# Hallmark discoveries in the biology of Wilms tumour

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## Abstract

**[Au: I have edited the first sentence of your abstract so the focus is Wilms tumour.]** The modern study of Wilms tumour **[Au: we only abbreviate specific two-word terms, Wilms tumour is not one, so I have edited accordingly throughout.]** was prompted nearly 50 years ago, when Alfred Knudson proposed the 'two-hit' model of tumour development. Since then, the efforts of researchers worldwide have substantially expanded our knowledge of Wilms tumour biology including **[Au: We do not signpost what will be discussed in an article within the abstract, instead we actually briefly describe it, so I have edited the following accordingly.]** major advances in genetics — from cloning the first Wilms tumour gene to high-throughput studies that have revealed the genetic landscapes of this tumour. These discoveries improve understanding of the embryonal origin of Wilms tumour, familial occurrences, and associated syndromic conditions. Many efforts have been made to find and clinically apply prognostic biomarkers to Wilms tumour, for which outcomes are generally favourable but treatment of some affected individuals remains challenging. Challenges are also posed by the intratumoural heterogeneity of biomarkers. Furthermore, preclinical models of Wilms tumour, from cell lines to organoid cultures, have evolved. Despite these many achievements, much still remains to be discovered: further molecular understanding of relapse in Wilms tumour and of the multiple origins of bilateral Wilms tumour are two examples of areas under active investigation. International collaboration, especially when large tumour series are required to obtain robust data, will help to answer some of the remaining unresolved questions.

## [H1] Introduction [Au: H1, H2 etc. refer to the heading level and are needed for Production, please don't delete.]

Wilms tumour, described for the first time in 1899 by Max Wilms[1], is the most common paediatric renal tumour: it affects approximately 1 in 10,000 children with a peak incidence at 3 years of age. Wilms tumour represents a paradigm for success in multidisciplinary paediatric oncology, with an overall survival (OS) of ~90% when treated under international protocols [2]. Worldwide, two main treatment strategies are in use: pre-operative chemotherapy followed by histological subtype, tumour stage and volume-based risk-stratified postoperative chemotherapy (and radiotherapy in selected subgroups) in International Society of Paediatric Oncology Renal Tumour Study Group (SIOP–RTSG) protocols and immediate surgery with postoperative risk-stratified treatment based on patient age, tumour stage and histology and molecular markers according to Children's Oncology Group Renal Tumor Committee (COG–RTC) protocols. These risk-based treatment strategies optimize event-free survival (EFS) whilst reducing risks of treatment-related early and late toxic effects whenever possible. This aim has largely been achieved, as following either strategy, OS is excellent for the majority of patients with Wilms tumour [3,4].

Biological research on Wilms tumour began almost 50 years ago; thus, telling the story of how the many discoveries in this field were made and tracing the timeline of the key scientific events is timely (FIG.1).

In this Review, we describe the history of key advances in the biology — and particularly the genetics — of Wilms tumour, from Knudson's description of the 'two-hit' model to the most current findings. We outline the scientific and historical importance of these findings, which will serve as starting points for new research opportunities.

## [H1] Wilms tumour genes

Before the discovery of any of the genes mutated in Wilms tumour, these tumours were proposed to arise from fetal kidney elements with dysregulated embryogenesis [5]. **[Au: Please reference this statement.]** Firstly, the histology of Wilms tumour bears striking similarity to embryonic kidney with a mixture of epithelial cells forming prototubules, primitive blastemal cells, and interspersed stromal elements [6]. Furthermore, Wilms tumours frequently arise within or in association with nephrogenic rests, which are islands of remnant embryonic renal elements within otherwise mature kidney (FIG.2) [5]. **[Au: A histological image of a nephrogenic rest might be useful to the reader here, if you can provide a previously unpublished high-resolution one.]** Hence, the discovery of genes associated with Wilms tumour has occurred alongside insights into the development controls of normal nephrogenesis.

## [H2] The Knudson model of tumour suppressor genes

In 1972, Knudson proposed that his 'two-hit' mutation model, which had been formulated originally for retinoblastoma [7]**[Au: Please reference this statement.]** , could also be applied to Wilms tumour [8]. Based on mathematical modelling of epidemiological data of familial and bilateral tumours versus unilateral and unselected tumours, the hypothesis is in modern terms interpreted to mean that two recessive, loss-of-function mutations in the same so-called 'tumour suppressor' gene are necessary for tumour development. The first mutation is presumed to be germline in children with familial and bilateral Wilms tumour, consistent with an earlier age of onset

than sporadic tumours, in which both mutations must happen by chance in the same cell during early kidney development. The model led to the use of molecular techniques to detect chromosomal regions showing loss of heterozygosity (LOH, also known as 'allele loss') to map Wilms tumour-associated gene loci.

Even before the first Wilms tumour-associated gene, *WT1* on chromosome 11p13, was identified in 1990 [9,10]**[Au: Please reference this statement.]** , allele loss and genetic linkage studies provided evidence for the existence of several different Wilms tumour-associated genes underlying familial, syndromic and sporadic Wilms tumours [11,12]. The preferential loss of the maternal allele also implicated genomic imprinting **[Au: brief explanation of genomic imprinting to answer PR2s comment at the first mention OK? Please add a reference.]** at 11p15 in the pathogenesis of Wilms tumour [13]. Genomic imprinting is an epigenetic process that causes only one copy of a gene in an individual (either from their mother or their father) to be expressed, whereas the other copy is suppressed [14]. Furthermore, the bimodal distribution of age-at-onset of bilateral Wilms tumour suggested underlying genetic heterogeneity [15]. Results of subsequent studies involving single-cell sequencing have shown that bilateral Wilms tumour can arise from early somatic mutations and epigenetic changes arising before divergence of the left and right kidney primordia [16]. Whole-exome sequencing of sporadic and familial Wilms tumours has now revealed a plethora of mutational drivers. Despite this genetic complexity, Knudson's two-hit model of inactivation of a tumour suppressor gene can still hold true for a considerable minority of Wilms tumours [17-19].

## [H2] Identification of the Wilms tumour suppressor gene *WT1*

The identification of *WT1* relied on a combination of classical syndromology, somatic cell genetics and reverse genetics approaches. Key early findings were cytogenetic deletions encompassing 11p13 in individuals with WAGR syndrome — a contiguous gene syndrome that includes **[Au: we do not add emphasis using underlining, bolding, italicizing etc. so I have edited accordingly.]** Wilms tumour, aniridia, genitourinary anomalies and a range of developmental delays [20]. Together with the two-hit hypothesis [8], this observation led to the suggestion of a predisposing Wilms tumour suppressor gene on chromosome 11p13. Separation of del11p13 alleles in somatic cell hybrids facilitated mapping of many anonymous markers and construction of megabase-scale restriction maps to characterize the crucial region [21-23]. Evolutionarily conserved genomic sequences were used to pinpoint candidate genes. A single, but complex, transcription unit was found in the smallest region of deletion overlap that encoded WT1, a C2H2-type zinc finger protein [9,10]. A strong indicator of its likely involvement in Wilms tumorigenesis was the finding that *WT1* expression is restricted to key cell types in the kidney and other tissues undergoing mesenchymal–epithelial transitions during embryogenesis [24]. Most *WT1* mutations were loss of functions and homozygous in somatic tumour cells; however, a minority were heterozygous [25]**[Au: Please reference this statement.]** , suggesting the potential for dominant-negative effects of the mutated protein [26]. The prevalence of somatic *WT1* mutations in patients with sporadic Wilms tumour unselected for clinical outcome or high-risk features (such as blastemal type Wilms tumour in SIOP protocol, or diffuse anaplastic Wilms tumour [3]) **[Au: such as? Please add and reference here]** ranges from 6% to 20% [19,27-31]. WT1 has since been shown to act in transcriptional regulation and RNA metabolism and to affect a broad range of biological processes, making it

an essential factor for tissue homeostasis and disease in multiple organs [32]. **[Au: why is this relevant? Please explain.]**

The consequences of *WT1* mutations are fascinating and complex: haploinsufficiency in patients with WAGR leads to male genitourinary anomalies with hypospadias, cryptorchidism and elevated risk of developing Wilms tumour [33]. Loss of *WT1* in kidney precursors can induce tumour formation, accounting for a minority **[Au: by 'a fraction' do you mean 'a minority of'?]** of Wilms tumours, with preferential **[Au:OK? Is this what you meant? If not, please clarify.]** stromal predominant histology [25]. Complete loss seems to be lethal as knock-out mice die prenatally owing to a lack of kidneys and multiple other organ defects [34]. Dominant-negative mutations disrupting the zinc fingers underlie Denys-Drash syndrome (DDS), characterized by disorder of sex development, diffuse mesangial sclerosis with early kidney failure and one of the highest risks of developing Wilms tumour [35]**[Au:OK? To address peer reviewer 2's comment.]**. Subtle heterozygous point mutations of the exon 9 alternative splice site increase production **[Au: expression level? Please specify.]** of the short -KTS isoform, leading to Frasier syndrome with male pseudohermaphroditism, focal segmental glomerulosclerosis and a risk of gonadoblastoma in streak gonads [36,37]. These alterations underscore the broad involvement of WT1 in development and homeostasis throughout adult life of many tissues and organs [32].

## [H2] The 11p15 Locus

The presence of a second Wilms tumour-associated gene distinct from *WT1* was first inferred from the observation that LOH at chromosome 11p in Wilms tumour is often limited to 11p15, excluding the *WT1* locus at 11p13 [38]. The 11p15 locus, initially

213 referred to as '*WT2*', was also linked to Beckwith Wiedemann syndrome (BWS), an
214 overgrowth disorder associated with macroglossia, umbilical hernia, hemihypertrophy,
215 neonatal hypoglycemia, and predisposition to Wilms tumour and other embryonal
216 cancers (Table 2) **[Au:OK?]** [39]. The 11p15 locus contains a series of genomically
217 imprinted genes clustered into two domains [40]. Imprinting centre 1 (IC1) includes *IGF2*
218 and *H19*. *IGF2* encodes a growth factor that is fundamental to embryonal growth and
219 development, and *H19* encodes a biologically active untranslated RNA that might act
220 as a tumour suppressor [41]. In **[Au:OK?]** cells of individuals not affected by BWS, the
221 paternal IC1 allele is methylated, leading to expression of *IGF2* and silencing of *H19.*
222 Imprinting centre 2 (IC2) contains several genes including *KCNQ1*, *KCNQ1OT1*, and
223 the tumour suppressor gene *CDKN1C* **[Au: what do these refer to and what does**
224 **the solidus denote? Are they alternative names? Please clarify.]**. In **[Au:OK?]**
225 cells of individuals not affected by BWS, the maternal allele of IC2 is methylated,
226 resulting in expression of *KCNQ1* and *CDKN1C* and repression of *KCNQ1OT1.* BWS
227 most commonly arises from gain of methylation of IC1 (and resultant gain of
228 expression of the growth promotor *IGF2),* loss of methylation of IC2 (and resultant loss
229 of expression of the tumour suppressor *CDKN1C*), or uniparental disomy of the
230 paternal allele (resulting in both gain of methylation at IC1 and loss of methylation of
231 IC2). All three mechanisms predispose to Wilms tumour, but the molecular alterations
232 leading to *IGF2* overexpression are associated with the greatest risk of Wilms tumour
233 [42-44].

234 In addition to its implication in Wilms tumour risk in the setting of BWS, *IGF2*
235 overexpression has been observed in up to 70% of sporadic Wilms tumours, with 3%
236 of patients presenting this alteration constitutionally [45], providing further evidence of
237 the importance of this locus in Wilms tumour biology [19,46,47]. *IGF2* is also

overexpressed in nephrogenic rests, considered the embryonic precursors of Wilms tumour,[48] as well as in multiple samples from the same tumour [49]. In contrast to Wilms tumour arising as a result of *WT1* mutations, where stromal predominant histology is often observed, Wilms tumour secondary to 11p15 alterations typically has a mixture of blastemal and epithelial cells. **[Au: what is the importance of tumours presenting a predominance of blastemal or epithelial cells in this context? Please explain here.]** [47].

