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Germline (epi)genetics reveals high predisposition in females: a 5-year, nationwide, prospective Wilms tumour cohort

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ABSTRACT

Background Studies suggest that Wilms tumours (WT) are caused by underlying genetic (5%–10%) and epigenetic (2%–29%) mechanisms, yet studies covering both aspects are sparse.

Methods We performed prospective whole-genome sequencing of germline DNA in Danish children diagnosed with WT from 2016 to 2021, and linked genotypes to deep phenotypes.

Results Of 24 patients (58% female), 3 (13%, all female) harboured pathogenic germline variants in WT risk genes (*FBXW7, WT1* and *REST*). Only one patient had a family history of WT (3 cases), segregating with the *REST* variant. Epigenetic testing revealed one (4%) additional patient (female) with uniparental disomy of chromosome 11 and Beckwith-Wiedemann syndrome (BWS). We observed a tendency of higher methylation of the BWS-related imprinting centre 1 in patients with WT than in healthy controls. Three patients (13%, all female) with bilateral tumours and/or features of BWS had higher birth weights (4780 g vs 3575 g; p=0.002). We observed more patients with macrosomia (>4250 g, n=5, all female) than expected (OR 9.98 (95% CI 2.56 to 34.66)). Genes involved in early kidney development were enriched in our constrained gene analysis, including both known (*WT1*, *FBXW7*) and candidate (*CTNND1, FRMD4A*) WT predisposition genes. WT predisposing variants, BWS and/or macrosomia (n=8, all female) were more common in female patients than male patients (p=0.01).

Conclusion We find that most females (57%) and 33% of all patients with WT had either a genetic or another indicator of WT predisposition. This emphasises the need for scrutiny when diagnosing patients with WT, as early detection of underlying predisposition may impact treatment, follow-up and genetic counselling.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The renal cancer Wilms tumour (WT) accounts for 1 in 14 malignancies in children.
⇒ Expanded availability and use of comprehensive genomic technologies have accelerated the discovery of WT predisposing genetic and epigenetic conditions, yet few studies have studied this systematically and unselectively.

WHAT THIS STUDY ADDS

⇒ Our study, a 5-year, nationwide comprehensive germline study of children with WT, identified either underlying (epi)genetic causes or highly suggestive phenotypic traits in 33% of participants.
⇒ A distinct phenotype, consisting of very high birth weight, perilobar nephrogenic rests, advanced tumour stage and, notably, female sex, emerged, possibly underlying the well-established over-representation of females among patients with WT.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study adds several novel insights contributing to a growing body of evidence that WTs are less sporadic than previously understood, with several clinical implications for prognosis, treatment and follow-up of patients, and tumour surveillance and family planning for both and their family members.

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INTRODUCTION

Wilms tumour (WT), or nephroblastoma, is a renal tumour of young children (median age: 3.5 years), accounting for 1 in 14 of all childhood cancers.[1 2] WT likely has embryonic origins, and, histologically, its tissue mimics the early stages in nephrogenesis possibly occurring as a product of abnormal renal development.[3–5] This type of histologically driven cancer aetiology is believed to underlie several early childhood malignancies.[6 7] Indeed, genetic alterations known to cause or drive WT often impact genes with key roles in fetal kidney development.[2]

With the advent of improved sequencing technologies, molecularly driven theories of WT aetiology have accelerated in recent decades (online supplemental figure 1). It is now appreciated that WT, despite being a generally non-familial disease, contains a significant component of underlying genetic and epigenetic causes.[8–15] These include both 'covert' cancer predisposition syndromes (CPSs) where increased risk of distinct tumour types is the sole manifestation (eg, WT caused by *REST*, *DICER1* and *FBXW7* germline alterations),

as well as more 'overt' CPSs where cancer risk is one phenotypic feature among several (eg, chromosome 11p15.5-related disorders with overgrowth, anatomical malformations and/or intellectual disability). Often, clinical suspicion of an underlying genetic predisposition syndrome may not be raised.

A recent germline genomics study[9] assessed the burden of CPSs among 91 familial and 799 singleton WT cases demonstrating that 8% had an identifiable pathogenic variant in a CPS gene and that this percentage is higher (~30%) in familial cases. Importantly, the study did not assess epigenetics, yet estimated that around 2% of WT cases had germline epigenetic origin, that is, Beckwith-Wiedemann spectrum (BWSp) disease. A more recent study,[16] focused on epigenetics, contests this latter estimate, showing that 6 (29%; all female) of 21 patients with WT (12 female; 9 male) had a low-mosaic gain-of-methylation (GOM) of imprinting centre 1 (IC1, H19-ICR) on chromosome 11p15.5 (an epimutation classically causative of BWS) in peripheral blood. These patients tended to have bilateral WT at diagnosis and nephrogenic rests on the tumour pathology.

