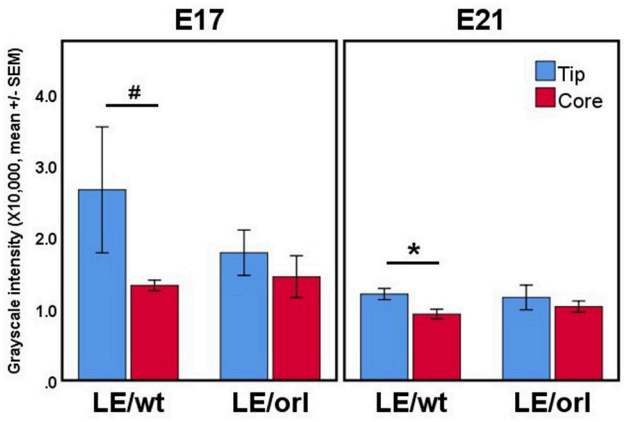

FIGURE 3 | Confocal volumes of AR immunostaining in LE/wt and LE/orl gubernacula ($n = 3$–8). The volumes were segmented into "tip" regions immediately inferior to the cord, and "core" regions of the mesenchymal bulk. In all cases, the tip region trended toward increased mean staining intensity, and this trend was significant in E21 LE/wt (*$p < 0.05$) and approached significance in E17 LE/wt (#$p = 0.06$) analyses. We did not observe increased AR expression at the tip relative to other regions in LE/orl gubernacula.

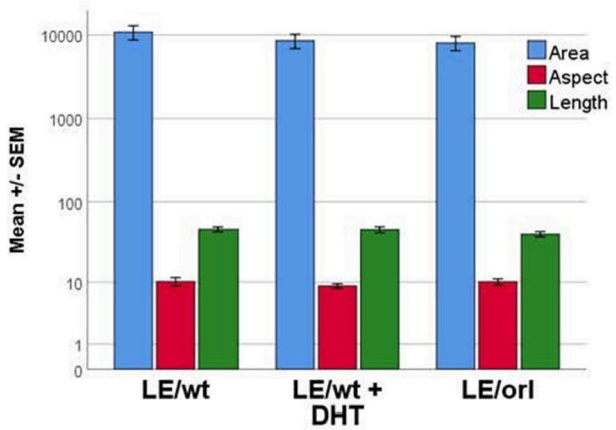

FIGURE 5 | Muscle cell morphology was assessed *in vitro* with fetal gubernacula immobilized in Matrigel to allow peripheral migration of myoblasts and subsequent formation of striated muscle fibers. Using this model, we found no differences in muscle cell morphology between LE/wt samples exposed ($n = 10$) and unexposed ($n = 10$) to 10 nM DHT and unexposed LE/orl ($n = 19$) fetal gubernaculum cells following cell migration and muscle fiber formation (results expressed using a logarithmic scale; area in μm^2; aspect and length in μm).

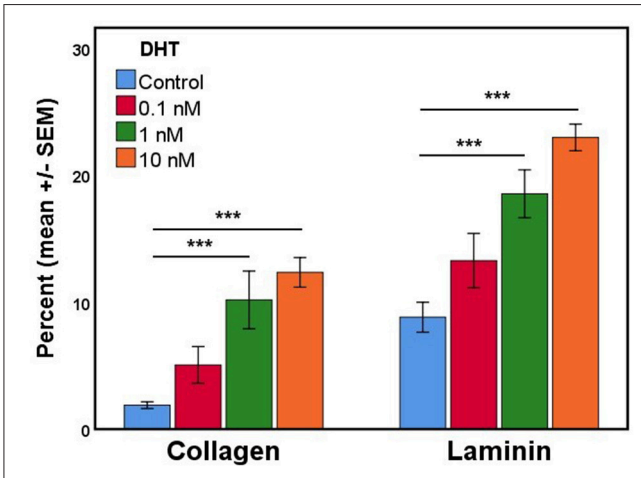

FIGURE 4 | The mean percentage of cultured gubernaculum mesenchymal cells expressing AR ($n = 6$ per exposure group) is enhanced by DHT in a dose- and substrate-dependent manner (original magnification, 40X; ***$p < 0.001$).

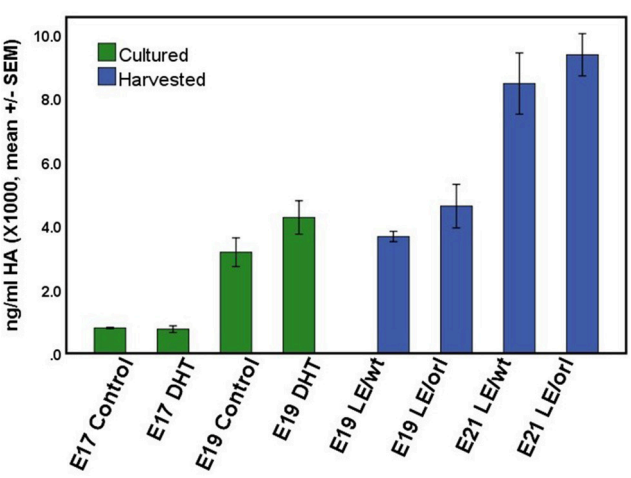

FIGURE 6 | HA concentration did not differ significantly between LE/wt E17 or E19 gubernacula cultured with and without exposure to 10 nM DHT (green bars; $n = 5$–6 per treatment group per time point) or between LE/wt and LE/orl gubernacula harvested at E19 or E21 (blue bars; $n = 4$ per strain per time point).

other qualitative strain- or hormone-dependent differences in muscle development.

DHT Does Not Stimulate Production of Extracellular Matrix Proteins

Using a sensitive rat-specific ELISA assay, we found that rat HA is easily measurable in the fetal gubernaculum, but levels are not influenced by exposure to DHT (**Figure 6**). Similarly, we found no significant differences in HA levels between LE/wt and LE/orl gubernacula at E19 or E21, during the outgrowth phase of the organ.

Our sensitive tandem mass spectrometry (LC-MS/MS) assay results (**Figure 7**) suggest that heparan sulfate (DiHS-NS, DiHS-0S) and mono- and di-keratan sulfate (DS-KS, MS-KS) levels are measurable and reproducible in fetal gubernaculum tissue and conditioned media. In membrane whole mount cultures, we measured GAG secretion into the media that exceeded baseline values, but did not observe any effect of DHT (**Figure 7A**). The concentration of the most abundant GAG, KS, is maintained

in LE/wt between E19 and E21, but reduced (although not significantly) by E21 in LE/orl gubernaculum (**Figure 7B**).

Stiffness Is Maximal in the Gubernacular Midportion in LE/wt but Not LE/orl Organs

Using micropipette aspiration, we determined the apparent elastic modulus (E_{app}, in kilopasquales, kPa) of the fetal gubernaculum at E19 and E21. To account for potential spatial variation, we measured 3 locations along the outer curvature: base (near the pelvic floor), midportion, and tip (near the gubernacular cord); additionally we completed measurements at E19 and E21 to capture changes in mechanics as the gubernaculum undergoes developmental remodeling. In LE/wt rat fetuses, we observed increased stiffness in the midportion at both E19 and E21 compared to the other regions measured ($p < 0.01$), and increasing stiffness between E19 and E21 ($p < 0.001$; **Figure 8**). In contrast, LE/orl fetal gubernacula did not show regional differences in stiffness or significant changes with increasing gestational age.