Genetically engineered mouse models suggest that *Igf2* overexpression by itself is insufficient for Wilms tumorigenesis and that cooperating mutations in genes such as in *Wt1* are required [50].

The high prevalence of *IGF2* overexpression in Wilms tumour makes it an attractive therapeutic target, in theory. However, results from preclinical models and a phase 2 study suggested that blockade of the insulin-like growth factor receptor 1 (IGF-1R), the receptor to which IGF2 binds, has not been effective for Wilms tumour or other paediatric solid tumours [51,52]. The lack of effect might be explained by bypass mechanisms such as activating mutations in the PI3K—AKT pathway, downstream of IGF signalling. Moreover, as IGF2 overexpression acts in conjunction with other genetic alterations to promote Wilms tumorigenesis, targeting more than one pathway could yield greater anti-tumour effects. Further investigation of IGF inhibitors, alone or in combination with other agents, is warranted before abandoning this therapeutic target.

**[H2] *TP53* mutation**

*TP53* was discovered in 1979, [53,54] with the first indications that mutations were involved with cell transformation being reported in the 1990s [55]. *TP53* has a tumour suppressor role, with TP53 presenting a short protein half-life resulting in low levels in nonmalignant cells and tissues where it coordinates DNA replication fidelity and genomic stability [56]. Nearly 73% of *TP53* mutations are missense, the majority of mutations stabilize the protein resulting in its accumulation in the nucleus [57] **[Au: Please reference this statement.]** . The process of TP53 accumulation is not fully understood, but in rare instances mutant TP53 protein might not only lose the tumour suppressive function but also gain new tumorigenic activity [56].

In Wilms tumours, most mutations occur in the DNA binding, oligomerization and transcriptional regulation domains [58-60]. Historically, *TP53* mutations have been linked to anaplastic Wilms tumour. Diffuse anaplasia is found in 5–10% of Wilms tumours and is defined morphologically as cells with marked nuclear enlargement with abnormal, multipolar mitotic figures (FIG.3)**[Au: a histological image of this would be useful to the reader if you can provide a previously unpublished high-resolution one.]** . Anaplasia can also be focal in a further 2% of cases. Diffuse anaplasia is universally considered a high-risk subtype of Wilms tumour. The first study in which various histologies were evaluated showed that eight of eleven anaplastic Wilms tumours carried a *TP53* mutation. However, the authors raised the possibility that other histologies might also harbour *TP53* mutations in a small number of cells below the threshold of the method used for detection (PCR-single strand conformation polymorphism **[Au: please define 'SSCP']** ) [61]. With the advent of high-throughput sequencing technologies, large studies have reported that *TP53* is mutated

in 50 to 60% of diffuse anaplastic Wilms tumours (DAWT) [58,59] , as well as in some Wilms tumours with non-anaplastic histologies with lower frequency [30,60,62].

TP53 protein expression, detected by immunohistochemistry and used as a surrogate for *TP53* mutation, is usually associated with anaplasia and poor prognosis [63-65]. The correlation between *TP53* mutations and immunopositivity might be dependent on the type of mutation. Wilms tumours with R175H, G245S/D, R273C, R342P, which are the most common amino acid changes, as well as other deleterious missense mutations, result in TP53 accumulation, whereas tumours with G105V, L194R and G226S, similar to neutral mutations (P309L and S362N), contained almost no TP53-positive cells [60]. In anaplastic Wilms tumours, *TP53*-mutated tumours have higher burdens of copy number aberrations than *TP53* wild-type tumours with the same histology, indicating a higher level of genomic instability [58]. Accordingly, when comparing gene expression profiles from anaplastic Wilms tumours with mutant and wild-type *TP53*, the differentially expressed genes were enriched for cell cycle, apoptosis and DNA repair, among other biological processes [58,59].

**[H2] *CTNNB1* mutation**

*CTNNB1,* located on chromosome 3p22.1, codes for a protein that is an integral part of the canonical WNT signalling pathway. The role of the *CTNNB1* proto-oncogene in the development of Wilms tumour was first reported in 1999 [66], when mutational activation of this gene was shown to be a recurrent event in Wilms tumour. The β-catenin protein was identified as a downstream target of WNT4 signalling, and dysregulation of mesenchymal–epithelial transition that occurs during normal nephron development by mutated forms of β-catenin might lead to development of Wilms

tumour. The WNT pathway regulates essential cellular functions, including proliferation, differentiation, migration, apoptosis, and stem cell renewal, and has a central role in kidney development [67]. Aberrant activation of WNT–β-catenin signalling has been found in many human cancers [68] and in Wilms tumours of mesenchymal lineage, which represent 15% to 20% of all Wilms tumours [69]. Genes from the canonical WNT signalling pathway expressed early in kidney development are over-expressed in Wilms tumours, indicating that the pathway is disrupted at least in part of the tumours [69]. In the WNT pathway, β-catenin serves as a transcriptional co-activator to promote target gene transcription [70]. Activating mutations of *CTNNB1* have been identified in about 15% of Wilms tumours [27,66]. Most *CTNNB1* mutations (~65%) affect exon 3 and result in the loss of crucial phosphorylation sites. This loss **[Au:OK?]** precludes ubiquitination and degradation of the protein, which then is stabilized, accumulates, and leads to constitutional activation of the WNT–β-catenin signalling pathway as well as to aberrant myogenesis [71]. Approximately one-third of *CTNNB1*-mutant Wilms tumours show alterations affecting exons 7–9 (Armadillo repeats, characteristic repetitive amino acid sequences ~40 residues in length that form a conserved 3D structure [72]). The mechanism of mutations in this region is not well characterized and they might act in a different way [29,30,73].

A highly significant (p-value = 3.6 x 10(-13)) **[Au: statistically significant? If so please add the p value, if clinically significant please specify, if neither, please change to 'important' or similar.]** association between *WT1* and *CTNNB1* mutations was observed in a study of 153 Wilms tumours [27], with 19 out of 20 *CTNNB1* mutant tumours also harbouring *WT1* mutations. The development of tegavivint, a β-catenin inhibitor now in phase 1/2 studies in children (NCT04851119) [74]**[Au: please add this trial to your reference list in the format 'ClinicalTrials.gov. US National Library**

**of Medicine. https://ClinicalTrials.gov/show/NCTXXXXXX (20XX)'.]** , might result in additional therapeutic options for a subgroup of patients with Wilms tumour, particularly those with aberrant WNT–β-catenin signalling.

### 1.6 *AMER1* *[Au: we use the UniProt names for genes so I have changed to AMER1 throughout this section]* mutation

*AMER1* was first described as *WTX* and was found to be involved in Wilms tumorigenesis in 2007 [75]. Somatic deletions targeting the chromosomal region Xq11.1 were found by genome-wide array comparative genomic hybridization **(**CGH) **[Au: please define 'CGH']** of primary Wilms tumour samples **[Au:OK?]** . The minimal area of overlap implicated a single, previously uncharacterized gene initially named Wilms Tumor gene on the X-chromosome (*WTX*) and subsequently given the official gene name APC Membrane Recruitment Protein 1 (*AMER1*). Besides deletions, frameshift and non-sense mutations were detected, resulting in AMER1 **[Au: gene OK?]** inactivation in 30% of tumours.

Additional screening of >500 samples revealed *AMER1* alterations in 7–18% of Wilms tumours [29,76,77] and demonstrated intratumour heterogeneity, suggesting that *AMER1* alterations occur late in tumorigenesis rather than as an initiating event. This hypothesis is supported by germline *AMER1* deletion or **[Au:OK?]** truncating mutations that cause osteopathia striata with cranial sclerosis, a severe bone malformation syndrome with male lethality, but that does not predispose to tumorigenesis [78].

*AMER1* ***[Au: gene OK or was it protein expression?]*** expression was detected in the condensing metanephric mesenchyme and in early epithelial structures during

mice **[Au: mouse or rat? Please specify]** kidney development. Subsequent functional studies revealed that AMER1 is part of the β-catenin-destruction complex, binds to AXIN1, β-TrCP (BTRC) and APC and antagonizes WNT–β-catenin signalling by promoting β-catenin ubiquitination [79]. Initially *AMER1* and *CTNNB1* mutations were described as mutually exclusive, already suggesting impairment of the same pathway. In subsequent analyses *AMER1* alterations were also found concurrently with *CTNNB1* mutations in Wilms tumour [29,73,77]**[Au: Please reference this statement.]** , but patient outcomes have not been reported **[Au: Added from your rebuttal to answer the peer reviewer's query.]** . *Wtx* deletion in mice causes neonatal lethality, somatic overgrowth and malformation of multiple mesenchyme-derived tissues [80]. In this mouse study, Wtx regulated mesenchymal progenitor cell fate specification and had an important role in embryonic development and organ differentiation **[Au: what are the implications of these observations clinically? Please comment here.]** .

**[H2] *MYCN* gain or mutation**

*MYCN*, which is mapped to 2p24.3, encodes a proto-oncogenic MYC family transcription factor that is involved in the control of important processes during embryonal development [81]. *MYCN* has been shown to be gained or amplified in several embryonal childhood cancers, and in some adult cancers [81-84]. Individual cases of *MYCN* gain in Wilms tumour have been reported since the late 1980s **[Au:OK?]** [85-87], and the analysis of large series with copy number arrays revealed that this aberration is relatively frequent, typically being a focal low copy number event [88-90]. Germline copy number gain in a patient with bilateral Wilms tumour, suggestive of a possible role for *MYCN* in Wilms tumour predisposition, has also been described [90]. *MYCN* is less frequently affected by somatic mutations that give rise to a recurrent

amino acid change, P44L, which is postulated to act as a gain of function alteration, potentiating the effect on downstream targets relative to the wild-type protein **[Au: comparator OK?]** , [62,90,91]. In tumours from patients treated according to SIOP protocols, *MYCN* gain is associated with anaplastic histology, and with poor outcome even in the absence of anaplasia [88,90,92]. *MYCN* overexpression is also associated with adverse outcome [90,93] and with specific DNA hypomethylation events at five loci mapped within the *MYCN* gene [90]. Other somatic changes might have an effect on the oncogenic activity of MYCN, including loss or mutation of *FBXW7* (which degrades MYCN) or *MAX* (a MYCN heterodimerization partner) [62,88].

### 1.8 *SIX1 and SIX2* ***[Au:OK? I understand 'SIX1/2' is common in the literature, but our Style is to refer to two separate entities individually. You also refer to a single one individually in your discussions below.]*** homeodomain mutation

Recurrent *SIX1* and *SIX2* (Q177R) homeodomain mutations were first reported in Wilms tumours in 2015 [62,94]. To define the genetics of high-risk, blastemal type, pre-operative chemotherapy-treated Wilms tumours **[Au: does pre-operative chemotherapy result cause any changes in gene expression? Please comment to address peer reviewer 2's comment.]**, a discovery set of 58 tumours was investigated, with a larger replication cohort of unselected Wilms tumours. Recurrent *SIX1* and *SIX2* Q177R mutations were identified in 18.1% of blastemal cases (and in 4.3% of all cases) [62]. In addition, as a component of the National Cancer Institute's therapeutically applicable research to generate effective treatments (TARGET) initiative, comprehensive categorization of a discovery set of 117 high-risk Wilms tumours, defined as favourable-histology Wilms tumour (FHWT) that relapsed or DAWT was performed, followed by evaluation of a validation set of 651 Wilms tumours

(533 FHWT and 118 DAWT) treated in the National Wilms Tumor Study-5 clinical trial [30]. In this cohort, *SIX1* and *SIX2* Q177R mutations were identified in 7% of FHWTs. The *SIX1* and *SIX2* Q177R mutation is located within the highly conserved *SIX* homeodomain, which is responsible for DNA binding and protein interaction [95]. These mutations likely result in gain of function compared to wild-type protein **[Au: than loss of function? Please add the comparator here.]** , as these tumours have a distinct gene expression profile from that of *SIX1* and *SIX2* wild-type tumours [94]. In pre-operative chemotherapy-treated Wilms tumours, *SIX1* and *SIX2* mutations are detected more frequently in blastemal than in regressive and necrotic tumours, pointing to a role in chemotherapy resistance [62]. *SIX1*-mutated and *SIX2*-mutated tumours exhibit over-expression of cell cycle and kidney development genes and down-regulation of cell adhesion, extracellular matrix secretion, inflammatory response and chemotaxis [62].