Previous aetiological studies of WT have mostly been focused on either germline genetics or DNA methylation—and the studies have mostly been conducted on selected cohorts with a disproportionate number of cases with high-risk and/or familial disease.[9 12 16] A key exception is a recent nationwide Dutch study,[17] in which one-third of children with WT (42 of 126) were found to harbour WT predisposing factors. Of note, this included 13 patients with pathogenic epigenetic aberrations isolated to kidney DNA or heterozygous DIS3L2 variants, which were suggested as a bona fide WT CPS within the same work. Here, we present a prospective, nationwide study, performing both epigenetics and whole-genome comprehensive germline genomics, with the aim of characterising the contribution of monogenic diseases to WT in children. Our work adds to the growing literature on WT predisposition and contributes key findings regarding female over-representation among patients with WT and a novel evolutionary approach to WT predisposition gene discovery.

METHODS

In the interest of reproducibility, detailed methods are available in the online supplemental materials and are only described briefly below (see overview in figure 1).

Histology and stage were assessed and centrally reviewed for all tumours according to the International Society of Paediatric Oncology (SIOP) staging after preoperative chemotherapy.[18] Following diagnosis patients consented to the *Sequencing of Tumour and Germline DNA—Implications and National Guidelines* project, with inclusion from 1 July 2016 to 1 July 2021. Inclusion procedures and germline sequencing protocols have been published elsewhere.[19] Whole-genome sequencing (WGS) was performed and rare variants in a panel of 390 selected genes[20–22] were reviewed by a multidisciplinary team.[23] Next, full genome predicted loss-of-function (pLoF) variants were filtered and subjected to constraint gene analysis, as previously presented.[24–26]

Additionally, pyrosequencing of IC1 was performed on peripheral blood and, when available, tumour DNA from individuals with WT and relevant controls, using methods described previously.[27] Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA, ME030-C3, MRC Holland, Amsterdam, The Netherlands) was conducted according to the manufacturer's instructions. All statistical tests were performed using R (V.3.6.1) and are indicated in-text whenever applied.

RESULTS

Over a period of 60 months, we prospectively included 596 Danish paediatric pancancer patients nationwide. Among all included patients, 24 (4%) had a diagnosis of WT (n=23) or nephroblastomatosis (NB) (n=1) (24 out of 28 eligible; inclusion 86%). Females comprised 58% of the participants (14 out of 24). The four patients with WT that declined inclusion were all female. Patient #11 had NB and only needle biopsy of the tumorous mass was performed (table 1). Nine patients (38%; 50% of females and 20% of males) showed perilobar (5) or intralobar (4) nephrogenic rests on pathology.

Panel of cancer-related genes

Among 390 genes known to be related to cancer (somatically, germline or both), we classified 696 SNVs or SVs (online supplemental table 1). Of these, five likely pathogenic variants were found in three patients (patients #1–#3). Variants in three genes (*REST, FBXW7* and *WT1*) were considered causative (figure 2 and online supplemental table 4).

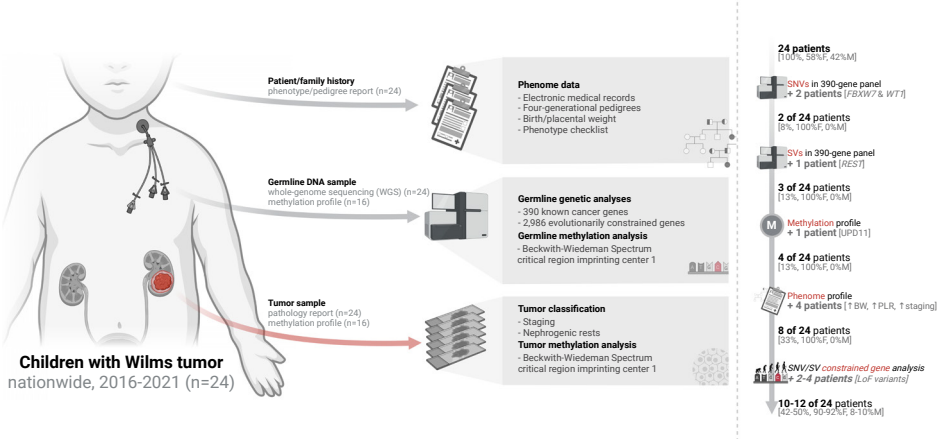

Figure 1 On the left: an overview of the population, materials and analysis methods. On the right: an overview of the aetiological findings using different analyses/modalities. BW, birth weight; F, female; LoF, loss-of-function; M, male; PLR, perilobar nephrogenic rests; SNVs, single nucleotide variants; SVs, structural variants; UPD11, uniparental disomy of chromosome 11.