DISCUSSION

The results of these studies are consistent with prior work defining mesenchymal rather than muscle cells as targets of androgens in the fetal gubernaculum (14, 16), cell which appear to contribute to formation of the cremaster muscle (25). Loss of AR expression once mesenchymal cells have differentiated into cremaster muscle may explain why conditional *Ar* deletion in cells that have already undergone myogenic commitment does not interfere with testicular descent (5). Enhanced nuclear AR expression in the tip and cord of the gubernaculum is also consistent with prior studies showing that failure of the cord to shorten and delayed or absent inversion of the gubernaculum occur with loss of AR signaling (36, 37). The present data further indicate that AR is expressed in the gubernaculum during a time frame that overlaps with susceptibility of the rat fetus to antiandrogen-induced cryptorchidism. Our prior expression profiling data showed that *Ar* transcript levels are suppressed in gubernacula exposed to DHT (10 or 30 nM for 24 h) *in vitro* (38), while the current studies show dose-dependent increased expression of AR protein in dispersed gubernacular mesenchymal cells after exposure to the same DHT concentration (10 nM) for 7 days. This may be due to differing durations of exposure in each experiment, although divergent responses of mRNA and protein to the same stimulus may occur, particularly in cases of transcript suppression or when analyzing

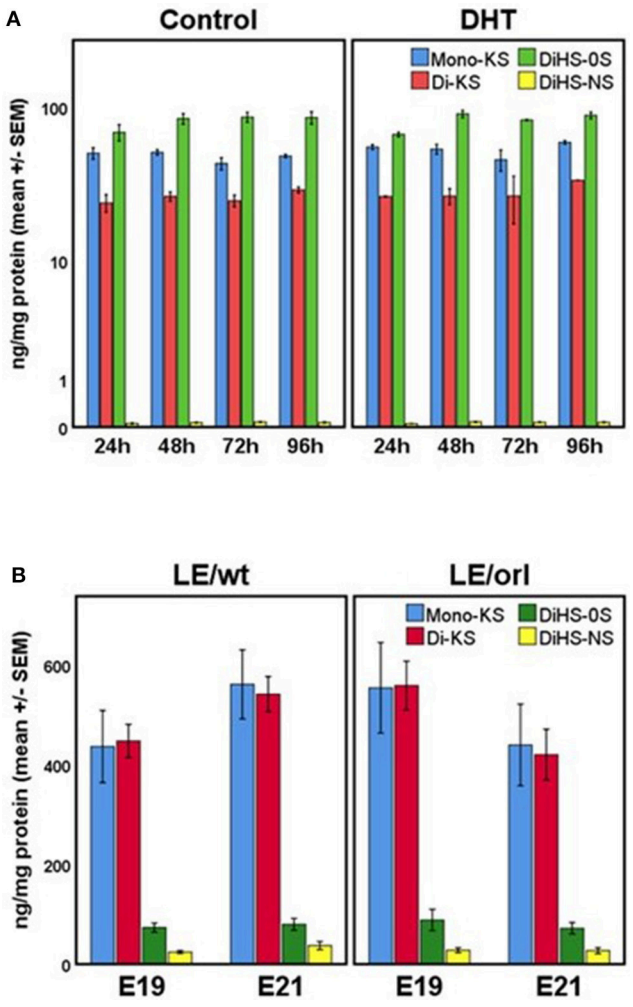

FIGURE 7 | (A) GAG concentrations in conditioned media from LE/wt gubernaculum membrane cultures show no differences between control and DHT-exposed samples (results expressed using a logarithmic scale; $n = 2$–3 per treatment group per time point). **(B)** Gubernaculum GAG expression revealed increasing mono- and di-keratan sulfate (Mono-KS, Di-KS) concentrations between E19 and E21 in LE/wt with a reverse trend in LE/orl fetuses, although differences between strains were not significant ($n = 10$–18 per strain per time point).

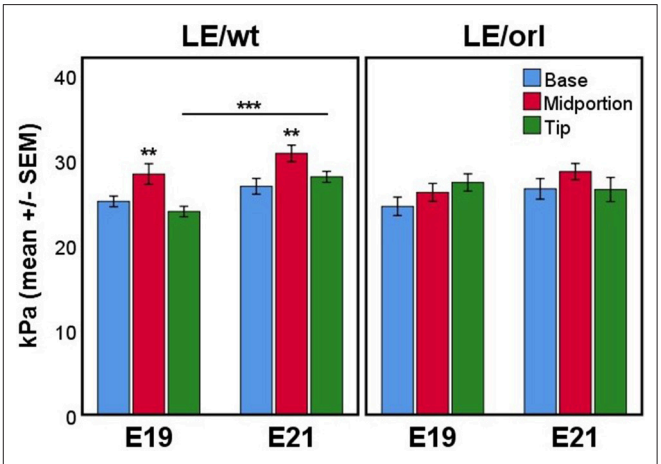

FIGURE 8 | Micropipette aspiration ($n = 13$–17 per strain at each time point) showed increased stiffness of the midportion relative to both base and tip at E19 and E21 in LE/wt gubernaculum (**$p < 0.01$, ANOVA) and a significant increase in stiffness at the tip between E19 and E21 (***$p < 0.001$, independent sample *t* test). Significant differences in stiffness between gubernaculum segments or gestational days was not observed in the LE/orl gubernaculum.

whole tissues that contain different cell types (39). Nevertheless, the observation that androgen is able to regulate expression of its receptor suggests that gubernacular AR is functional, and that the effect of androgens in stimulating testicular descent has an innervation-independent mechanism.

The role of androgens in each phase of testicular descent remains unclear. Available data do not consistently support the theory that regulation of transabdominal (Phase 1) and transinguinal (Phase 2) descent by INSL3 and androgens, respectively, is distinct (11). The majority of rats exposed to the antiandrogen flutamide during Phase 1 have undescended, and sometimes perirenal testes, while flutamide exposure during Phase 2 does not inhibit testicular descent (40–43). We found consistent AR expression in the gubernacular cord, also known as the cranial suspensory ligament (CSL). This finding is consistent with evidence from prior expression studies and from rodent models of AR deficiency in males and androgen exposure in females, which suggest that shortening of the cord is androgen dependent (7, 10, 37, 44–47). INSL3 and DHT regulate many of the same E17 gubernacular transcripts *in vitro* and both target WNT signaling, but the effects of DHT occur after 24 h, but not 6 h, of stimulation (38). A "delayed secondary response" (36, 48) to AR signaling may occur via crosstalk with other signaling pathways, and could explain why the gubernaculum is less sensitive to antiandrogens than other fetal reproductive organs (49, 50).

The current experiments suggest that androgens do not have a major role in stimulating production of CM or defining muscle fiber size in the rat gubernaculum. These results are consistent with observations that swelling of the gubernaculum still occurs to some extent in mice with spontaneous (*Tfm*) or transgenic (ARKO) *Ar* inactivation, or in the LE/orl rat, which shows reduced fetal androgen synthesis and action (10, 21, 37). Swelling of the gubernaculum affects its shape, and likely its mechanical properties and function. ECM components facilitate swelling due to osmotic pressure but are also involved in extracellular signaling pathways and cellular differentiation (51). In larger mammals such as pig and man, the gubernaculum consists primarily of mesenchymal cells embedded in ECM. In these species, ECM deposition and degradation are thought to drive Phase 1 and 2 of testicular descent, respectively (52–54). The GAG content of the fully developed porcine fetal gubernaculum consists of dermatan sulfate (50%), hyaluronic acid (30%), and chondroitin/keratan and heparan sulfates (10% each) (54). While there are no prior studies for comparison, our data suggest that KS is the most abundant GAG in rat fetuses, possibly because muscle is a much more prominent component of the rodent gubernaculum (34).

A known role for androgen and INSL3 is stimulation of cellular proliferation via additive effects that were demonstrated using the *in vitro* model employed in the current study (17, 18). We previously reported data suggesting that proliferating myogenic precursors contribute to enlargement of the mesenchymal core and cremaster muscle, and that gubernacular mesenchymal cells exhibit characteristics of myofibroblasts (25), a cell type that produces ECM and contributes to tissue remodeling (55). If the main function of androgens is to stimulate cellular proliferation in the gubernaculum, we would not anticipate relative changes in GAG or HA concentrations following exposure to DHT.

Our genetic and genomic studies of the LE/orl rat, a model of inherited cryptorchidism, suggest that dysfunctional AR signaling contributes to failure of testicular descent in this strain. In both LE/orl and flutamide-exposed rats, undescended testes become misdirected and reside in the superficial inguinal pouch (21, 36, 41). Testosterone levels and muscle-specific transcript levels are reduced, DHT-responsive transcripts and cremaster muscle patterning are altered, and gubernacular inversion is delayed or abnormal in LE/orl fetuses (21, 23, 56). We used linkage analysis and whole genome sequencing of the LE/orl strain (22) to identify homozygous variants in *Syne2* and *Ncoa4,* two genes encoding AR-interacting proteins that are also implicated in myogenesis (57–60). Our data suggest genetic interaction between *Syne2* and *Ncoa4,* and by selective breeding to enrich for LE/orl *Syne2* and *Ncoa4* alleles in outbred strains, we were able to reproduce the cryptorchid phenotype (22). The muscle defects and altered shape of the fetal LE/orl gubernaculum (21) are consistent with the abnormal elastic modulus that we identified in the present studies. Consistent with a role for abnormal AR signaling in the LE/orl strain, the present data suggest that neither DHT nor the LE/orl background appeared to influence muscle fiber morphology or ECM composition in fetal gubernacula.