Homologous *SIX1* and *SIX2* are known to have a crucial role in renal development [95,96]. In fact, *Six1*-knockout mice exhibit renal hypoplasia or kidney agenesis [97]. Mechanistically, mutations in *SIX1* and *SIX2* perpetuate the progenitor state, potentially leading to Wilms tumour development [30].

**[H2] microRNA processing gene mutation**

Recurrent hotspot microRNA processing gene (miRNAPG) mutations in *DROSHA* (RNAse III domains) and *DGCR8* (E518K) were reported in Wilms tumours in 2014 and 2015 [62,91,94,98]. During the investigation of the discovery set of 58 high-risk, blastemal type, pre-operative-chemotherapy treated Wilms tumours and replication cohort, mutations in the *DROSHA* and/or ***[Au: what does the solidus denote here? 'and', 'or' or 'and/or'? Please specify]*** *DGCR8* gene were noted in 18.2% of

blastemal cases (and in 8.1% of all the cases)[62]. During the TARGET initiative, recurrent *DROSHA* RNAse III domain mutations were identified in 10% of patients with FHWT, with *DGCR8* E518K mutations occurring in 4.5% [30,94]. Given the many cellular pathways microRNAs (miRNAs) are known to affect combined with their complex interactions and feedback loops, the range of effects associated with miRNAPG mutations is heterogeneous and complex [99]. Mutations in different genes result in different miRNA profiles, suggesting additional functions of these genes [30,91]. Impaired miRNA synthesis has been shown to accelerate oncogenic transformation by deregulating target oncogenes [100]. miRNAPG-mutant tumours were shown to have reduced expression of crucial miRNAs [62,94]. Mechanistically, mutations in miRNAPGs also perpetuate the progenitor state, potentially leading to Wilms tumour development [30]. Rarely, mutations in the *SIX1 and SIX2* pathway and in *DROSHA* or***[Au: what does the solidus denote here? 'and', 'or' or 'and/or'? Please specify]*** *DGCR8* have been shown to occur in combination and result in poor outcomes [94]. In addition, in evaluations of small cohorts of paired primary and relapsed Wilms tumours, investigators have noted that mutations of *SIX1* and *DROSHA* were more frequent in tumour recurrence [101,102]. Functionally, both pathways are active during early renal development, with mutations in both the *SIX1* and *SIX2* and the miRNAPG pathway leading to continued perpetuation of the progenitor state, which can potentially result in Wilms tumour development [30]. Whether this combination of driver mutations can serve as prognostic markers in the future remains to be determined.

## [H2] Genes from the TARGET initiative

In addition to genes previously reported to harbour mutations in Wilms tumours (*WT1, CTNNB1, WTX, DROSHA, DGCR8, XPO5, DICER1, SIX1, SIX2, MYCN, and TP53*), the TARGET study identified mutations in genes that had not previously been associated with Wilms tumour (Table 1) **[Au: Reference to table formatted to journal style.]** . The most frequent novel recurrently mutated genes in TARGET and the percentage of patients harbouring these mutations were *BCORL1 (3.8%), COL6A3 (3.2%), MLLT1 (3%), NF1 (2.9%), BCOR (2.6%), NONO (2%), ARID1A (1.8%), MAX (1.7%), MAP3K4 (1.7%), and ASXL1 (1.7%)*. Germline mutations were identified in *PALB2* (1.2%) and *CHEK2* (1.2%). Unsupervised hierarchical clustering of 76 FHWT TARGET discovery set samples based on gene expression showed that samples with *DROSHA, DGCR8, SIX1*, or *SIX2* mutations and samples with *MLLT1, WT1, CTNNB1*, or *WTX* mutations resided in distinctly different clusters, revealing differential gene expression cluster membership according to common recurrently mutated genes[30]. However, positive enrichment of genes associated with the pre-induction metanephric mesenchyme and low expression of genes associated with post-induction were found in the majority of samples, regardless of candidate driver gene mutation, suggesting that the numerous genetic changes found in Wilms tumour result in a similar outcome, namely disrupted early renal development.

Overall, the data indicate that Wilms tumours do not share a small number of common driver mutations, but rather arise from numerous candidate driver genes, most of which individually account for ≤5% of Wilms tumours. However, many of the recurrently mutated genes in Wilms tumour share common functional pathways, with involvement in either epigenetic pathways such as transcriptional elongation and/or **[Au: what does the solidus denote here? 'and', 'or' or 'and/or'? Please specify]** chromatin modification (*MLLT1*, *BCOR,* **[Au: what does the solidus denote here?**

**'and', 'or' or 'and/or'? Please specify]***BCORL1, BRD7, CREBBP, ARID1A,* and *EP300* [30,103]) and miRNA processing (*DROSHA*, *DGCR8*, *DICER1*, and *XPO5* [62,91,94,98]) or early renal development (*SIX1 and SIX2* [62,94]). Because epigenetic pathways have key roles in progenitor cell preservation and differentiation, mutations in genes in these pathways could interrupt standard development. Several of these genes have been shown to have a role in early renal development. *SIX1* and *SIX2* have well-established roles in renal development [95-97,104]**[Au: we do not refer back, so please add a relevant reference here.]** . Crebbp and Ep300 are essential for Wt1-associated activation of *Wnt4*, which is required for the mesenchymal-to-epithelial transition in mouse embryonic kidney mesenchymal cells [105], and *Dicer* ablation in the metanephric mesenchyme results in increased apoptosis and renal dysgenesis in mice [106]. Additionally, the non-frameshift *MLLT1* mutations found in a subset of Wilms tumours were subsequently shown to affect cell fate in human and mouse cells and to result in undifferentiated structures in mouse nephrogenesis assays [107]. These observations suggest that different genomic abnormalities in Wilms tumour converge upon disruption of early renal development, and that Wilms tumour therapies targeting epigenetic or developmental pathways might be more efficient than targeting the individual candidate driver genes.

## [H1] Associated syndromic conditions **[Au: heading edited for length requirements.]**

Several genetic syndromes have long been known to be associated with an increased risk of Wilms tumour development (Table 2) **[Au: Reference to table formatted to journal style.]** . The most well-known examples are WAGR and BWS, although many other syndromes have also been reported in smaller numbers. Published guidelines

agree that children with increased risk of developing Wilms tumour should undergo a surveillance protocol with abdominal ultrasonography until 7–8 years of age. However, debate is ongoing as to the optimal risk threshold at which surveillance should be recommended [108-111].

The description of the molecular basis of WAGR syndrome — a micro-deletion of chromosome 11p13 [112] — was one of the first for a cancer predisposition syndrome. The key genes in the deleted region are *WT1,* which is related to genitourinary development and Wilms tumour risk, and *PAX6,* which is related to aniridia and neurodevelopmental issues [113]. Missense, nonsense, and splice site variants of *WT1* are also associated with Wilms tumour development, genitourinary malformations, and renal dysfunction with a spectrum of severity of renal disease and tumour risk [114,115].

Children with BWS are at risk of Wilms tumour development, but are also at risk of other childhood tumours — particularly hepatoblastoma [116]. BWS is driven by genetic and epigenetic alterations at the imprinted 11p15.5 locus and the clinical features and tumour risk vary depending on the particular alteration [117]. However, an elevated risk of Wilms tumour remains even in the lowest risk molecular category [118]. Children with BWS can exhibit somatic mosaicism leading to subtle features — up to 3% of children **[Au: by 'non-syndromic children' do you mean children with no syndrome? Please clarify. We try not to describe people in this manner, so could we change to 'children with Wilms tumour but no syndrome'?]** with Wilms tumour but without other phenotypic features of BWS have been found to harbour pathogenic alterations at 11p15.5 in blood [45]. An even higher proportion than 3% of children without classic phenotypic features of Wilms tumour predisposition syndromes **[Au: children with no syndrome?]** could harbour 11p15 variants in some cell types but, owing to tissue mosaicism, these variants might be below **[Au: what might be below specifically?**

**At the moment it reads as if it is the children themselves, but I'm not sure this is what you mean, do you mean the level of variant expression?]** the level of detection by standard clinical testing methods. The clinical implications of these latter findings have yet to be elucidated [119].

Analyses of families with multiple Wilms tumours have uncovered other predisposition genes [18,120,121]. No consistent syndromic features are associated with variants in these genes, but these descriptions are expected to evolve along with the literature. In total, ~10% of Wilms tumours are associated with a known predisposing syndrome or constitutional genetic variant.

## [H1] Familial loci and genes

Only 1–2% of Wilms tumours cluster within families, and the genetic causes underlying these rare pedigrees are heterogeneous. The majority of Wilms tumour pedigrees are consistent with an autosomal-dominant mode of inheritance with incomplete penetrance. Some families with Wilms tumour **[Au:OK?]** have been associated with syndromic conditions [122]. *WT1* germline abnormalities without syndromic signs have been reported in four families [122]. Segregation analyses involving different families identified two main familial Wilms tumour loci: *FWT1,* mapped to chromosome 17q12-q21, and *FWT2* mapped to chromosome 19q13.5 [123-125]. Wilms tumours in familial *FWT1*-linked pedigrees tend to be diagnosed at a later age than sporadic cases, the penetrance of *FWT1* mutation is ~30%, and tumours do not show loss of the wild-type allele, indicating that *FWT1* does not behave as a classic tumour suppressor gene [123,124]. Wilms tumours in familial *FWT2*-linked pedigrees do not show loss of the wild-type allele, whereas 19q loss was observed in Wilms tumours in two families in which predisposition was not due to *FWT2*, suggesting a two-locus mutational model for the

ethiology of familial Wilms tumours [125]. **[Au: what does this observation suggest and what implications does it have? Please comment here.]**

Whole-exome sequencing studies have disclosed new familial Wilms tumour genes, *CTR9*, *REST* and *TRIM28,* all of which showed an incomplete penetrance of the germline mutation for the development of Wilms tumour [17,18,120,121,126,127]. Biallelic truncating mutations in *NYNRIN* (for which very little concerning its function is known), inherited from heterozygous parents, were also identified [18]. Mutations in *CTR9*, a gene coding for a component of PAF1c complex, have been identified in four families with Wilms tumours. All investigated *CTR9*-mutated Wilms tumours showed a second-hit possibly inactivating the wild-type allele [121,126]. *REST* codes for a transcriptional repressor with a crucial role during embryonic development and neurogenesis [120] **[Au: Please reference this statement.]** . In total, eleven different inactivating mutations have been identified in 16 individuals from four familial WT pedigrees [120]. *TRIM28* codes for a transcriptional co-repressor, located at 19q13.4 in the proximity of the putative familial *FWT2* locus [127] **[Au: Please reference this statement.]** . Alterations in *TRIM28* in familial Wilms tumour pedigrees have been identified in three studies [17,18,127]**[Au: Please reference this statement.]** . *TRIM28* mutations showed a strong parent-of-origin effect, being maternally transmitted in all informative cases. Furthermore, all investigated *TRIM28*-mutated Wilms tumours also showed loss of the wild-type allele and a predominantly epithelial histology [17,18,127]. However, in 60–70% of families with Wilms tumours, no causative molecular variant has been uncovered, suggesting that the number of children with Wilms tumour caused in part by constitutional genetic changes is even higher than reported [18].