Table 1 Summary characteristics for 24 patients with Wilms tumour

Characteristic	N=24*
Sex	
Female	14 (58%)
Male	10 (42%)
Age (month)	42 (29, 58)
Tumour side	
Bilateral	2 (8.3%)
Left kidney	14 (58%)
Right kidney	8 (33%)
Stage	
NB	1 (4.2%)
I	7 (29%)
II	5 (21%)
III	7 (29%)
IV	2 (8.3%)
V	2 (8.3%)
Nephrogenic rests	
–	15 (62%)
Intralobar	4 (17%)
Perilobar	5 (21%)

*N (%); median (IQR).
NB, nephroblastomatosis.

Patient #1 harboured a 1.62 Mb structural germline deletion (chr4:57,761,129-59,377,004) that spanned seven genes including all the coding regions of the *REST* gene (and *POLR2B*, see below). The deletion was shown to be inherited paternally matching the patient's family history with two paternal relatives (second and fourth degree) with WT in childhood. The first relative died of the disease at age 6 years and biological material was unavailable for study. The second, more distant relative was alive and microarray revealed that he was a carrier of the same 1.62 Mb deletion, which thus segregated with WT. The proband's father and the two obligate carriers were unaffected (figure 2).

Patient #2 harboured a nonsense germline variant in *FBXW7* (NP_361014.1:p.Arg278Ter) shown to be inherited from the mother who had an otherwise unremarkable medical history. The proband initially achieved remission, but relapsed with treatment-refractory metastatic disease which was ultimately fatal.

Patient #3 harboured a causative frameshift variant in *WT1* (NP_077744.3:p.Pro111Argfs*47) in addition to two likely pathogenic variants in *SDHC* and *BUB3*. The *SDHC* variant (NP_002992.1:p.Arg50Cys) was classified as likely pathogenic in ClinVar as it has been observed in several patients with hereditary paraganglioma-pheochromocytoma syndrome and is absent from gnomAD. The same variant was observed in 2 of 572 Danish children with cancers other than WT (acute myeloid leukaemia and osteosarcoma). The *BUB3* splice acceptor variant