Our data suggest regional differences in stiffness of the gubernaculum characterized by peak stiffness of the midportion, and increasing stiffness at the tip between E19 and E21. The reduced swelling that we observed in the LE/orl gubernaculum herein and in prior studies (21, 23) may account for differences in its biomechanical properties. In a prior study using histological sections, matrix metalloproteinase (MMP)-expression was reported in cells adjacent to the inverting perinatal rat gubernaculum, suggesting that active ECM remodeling may facilitate gubernacular motility (61). Areas of clearing that we observed in the region between the central mesenchyme and peripheral muscle layers may be comparable to previous anatomical studies of the human fetus, which revealed loosening of the connections between the gubernaculum and surrounding cremaster-lined inguinal canal just prior to testicular descent (62). The increasing elastic modulus of the gubernaculum at the midportion and tip, along with symmetrical detachment of the mesenchymal core from the surrounding muscle, may enable force generation in a scrotal direction and transinguinal passage of the testis. Swelling of the mesenchymal core is subtle in rodents but if insufficient, may affect the shape and mechanical properties of the gubernaculum and its capacity for correct inversion.

In summary, current and prior data suggest that androgens enhance AR expression and proliferation of mesenchymal cells of the fetal gubernaculum, and that AR expression is lost with myogenic differentiation. Androgens may also have indirect effects on ECM production by stimulating the growth of AR-expressing myofibroblasts. Susceptibility to cryptorchidism in the LE/orl rat is associated with altered stiffness, but not with changes in ECM composition or muscle fiber morphology of the fetal

gubernaculum. Regional differences in stiffness and localized ECM remodeling may be required for successful transinguinal passage of the gubernaculum and testis.

DISCLAIMER

This work was prepared while Dr. Julia Barthold was employed at Nemours. The opinions expressed in this article are the author's own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

ETHICS STATEMENT

This study was carried out in accordance in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, based on the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Nemours Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. JM and JG performed mechanical testing and quantitative image analysis. AR, AM, and DG performed dissection, cell and organ culture, and staining experiments. KS and ST performed matrix analysis. JSB, AR and JM were responsible for drafting the manuscript. All authors contributed to the development and interpretation of the experimental data as well as manuscript editing.

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Endocrine Disrupting Chemicals Interfere With Leydig Cell Hormone Pathways During Testicular Descent in Idiopathic Cryptorchidism

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Cryptorchidism, a frequent genital malformation in male newborn, remains in most cases idiopathic. On the basis of experimental, epidemiological, and clinical data, it has been included in the testicular dysgenesis syndrome and believed to be influenced, together with genetic and anatomic factors, by maternal exposure to endocrine disrupting chemicals (EDCs). Here, we analyze how EDCs may interfere with the control of testicular descent, which is regulated by two Leydig cell hormones, testosterone, and insulin like peptide 3 (INSL3).

Keywords: cryptorchidism, endocrine disrupting chemicals, testosterone, insulin like peptide 3, leydig cells

IDIOPATHIC CRYPTORCHIDISM

Undescended testis (UDT), also called cryptorchidism, is the most frequent congenital malformation in males, occurring in 2–8% of full-term male births (1–3). In young adults, it is associated with a higher risk for male infertility and testicular cancer (4, 5). Cryptorchidism cases can be characterized as unilateral or bilateral, transient (when spontaneous descent of the testis occurs within the first year of life) or persistent, and palpable or non-palpable according to the position of the undescended testis, following Scorer classification (6). With the exception of complex syndromes with multiple congenital abnormalities (7), most cases of UDT are unilateral, often transient and are considered as idiopathic (7). Idiopathic UDT is believed to be a multifactorial disease with anatomical, genetic and environmental risk factors (7–10). Anatomical factors could explain the frequent unilateral cases (10). Genetic causes such as mutations of INSL3, testosterone or their receptor genes (7, 11, 12) are rare in case of "idiopathic" UDT. Environmental factors, including *in utero* exposure to EDCs, have been proposed as co-factors for the occurrence of idiopathic UDT and other male reproductive developmental abnormalities (9). This environmental hypothesis is supported by: 1/ epidemiological studies showing, for example, temporal (13) or geographical differences (14), 2/ observations made in wildlife after environmental accidents, and 3/ experimental results in rodents, showing that exposure to several EDCs with estrogenic or anti-androgenic effects during fetal life, disturbs testicular descent (15).

However, epidemiologic evidence in humans, remain scarce and the mechanisms which could link the EDCs exposure with UDT remain incompletely understood. In this review, we will analyze the data which support that EDCs with estrogenic or anti-androgenic effects may influence the occurrence of cryptorchidism, and how they may interfere with the hormonal control of testicular descent.

HORMONAL CONTROL OF TESTICULAR DESCENT

Physiological descent of the testes during fetal development is quite well-understood, and has been described in several reviews (10, 16). Briefly, it includes two successive phases involving the participation of two ligaments: the cranial suspensory ligament (CSL) and the gubernaculum. The first phase, called the trans-abdominal phase, occurs in humans, between weeks 10 and 23. Due to the regression of the CSL and the growth of the gubernaculum (10), the testis migrates from the uro-genital ridge to the inguinal region. The second phase, inguino-scrotal, occurs after 28 weeks gestation. During this second phase, the regression of the gubernaculum will allow the testis to reach its definitive scrotal position. This will occur before birth in most cases, or during the neo-natal period for some of them (transient cryptorchidism). As supported by observations made in genetically modified rodents (17–19) or human genetic syndromes (7, 11, 12), the two-phases testicular descent is regulated by two testicular hormones: INSL3 and testosterone (18), which are produced by the differentiated Leydig cells (20). Classically, INSL3 is the regulator of the abdominal phase, and testosterone is necessary for the inguino-scrotal phase; but experimental data also support a role for INSL3 during the second phase, in association with androgens. INSL3 is a peptide hormone belonging to the relaxin family, specifically produced in the testis. Its receptor, RXFP2 (relaxin family peptide receptor 2), is developmentally expressed in the gubernaculum (21). Bilateral UDT and abnormal gubernaculum are present in INSL3 and RXFP2 knockout mice models (18, 19). Mutations of one of these two genes have been found in 4.7% of cryptorchid boys (7). The gubernaculum expresses the androgen receptor. Its regression is induced by androgens during the inguino-scrotal phase, as demonstrated by both animal models and human genetic syndromes (19–21). Impaired hypothalamic-pituitary axis leading to lack of testicular testosterone production or impaired androgen sensibility by lack of receptor expression, are associated with persistent, bilateral UDT, but they remain, like mutated INSL3/RFXP2 gene, very rare (17, 22, 23). Nevertheless, in the absence of mutations, impaired secretion of INSL3 and/or testosterone may influence testicular descent.

LEYDIG CELL HORMONES AND IDIOPATHIC CRYPTORCHIDISM

Two longitudinal case-control studies have tried to assess during the neonatal period, the Leydig cell hormones involved during testicular descent, in cryptorchidic boys. Bay et al. (24) were the first to report that INSL3 was decreased in idiopathic UDT (24). In their prospective study including 3 groups (control, Danish and Finnish cryptorchidic boys), they could first clearly establish the physiological ontology of testicular INSL3 secretion in boys. Levels were higher at birth and at 3 months, than in older pre-pubertal boys and significantly correlated to LH (24). They suggested that INSL3 is regulated at this period by the transient post-natal wave of gonadotropins. Secondly, they showed that INSL3 cord blood levels were reduced in persistent cryptorchidic boys and in the Finnish transient cryptorchidic subgroup when compared to controls. Thirdly, they observed that individual INSL3 levels in cryptorchidic boys increased significantly when assessed at birth and at 3 months for both transient and persistent cryptorchidism. However, at 3 months, they still observed a reduced level of INSL3 and an increased LH to INSL3 ratio in persistent cryptorchidic boys when compared to controls, while no more significant difference was noticed at that time in the transient group (24). Regarding the results of the persistent group, the authors suggested that in persistent cryptorchidism, Leydig cell dysfunction was already present in the perinatal period. As for the transient group, they suggested that the postnatal surge, which seems to physiologically stimulate INSL3 secretion, was able to normalize INSL3 secretion, contributing to the spontaneous testicular descent between birth and 3 months of a still normal testis (24).

From a prospective case-control study performed in Nice area (25), 180 boys born after 34 weeks of gestation, were assessed at birth and followed clinically during 1 year: 52 cryptorchid boys (48 unilateral, 4 bilateral; 26 transient, 26 persistent), and 128 controls matched for term, weight and time of birth. Cord blood INSL3 levels were significantly decreased in the total cryptorchidic group when compared to controls; this was mainly due to the transient cryptorchid subgroup, since the persistent group had values not significantly different from control (**Figure 1**). INSL3 was more significantly decreased in the group of 20 boys with non-palpable testes compared to the group of 21 with palpable testes, according to Scorer classification (25). In the whole population, INSL3 was positively correlated with LH and negatively with AMH, but with no other measured hormones.