## [H1] Links with kidney development [Au:OK?]

Wilms tumours variably display a unique histology consisting of cells similar to undifferentiated metanephric mesenchyme (blastema) and also more differentiated cells (stroma and tubular epithelial cells normally derived from metanephric mesenchyme), suggestive that tumours arise as a result of aberrant kidney development [128,129]. Genetic, gene expression, methylation and mouse model studies support this model of tumorigensis [16,47,48,50]. **[Au: We do not stack headings one on top of the other, so please provide a brief introductory statement to this section, avoiding phrases such as 'below' and 'in this section' as we do not signpost.]**

### [H2] Embryonal precursors of Wilms tumour

Modern molecular findings support the embryonic renal origins of Wilms tumours. Many of the recurrently mutated genes within Wilms tumours have crucial roles in renal development [30,62,94,130]. Additionally, several independent groups have described RNA expression profiles in Wilms tumours that reflect various stages of renal development including the uninduced metanephric mesenchyme, cap mesenchyme, and epithelium after the mesenchymal–epithelial transition [47,131]. Genes overexpressed in embryonic kidney and Wilms tumours include *OSR1, SIX1, HOXA11, and WT1.* In keeping with this RNA expression profile, immunostaining patterns in Wilms tumours suggest an embryonic renal origin — particularly the combined $CD56^{+}$ and **[Au: what does the solidus denote here? Please clarify.]** $WT1^{+}$ pattern [132].

A detailed investigation into the RNA expression landscape of Wilms tumours on a single-cell basis has been published [133]. Tumours were investigated along with a set of adult, paediatric, and fetal non-neoplastic kidneys. Malignant cells within Wilms

tumours clustered into four major groups: one cluster was consistent with differentiated fibroblast-like cells, whereas the other three clustered with fetal kidney elements; two of these clusters reflected specific embryonal renal elements — the ureteric bud and primitive vesicle. This embryonal pattern of gene expression was unique to Wilms tumours compared with other renal tumour types including renal cell carcinomas, rhabdoid tumours, and congenital mesoblastic nephromas.

Results of a second landmark study supported the early embryonal origins of Wilms tumours, showing that ~60% of Wilms tumours arise within a background of clonal nephrogenesis — non-neoplastic kidney cells expanded from a clone that is not present in other tissues [16]. Kidneys exhibiting clonal nephrogenesis often had gains of methylation at the *H19* imprinting control region that leads to overexpression of the growth factor *IGF2* **[Au: why is this important in context? Please explain. What implications does it have? Please comment.]** [134]. Evidence of clonal nephrogenesis was found in all four instances of bilateral Wilms tumours in this study **[What implications does it have? Please comment.]**. These findings support the role of clonal nephrogenesis in tumour development as it is associated with over expression of a known Wilms tumour oncogene (*IGF2*) and is associated with the specific phenotype of bilateral disease.

In summary, findings in the past decade have supported the long-held supposition that the origins of Wilms tumour are embryonal rather than mature renal tissues.

**[H2] Clinically Relevant Tumour Subsets and a Revised Ontogenic Model** **[Au: heading edited for length.]**

633 Gene expression analyses of a population-based set of 224 FHWT **[Au: do you mean**
634 **FHWT here? If not please define 'FH']** treated with immediate surgery (65 of which
635 were from patients who subsequently experienced relapse) delineated five subsets
636 with characteristic gene expression patterns [47]. These subsets were further
637 characterized by tumour histology; frequency of *WT1*, *CTNNB1* exon 3, and *WTX*
638 mutations and 11p15 LOH and/or **[Au: What does the solidus denote here, 'and',**
639 **'or', or 'and/or'? Please clarify.]** loss of imprinting (LOI) **[Au: please define 'LOI']** ;
640 frequency of associated nephrogenic rests; age at tumour diagnosis; and frequency
641 of relapse. Subset 1 (S1) tumours were a distinct set, distinguished by a decreased
642 expression of genes usually expressed during kidney development and an increased
643 expression of genes expressed at late stages of renal epithelial differentiation. These
644 tumours were diagnosed at an early median age (14 months), but were not associated
645 with nephrogenic rests. *WT1*, *CTNNB1*, and *WTX* mutations were not detected, and
646 11p15 LOH and/or **[Au: What does the solidus denote here, 'and', 'or', or 'and/or'?**
647 **Please clarify.]**LOI was rare (9%). No relapses were observed in this subset of
648 patients. Tumours from subsets 2, 3 and 4 (S2, S3, and S4, respectively) all had a
649 mixed histology. S2 tumours displayed increased expression of genes expressed very
650 early in kidney differentiation (intermediate mesoderm and metanephric mesenchyme)
651 **[Au: what was the clinical outcome for patients with this tumour subset? Please**
652 **add.]** . Over half displayed *WT1* and *CTNNB1* mutations (54% and 55%, respectively)
653 and 11p15 LOH and/or **[Au: What does the solidus denote here, 'and', 'or', or**
654 **'and/or'? Please clarify.]**LOI (68%) and were associated with intralobar nephrogenic
655 rests (ILNR, 74%). S3 tumours were very similar to S2 with respect to *WT1* mutation
656 and ILNR frequency, but did not display an increased expression of intermediate
657 mesenchyme genes, and had a lower frequency of 11p15 LOH and/or **[Au: What**

**does the solidus denote here, 'and', 'or', or 'and/or'? Please clarify.]**LOI and *CTNNB1* mutations than S2 tumours **[Au: what was the clinical outcome for patients with this tumour subset? Please add.]**. The S4 tumours displayed an increased frequency of 11p15 LOH and/or **[Au: What does the solidus denote here, 'and', 'or', or 'and/or'? Please clarify.]**LOI (80%) and a reduced frequency of *WT1* and *CTNNB1* mutations **[Au: what was the clinical outcome for patients with this tumour subset? Please add.]**. S5 tumours, comprising the largest subset, also expressed genes characteristic of metanephric mesenchyme, but had a more variable histology and a higher frequency of 11p15 LOH and/or **[Au: What does the solidus denote here, 'and', 'or', or 'and/or'? Please clarify.]**LOI than S2 and S3 tumours and a lower frequency of associated ILNRs than subsets S2, S3, and S4. Interestingly, perilobar nephrogenic rests (PLNR) were uniquely observed in S5 tumours (25%). From these data, S2 tumours were suggested to arise early in kidney development in the intermediate mesoderm and S3, S4 and S5 tumours to arise at a slightly later stage (in the metanephric mesenchyme), whereas S1 tumours were suggested to arise from post-induction epithelial precursors. Subsequent work identified mutations in genes (such as *SIX1and SIX2*, *DGCR8, DROSHA* and *DICER1*) that have important roles in the regulation of self-renewal and differentiation of the nephrogenic zone [62,91,94,98,135,136] **[Au: Please reference this statement.]**. Many of the S5 tumours were subsequently found to carry mutations in these genes. Tumours with these mutations were associated with PLNR, blastemal-predominant histology, and a poor clinical outcome **[Au: please add the p values]** . While in this study the relapse rates in the five subsets showed clear differences, they did not achieve statistical significance [47]. **[Au: Please draw a conclusion from these results. What are their implications? Please comment.]**

## [H2] DNA methylation and developmental pathways

DNA methylation is a powerful and accessible marker reflecting cell of origin and developmental pathways [137]. An increase in DNA methylation at the *H19* locus is also the most common recurrent molecular change found in Wilms tumours [19]. This background spurred several independent groups to look at genome-wide DNA methylation profiles in Wilms tumours to describe subgroups. Considerable differences exist between the approaches and subgroups presented in each paper, but all described a subset of Wilms tumours with similar features to non-neoplastic tissue [48,138,139]. Importantly, this finding was consistent between SIOP and COG groups, indicating that these features are not influenced by chemotherapy exposure.

DNA methylation subgroups hold promise for future clinical use as biomarkers of tumour behaviour. The subgroup with non-neoplastic molecular features has unique clinical characteristics, including a high frequency of tumours from patients with bilateral disease [48,138] and, in one study, a trend towards a reduced risk of relapse [138]. Interestingly, the one large study investigating DNA methylation profiles that did not describe a non-neoplastic-like subgroup only included cases from children whose tumours eventually recurred or had diffuse anaplasia [30]. The absence of this non-neoplastic-like subgroup only within a cohort of patients with poor outcomes supports the hypothesis that this subgroup represents tumours with low relapse potential.

The results of these studies suggest that a differentiation process that is associated with a reduced risk of relapse might be occurring in some Wilms tumours. These tumours might eventually be candidates for a trial of therapy reduction — an approach that could be especially important for children with bilateral disease who are at increased risk of renal failure [140]. Further studies of this subgroup of tumours —

particularly the mechanisms by which they differentiate — might result in discovery of a pathway that can be targeted by a novel therapeutic approach.

## [H2] Endogenous Mouse Models

The first genetically engineered mouse model relevant for Wilms tumour was the *Wt1*$^{-}$ mouse (strain *Wt1*$^{tm1Jae}$) carrying a large intragenic *Wt1* deletion [34]. Mice heterozygous for this deletion (*Wt1*$^{+/-}$)**[Au: It is currently unclear whether you are referring to the same or a different model to the one mentioned in the previous sentence. Please clarify. Please be consistent with terminology where relevant to aid reader understanding.]** disappointingly do not develop Wilms tumours in the same way children heterozygous for a germline *WT1* inactivating allele do [20,34]**[Au: Please reference this statement.]** . Nevertheless, the *Wt1*$^{tm1Jae}$ **[Au: Is this the same starin as in the previous sentence, please clarify.]** strain has provided invaluable insight regarding the role of *Wt1* in kidney development, notably that *Wt1* is essential for the survival of intermediate mesoderm and that its loss affects kidney development at all developmental stages [34,141]. The generation of a conditional *Wt1*-null strain, *Wt1*$^{tm2Vih}$, resulted in the first endogenous mouse model for Wilms tumour [50]. This model was created by combining somatic ablation of *Wt1* at early stages of kidney development with loss of *IGF2* imprinting, two genetic alterations observed in some human Wilms tumours, including those developing in children with WAGR [50]. The tumours arising in the mice display the triphasic histology typically (but not universally) observed in patients with Wilms tumour, consistent with the induced loss of *Wt1* early in metanephric mesenchyme development. Subsequently, targeting of *Wt1* loss to committed renal epithelial progenitors at different stages of nephric differentiation and pairing this loss with either *Igf2* loss **[Au:OK?]**of imprinting or *Ctnnb1* activation

(observed in a subset of human Wilms tumours) resulted in mouse tumours with increased epithelial histology, consistent with the targeting of increasingly differentiated epithelial progenitors [142]. Notably, targeting of these mutations to murine **[Au: mouse?]** stromal progenitors did not result in tumours, despite stromal elements frequently being histologically observed in human Wilms tumours. These data suggest that such elements arise from malignant mesenchyme that subsequently differentiates along a stromal pathway. In a subsequent mouse model, *Ctnnb1* activation paired with an activated *Kras* allele resulted in primitive renal epithelial tumours similar to the epithelial components of human Wilms tumours [143]. These data suggest a synergy between *Ctnnb1* and *Kras* for mouse tumour development, although *KRAS* mutations are rare (<1%) in human Wilms tumours and are not observed in association with *CTNNB1* alterations [30].