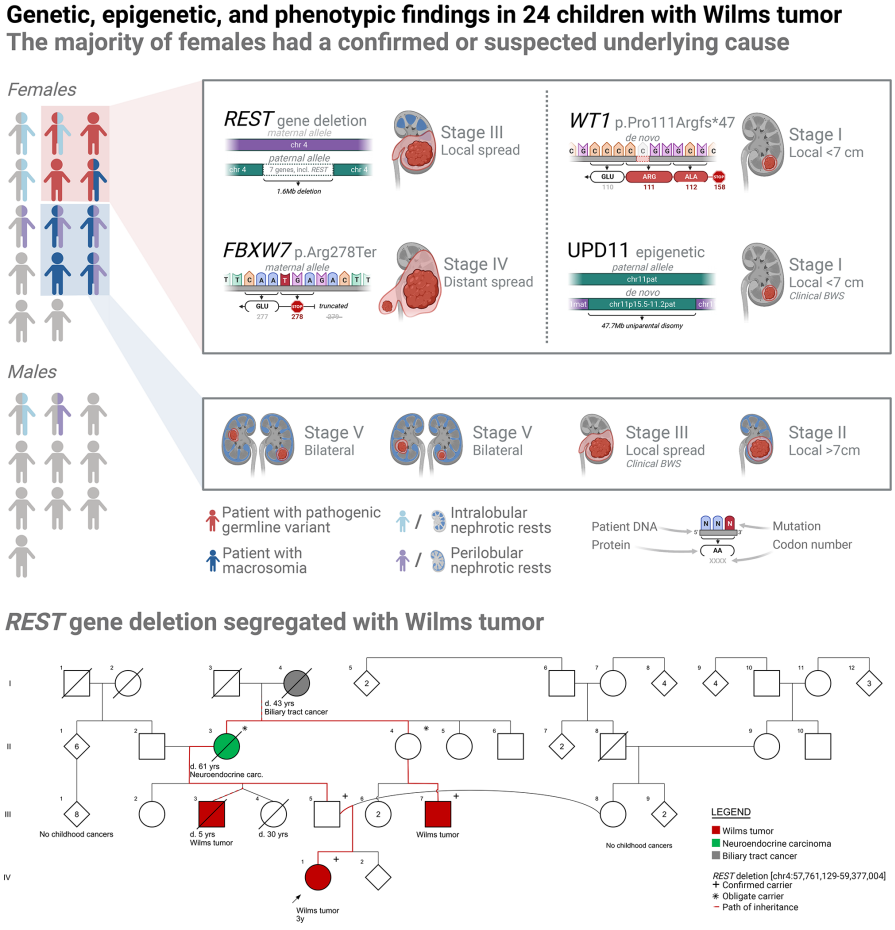

Figure 2 Top: visual representation of the main genetic, methylation and phenotypic findings. BWS, Beckwith-Wiedemann syndrome; UPD11, uniparental disomy of chromosome 11. Bottom: four-generational pedigree, indicating the proband (IV.1; patient #1) in whom the microdeletion of chromosome 4, including the REST gene, was found and her family, in which two members developed Wilms tumour (one shown to carry the same microdeletion and one is a probable carrier, but had no tissue available for sequencing). d., age of death.

(NM_004725.3:c.755–2A>T) was absent from gnomAD. The proband had no family history of early cancers in four generations.

More than a 100 variants of unknown significance (VUSs) were identified. Yet, 13 VUSs in 10 genes were considered of interest as they affected genes previously linked to WT predisposition (online supplemental tables 1 and 4). Of note, four missense variants in the *NYNRIN* gene were detected. This constituted a significantly higher rate than in all other in-house germline sequencing data from children with cancer (Poisson's exact test; 4/24 vs 10/572, p=0.002). Two *NYNRIN* missense variants (NP_079357.2:p.Thr1172Met and p.Glu420Met) were identified in patient #15 and subsequent Sanger sequencing revealed that they were inherited from the father and mother, respectively, indicating compound heterozygosity. The proband had stage I WT of triphasic histology at <36 months of age as well as mild motor delay and a single café-au-lait macule (online supplemental table 1). Additionally, a 183 kb structural deletion of unknown significance spanning *BARD1* (hg19; chr2:215591264-215774591) was observed in patient #9: the deletion included the first 11 exons of the gene and hereby the patient was hemizygous for the A allele in SNP position rs7585356, associated with nephroblastoma risk.[28] The same patient also harboured a heterozygous *NYNRIN* missense variant (NP_079357.2:p.Gly353Arg).

Epigenetics and BWS phenotypes

Clinical epigenetic testing (MS-MLPA) revealed uniparental disomy of chromosome 11 (UPD11) in patient #4 with several non-cancer phenotypic features, including lateralised overgrowth and macroglossia, all highly suggestive of BWS (online supplemental table 2). This patient had a birth weight of 4920 g (>98 percentile). Patient #7 likewise had features suggestive of BWSp, with mild lateralised overgrowth of the right leg and arm, minor umbilical hernia and neonatal hypoglycaemia in addition to WT. This alone establishes a clinical diagnosis of BWS, however, MS-MLPA and *CDKN1C* sequencing did not reveal a detectable causative finding. Nevertheless, in line with the clinical BWS diagnosis, this patient also had a high birth weight (4850 g, >98 percentile). Electronic medical records noted birth weights for 19 of 24 patients. Of these, a total of five patients had macrosomia with birth weights >4250 g; two (#4 & #7) with a BWS diagnosis and two (#5 & #6) with stage V WT, that is, bilateral. Together, patients with BWS or bilateral WT were all female and had significantly higher birth weight than the rest of the cohort (t-test; 4780 g vs 3575 g (95% CI 504 g to 1906 g), p=0.002). Of note, patient #8 also had macrosomia (birth weight=4500 g), yet she presented with stage II WT and without further evidence of BWS. Collectively, the number of females with macrosomia in our cohort (n=5) was significantly higher than that expected based on publicly available birth weight data (Fisher's exact test; 5/13 vs 1790/30 373 OR 9.98 (95% CI 2.56 to 34.66), p<0.001). This association remained significant even when patient #4 (epigenetically confirmed BWS), patient #8 (clinical BWS) or both were excluded (p=0.004, p=0.02, respectively). Of note, patient #8 showed perilobar nephrogenic rests on the WT pathology and so did the two patients with macrosomia and bilateral disease (figure 2). Again, excluding the patients with known BWS, macrosomia was significantly associated with perilobular nephrotic rests (Fisher's exact test; 3/3 vs 2/19, p=0.007).

As introduced at the beginning of this paper, the phenotype that emerged (females with WT and one or more features of BWS, perilobar nephrogenic rests and/or bilateral/extensive disease) was recently suggested as a distinct aetiological group by Fiala *et al*,[16] although birth weight and location of nephrogenic rest were not reported in their study. We therefore undertook a replication experiment based on the hypothesis that the females in our cohort with macrosomia and the other features mentioned would display equivalent DNA methylation changes to those reported by Fiala *et al*.