Those two prospective studies on INSL3 in cryptorchidic boys confirm that neonatal INSL3 levels are decreased, but they seem to differ somewhat concerning the transient and the persistent cryptorchidic subgroups. However, when analyzed in details, they are rather concordant and complementary (25). Both teams found a relative large dispersion of the INSL3 values in all groups and the sizes of their subgroups were relatively small (24, 25). First, concerning the transient cases, the French team found a very significant decrease at birth and the Nordic team only in the Finnish subgroup (24); but the authors indicated that this subgroup had more severe (suprascrotal or worse) than mild (high scrotal) UDT, as opposed to the Danish subgroup, suggesting that this difference could explain the lower INSL3 levels. This hypothesis was confirmed later by the French study as reported above (25). One may now consider that transient cryptorchidism has a reduced secretion of INSL3 at birth, and that thanks to the postnatal LH wave (that correlates with it in

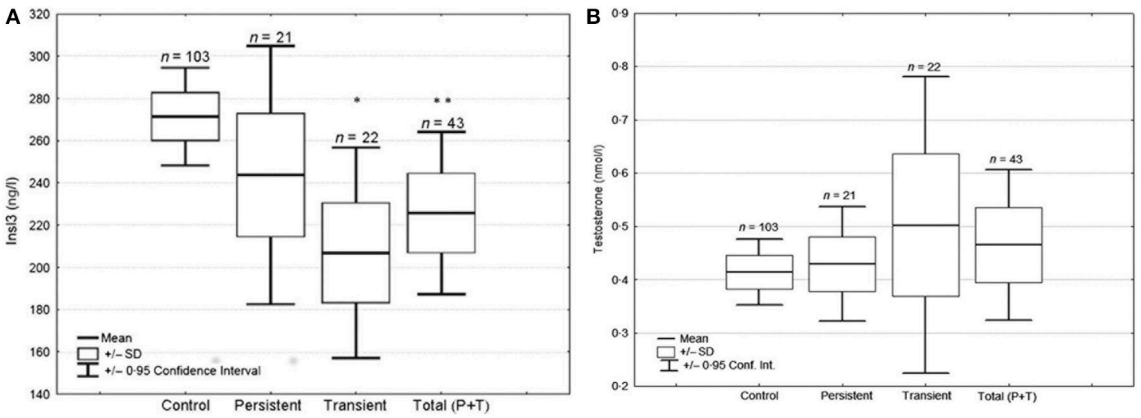

FIGURE 1 | Testosterone **(A)** and INSL3 **(B)** cord blood levels in UDT and control groups. Boxand-whisker diagram of testosterone and INSL3 cord blood levels in controls, transient and persistent cases of cryptorchidism. The "total group" corresponds to the sum of transient and persistent cases. $^{*}P = 0.029$, $^{**}P = 0.031$ when compared to control group by logistic regression test. In Fénichel et al. (25).

both studies), it will be normalized at 3 months, contributing with normal Leydig cells, to the spontaneous testicular descent, as illustrated and proposed by Bay et al. (24). Second, regarding the persistent group, it was clearly associated in the Nordic study, with decreased INSL3 levels at birth still present at 3 months, in spite of the LH peak, while in the French study no significant INSL3 decrease was observed in cord blood (25). Tight analysis of the individual values showed (**Figure 1**) a wider dispersion suggesting a greater heterogeneity of the causal factors and/or of the degree of Leydig cell impairment.

Both studies found normal ranges of testosterone concentrations and LH/testosterone ratio in cryptorchidic newborn (25). The Nordic have reported an increased LH/testosterone ratio at 3 months of age (26, 27) or a decreased testosterone at 6 months, suggesting secondary Leydig cell dysfunction. Normal testosterone levels at birth contrast with lower INSL3 which appears at this time as a specific and sensitive marker of fetal Leydig cell impairment as proposed by Bay and Anand-Ivell (28). Fénichel et al. (25) reported in the cryptorchid group at birth, a positive correlation between LH and INSL3, but not between LH and testosterone. This suggest that INSL3, before and at birth, could be regulated by LH in a different way compared with testosterone as already proposed (20).

The reported INSL3 levels during neonatal period in cryptorchid boys triggers two related questions: 1/Is decreased cord blood INSL3 a cause or a consequence of UDT? 2/Could cord blood levels of INSL3 reflect what happened during fetal development and testicular descent? Classically, INSL3 is considered as regulating the first phase of testis migration during the second trimester of pregnancy. However, its contribution to the inguino-scrotal phase, has more recently been suggested. First, in the LH receptor knock-out mouse, testosterone administration causes an up-regulation of gubernaculum RXFP2 expression acting via the androgen receptor (29). Secondly, an INSL3 antagonist can inhibit the testosterone-induced inguinoscrotal descent (30). Last, as mentioned before, the higher levels of cord blood INSL3 in normal male newborns (24, 25) and the LH-dependent increase of INSL3 associated to spontaneous testicular descent in transient cryptorchidism, support a role for INSL3 during the inguino-scrotal phase (24). Moreover, there are several clues that support the INSL3 decrease in cryptorchidism as a causal factor, rather than a consequence. Briefly, as discussed in Fénichel et al. (25): "First, experimental induction of cryptorchidism in mice does not significantly alter the expression of INSL3 mRNAs in the testis (29). Secondly, testosterone, another Leydig hormone, was not affected in our cohort. Thirdly, if reduced cord blood INSL3 was a consequence of UDT, then the extent of the decrease might have been similar or even more marked in the persistent UDT boys, and this was not the case in our study (25)." This was in fact the case in the Nordic study for persistent cryptorchidism (24). One could integrate the different data in the following concept: persistent cryptorchidism is associated with low INSL3 levels already present at birth, persistent at 3 months, with a high LH/INSL3 ratio and altered testosterone or LH/testosterone ratio, suggesting impaired Leydig cells functioning as a consequence of UDT. In the transient forms of UDT that can be corrected after birth, lower reversible INSL3 levels suggest a functional causal effect with a down-regulation of INSL3 expression rather than a true testicular injury.

What could be the mechanism leading to fetal INSL3 decrease? Mutations or polymorphisms in the INSL3 and its receptor genes, in human patients with idiopathic UDT have been actively researched. Ferlin et al. (12) in a study involving 600 isolated cryptorchid infants, found only 1.1% of such mutations. On the other hand, INSL3 gene expression is negatively regulated by estrogens and positively by androgens, as shown in Leydig cells *in vitro* (30, 31). Thus, fetal exposure to estrogenic or anti-androgenic EDCs may be involved in the decrease of fetal INSL3 levels.

Moreover, the normal testosterone levels observed at birth in cryptorchidic boys, do not exclude an antagonistic action

at the androgen receptor level mediated by an anti-androgenic EDC, which could indirectly impair the testosterone effect. Such an effect of antiandrogens on testicular descent has been demonstrated in animal models for several EDCs, like flutamide, vinclozolin, or phthalates.

What about human epidemiological data ?

IDIOPATHIC CRYPTORCHIDISM AND ENDOCRINE DISRUPTORS

Relationship Between Exposure to Endocrine Disruptors and Cryptorchidism

Maternal exposure to diethylstilbestrol (DES), a potent synthetic estrogen which was given to prevent miscarriages (32), has been associated with an increased risk of urogenital abnormalities in male newborn. Results from an American cohort estimated a doubling of the risk for cryptorchidism after *in utero* exposure to DES, with a higher risk when exposure occurred before week 11 of pregnancy (15). Several case-control studies have tried to link fetal exposure to EDCs and cryptorchidism, but prospective, longitudinal studies with a right methodology, are scarse. In a meta-analysis, Bonde et al. (33) could select 10 case-referent studies, addressing the risk of cryptorchidism following prenatal and post-natal exposure to endocrine disrupting chemicals. Summary Odds Ratio (OR) was not significantly increased. Only two studies (1, 34) and three risk estimates for beta-hexachlorocyclohexane (HCCB), p-p'- 1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) and Polychlorinated Bisphenyls (PCBs) measured in maternal serum or milk, were significant. One of these prospective studies performed in Nice area (France) reported (1) an increased OR for PCBs (OR 2.74 [1.15, 6.53]) and for DDE concentrations (OR 2.16 [0.94, 4.98]) measured in maternal colostrum. More recently, a case-control study examined whether there was a link between maternal hair polybrominated diphenyl ether (PBDE) concentrations and the risk of UDT in male infants (35) and found that every 10-fold increase of the concentration of maternal hair BDE-99 or BDE-100, was associated with more than a doubling in the risk of UDT (35). Fernandez et al. (6), in a small cohort, correlated BPA and propyl-paraben concentrations in the placenta and the occurrence of hypospadias or cryptorchidism. Levels of two pesticides, heptachloroepoxide (HCE) and hexachlorobenzene (HCB), were found significantly higher in the fat taken during surgery for orchidopexy in a group of cryptorchid boys, when compared with controls (36). All these case-control prospective longitudinal studies report only indirect links; they are difficult to perform, have a limited sample size (37), are expensive, and usually assess only a small number of chemicals. As reviewed by Virtanen et al. (38), it is also hazardous to link UDT with a single chemical product. Fetus and newborn are exposed to many chemicals, which may present additive, antagonistic and/or synergistic effect. This was also shown *in vivo* in humans for UDT by Damgaard et al. (39), who found a correlation with a "cocktail" of several pesticides in breast milk, but not with any single pesticide, and by Brucker-Davis et al. (1) who built a score associating colostrum concentrations of DDE and several PCBs. In a study associating Danish and Finnish patients, seven PBDEs, all flame retardants, were detectable in milk and their sum was significantly higher in the group of Danish cryptorchid boys than in controls (40). Moreover, cord blood or maternal milk levels do not directly reflect fetal exposure during the window of testis descent. Amniotic fluid collected around 18 weeks of gestation has been proposed to evaluate INSL3 secretion at a time closer to this period (41). However, chemical concentrations are difficult to analyze, because they depend on varying dilution (41). Nevertheless, both epidemiological and experimental data, including those studying cryptorchidism, hypospadias and/or testicular cancer, support the hypothesis of a deleterious role for fetal exposure to EDCs.