The initial observation of *DROSHA* mutations in human tumours [98] implicated the miRNA processing pathway in both kidney development and Wilms tumour. Overexpression of Lin28B, a component of this pathway, in intermediate mesoderm was found to result in triphasic tumours and also continued proliferation of cap mesenchyme in adult animals. Targeting *Lin28B* overexpression to increasingly differentiated cell populations was not tumorigenic [136]. These data are consistent with the role of DICER, critical for the biogenesis of miRNAs, in regulating cap mesenchyme progenitor self-renewal and differentiation [144]**[Au: Please reference this statement.]** .

In summary, the various Wilms tumour mouse models have greatly increased our understanding of the genes and cellular pathways that are crucial for kidney

development and how perturbations of these pathways can result in malignancy. Many of the genes most frequently altered in human tumours are known to influence murine **[Au: mouse?]** kidney development and, in some cases, result in tumours. These data suggest that the mouse will continue to be a relevant model for understanding the mechanism by which other mutations identified in human tumours dysregulate kidney development and result in malignancy.

## [H1] The history of prognostic markers

The current treatment strategies focus on optimizing EFS and reducing the risk of treatment-related toxicities, wherever possible [3,4]. Biomarker studies aim to improve outcomes of treatment by identifying patients at higher risk of relapse (and thus also avoiding the overtreatment of patients at lower risk of relapse), to monitor them during treatment in order to detect recurrences, and to develop biomarkers evaluable by liquid biopsies.

**[Au: We do not stack headings one on top of the other, so please provide a brief introductory statement to this section, avoiding phrases such as 'below' and 'in this section' as we do not signpost.]**

### [H2] LOH of chromosome arms 1p and 16q

In 1989, the discovery was made that the gene predisposing to familial Wilms tumour did not map to the expected locus at 11p13 in two large families [11,145] as predicted by the region of deletion in patients with the Wilms and aniridia syndrome. Subsequently, somatic LOH in Wilms tumours genomes were postulated to point to the location of

another Wilms tumour suppressor gene in familial, and perhaps some sporadic Wilms tumours. An analysis of an unselected series of tumours, using Southern blot analysis of genetic polymorphisms near the telomeres of each chromosomal arm revealed LOH in 20% of cases at 16q, and at 1p in 12% in addition to the expected 40% rate at 11p with none or fewer than 5% losses observed at loci near the telomeres of other chromosomes [146]. These isolated instances of LOH were considered random events and were too infrequent to be predictive of any tumour-specific characteristic.

Supporting evidence for this finding was sought by analysing an increased number of tumours and to determine whether any clinical correlates to LOH of the putative underlying tumour suppressor genes exist. Such a correlation would add to the evidence that the LOH was clinically **[Au:OK?]** significant, rather than being a random event, and could possibly provide a marker to guide clinical management. An analysis of 232 Wilms tumours collected through the Pediatric Oncology Group using PCR-based polymorphic loci confirmed the non random losses of 16q and 1p. No correlations between LOH and histology or stage were found, but an at least a three-fold increase in relapse and deaths in those with LOH was observed [147]. The reduced incidence of 1p loss limited the statistical power to demonstrate associations.

The fifth National Wilms tumour Study (NWTS-5) tested the hypothesis that LOH 16q and/or 1p was associated with an inferior EFS and/or OS. An analysis of >1,700 patients with FHWT accrued over 5 years examined the effect of LOH among patients grouped as stage I/II favourable-histology **[Au:OK?]** disease (who were treated similarly with a two-drug chemotherapy regimen of vincristine and actinomycin D, EE4A). The risks of relapse and death were increased for LOH 1p, and for combined

1p and 16q LOH, in comparison with patients with no LOH at either locus **[Au:OK?]** . Patients with LOH 16q alone had an inferior EFS but not OS. The risks of relapse and death for patients with Stage III or IV favourable-histology tumours (also treated similarly but with the addition of a third drug, doxorubicin, and usually radiotherapy) were increased only with LOH for both regions. Subsets of patients whose tumours have LOH 1p and/or 16q are at increased risk of relapse and/or death. The association is particularly marked for patients with tumours with combined LOH 1p and 16q, when examined by clinico-pathological groupings of stage I/II and stage III/IV[148]. Results of an investigation of 426 FHWT treated according to the UKW1–3 clinical trials supported that 16q LOH is an adverse risk factor [149], and the results of analysis of 125 FHWT enrolled in the Italian Association of Pediatric Hematology and Oncology (AIEOP)-TW-2003 protocol supported that 1p LOH is a marker of poor prognosis [150]. While rare combined LOH 1p and 16q was not analyzed in the SIOP cohorts, simultaneous copy number loss at both chromosome arms was infrequent and not prognostically significant, perhaps due to limitations in cohort size [92].

Whether the outcome for patients with tumours with LOH 1p and 16q could be improved by augmenting therapy was then investigated. Treatment was augmented for low-stage (I, II) tumours by adding doxorubicin to vincristine and actinomycin (regimen DD4a), and for high-stage (III, IV) by adding cyclophosphamide and etoposide to the previous standard therapy (regimen M)[151] . The results of these studies by COG demonstrated that the 4-year EFS for patients with stage I or II disease was improved to 87.3% from 68.8% and for patients with stage III or IV disease to 90.2% from 61.3%. **[Au: Peer reviewer 1 asked about SIOP data regarding LOH at 1p and/or 16q to which you replied "data were negative and**

**published by Chagtai T. et al. J Clin Oncol 2016. It wasn't quite LOH but it was copy number loss, which accounts for 90% of the cases with 1p and 16q LOH" but you don't seem to mention this in this section. For completeness and reader understanding I think it important to add this a discuss it the context of the other results. Please do here.]**

Thus, LOH at 1p and 16q provides a useful marker of adverse prognosis that can be demonstrably overcome by augmentation of therapy. In addition, an analysis of patients with stage III FHWT enrolled in AREN0532 demonstrated that LOH 1p or 16q in the presence of positive lymph node involvement by Wilms tumour resulted in an inferior EFS to those without [152]. This finding will be used in the upcoming COG FHWT risk stratification schema. However, the greatest limitation of these prognostic factors is they identify only a minority of patients at increased risk of relapse, which has driven the search for more effective markers.

**[H2] 1q gain**

Gain of 1q is a recurrent chromosomal aberration in Wilms tumour. Results of cytogenic studies in the 1980s and 1990s showed relatively frequent full or partial 1q trisomy in Wilms tumour series, most commonly the result of unbalanced translocations **[Au: Please provide the primary references for these studies.]** [153-155]. Results of a metaphase comparative genomic hybridisation (CGH) analysis of tumours from 46 relapsing and 21 non-relapsing Wilms tumours [156] showed that 1q gain was significantly associated with relapse (p-value = 0.019) **[Au: please add the p value]** and was detectable in specimens sampled at the time of initial diagnosis, suggesting that this aberration might be a biomarker of prognostic relevance. Partial

gain of 1q overlapping with the 1q21-25 region, was found in several samples, but most tumours with 1q gain had whole-arm gain, rather than focal events that might implicate a specific driver gene [156]. **[Au: Please reference this statement.]** . **[Au: what do these observations suggest, what implications do they have? Please comment.]**

Early analyses of gene expression in Wilms tumour also highlighted the potential clinical significance of the 1q region in tumour recurrence. In a small series analysed using comparative expressed sequence hybridisation (CESH) on metaphase spreads [157], overexpression of 1q seemed to be associated with relapse. A specific association between relapse and 1q expression in general did not emerge in a subsequent expression array study [158], but classifiers that distinguished between tumours with and without 1q gain were enriched in genes that mapped to the 1q region, suggesting that the increased copy number was able to drive overexpression.

Analyses with high resolution array CGH platforms supported and extended the metaphase CGH results. In a genome-wide bacterial artificial chromosomes (BAC) **[Au: please define 'BAC']** array analysis of 76 Wilms tumours, including 37 that subsequently relapsed, 1q gain was found to be one of the most significant events **[Au: please add the p value]** associated with recurrence (p-value < 0.001) [159]. Similarly, in a single-nucleotide polymorphism (SNP) array study of 77 Wilms tumours including 17 relapsing cases, 1q gain in the broad 1q21.1-1q31.3 region was significantly associated with relapse (Q-bound=0.006 for the 1q region with the highest level of association with relapsing patients, where Q-bound is the p-value corrected for multiple testing) **[Au: please add the p value]** [160]. These results were consistent

with a large nationwide cytogenetic analysis of 331 Wilms tumours [161], in which 1q gain was found to be independently associated with poor EFS and OS in multivariable analyses that took account of age at diagnosis, tumour stage, anaplasia and other common cytogenetic abnormalities. The results of this study also supported that 1q gain in Wilms tumour is predominantly the result of unbalanced chromosomal translocations, with 16q the most frequent partner, and isochromosome 1q. These events are not necessarily clonal aberrations found throughout the tumour. In a SNP array analysis of tumours sampled at multiple spatial positions [162], intra-tumour heterogeneity of 1q gain was detected in the majority of cases, which has considerable implications for tumour evolution (as it suggests that 1q gain is not one of the earliest events in tumorigenesis) and sampling (as biomarker studies might require at least three samples per tumour to be reasonably certain of detecting gain when it is heterogeneous).

As 1q gain is a relatively common aberration, associated with poor outcome but not restricted to tumours with high-risk histology, it is potentially an attractive biomarker for treatment stratification, which is still largely dependent on histopathological and clinical staging criteria. Several studies have now been conducted to rigorously test the prognostic value of 1q gain. Using multiplex-ligation dependent probe amplification (MLPA) assays, the COG RTC and the SIOP–RTSG have analysed large panels of samples from patients treated predominantly with immediate nephrectomy (COG) or pre-operative chemotherapy (SIOP–RTSG). In an initial COG study including 212 patients with FHWT[163] and a follow-up study of 1,114 patients [164], 1q gain was significantly associated with poorer EFS (p-value < 0.001 in the larger cohort) and OS (p-value < 0.001 in the larger cohort) in patients who received immediate nephrectomy

**[Au: please add the p value]** , and significance was retained for EFS in a multivariable analysis (p-value < 0.001 in the larger cohort) **[Au: please add the p value]** . In the SIOP–RTSG study involving 586 patients, univariable analyses showed a significant association between 1q gain and poor EFS (p-value < 0.001) and OS (p-value = 0.01) in patients who had received neoadjuvant chemotherapy **[Au: please add the p value]** , and significance was retained for EFS in subsets with SIOP intermediate-risk localized disease (p-value = 0.004) **[Au: please add the p value]** or non-anaplastic localized disease (p-value = 0.001) **[Au: please add the p value]** , and in a multivariable model that included stage, histology, sex, age and loss of 1p or 16q (p-value = 0.002) [92]. **[Au: Peer reviewer 1 asked about SIOP data regarding gain of 1p to which you replied "This is in the addendum of the paper by Chagtai T. et al. J Clin Oncol 2016 (only raw data, but it was not significant, likely due to small sample size)" but you don't seem to mention this in this section. For completeness and reader understanding I think it important to add this a discuss it the context of the other results. Please do here**.] Subgroup analysis for clinical stages was not informative in this cohort [92].

The utility of 1q gain as a clinical biomarker is becoming clearer, but the fundamental mechanism of the influence of this aberration on disease outcome remains unknown. To date, no compelling 1q candidate gene has been identified as a specific driver of poor outcome, and multiple genes in this large chromosomal region, modulated by increased copy number, could have a role in the phenotype.