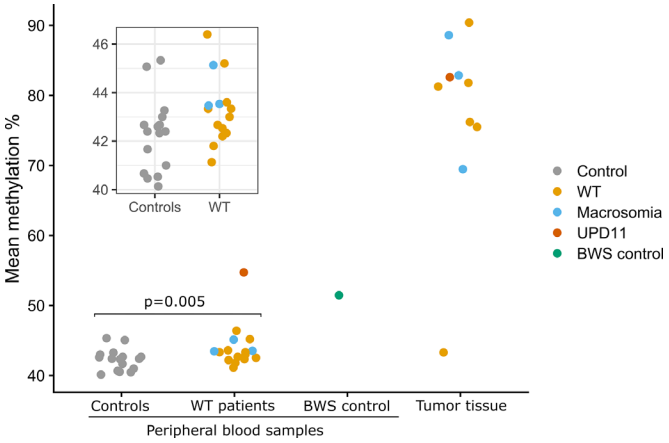

Figure 3 Jitter plot showing IC1 methylation levels using pyrosequencing of all individuals included in the methylation analysis. The methylation levels are calculated as an average of the five CpGs analysed, and the mean of the triplicates. The plot within the rectangle in the upper left corner shows only blood IC1 methylation levels in patients with WT and controls in a detailed scale. BWS, Beckwith-Wiedemann syndrome; IC1, imprinting centre 1; UPD11, uniparental disomy of chromosome 11; WT, Wilms tumour.

Pyrosequencing analysis of IC1 in peripheral blood revealed clear GOM in patient #4 (with UPD11). The remaining patients had IC1 methylation levels within the normal range, although slightly higher for the children with WT compared with controls (linear mixed-effects model, p=0.005, figure 3). Similarly, there was a tendency of higher methylation in individuals with macrosomia compared with those without, but the difference was not significant (linear mixed-effects model, p=0.143). IC1 was hypermethylated in the tumour samples of 9 out of 10 individuals (figure 3). We did not observe any correlation between methylation levels in blood and tumour tissue (Pearson's correlation test, r=0.35, p=0.49).

MS-MLPA revealed borderline GOM of IC1 in the blood of an additional individual (patient #19, IC1 mean methylation=0.65, threshold indicative of GOM is set as >0.65 in the clinical set-up), who also had the highest IC1 methylation with pyrosequencing among those within the normal range (46.4%, online supplemental table 4). Furthermore, MS-MLPA detected UPD11 in the tumour tissue of an additional individual (patient #16, IC1 GOM and IC2 loss-of-methylation). With MS-MLPA, a tendency of higher blood IC1 methylation was observed in children with WT (online supplemental figure 2), but the difference was not significant (Welch's t-test, p=0.054). In contrast to pyrosequencing, MS-MLPA showed a non-significant tendency towards lower blood IC1 methylation in individuals with macrosomia compared with those without (online supplemental figure 2, Wilcoxon rank-sum test, p=0.14). A moderate correlation was observed between pyrosequencing and MS-MLPA results (Pearson's correlation test, r=0.46, p=0.01). For the tumour samples, MS-MLPA showed GOM in the same patients as pyrosequencing.