How could EDCs disrupt testicular descent?

Interference Between Exposure to Endocrine Disruptors and Leydig Cell Hormones

However, while it has been clearly shown that maternal exposure to estrogenic or anti-androgenic EDCs could induce cryptorchidism in rodents, it remains unproven that such environmental factors are operating in human idiopathic UDT, even if epidemiological studies with statistical correlations do exist as shown above (1, 6, 33–35). What could be the mechanism involved? Although cord blood levels of bisphenol A were not significantly increased in cryptorchidic boys (1.26 + 0.17 ng/ml vs. 1.14 + 0.13 ng/ml) when compared to control boys (42), when we looked for correlations between hormones and xenobiotics in the whole population (**Figure 2**) (43), we found a significant negative correlation between bisphenol A and INSL3 levels ($p < 0.01$). No significant correlation was found for testosterone or between both hormones and the other xenobiotics assessed (43). While the participation of BPA in this decrease remained small ($R_2 = 0.05$), the statistical link was significant; this was consistent (negative effect at low dose) with the reported decrease of fetal INSL3 production observed by N'Tumba-Byn et al. (44) on human explanted fetal testes, cultured with low doses of BPA, even though these results were confirmed by Ben Maamar et al. (45) only in special culture conditions, omitting Human Chorionic Gonadotropin. From a mechanistic point of view, it is also in agreement with what is known from experimental data on the regulation of INSL3 gene expression and also on the disrupting effect of BPA. INSL3 gene expression is negatively regulated by estrogens, as shown in Leydig cells *in vitro* (31), and positively by androgens (46). In mice, maternal exposure to xenoestrogens, including the potent synthetic estrogen diethylstilbestrol (DES), results in down-regulation of INSL3 (but not testosterone) mRNA expression levels in Leydig cells (47, 48), and is associated with intra-abdominally located testes. In humans, an increased risk of cryptorchidism has been reported after fetal exposure to DES given as maternal treatment to prevent miscarriages (32). BPA, like DES, was initially designed as a synthetic estrogen, but it rapidly came to be widely used in the manufacture of plastics and epoxy resins. Because of its low affinity for the classical nuclear estrogen receptors ERα and ERβ (49), the classification

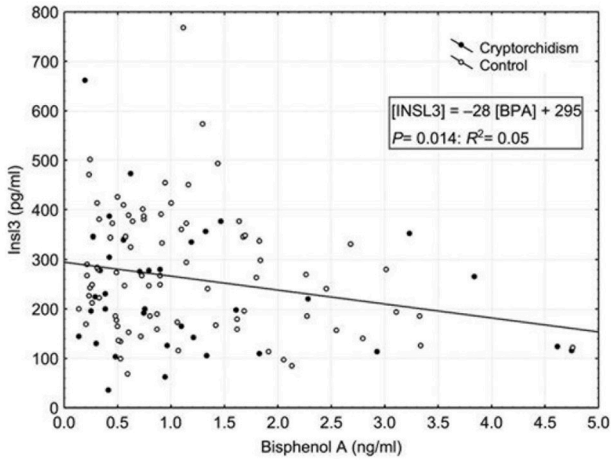

FIGURE 2 | Insulin-like peptide 3 (INSL3) in relation to bisphenol A (BPA) in the whole study population of boys. Filled circles correspond to cryptorchid cases ($n = 52$), while open circles correspond to controls ($n = 128$). The linear regression is: Insl3 = −28 [BPA] + 295; $p = 0.014$, $R^2 = 0.05$. In Chevalier et al. (43).

of BPA as a xenoestrogen has also been debated (50). To explain BPA mechanisms of action, other receptors have been proposed, such as androgen receptor (51), estrogen related receptor gamma ERRγ (52) or membrane non-classical estrogen receptors (53–55). N'Tumba-Byn et al. (44), in reporting the negative effect of BPA on INSL3 Leydig cell secretion during human fetal testis culture, were able to exclude the ERα pathway by gene invalidation, and they suggested the participation of non-classical ERs (44). We have identified, in human testis, including Leydig cells, one of these membrane receptors, GPR30/GPER (G protein coupled estrogen receptor) for which BPA has a high affinity (53, 54). An anti-androgenic effect of BPA (51) has also been reported which could interfere with the positive regulation of testosterone on INSL3 gene expression (46). The lack of correlation between BPA and testosterone concentrations is not completely surprising since INSL3 and testosterone have been shown to be differentially regulated at the Leydig cell level. INSL3 secretion is dependent on the pituitary axis in a less acute way than testosterone (20) and synthesis of both hormones is also distinctly regulated (24, 35). Indeed, maternal BPA easily crosses the placenta (56, 57), and will be less easily conjugated and cleared by the fetus because of immature hepatic glucuronyl-transferase enzymes (58, 59) and active placental or fetal glucuronidases or sulfatases (58).

As in our previous report (41), there was no significant increase of BPA in boys with UDT when compared with controls (42). However, mean levels of BPA were higher in the cryptorchidic group, and strikingly more in the non-palpable vs. palpable subgroups, suggesting a link with the degree of migration defect. We have already reported a similar trend for INSL3 decreased levels (25). On the other hand, a single blood or spot urine BPA or conjugates test reflects short term exposure and not chronic exposure (60). Therefore, although exposure through diet is likely to be continuous, it cannot be concluded from this study, performed at the time of delivery, whether chronic fetal exposure to maternal BPA could disturb testicular descent at the time when INSL3 is most likely to be acting directly on the testis, in the first phase of testicular descent (gestational week 12–16). However, our data support the hypothesis that INSL3 is a target for endocrine disruption. Anand-Ivell and Ivell (41, 61) have even proposed that INSL3 could be a "monitor of endocrine disruption." Indeed, INSL3 could be influenced by fetal exposure to several estrogenic and/or anti-androgenic EDCs acting as a "cocktail," as suggested by epidemiological studies in idiopathic UDT (1, 37, 38).

Beside BPA, phthalates are among the strongest candidates for affecting the testis (62). There are robust data in rodents (23) and more recently in humans (63) supporting the deleterious effects of phthalates on testicular descent (23) and function (63). They may act on INSL3 gene expression/ action, on steroid hormone production or as an androgen antagonist (23). Effects of phthalates on INSL3 are sometimes contradictory, with some data showing an impact (62, 64), and others not (65). This discordance is likely due to a differential effect according to time of exposure or species (62, 64, 65).

In order to approach fetal exposure during specific windows of development, the assessment of phthalates in amniotic fluid has also been recently proposed with, however, the well-known technical difficulties associated with such studies (60, 61). Phthalates are able to interfere with the androgenic function of Leydig cells like DDE or PBDE (66) which have been both associated with cryptorchidism (1, 18/1, 34). This impairment of the androgenic action by phthalates may be involved in the experimental or epidemiological link reported with UDT (1, 35, 62), though the molecular mechanisms remain still largely unclear. but it is more difficult to demonstrate directly an antagonistic effect than a decreased peripheral blood level.

Acetaminophen (Paracetamol*) given to pregnant women has been suspected to increase the risk for male fetus to develop cryptorchidism (67, 68). In a xenograft model, it has been shown that prolonged exposure to acetaminophen reduces testosterone production by the human fetal testis (69). In another model of *ex vitro* culture of human fetal testis, exposure to acetaminophen was able to decrease INSL3 (but not testosterone) production during the critical window of the first abdomino-inguinal phase, (70), this could represent the mechanism by which this analgesic drug increases cryptorchidism risk.