**[H2] 1p and 16q LOH and 1q gain in clinical protocols**

Patients with LOH of 1p and 16q were found to have a poor prognosis in multiple studies. Importantly, this adverse prognosis can be overcome with intensified therapy, as shown in COG AREN0532 [151]**[Au: please add the trial as a numbered reference to your reference list and cite here.]** (for patients with stage I/II disease) and COG AREN0533 (for patients with stage III/IV disease) [151]**[Au: please add the trial as a numbered reference to your reference list and cite here.]** . However, patients with combined LOH of 1p and 16q only account for ~6% of all Wilms tumours. By contrast, chromosome 1q gain is considerably more prevalent than LOH of 1p and 16q. Depending on disease stage, 1q gain is present in 20–40% of patients with Wilms tumour [92,161,163,164]. Also, prevalence of 1q gain increases with tumour stage [163], making this marker particularly desirable for stratification. The currently open SIOP–RTSG UMBRELLA 2016 protocol will validate the importance of 1q gain [165]. In the next COG favourable-histology protocol, 1q gain will be used to stratify treatment. Patients with 1q gain will receive augmented therapy, whereas those without 1q gain will be candidates in some situations for less intensive therapy. If successful, this strategy will improve survival for patients with high-risk disease, and reduce the risk of toxic or late effects for patient whose disease has a more favourable risk.

Early evidence suggests that these adverse biomarkers (LOH 1p, 16q and 1q gain) can be detected as circulating tumour DNA (ctDNA) in blood and urine **[Au: please add the details of this evidence to more fully address the peer reviewer comment. What did the study show specifically?]** [166] . Current studies both in the COG and SIOP context are still exploratory: validation of ctDNA in future studies might overcome challenges posed by tumour molecular heterogeneity and enable this technology to be used in future risk stratification.

### [H2] *TP53* mutation and prognosis

In one of the first studies in which the *TP53* status was evaluated in Wilms tumour , matched anaplastic and non-anaplastic areas of seven Wilms tumours were analysed: six had *TP53* mutations, with five having mutations identified only in the anaplastic area [167], suggesting an association between *TP53* mutation and anaplastic cells. In Wilms tumours, anaplasia is associated with poor prognosis [3,168]**[Au: Please reference this statement.]**, so whether the presence of *TP53* alterations is an additional adverse prognostic factor was investigated. Initially, a cohort of 40 Wilms tumours in which the anaplastic area was evaluated suggested that DAWTs with wild-type *TP53* had a better outcome than DAWT with *TP53* alterations [58]. Similarly, in tumours treated in the SIOP 2001 trial, an exploratory analysis of *TP53* (17p) by MLPA showed an association between *TP53* loss and inferior EFS and OS in a series of 586 Wilms tumours with various histologies, and in subsets excluding high-risk histologies or all anaplastic tumours [92]. Furthermore, an analysis of 118 tumours suggested that in addition to *TP53* alterations, stage should be considered for patient stratification. Patients with stage III or IV DAWTs containing *TP53* abnormalities experienced relapse and death at higher rate (61%) than those with stage III and IV DAWT without detectable *TP53* abnormalities (13%), whereas in patients with stage I and II disease no difference was observed in outcome based on *TP53* status [59]. In another study, molecular characterization of Wilms tumours of any histology from patients who died indicated that 90% of anaplastic tumours and 26% of non-anaplastic tumours harboured *TP53* mutations [60]. In a cohort of 344 patients enrolled in the Japan Wilms tumour study group, < 5% (17) were diagnosed with anaplastic Wilms tumour and only two had *TP53* mutation, both with diffuse anaplasia. The 4-year EFS and OS rates

were 90.9% and 86.7%, respectively. The good outcome **[Au: which was what? Please add the data that supports this statement]** of patients with anaplastic Wilms tumours in this cohort is probably related to most cases being classified in the early stages of disease **[Au: of disease? What stage were they? Please add this information for reader understanding.]** rather than genetic alterations [169]. Thus, in Wilms tumour, the effect of anaplasia on outcome is clear, but the question of whether *TP53* mutation provides additional information is not and, therefore, its use in risk stratification remains exploratory. **[Au: Added from your rebuttal to more completely address the peer reviewer comment in text.]** Intratumoral somatic heterogeneity of *TP53* mutation can potentially be overcome by the use of liquid biopsies, as suggested by the detection in ctDNA of children with anaplastic Wilms tumours of mutant *TP53* even in presence of intratumour heterogeneity **[Au: how? Please explain for reader understanding.]** [170]. Identification of therapies that target p53 remains a priority for advancing the care of children with anaplastic Wilms tumour. As yet, no definable therapies taking advantage of this biological finding have proven beneficial. **[Au: Added from your rebuttal to more completely address the peer reviewer comment in text.]**

**[H2] *MYCN* gain and prognosis**

SNP array copy number profiling of Wilms tumours from patients treated under SIOP protocols suggested an association between *MYCN* status and anaplasia, with copy number gain detected in nearly a third of high-risk diffuse anaplastic tumours [88]. This finding was subsequently supported by results of a large-scale copy number biomarker study using MLPA, in which 40% of diffuse anaplastic tumours analysed had 2p gain, three-quarters **[Au:OK?]** of which had gain restricted to a narrower region

of 2p that included the *MYCN*-specific probe on 2p24 but not a *DYSF* probe on 2p13.2 **[Au: than what? Please add the comparator here for clarity.]** [92]. *MYCN* gain as an independent prognostic factor for outcome was also investigated in this study[92]. *MYCN* gain was significantly associated with poor EFS (p-value = 0.01 for 2p gain in general; p-value = 0.002 for the narrower region) **[Au: please add the p value]** and OS (p-value = 0.04 for 2p gain in general; p-value = 0.003 for the narrower region) **[Au: please add the p value]** in the complete series of 586 tumours of all subtypes, as well as in subsets including only SIOP intermediate-risk or non-anaplastic tumours. These results, together with reports suggesting an association between *MYCN* expression levels and adverse outcome in series including fewer samples [90,93] indicate that *MYCN* status is a promising prognostic biomarker for Wilms tumour, as it is in several other paediatric tumours [81-84], and that the *MYCN* pathway is potentially a target for therapeutic interventions.

### 5.6. Blastemal volume

Approximately 40% of Wilms tumours show blastemal predominance after primary surgery, whereas after preoperative chemotherapy only 10% of Wilms tumours belong to the blastemal subtype [171]**[Au: Please reference this statement.]** . Neither pathology nor imaging studies are, at the moment, helpful to distinguish between blastema that will or will not respond to chemotherapy. In SIOP-9 (the 5$^{th}$ clinical trial run by the SIOP–RTSG), the influence of histological subtypes after pre-operative chemotherapy in non-anaplastic nephroblastoma was shown retrospectively for the first time [171]. Viable blastemal content correlates with a worse outcome [172]. Blastema itself is characterized as a tumour component composed of primitive undifferentiated cells showing no specific differentiation pattern (FIG.4) **[Au: have you got a**

**previously unpublished histological image of this we could add a figure? it would be a useful tool for the reader.]** [173]. A comparison of data from the 6th trial (SIOP 93-01) and the 7th trial (SIOP 2001), in which, for the first time, the blastemal subtype being treated as a high-risk tumour with intensified chemotherapy showed improved outcomes for these patients in SIOP 2001 **[Au:OK?]** . The 5-year EFS in SIOP 2001 was 80% compared with 67% in SIOP 93-01 (p-value = 0.006) [174]. Such an improvement based on a histological subtype highlights the need for improved classification of treatment-resistant blastema. According to the revised SIOP 2001 working classification of renal tumours, blastemal type Wilms tumour is based on relative volume measures, of which >66% of the viable tumour component consists of blastema in a tumour with more than one-third viability [175]. This classification neglects the absolute volume of viable blastema, where the same blastemal volume can end up in different histological risk groups resulting in more or less intensive treatment for patients (for example, volume after preoperative chemotherapy of 200 ml in two different tumours, one tumour with 70% necrosis and 90% surviving blastema and the other one with 60% necrosis and 70% blastema: the blastemal volume in these tumours is 54 ml vs 56 ml nearly the same, but the one with 70% necrosis is per definition a regressive type and the other one a blastemal type high risk tumour) **[Au: meaning what? What implications does this have? Please comment.]** . In a retrospective analysis of SIOP 2001, a threshold of ~20 ml residual blastemal volume in localized nephroblastoma was suggested as a poor prognostic marker [176]. In the ongoing SIOP–RTSG UMBRELLA 2016 protocol [165,173]**[Au: Please reference this statement.]** , such a prognostic threshold will be prospectively analyzed. Besides volume aspects, correlations of blastema with specific molecular findings are currently under investigation.

## [H2] Prognostic factors in very specific risk groups

Different studies provide a biological explanation for the clinical and pathological heterogeneity seen within Wilms tumour and support a model of Wilms tumour ontogeny in which both the type of initiating genetic event and the developmental stage in which it occurs are important determinants. One novel subset included epithelial Wilms tumours in infancy that lacked nephrogenic rests and did not recur; these displayed a gene expression pattern similar to **[Au:OK?]** the post-induction nephron [47,177]. This subset has now been shown to be characterized by recurrent mutations in *TRIM28* [178]. This subset is, in part, responsible for the excellent outcome of infantile, stage I Wilms tumour, as well as those with epithelial predominance [179,180]. A cluster of three Wilms tumour subsets are characterized by low *WT1* expression (often accompanied by *WT1* mutation) and ILNR [47]. Recurrent mutations in *MLLT1* and *WTX* have been identified within these subsets, pointing toward interference in mesenchymal–epithelial transition as an underlying mechanism for Wilms tumour development [30]. A final Wilms tumour subset comprises over 70% of Wilms tumours and is characterized by biallelic methylation of IC1 on 11p15, and by a pre-induction gene expression profile [47]**[Au: Please reference this statement.]** . Subsequent studies have demonstrated recurrent gain of *LIN28*, or recurrent mutations in *DROSHA, SIX1, SIX2*, and *DGCR8* within Wilms tumours showing biallelic methylation of IC1 **[Au: how many?]**. These genetic changes point toward the important role of microRNAs in preserving the undifferentiated state, resulting in Wilms tumour development.

In other studies potential predictors of relapse based on gene expression were also assessed [181]. Prediction was successful only in patients with stage III disease, and a set of genes was not proposed as a specific predictor. However, several of the genes for which increased expression most reliably predicted relapse in patents with stage III disease were located on chromosome 1q **[Au: with what implications? Please comment here.]** . Furthermore, although *IGF2* overexpression does not have adverse prognostic importance in Wilms tumours treated with chemotherapy [181], evidence suggests that 11p15 LOH or LOI are associated with tumour relapse in young patients (<2 years old) with small (tumour weight < 550 grams) stage I FHWT whose disease was classified as very-low risk (VLR) and not treated with chemotherapy [177,180,182]. In the COG study AREN0532, relapse was observed in only 3% of patients with VLR disease with retention of imprinting of 11p15, but was observed in 20% of those with LOH and 25% of those with LOI [180].