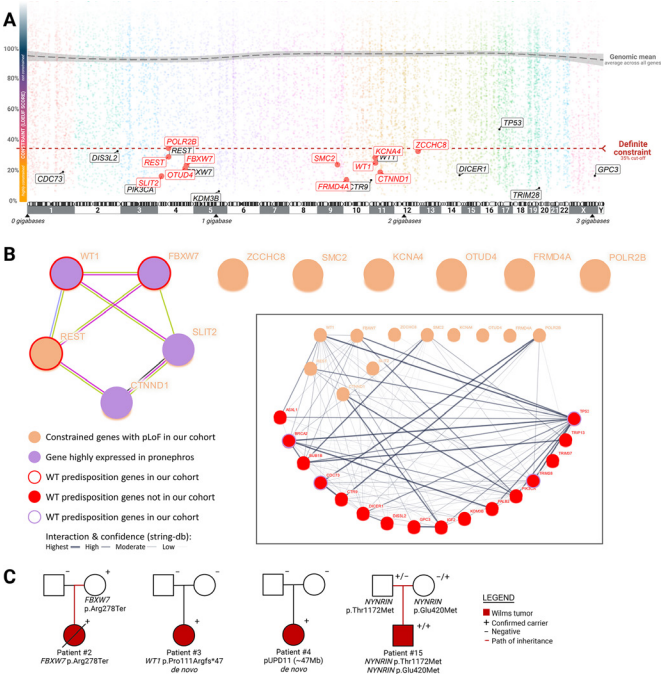

Figure 4 Constrained genes and Wilms tumour (WT) risk. (A) Genomic view of all genes visualised with a chromatic scale; arranged according to chromosomal position on x-axis and loss-of-function observed versus expected upper fraction (LOEUF) score on y-axis. Genes monoallelically associated with high WT risk are highlight with names in black. Constrained genes with loss-of-function mutations found in our cohort are highlighted in red. (B) Eleven constrained genes found to harbour predicted loss-of-function (pLoF) variants in our cohort. On the left: a cluster of interactions is seen with textmining in green (co-mentioned in abstract), experimentally determined interaction in magenta (including in non-human organisms), co-expression in dark blue (including in non-human organisms) and protein homology in light blue. The cluster involves all the genes either known to cause WT, highly expressed in pronephros or both. The box illustrates the same 11 genes along with 16 other genes known to cause WT. Two genes (SMC2, POLR2B) from our cohort, which had no interactions in isolation, emerge as having several interactions with other WT predisposition genes. (C) Parent-of-origin analyses in select cases (figure 2, online supplemental tables 1 and 4).

Constrained gene analysis

As we have previously described, genes associated with high risk of cancer in childhood show highly significant constraint.[24] This also holds true for genes associated with high WT risk, in which only 8% of expected pLoF occur in adult populations; markedly different from other genes (t-test; genes associated with monoallelic risk of WT vs all other genes, loss-of-function observed versus expected upper fraction (LOEUF) score 20.5% vs 95.3%, p<0.001) (figure 4A).

In our cohort, 8 patients harboured a total of 12 predicted pLoF variants in 11 constrained genes, that is, genes exhibiting evolutionarily intolerance of such damaging alterations (figure 4A,B and online supplemental table 2).[25] No pLoF variants were found in these 11 genes among 572 Danish children with cancers other than WT. Our constrained gene analysis (CGA) reidentified all three causative genetic variants found on the gene panel-based approach described above. In other words, 25% of the 12 variants found using CGA were immediately appreciated as pathogenic. This raises the question of whether any of the remaining nine variants may play a role in WT aetiology.

Functional interaction, Gene Set Enrichment Analysis and Gene Ontology (GO) enrichment analysis were performed for constrained genes with pLoF variants using the String Database (String-db V.11) and the GO knowledgebase.[29 30] String-db analysis of all 11 genes showed enrichment only for genes highly expressed in pronephros[29] (four genes, 36%; *FBXW7*, *WT1*, *SLIT2* and *CTNND1*, false discovery rate; o/e ratio 36.3 (95% CI 8.66 to 125.86), p=0.009). Excluding the three genes already known to be associated with high WT risk, pronephros expression in our findings remained significantly higher than expected, even among constrained genes only (Fisher's exact test; 2/8 vs 95/2966, p=0.026). Additionally, interactions were seen between 2 constrained genes (*SMC2* and *POLR2B*) and 16 other known WT predisposition genes. The pLoF variants in *SLIT2* and *POLR2B* were seen in patient #4 with UPD11 and in patient #1 with haploinsufficiency of *REST*, respectively; both patients are described above.

Patient #20 harboured a *CTNND1* pLoF variant (NP_001078927.1:p.Ser847Ter). This child was diagnosed with stage III WT at >72 months of age and showed perilobar nephrogenic rests on pathology. Extended phenotyping and EMR mining revealed that the patient had a wide fontanelle with abnormally delayed closure, blue hue of the sclera and that the proband's father was born with unilateral renal agenesis. The same patient also harboured a pLoF variant (NP_001305266.1:p.Gln48Ter) in the constrained gene *FRMD4A*, and patient #12 carried another pLoF variant (NP_001305266.1:p.Leu227Thrfs*51) in that same gene (online supplemental tables 2 and 4). Thus, two children (8%) in the WT cohort carried a pLoF variant in *FRMD4A*, versus none among the 571 children with cancers other than WT, and 11 (>0.01%) among 141 456 adults in gnomAD.[25] Patient #12 also carried an *SMC2* pLoF variant (NP_006435.2:p.Ser133Trpfs*3) and was diagnosed with stage III WT at 36–72 months of age. No nephrogenic rests were noted on pathology. Patient #12 had small stature (−2 SD weight; −2.5 SD height) and had been evaluated for Turner syndrome and coeliac disease prior to WT diagnosis.

DISCUSSION

In this prospective nationwide study, we show that patients with WT have a high rate of underlying CPS and that females with WT have a significantly higher birth weight than expected. The females with macrosomia had a tendency to have bilateral disease, and were significantly more likely to have perilobar nephrogenic rests on pathology. Nordic registry studies have previously found significantly higher birth weights in children with WT, an association shown to be limited to females.[31] Our results suggest that this observation is linked to the low-mosaic 11p15.5 GOM phenotype presented by Fiala *et al*,[16] which we expand to include high birth weight and perilobar nephrogenic rests.