CONCLUSION

To conclude, experimental and epidemiological studies support the hypothesis of a deleterious role for fetal exposure to a cocktail of endocrine disruptors during the testicular descent; those compounds, acting as xenoestrogens and/or antiandrogens, may disrupt the secretion and/or action of INSL3 and testosterone, the two Leydig cell hormones, regulating testis descent, and lead to cryprorchidism in case of a genetic susceptibility context as recently suggested by Barthold and Ivell (71). However, direct evidence to support such a pathophysiological link explaining idiopathic UDT, remain scares. More prospective, longitudinal epidemiological studies

and experimental models are necessary, exploring a more complete cocktail of common EDCs with possible estrogenic and/or anti-androgenic effects.

AUTHOR CONTRIBUTIONS

PF conceived and wrote the paper. NL preformed INSL3 and testosterone assay and discussed the results. NC participated to the discussion. PC made the statistical study. MP performed bisphenol assay. PP-F performed hormonal assays. KW-M supervised the clinical studies. FB-D directed the prospective study, discussed the results and participated to the writing of the paper.

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Insulin-Like Peptide 3 (INSL3) Serum Concentration During Human Male Fetal Life

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Context: Insulin-like peptide 3 (INSL3), a protein hormone produced by Leydig cells, may play a crucial role in testicular descent as male INSL3 knockout mice have bilateral cryptorchidism. Previous studies have measured human fetal INSL3 levels in amniotic fluid only.

Objective: To measure INSL3 serum levels and mRNA in fetal umbilical cord blood and fetal testes, respectively.

Design: INSL3 concentrations were assayed on 50 μl of serum from male human fetal umbilical cord blood by a non-commercial highly sensitive and specific radioimmunoassay. For secondary confirmation, quantitative real-time PCR was used to measure INSL3 relative mRNA expression in 7 age-matched human fetal testes.

Setting: UT Southwestern Medical Center, Dallas, TX and Medical University of South Carolina, Charleston, SC.

Patients or other Participants: Twelve human male umbilical cord blood samples and 7 human male testes were obtained from fetuses 14–21 weeks gestation. Male sex was verified by leukocyte genomic DNA SRY PCR.

Interventions: None.

Main Outcome Measures: Human male fetal INSL3 cord blood serum concentrations and testicular relative mRNA expression.

Results: INSL3 serum concentrations during human male gestational weeks 15–20 were 2–4 times higher than published prepubertal male levels and were 5–100 times higher than previous reports of INSL3 concentrations obtained from amniotic fluid. Testicular fetal INSL3 mRNA relative expression was low from weeks 14–16, rose significantly weeks 17 and 18, and returned to low levels at week 21.

Conclusions: These findings further support the role of INSL3 in human testicular descent and could prove relevant in uncovering the pathophysiology of cryptorchidism.

Keywords: insulin-like peptide 3, relaxin-like factor, cryptorchidism, fetal, human, testis

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INTRODUCTION

An undescended testis or cryptorchidism, is one of the most common congenital anomalies, occurring in 1–4% of full-term and 1–45% of preterm male neonates (1). The pathogenesis of isolated cryptorchidism remains largely unknown but is most likely multifactorial, involving both genetic, and environmental risk factors (2). The majority of what is known of the physiology of normal testicular descent in humans is inferred from animal models, primarily rodents (2). In the male fetus, the undifferentiated gonad forms high in the abdomen and descends through the abdomen and inguinal canal to eventually reside in the scrotum by birth. Testicular descent occurs in two phases—transabdominal and transinguinal (3).

The gubernaculum, a cordlike organ spanning from the testis and vas to the scrotum, plays a key role in testicular descent. Swelling of the gubernaculum is critically important to allow enlargement of the inguinal canal and testicular passage (2) and occurs in human male embryonic development during weeks 12–17 of gestation. Rodent studies provide strong evidence that the hormone INSL3 and androgen signaling pathways are the primary contributors to gubernacular development and testicular descent (4). During embryogenesis, testicular Leydig cells are the sole producers of INSL3, which circulates and binds to relaxin family peptide receptor 2 (RXFP2) receptors on gubernacular cells, causing proliferation of the gubernaculum through alterations in extracellular matrix, Wnt, bone morphogenetic protein, β-catenin, Notch, neural and cytoskeletal/muscular patterning gene pathways (5–11). With retraction of the gubernaculum, the testis then migrates from its retroperitoneal/abdominal location to the scrotum. Mice with targeted homozygous deletions of INSL3 or RXFP2 manifest bilateral cryptorchidism, with the testes remaining high in the abdominal position, as the result of gubernacular deficiencies (4, 12, 13).

While the importance of INSL3 in murine fetal testicular descent has been demonstrated, the role of INSL3 in human fetal testicular descent and maldescent is less clearly delineated. It is true that both INSL3 and RXFP2 are highly conserved at the gene and protein levels between mice and humans. Other data from human fetal testes are limited by small numbers of samples with variance between samples. Human testis INSL3 mRNA transcripts have been detected as early as 12 weeks gestation (14) and INSL3 protein was first noted by INSL3 immunohistochemistry in a few polygonal interstitial cells at gestational week 10 (15). During the time interval of human fetal gubernacular swelling, human fetal serum testosterone concentrations decline, being significantly higher at gestational weeks 11–13 than at weeks 17–19 (14). However, to our knowledge, human fetal serum INSL3 levels have not been directly measured in fetal blood during these gestational ages. As an indirect attempt, human INSL3 levels have previously been measured in the amniotic fluid of male fetuses between gestational weeks 12–22 in three publications (16–18). In 2008, Bay et al. measured amniotic fluid INSL3 concentrations between <0.02 and 0.36 ng/ml with a non-commercial semi-competitive time resolved fluorescent immunoassay (TRFIA). In 2018, using a modified TRFIA, Anand-Ivell et al. measured human amniotic fluid INSL3 concentrations in control, hypospadic and cryptorchid males, detecting levels between undetectable and 0.9 ng/ml (18). In contrast to these fetal amniotic fluid levels, normal male puberty yields a significant progressive rise in INSL3 serum levels (19–21), ultimately achieving adult male circulating INSL3 concentrations between 0.5 and 2.0 ng/ml (22). However, since amniotic fluid is a diluted source of INSL3, we hypothesize that INSL3 concentrations in fetal cord blood will be higher than measured in amniotic fluid. These findings will thus provide a more accurate assessment of circulating INSL3 levels throughout human male fetal life and improve the understanding of the physiology of testicular descent and pathophysiology of cryptorchidism.

Abbreviations: DNA, Deoxyribonucleic acid; gw, Gestational week; INSL3, Insulin-Like Peptide 3; PCR, Polymerase Chain Reaction; qRT-PCR, Quantitative reverse transcription-polymerase chain reaction; RIA, Radioimmunoassay; RXFP2, Relaxin family peptide receptor 2; mRNA, Messenger Ribonucleic acid; EIA, Enzyme immune assay; ELISA, Enzyme Linked Immunosorbent Assay; SRY, Sex-determining region of the Y gene; TRFIA, Time-Resolved Fluorescence Immunoassay; SD, Standard deviation; LC-MS/MS, Liquid Chromatography with tandem Mass Spectrometry.

MATERIALS AND METHODS

Human Samples

This study on de-identified human samples obtained from outside sources was deemed exempt by our institutional IRB. Serum umbilical cord blood samples and fetal gonads from reported normal, electively, and legally terminated pregnancies between 14 to 21 weeks gestation were provided by Advanced Bioscience Resources, Inc., (Alameda, CA). The fetal gonad tissue and umbilical cord blood were obtained from separate cases. One human adult testis sample was obtained from our institutional biobank. Clotted fetal cord blood samples (0.2–0.8 ml volume) were received in dry ice. After spinning the tubes at 2,500 rpm/min for 10 min in 4°C, the sera were drawn out and stored at −80°C for radioimmunoassay (RIA). The clotted blood in the bottom of the serum-separated gel was removed for DNA extraction. DNA was extracted from clotted whole blood samples using salting-out method (23).

Verification of Sex in Fetal Cord Blood by SRY PCR

Male sex was verified on each fetal cord blood sample (15–20 weeks gestational age) by determination of the SRY gene by polymerase chain reaction (PCR) of leukocyte genomic DNA. Two primer pairs were used for PCR. The forward primer X1 (aatcatcaaatggagatttg) and reverse primer X2 (gttcagctctgtgagtgaaa) were used to amplify a fragment of 131 base pairs in the X chromosome. The forward primer Y11 (atgatagaaacggaaatatg) and reverse primer Y22 (agtagaatgcaaagggctc) were used to amplify a fragment of 172 base pairs in the Y chromosome (24). The PCR program included denaturing at 95°C for 3 min, followed by 30 cycles at 94°C for 40 s, 54°C for 1 min and 72°C for 50 s. PCR products were separated on a 2% agarose gel.