**[Au: please add a brief summary statement for this section.]**

**[H2] Urine Tumour Markers**

Given the notable differences in the initial management of renal tumours using the SIOP or COG approach, considerable efforts have been made to identify non-invasive biomarkers at diagnosis. Early studies investigating the prognostic utility of urine biomarkers nominated basic fibroblast growth factor and hyaluronidase, as these were both elevated at diagnosis in patients with Wilms tumours and correlated with disease stage [183-185]. Ultimately, neither urine biomarker was incorporated into the current risk stratification paradigm owing to inadequate sensitivity and specificity. Subsequent urine proteomic studies leveraged high accuracy mass spectrometry to reveal that elevated diagnostic urine prohibitin (PHB) was a prognostic marker of relapse,

particularly local relapse, in patients with FHWT[186]. PHB was shown to be highly expressed in the mitochondria of Wilms tumours [186]. Using functional genomic techniques, investigators found that this excess mitochondrial PHB impairs Opa1-mediated mitochondrial apoptosis, leading to chemoresistance and ultimately treatment failure [186]. Additional pilot studies have demonstrated that the cell-free portion of urine specimens may be valuable in the detection of somatic Wilms tumour mutations beyond blood testing alone and may help identify patients at risk for developing Wilms tumour by virtue of nephroblatomatosis identification in patients with PIK3CA-related overgrowth spectrum **[Au: how? What is in this portion that is valuable in this context? Please expand briefly.]** [187,188]. Indeed, beyond aiding in initial diagnosis and identification of high-risk biomarkers, cell-free DNA (both urine and blood) might ultimately prove valuable as an early biomarker of minimal residual disease [166,170,188]. **[Au: following text added from your rebuttal to more completely address peer reviewer 2's comments. Please reference accordingly.]** Trending these biomarkers prospectively during therapy could be valuable to determine if, and if so, how, they can be used as evidence of minimal residual disease. Indeed, different biomarker assays could have specific utility for different aims. For example, assessing cell-free DNA using a broad Next Generation Sequencing panel that also includes shallow whole-genome sequencing to pick up 1q at diagnosis could help with initial histology identification and risk stratification [166]**[Au: Please add the reference number here]** whereas perhaps a focused digital droplet PCR aimed at one or a few canonical drivers would be better for picking up minimal residual disease [170]. **[Au: Please add the reference number here]**

**[H2] microRNAs**

miRNAs are a class of small, single-stranded RNAs originally identified in *C. elegans* in 1993 and, subsequently, in higher vertebrates including humans, which have a pivotal role in regulating gene expression on a post-transcriptional level by inhibiting protein translation of target genes [189,190]. Deregulation of miRNA expression can lead to a variety of diseases, including cancer [191]. Moreover, cells can release miRNAs into their surroundings and the bloodstream as a means of intercellular communication, where they can be exploited as biomarkers [192].

The first evidence of deregulated miRNA expression in Wilms tumours was found in 2008, upregulation of the Onco-miR-1 cluster was reported [193]. Subsequently, a number of deregulated miRNAs in Wilms tumour have been identified, some of which, most notably miR483-3p and let7b-5p, **[Au: please add some examples here]** might have prognostic potential as indicators of chemoresponsiveness, particularly of the Wilms tumour aggressive blastemal component [194]. A reasonable explanation for the disrupted miRNA expression patterns, at least in a subset of Wilms tumours, might be germline and somatic mutations in components of the microprocessor complex necessary for miRNA maturation, such as *DROSHA* and *DGCR8* [62,98,195]. In addition, several studies showed differential expression of some miRNAs in blood or serum of patients with Wilms tumour, emphasising the possible use of miRNA as minimally invasive biomarkers for Wilms tumour in blood, tumour, and urine samples in the future [196-198].

**[H1] Evolution and intratumour heterogeneity [Au: heading edited for length]**

Most Wilms tumours commence with somatic mutations already obtained in fetal life [16,199]**[Au: Please reference this statement.]** . This process either manifests as

microscopically visible nephrogenic remnants [199], or as clonal expansions in morphologically nonmaligant kidney tissue [16]. Like most cancers, a Wilms tumour emerges when a somatic cell lineage branches off from the germline genome to form a premalignant lesion. When such lesions transit into irreversible clonal expansion to manifest as Wilms tumour, this branching continues and causes genetic intratumour heterogeneity [162,200]. In such a branching evolutionary process, the distribution of a given mutation across the anatomic space of a Wilms tumour will depend on when in tumour evolution it occurs (FIG. 5A). For example, when LOH of the *IGF2* and ***[Au: what does the solidus denote here, 'and', 'or', or 'and/or', please clarify.]*** *H19* gene cluster in 11p is detected it is usually found in all Wilms tumour cells, reflecting its occurrence in precursor lesions [201]. In contrast, gain of 1q shows variability across tumour regions in ~50% of tumours in which it is ascertained [162,201], indicating that 1q gain in these cases does not emerge until tumour growth is well underway. *TP53* mutations are almost invariably regional, usually confined to anaplastic regions, and linked to the emergence of complex chromosomal rearrangements [60,201]. This observation indicates that they are late steps in tumour evolution. *TP53* mutations, 17p LOH, and anaplastic features can occur multifocally in a tumour, in a pattern mimicking convergent or parallel evolution [201]. However, whether these foci are, in fact, continuous in 3D is an open question in need of further elucidation. Chromosomal rearrangements within anaplastic regions in turn vary extensively, translating into a high frequency of local phylogenetic branching that can result in highly diverse genome profiles in different parts of Wilms tumours with diffuse anaplasia.

Branching evolution can give rise to a broad range of genetic variants among tumour cells, but the frequencies of these variants will vary across the tumour parenchyma as

a function of Darwinian selection and genetic drift (FIG. 5B). If most mutations are neutral, the genetic variation in a tumour will be in proportion with the mutation rate. Selection, on the other hand, will narrow down genetic variation by enrichment of the fittest genetic variant or variants. That such enrichment occurs during Wilms tumour evolution is supported by the presence of regional clonal sweeps, which are situations in which a daughter clone outgrows its mother population and rises to dominate a geographic region [202]**[Au: Please reference this statement.]** . That regional clonal sweeps can be caused by selection in Wilms tumour is supported by their association to mutations in driver genes such as *MYCN* and *SIX1* [201].

Variability of biomarkers owing to evolutionary branching can confound clinical treatment decisions. Sampling procedures in clinical protocols must take this variability **[Au:OK?]** into account. Indeed, in the SIOP–RTSG UMBRELLA 2016 protocol for renal tumours, multiregional sampling to cover genetic variation is recommended [173]**[Au: Please reference this statement.]** . However, how to use genomic information obtained across multiple samples remains unclear. **[Au: we do not have questions in text, therefore, I have edited to remove them.]** Whether a high-risk marker detected in just one region is sufficient to influence treatment is not certain, and if it is not sufficient, then the number or proportion of regions that will be enough needs to be determined. One way to solve this dilemma is to use evolutionary patterns for clinical risk assessment. This approach might have some promise, as a high degree of phylogenetic branching correlates with high-risk histology in Wilms tumours and to inferior relapse-free survival in solid paediatric cancers overall [203].

**[H1] Wilms tumour models**

To facilitate development of future Wilms tumour studies, effective preclinical models for functional analysis of genetic drivers and for testing of new treatment options are much needed. Establishement of cancer cell lines is difficult and immortalized cell lines undergo strong clonal selection [204,205] **[Au: Please reference this statement.]** . Consequently, cancer cell lines are typically very poor representatives of native tumour tissues [205].In vitro cell cultures, organoid models, and patient-derived xenografts represent complementary models currently available in this pathology.

**[Au: We do not stack headings one on top of the other, so please provide a brief introductory statement to this section, avoiding phrases such as 'below' and 'in this section' as we do not signpost.]**

**[H2] Cell culture models**

*In vitro* cell culture systems are cost-efficient and time-efficient models. Representation of the phenotypic and genetic heterogeneity of Wilms tumour *in vitro* requires a diverse collection of primary cultures derived from patient tumour material and it might be difficult, or even impossible, to find universal culture conditions that support proliferation of all different Wilms tumour cell types equally **[Au: the same extent as what? Please clarify.]** .

Results of early studies showed the possibility of short-term culture from primary Wilms tumour material or mouse xenografts [206-208], but these models were either short-lived or difficult to handle. Over the years, a few spontaneously immortalized Wilms tumour cell lines have been established from anaplastic tumours, but they represent only the rare and specific subtype with p53 alteration **[Au: what are the benefits and limitations of these models? Please comment.]** [209-211]. In addition, primary stromal

cells, often derived from *WT1*-mutant tumour samples, and epithelial cells can be cultivated as classic adherent cultures but with restricted life-span **[Au: what are the benefits and limitations of these models? Please comment.]** [212-215]. The challenging blastemal subtype could not be propagated under these conditions. This finding is in agreement with the observation that blastemal tumours lose their nude mouse engrafting capacity even upon short-term cultivation [216].

3D growth of tumour cells overcomes some limitations of 2D cultures on plastic surface as they intrinsically provide more physiological interactions. Blastemal Wilms tumour cells can be grown in suspension culture as spheroids in medium containing ROCK inhibitor (Y-27632) to avoid anoikis [217]. These 3D spheroid cultures represent the blastemal Wilms tumour component with respect to phenotype and marker expression [217]**[Au: Please reference this statement.]** . In addition, certain epithelial and immature stromal Wilms tumour elements can be propagated as spheroids and they maintain features of the initial tumour after long-term cultivation [217]**[Au: Please reference this statement.]** . 2D and 3D Wilms tumour cultures are amenable to genetic manipulation by viral transduction and/or transfection [217]**[Au: Please reference this statement.]** and, therefore, enable functional studies of candidate genes as well as high-throughput compound screening in multi-well formats. **[Au: what are the limitations of these models? Please comment.]**

**[H2] Organoid models**

Innovations in 3D culture technology, such as organoids, have revolutionized cancer research. Organoid technology enables efficient generation of *in vitro* culture models

from patient material, which can be propagated long term while retaining crucial characteristics of the tumour tissue from which they were derived [218]. **[Au: this highlighted section should be specifically mentioned in the above section on cell culture models. Please add there.]** Protocols have been developed for culturing organoids from a wide spectrum of different adult tumours, including colon [219], breast [220], ovarian [221], pancreas [222] and liver [223]. Results of several studies have demonstrated that patient-derived tumour organoids recapitulate patient drug responses [224-227], suggesting that organoids can be used for the development of individualized therapies.

In two recent studies, organoid models of paediatric renal tumours, including Wilms tumours were developed [228,229]. Wilms tumour organoid cultures were demonstrated to capture the genetic and cellular heterogeneity of Wilms tumour tissue to a large extent, including blastemal, stromal and epithelial cells, over multiple passages. Yet different Wilms tumour cell types will probably still favour different culture conditions for their optimal growth to be fully representative of the predominant subtypes found in the original tumour. Moreover, Wilms tumour organoids can be cryopreserved, enabling the generation of large collections of pre-clinical models in so-called 'living biobanks', which can be used for drug screens, and are amenable to genetic modification. This technology is still in its infancy, but the use of organoid technology to study Wilms tumour biology so far seems promising and might pave the way for development of new therapeutic strategies. **[Au: what are the limitations of these models? Please comment.]** Further development of organoid technology by including components of the tumour microenvironment (for example immune cells) will

be critical to make these in vitro models resemble patient tumours even better and suitable for testing, among others, immunotherapy approaches.