Our results support a distinct syndromic subentity on the BWSp, and we hypothesise that a significant portion of the overrepresentation of neonatal macrosomia in females with WT is due to discrete and/or low-mosaic epimutations of the IC1 locus. However, the present DNA methylation data obtained by pyrosequencing and MS-MLPA did not replicate the findings of low-mosaic epimutations previously reported.[16] Of note, the method used for methylation analysis in the present study differ from the methylation-sensitive, quantitative, real-time PCR used by Fiala *et al*. It is thus possible that the GOM previously

observed in patients with WT with bilateral tumours is restricted to regions of IC1 not analysed in the present study. As a whole, we observed slightly higher methylation levels in the peripheral blood in the patient group than in age-matched controls, but the difference was only statistically significant ($p<0.05$) with the pyrosequencing analysis. This could be due to different regions of IC1 being analysed with pyrosequencing and MS-MLPA, but it has also been reported that pyrosequencing is more sensitive in detecting partial or mosaic methylation changes than MS-MLPA.[32] Furthermore, the pyrosequencing was conducted in triplicates, which increased the amount of data, and thus the statistical power of the analysis. The methylation levels for both controls and patients with WT were overlapping, illustrating that any true difference is unlikely to be useful for presymptomatic clinical identification of high-risk individuals.

Collectively, the pathogenic and likely causative genetic/epigenetic events were de novo in two of four individuals (50%). As expected, none of these patients had any family history of WT. Another patient had a heterozygous whole gene deletion of the *REST* gene, shown here to segregate with WT in the family. We report another patient, who succumbed to WT, carrying a pathogenic LoF variant in *FBXW7* in the germline. Among the three other WT cases with such variants reported in the literature, two have died (one from primary disease and one from a second primary osteosarcoma in adulthood, and the third has relapsed 2 years after initial diagnosis).[9] In a cancer with very high cure rates, such findings may indicate that WT caused by an underlying pathogenic *FBXW7* variant has a poor prognosis. Data were not available for proper survival analysis, but *FBXW7* germline status showed a significant, although entirely uncorrected, correlation with death (Fisher's exact test; 3 of 4 vs 1 of 38, $p=0.001$), when our data were combined with those of Mahamdallie *et al* (carriers of germline variants only).[9] This tentative correlation warrants further study, but somatic FBXW7 loss in several tumour types is correlated to resistance to chemotherapeutic agents and poorer disease outcome.[33]

We report a patient #15, with compound heterozygous missense mutations in the autosomal recessive WT predisposition gene *NYNRIN*. The three previously reported cases were compound heterozygous for LoF variants.[9] Our observation of two missense variants located in trans in a patient with WT is novel and merits further investigation of *NYNRIN* variants, including non-LoF, within WT cohorts.

We show that pLoF variants in evolutionarily constrained genes were significantly more likely to play a role in kidney development; including *CTNND1*, not previously linked with WT predisposition. Today, the long-term survival of children with WT in high-income nations is high (~90%).[26 34] Yet, throughout human[35] and prehuman[36] evolution, the cancer, typically presenting in the first 5 years of life, presumably meant that affected individuals did not survive into adulthood. Consequently, any genetic event causing the disease would be subject to natural selection. Based on this reasoning, we undertook an analysis of the 2986 genes found to be evolutionarily constrained in the human gene pool.[25] Three of the 11 genes found to be affected by pLoF variants in our CGA were already known to be WT predisposition genes, highlighting the utility of this analysis for variant prioritisation. Unquestioningly, not all of the remaining eight genes will impact WT risk, yet, as detailed below, the literature indicates that quite a number of the genes are central in processes related to kidney and WT developments.

Patient #20 harboured a nonsense variant (NP_001078927.1:p.Ser847Ter) in the *CTNND1* gene and showed perilobar rests on pathology—a phenotype typically associated with 11p15 locus dysregulation.[37] No *CTNND1* pLoF variants were seen among the 571 non-WT childhood patients with cancer of this study, and just six pLoF variants, mostly splicing, were observed in 141 456 adults in gnomAD.[25] *CTNND1*, highly expressed in pronephros, encodes the p120-catenin protein, which, in turn, interacts with the E-cadherin protein, aiding cell adhesion and tissue formation.[29] Gain-of-function variants in the paralogous gene *CTNNB1,* encoding β-catenin of the catenin family, occur in 15%–50% of WTs and are known to drive paediatric cancers related to BWS, highlighting this pathway's key role in WT formation.[38 39] Germline *CTNND1* LoF variants are known to cause the Mendelian autosomal dominant blepharocheilodontic syndrome (BCD, MIM: 617 681). The phenotype of patient #20 was abnormal, yet did not resemble BCD. Recently, a case series[40] revealed that pathogenic *CTNND1* variants in 13 patients with BCD were associated with broad and sometimes discrete phenotypes driven both by p120-catenin's epithelial function and by distinct mesenchymal molecular functions—with the latter regulating the WNT signalling pathway. Of note, one of the 13 patients with BCD had childhood cancer (ovarian dysgerminoma). Patient #20 represents the first association between germline *CTNND1* variants and WT.