INSL3 RIA

INSL3 serum concentrations were measured on 50 μl of 12 proven male serum samples (assayed once, in duplicate, or in triplicate, depending on available serum volume) by a non-commercial highly-sensitive and specific human INSL3 RIA as previously described (25). For each assay, new ^{125}I tracer was made just prior to the measurements to achieve the highest sensitivity. Standard curves were obtained using human INSL3 labeled with ^{125}Iodinated INSL3-mono-oxide tracer and 100 μL of 1:10,000-diluted anti-human INSL3 antibodies raised in albino rabbits. Lower and upper limits of detectability were 0.3 and 3 ng/ml. Individual samples were blinded to the person performing the assay and γ counts of the washed pellets were compared to dose-response curves of human serum INSL3.

Verification of Fetal Sex by Gonadal Histology

Male sex was confirmed histologically on each fetal gonad (15–20 weeks gestational age) by analyzing a portion of the testis. After formalin fixation, testes were paraffin embedded, sectioned to 5 μm, stained with hematoxylin and eosin and confirmed to be testis by light microscopy.

INSL3 mRNA qPCR

To secondarily confirm the trends of INSL3 concentration observed, INSL3 mRNA expression was assayed on 7 proven fetal testes by qRT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), treated with DNase, and purified using Aurum Total RNA Mini Kit (Bio-Rad, Hercule, CA), according to manufacturers' instructions. Due to small tissue size and cDNA output, cDNA was amplified using the TaqMan PreAmp Master Mix Kit (Applied Biosystems, Foster City, CA). RNA was reverse transcribed to cDNA using SuperScript III Reverse Transcription kit (Invitrogen). Probes for INSL3 (Hs01895076_s1) or CDKN1B (Hs00153277_m1) (Life Technologies) were mixed with Taqman Universal PCR Master Mix (Applied Biosystems) and amplified in triplicate on an iCycler thermocycler (Bio-Rad). Expression levels of INSL3 were normalized to CDKN1B per the Applied Biosystems T-PreAmp uniformity reference gene assay (26) and fold differences were calculated using the $\Delta\Delta C_T$ method.

Statistics

Because of the small number of samples, statistical analysis was limited. Shapiro-Wilk test for normality did not reject the hypothesis of normally distributed data ($p = 0.9$). Data on the serum INSL3 levels and INSL3 expression levels were presented as group mean ± standard deviation (SD) and were analyzed between groups using repeated measures ANOVA. A hierarchical linear regression model was used to test for trend of serum INSL3 levels over gestational ages 15–20 weeks. In this model, fixed effect was the measured INSL3 level and random effect was fetus, as some feti had repeated serum INSL3 measures obtained. $P < 0.05$ was considered statistically significant.

RESULTS

Human fetal cord blood samples from 12 males were obtained between the ages 15–20 gestational weeks (gw 15, $n = 1$; gw 16, $n = 3$; gw 17, $n = 1$; gw 19, $n = 3$; gw 20, $n = 4$) (**Supplementary Figure 1**). All 12 samples had measurable INSL3 levels (range 0.44–2.04 ng/ml) and all levels assayed were within the limits of detection for this RIA. Most of the feti had enough cord blood for multiple measures of serum INSL3 levels. Combining all fetal samples, the mean ± SD serum INSL3 concentration was 1.26 ± 0.43 ng/mL (**Figure 1**). When segregated by gestational age groups, there was no overall statistical difference found between the serum INSL3 concentrations by repeated measures ANOVA. However, there was an upward trend to the fetal serum INSL3 concentrations from 15 to 20 weeks of gestation by hierarchical linear regression ($p = 0.02$; **Figures 1, 2** and **Supplementary Figure 1**).

A comparison of these fetal cord blood serum INSL3 concentrations with previously reported INSL3 levels reveals that fetal cord blood serum INSL3 levels are 5–100 times higher than fetal amniotic fluid levels, are 2–4 times higher than prepubertal levels, and are similar to the high levels seen in young adulthood (**Figure 2** with references cited). To secondarily evaluate the trends of INSL3 concentration observed, quantitative real-time PCR was used to measure INSL3 relative expression in 7 age-matched fetal testes 14–21 weeks gestation with 1 adult testicle to serve as a comparison. INSL3 expression was detectable in all 7 fetal testes and 1 adult testes. While minimal INSL3 relative expression was observed in gestational weeks 14, 16, and 21, there was a robust peak of maximal INSL3 relative expression during weeks 17 and 18, which was over 5 times higher than observed in the adult testis (**Figure 3**).

DISCUSSION

Previous reports have shown INSL3 is only detectable in the amniotic fluid of pregnancies with a male fetus (16–18).

FIGURE 1 | Human male fetal umbilical cord serum INSL3 concentrations during gestational weeks 15–20. Each data point represents the means ± SEM for all samples tested at that age. At gestational weeks 15, 16, 17, 19, and 20, there were 1, 3, 1, 3, and 4 fetal serum samples, respectively. No fetal cord blood was collected for age 18 weeks. All feti were assayed in duplicate or triplicate when possible. Fetus #9, 10, and 11 did not undergo repeat measure due to insufficient serum volume for repeat testing.

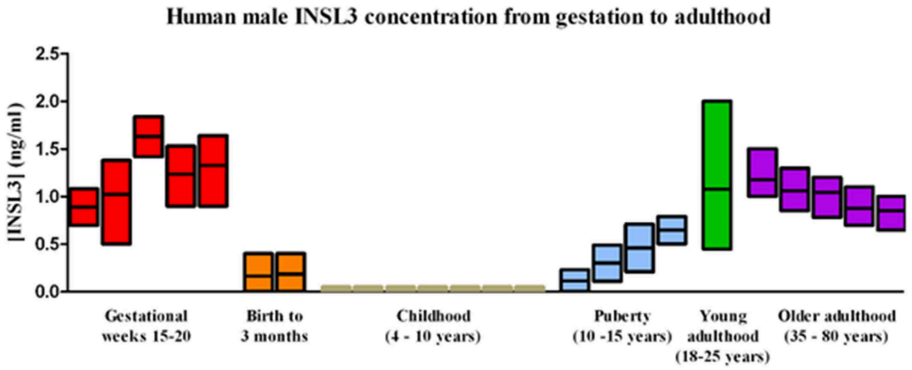

FIGURE 2 | Normal human male serum INSL3 concentrations from gestation to adulthood. INSL3 concentrations were measured in fetal cord blood from gestational weeks 15–20 (this study), cord blood from newborn male infants (27–30) and in serum from 3-month-old male infants (31), prepubertal and pubertal boys (19–21), young adult males (22), and older adult males (32).

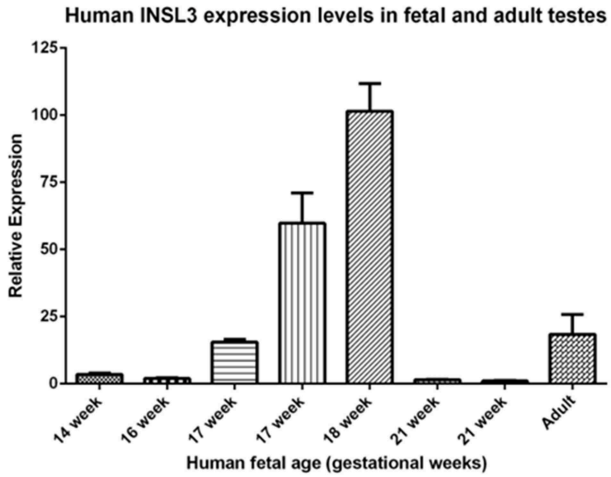

FIGURE 3 | Human INSL3 expression levels in fetal and adult testes. Human INSL3 relative expression was measured by real-time quantitative PCR in fetal gonadal tissue from gestational weeks 15–21 and adult testes tissue.

Though these previous reports have quantified fetal INSL3 concentrations in amniotic fluid as early as week 12 of pregnancy (16–18, 31), to our knowledge this is the first report of fetal INSL3 levels obtained directly from fetal umbilical cord blood. The fetal INSL3 cord blood data herein is supported by the human fetal testis INSL3 mRNA, which we and O'Shaughnessy (14) both found to be maximal at gestational week 18. As INSL3 is produced by no other fetal organ than the testes and does not appear to be produced by the female fetus, it has the potential to be a highly specific monitor for the development and differentiation of the fetal testis (33). Our findings that INSL3 levels in fetal umbilical cord blood during human male gestational weeks 15–20 are higher than previous reports of INSL3 concentrations in amniotic fluid during gestation only strengthen the role of INSL3 in testicular descent.