Curiously, the same patient harboured a pLoF variant in the highly constrained *FRMD4A*, a gene also known to play a role in cellular adhesion, presenting a possible oligogenic cause. This was somewhat strengthened by the finding that patient #12 also carried a pLoF variant in *FRMD4A*. She too carried an additional pLoF variant in another gene, namely *SMC2,* which provides an integral subunit to the human condensin complex[41]; playing a driving role in human cancers. The gene is regulated directly by β-catenin and its expression is thus enhanced as part of the transcriptional activity of the WNT pathway. In isolation, these early findings in genes with high evolutionary intolerance to LoF variants in patients with WT cannot infer causality. Yet, incorporating constraint gene analysis in future studies should inform which genes merit further/functional studies. Individual findings may also be further investigated through somatic sequencing, which was outside the scope of the current study.

Our findings should be compared with a recent nationwide Dutch study by Hol *et al*,[17] which represents the only other published comprehensive, unselected study of germline (epi) genetics of patients with WT. Comparable to our sex ratio, the Dutch study included 56% females (71 of 126 participants). First, Hol *et al* did not focus on sex differences or birth weights. In the Dutch study, twice as many females as males had WT predisposing factors (28/71 vs 14/55, Fisher's exact test; OR 1.90, 95% CI 0.83 to 4.49, $p=0.128$). This too mirrors our data, which, when considering only the (epi)genetic diagnoses, did not yield a significantly skewed sex ratio (5/9 vs 0/10, Fisher's exact test; OR Inf, 95% CI 0.75 to Inf, $p=0.053$). Only after considering macrosomia as a WT predisposition factor did our results reach significance. As Hol *et al* neither analysed nor reported birth weight data, it is unknown if the same tendency is present in their cohort. Second, Hol *et al* included a meta-analysis of variants in selected genes for the 42% (n=53) that had exome sequencing done. Of note, the selection had an inclusion criterion for pLoF variants in genes with a high probability of being LoF intolerant (>0.5 pLI score)[42]. The pLI score (now superseded by the LOEUF score[25]) cut-off used by Hol *et al* results in a more inclusive analysis (5451 vs 2986 genes with the latter including 174 genes now considered constrained not captured by pLI). Supplementary results from the Dutch cohort indicate that 18 variants in 17 patients were retained based on, at minimum, this inclusion criterion. Despite being more inclusive,

this yielded a mutation rate 32% lower than our results. Indeed, only nine variants reported by Hol *et al* would have met the LOEUF criteria applied in this study, a significantly lower yield than ours (Poisson's exact test; 9/24 vs 9/53, p=0.022). This is likely a reflection of the fact that performing WGS instead of WES led to increased variant detection in our study. Of note, the nine constrained genes with variants reported by Hol *et al* had no overlap with those identified in the constrained gene analysis of our cohort.

Strengths of this prospective study include population-based inclusion as well as comprehensive genomics, epigenetics, deep phenotyping and novel evolution-based bioinformatic analyses. The two main limitations were (1) the cohort size, that, although representing 5 years of nationwide inclusion, only numbered 24 patients and (2) the discrepancies in the methods used to detect low-mosaic epimutation at IC1 on chromosome 11p15.5 between our study and that of Fiala *et al*.[16] The former may inadvertently skew the sex distribution of genetic variants, and indeed pathogenic variants in CPS genes have been reported in both sexes[9] and in our cohort too, a pathogenic *REST* variant segregated with WT in a family where the two affected family members were male. However, we consider the finding of macrosomia in females, which builds on independent lines of existing evidence, to be a true difference in sex disparity in the phenotypes of patients with WT.

In conclusion, combined genomics and epigenetics directly detected the aetiology in 17% of children with WT. For females, we expanded the emerging picture of a sex-specific discrete BWSp phenotype, including macrosomia and perilobular rests on pathology, possibly explaining the epidemiologically observed over-representation and neonatal macrosomia of females with WT. Furthermore, we introduced constrained gene analysis for the study of genetic WT predisposition, successfully identifying known and novel candidate genes. In total, 50% had either phenotypic traits and/or (epi)genetics directly or possibly linked to WT risk. Notably, in our study, the majority of the females with WT evidently had an underlying (epi)genetic condition.

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