During embryogenesis, Leydig cells produce INSL3, which circulates and binds to the G protein coupled receptor RXFP2 on the gubernaculum (34). This interaction results in proliferation of the embryonic gubernaculum, which in turn retracts the testis from the abdominal cavity to the scrotum. Gestational weeks 15–18, in which INSL3 levels are at their highest, coincide with gubernacular outgrowth and the initial abdominal phase of testicular descent. The findings of increased production of INSL3 after week 15 suggests that INSL3 is not only required for the transabdominal phase of descent but possibly in the inguinoscrotal phase of testicular descent, perhaps by dilating the inguinal canal (16).

It was previously believed that the second phase of testicular descent, the passage of the testes through the inguinal canal and into the scrotum, was largely under androgen control. However, a combination of androgen and INSL3 receptor antagonists in mice has shown that the remediation of testicular descent in cryptorchid mutant mice by testosterone replacement therapy required the induction by androgens of the INSL3 receptor RXFP2 (34, 35). Thus, as concluded by Ivell et al. it is reasonable to hypothesize that gonadotropin induced testicular descent may not be entirely due to the induction of testicular androgen production but rather to the stimulation of Leydig cell differentiation and thus secretion of INSL3 (34). The findings of even higher fetal INSL3 levels than previously reported via amniotic fluid at gestational weeks 18–20 further confirms the role of INSL3 in all phases of testicular descent.

In the fetal amniotic fluid level studies, the maximum concentrations of INSL3 were between 12 and 16 weeks with a decline thereafter to below detection levels (16, 17). The authors have stated that it is not clear whether this decline was secondary to a down-regulation of INSL3 expression, an increasing dilution effect, decreasing fetal skin permeability, or catabolism (33). During the first and early second trimester, amniotic fluid can be regarded to a degree as an exudate of fetal serum, however later in gestation, when the fetal skin keratinizes, the composition of amniotic fluid becomes more representative of fetal urine (36). Fetal cord blood INSL3 levels, as reported in the present study,

thus provide a more accurate representation of INSL3 with levels persisting through 20 weeks gestation.

In addition, fetal cord blood INSL3 levels were found to be higher than reported INSL3 concentrations in pre-pubertal males and throughout most of adult life (**Figure 2**). Circulating levels of INSL3 in postnatal life are characterized by an increase early (birth to 3 months), followed by very low levels during childhood (4–10 years), with a spike in INSL3 production at puberty that leads into high levels in young adulthood before decreasing again in later adulthood (21, 22, 31, 32). As INSL3 is secreted by Leydig cells, Leydig cell differentiation influences INSL3 production and the triphasic nature of Leydig cell development coincides with the peaks of INSL3 levels seen in human life. The three populations of Leydig cells include the fetal Leydig population which reaches its maximal size around gestational weeks 14–18 (14), a perinatal population that peaks in size 3 months after birth, and the adult Leydig population which begins increasing in size at puberty.

All types of Leydig cells produce INSL3. It is believed by some that the fetal Leydig cell population responsible for fetal INSL3 production for testicular descent is completely separate from the adult Leydig population which develops from stem cells at puberty (34, 37), while others believe the human fetal Leydig cell population involutes and re-differentiates with pubertal onset. It has been suggested that INSL3 is produced constitutively, independent of acute regulation by the hypothalamo-pituitary gonadal axis, in amounts which reflect the numbers and differentiation status of the Leydig cells (33). Serum INSL3 levels may thus be a better indicator for Leydig cell function than serum testosterone concentrations in the male (38, 39). In addition, as fetal Leydig cells are a separate population from adult Leydig cells, it is possible that alterations in fetal Leydig function or INSL3 production or secretion may underlie the pathophysiology of cryptorchidism. In humans, this has been challenging to prove, with some contradictory findings. Three relatively large human studies compared serum human INSL3 cord blood levels at birth in male infants with descended and undescended testes, finding reduced INSL3 serum levels in cryptorchid boys and suggesting impaired Leydig cell function in boys with persistent cryptorchidism already in early postnatal life (27, 28, 31). The major criticism of these three studies is that serum levels at birth or 3 months postnatally may not reflect second trimester levels. To address this concern, Jensen et al studied 270 cryptorchid and 300 control second trimester amniotic fluid samples' INSL3 and phthalate levels and found no clear association (40). In addition to other estrogenic and anti-androgenic compounds, fetal testes cells exposed in culture to paracetamol and ketoconazole have decreased INSL3 levels which may be the mechanism by which analgesics, and possibly other endocrine disruptors, increase the risk of cryptorchidism (41).

Nevertheless, serum levels of a hormone do not necessarily correlate with required tissue levels for proper development and function. For example, it is known that postnatal rat testosterone concentrations assayed from either the testicular vein (42) or the testicular interstitial fluid (43) are 20 times and 100 times higher than systemic circulating serum concentrations, respectively. In addition, varying serum and tissue testosterone concentrations on fetal mammalian paratesticular tissues (directly on the vas, epididymis, seminal vesicles, gubernaculum, and indirectly via 5-alpha-reductase/dihydrotestosterone on the penis/urethra, prostate, and scrotum) via endocrine and paracrine mechanisms mediate the phenotypic spectrum of male birth defects seen in disorders of sex development (44–46). Similarly, it has been shown that rat fetal testicular INSL3 mRNA expression probably requires at least 40% reduction before a change in phenotype occurs (47). Hence, human fetal amniotic fluid and even human fetal cord blood INSL3 concentrations likely do not truly measure and reflect the threshold of INSL3 fetal tissue concentration required to induce tissue level maldevelopment of the gubernaculum and maldescent of the testis during fetal life. They are indirect measures at best.

The main limitation to this study is the small sample size secondary to limited access to umbilical cord blood from human fetuses aged 12–20 gestational weeks. A second potential limitation of this study is the INSL3 assay used. Nowadays, INSL3 concentrations can be measured by several assays, including RIA, EIA, ELISA, TRFIA, and LC-MS/MS. Our INSL3 RIA assay was performed several years ago to generate our INSL3 data, explaining the older methodology. As a result, this older assay has not actually been fully validated, hence it is not known if the assay cross-reacts with other plasma components. The plasma samples did parallel the standard curve for both fetal and adult plasma samples, suggesting that the endogenous INSL3 peptide is similar in adult and fetal blood. Unfortunately, the quantity of each serum sample was insufficient to permit replication of the serum Insl3 concentrations by one of the newer assays that have a lower limit of detection than our RIA. Given the lower and upper limits of detectability of our RIA were 0.3 and 3 ng/ml, the RIA is at least one order of magnitude less sensitive than the assays in current use, which have lower limits of detection ranging from 0.01 to 0.06 ng/ml (18, 19, 27–29, 48, 49). Less sensitivity can increase intra-assay coefficient of variation. While this would be most problematic if the serum levels were quite low, the mean serum umbilical cord INSL3 levels were well within the midrange of the limits of detection of the RIA assay and the mRNA data is corroborative. Additionally, while the fetuses were labeled as normal based on prenatal investigation prior to elective termination, the ultimate phenotype as a term infant is unknown, and the study could theoretically have included samples that might have gone on to develop cryptorchidism or a disorder of sex development, thus skewing results. Lastly, we did not measure fetal cord serum levels of H2 relaxin (RNL2). While there is theoretical concern that H2 relaxin is able to activate the RXFP2 receptor given this occurs *in vitro*, it occurs only at supra-physiological concentrations which are unlikely to occur in the fetus since no major relaxin expression has been observed on tissue microarray studies before puberty. Thus, most evidence suggests H2 relaxin is unlikely to play a major role in testicular descent (50).

CONCLUSIONS

Human fetal levels of INSL3 have previously only been studied in amniotic fluid, which underestimated the circulating

concentration of INSL3. This study of INSL3 levels obtained from fetal cord blood and testes indicates that INSL3 serum concentration during male fetal life is 5–100 times higher than INSL3 levels observed in amniotic fluid, is 2–4 times higher than serum levels of prepubertal boys, and is amazingly similar to that of young adult men. These findings lend further support to the role of INSL3 in human testicular descent during fetal life in males.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00596/full#supplementary-material

Supplementary Figure 1 | Human Fetal umbilical cord serum INSL3 concentrations: raw data. Human umbilical cord serum INSL3 concentrations raw data, reporting individual and repeated measures from each fetus by gestational age.

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