## [H2] Patient-derived xenografts

Patient-derived xenografts have been crucial for Wilms tumour research, owing to the paucity of available *in vitro* cell lines. In 1985, human Wilms tumours heterotransplanted into the flanks of nude mice were shown to closely resemble the surgically resected human tumours from which they were derived [230,231]. Tumour cells injected in the flank resulted in blastemal predominant xenografts, whereas intraperitoneal injections of tumour cells from the same source demonstrated tubular epithelial differentiation [231]. In 1987, serially passaged Wilms tumour xenografts showed accumulation of blastema [232]. The accumulation of blastema in serially passaged heterotopic Wilms tumour xenografts was associated with accelerated cellular proliferation, upregulation of cell cycle genes, loss of imprinting for a multitude of paternally expressed genes, and **[Au: what does the solidus denote here, 'and', 'or', or 'and/or'? please specify.]** increased expression of genes associated with differentiation blockade and maintenance of cellular self-renewal [233]. Serial passaging of Wilms tumour xenografts was subsequently demonstrated to be associated with *WT1*, *PAX2*, and *SALL1* promoter hypomethylation and upregulated expression of these renal progenitor genes [234]. This enrichment of nephron progenitor genes was exploited to expand and isolate Wilms tumour cancer initiating cells **[Au: what does the solidus denote here, 'and', 'or', or 'and/or'? please specify.]** (also known as cancer stem cells) characterized by the expression of the *NCAM1* gene **[Au: please define 'NCAM']** , and later, more specifically, combined expression of the genes *NCAM1* and *ALDH1* **[Au: please define 'ALDH1']** [216,235]. A panel of four Wilms tumour

heterotopic xenografts investigated in the Pediatric Preclinical Testing Program have been used to successfully guide prioritization for phase 1 clinical trials that include patients with Wilms tumour with relapsed or refractory disease [236]. Orthotopic Wilms tumour xenografts derived from the WiT49 anaplastic Wilms tumour cell line implanted in the kidney subcapsule, but not heterotopic subcutaneous xenografts, demonstrated a single-agent response to IGF1R inhibition, demonstrating the potential importance of the tumour microenvironment in xenograft biology and even therapeutic response [237]. A panel of orthotopic Wilms tumour xenografts was established and is freely available from the Childhood Solid Tumor Network [238]. A direct comparison of genetics and transcriptomics between orthotopic and heterotopic Wilms tumour patient-derived xenografts has not yet been performed. In 2019, a comprehensive genomic characterization of 45 Wilms tumour patient-derived xenografts **[Au: than what? Please add the comparator here.]** was performed to capture the clinical and biological heterogeneity of this disease more completely than previous xenograft libraries. Patient-derived xenografts from this effort included models from patients with unfavourable histology Wilms tumour, bilateral disease, and from those who went on to develop disease relapse [239]. These heterotopic xenografts maintained genetic variants of the original patient primary tumours, phenocopied predicted chemotherapy response according to histology and were enriched for gene expression characteristic of Wilms tumour blastema and the cap mesenchyme in kidney development [239]. Overall, Wilms tumour patient-derived xenografts have provided a critical resource for preclinical treatment studies; however, these models are all established in immunodeficient mice and therefore lack the ability to appropriately test many types of cancer immunotherapies. Furthermore, the models are difficult to genetically modify and therefore have limited ability to provide functional insights into Wilms tumour

related genes using controlled experiments. **[Au: what are the benefits and limitations of these models? Please comment.]**

**[Au: Please add a brief, overarching summary statement concerning Wilms tumour models here.]**

Cell cultures, organoid models, and patient-derived xenografts are complementary model systems in the study of Wilms tumour biology and treatment. When used together, these approaches can allow for functional genetic insights, screening of novel therapeutic compounds, and formal preclinical treatment studies.

## [H1] Progression and resistance

**a**

Half of patients who experience relapse develop resistance to therapies and die [2]. Thus, understanding of the molecular features underlying tumour resistance and recurrence is urgently needed.

### [H2] Cancer Stem Cells

Classic Wilms tumours exhibit a triphasic histology consisting of undifferentiated mesenchyme, stroma, and renal tubular epithelia [128]**[Au: Please reference this statement.]** . However, histology varies widely and can also include differentiated cells such as muscle and cartilage that also derive from mesenchymal precursors [129]**[Au: Please reference this statement.]** . This fascinating histology has led to the well-accepted idea that Wilms tumour arises from undifferentiated renal mesenchyme [128]. Studies in mice in which triphasic histology tumours develop following the

introduction of genetic alterations in fetal metanephric mesenchyme support this idea [50].

This aetiological model of Wilms tumour is consistent with the continued presence of aberrant mesenchymal cells that act as cancer stem cells (CSCs) or tumour initiating cells (TICs) [129]**[Au: Please reference this statement.]** within tumours. In studies in which human primary Wilms tumours were serially passaged in NOD–SCID mice, cells were identified that were increasingly able to initiate xenografts that histologically displayed the blastemal, epithelial, and stromal elements commonly observed in Wilms tumours. The proportion of blastemal elements in the xenografts increased with passage, suggesting that they were CSCs [216].

Whether this work has enabled identification of a CSC unique to tumours serially passaged in immune-compromised mice or a CSC operative in human tumours *in vivo* is still an open question. The identity of such a CSC is certainly consistent with long-held models of the cellular aetiology of Wilms tumours. However, mutations in primary Wilms tumours have been identified in several genes with diverse functions at various stages of kidney development [30,62,66,90,91,94,98] **[Au: Please reference this statement.]** . Additionally, tumours bearing these different mutations are associated with differing clinical features and outcomes. This observation suggests that, if Wilms tumours are propagated from CSCs, those CSCs differ between different genetic and clinical subsets of tumours.

**[H2] Relapsed Wilms tumour**

In both COG and SIOP treatment algorithms, treatment intensity is based on a patient's risk classification which is further defined by known prognostic variables, the most common being patient tumour stage and histology (COG: favourable vs unfavourable; SIOP: low, intermediate vs high risk), and increasingly molecular biology. Commonly stage I and II and some stage III patients with favourable or low and intermediate risk histology Wilms tumours are treated with 2-drugs only, whereas most other patients are treated with 3-5 chemotherapy agents, pending risk characterization [3,240]. With such a risk stratified approach, relapse rates continue to vary based on stage and histology (favourable vs anaplastic or post-treatment blastemal type), as well as other prognostic factors such as biology, with relapse lowest for stage I with favourable or low and intermediate risk histology treated with 2-drugs (<10%) and highest for stage IV (~ 20% for favourable histology and 50-60% for anaplastic or post-treatment blastemal histology) despite more intensive treatments [3,241]. Treatment of patients with relapsed Wilms tumour is based on initial histology and first-line treatment (choice of chemotherapy and radiotherapy yes or no) in SIOP, and the ongoing COG protocol has a similar definition of relapse risk groups according to upfront drug treatment **[Au: Peer reviewer 2 also asked if there are any differences between stages and recurrence rates based on therapies received, could you please add if this information is available with appropriate references.]** [2].

Genetic anomalies in recurrent Wilms tumours, and the events leading the primary tumour to relapse, have been poorly investigated, mainly owing to the difficulty in obtaining new sample material at relapse to match with the corresponding primary sample. A study of 10 such paired samples revealed gains at chromosomes 5p, 8p12, 15q, 16p and 20q, and losses of 11q and 17p as events acquired in two recurrent

tumours [242]. Analysis of eight paired primary and relapsed tumours showed chromosomal anomalies at 1q, 3, and 16q in Wilms tumour recurrences, and the co-occurrence of *SIX1* and *DROSHA* mutations in the recurrence in three patients [101].

Targeted sequencing of *SIX1* and *SIX2* and miRNAPGs of a further 19 paired tumour cases in which multiple samples were investigated for each tumour, showed co-occurring *SIX1* and *DROSHA* mutations in one case and miRNAPGs mutations in five cases [102]. Intriguingly, in all cases in which mutations were observed in the primary Wilms tumour, although not necessarily in all the blocks, and not necessarily heterozygously (not in all the cells of the sample), the same mutations were invariably present in the matched relapsed disease, being present in all examined blocks and in all tumour cells, indicating that they were positively selected during tumour progression. In particular, among the four patients with co-occurring *SIX1* and *DROSHA* mutations in the relapse, two displayed positive selection of the primary tumour cell clones bearing both events, whereas in the other two, the *SIX1* mutation was not detected in the primary disease [101,102]. These data suggest that *SIX1* and miRNAPGs mutations might provide an advantage during the progression to recurrence and can represent oncogenic drivers in Wilms tumour.

A study of FHWT investigating 45 trios of samples (germline, primary and relapsed tumour), 6 germline-relapsed samples and 31 relapse samples showed that >40% of relapse samples displayed mutations in *SIX1* or genes of the MYCN network. Intriguingly, in some cases, *SIX1* and *MYCN* hot-spot mutations were present in the relapse, but not in the primary tumour sample, suggesting their involvement in tumour progression **[Au: why is this intriguing? Please expand and explain.]**. Other mutations not previously found in primary Wilms tumours affected *DIS3* and *TERT*. Furthermore, 75% of relapse samples had 1q gain [243]. Results of these studies

suggest that combinations of mutations or structural changes, rather than the temporal order of their acquisition, might be important for tumour progression. The co-occurrence of mutations in genes that support continued progenitor proliferation with those preventing differentiation might be crucial. In addition, the evidence of occurrence of 1q gain in 75% of relapses strongly supports the importance of this biomarker [243,244].

**[Au: Please add a summary statement regarding Wilms tumour progression and relapse.]**

**[H1] HARMONICA**

The task force harmonization and collaboration for paediatric renal tumours (HARMONICA) was established in 2015, (Chairs: Prof. dr. J. Geller and Prof. dr. MM van den Heuvel-Eibrink) representing efforts of the COG-RTC and SIOP–RTSG. The combined multidisciplinary intellectual resources and organizational structure of the two committees brings together the world's clinical and research experts in paediatric renal tumours inclusive of all disease disciplines.

Building on such expertise and historic cooperative group renal tumour studies and trials with defined and harmonized data variables and end points, further collaboration in the form of various research efforts has been facilitated. For example, performing biological studies and systematic reviews has guided targeted treatment development for adverse prognostic subgroups and evidence-based international treatment and surveillance consensus guidelines, respectively [4]**[Au: Please reference this statement.]**. Collaborative formal prioritization of therapeutic targets of interest for

pre-clinical investigation in Wilms tumour, harmonized risk stratification for relapsed Wilms tumour, and harmonization of definition of pulmonary metastases are three of many unpublished work products affecting current research and clinical care resulting from the task force [4] **[Au: we still need this statement to be attributed to an author or authors. Please add an attribute and provide permission to use it from the relevant authors, an email to me will be sufficient.]**.

An important guiding principle behind HARMONICA is that investing in future developments in the field will benefit from programmes focused on collaborative training, mentoring and transatlantic exchange of junior clinicians and investigators (SIOPe and COG Young investigator initiatives). A second driving principle guiding the strategy of HARMONICA's collaborative efforts is that it aims to advance research that cannot be successfully achieved by either cooperative group separately (replication, validation, and power issues). So far, several studies on harmonization of standard-of-care approaches, reviews and biological studies have been published [2,3,59,245-251]. The present paper is also the result of a HARMONICA effort.

## [H1] Conclusions

Our collective understanding of Wilms tumour biology has evolved considerably over the past five decades. From early studies using techniques such as chromosome mapping to those exploiting cutting-edge techniques including single-cell sequencing and organoid models, collaboration has been a key element in all aspects of this body of work. Through continued global collaboration and building on previous work, we expect that the next five decades will be as fruitful for understanding this disease. Most importantly, we believe that approaches that build on this growing body of research,

such as improved stratification of therapy according to prognostic biomarkers, or targeted therapy based on emerging molecular targets, will further improve care for our patients.

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## Competing interests

The authors declare no competing interests

## Key Points

- The genetic landscape of Wilms tumours has been deeply investigated, but their relationship to intratumoral heterogeneity and how abnormal nephrogenesis relates to malignant transformation to Wilms tumour are still being explored.
- Genetics of the classical syndromic conditions associated with Wilms tumour development have been described in detail, but whole-exome sequencing is revealing a wide range of constitutional mutations in children lacking any clear phenotype.
- Prognostic biomarkers are starting to be used in clinical practice and for risk stratification in trials, but more need to be identified and validated.
- Many complementary preclinical Wilms tumour models are now available.
- Efforts to explore the events leading to aggressive and/or recurrent disease are needed.

- Worldwide international collaborations among experts in the field have been established.

Table 1: Recurrent Wilms tumour mutations

| Hugo Symbol | Chromosome location | Number of patients with FHWT with mutations (n = 533) | Number of patients with DAWT with mutations (n = 118) | Percent of total (n = 651) |
|---|---|---|---|---|
| CTNNB1 | 3p22 | 86 | 2 | 13.52 |
| DROSHA | 5p13 | 61 | 5 | 10.14 |
| TP53 | 17p13 | 9 | 56 | 9.98 |
| WT1 | 11p13 | 40 | 1 | 6.30 |
| FAM123B | Xq11 | 34 | 4 | 5.84 |
| DGCR8 | 22q11 | 22 | 7 | 4.45 |
| SIX1 | 14q23 | 23 | 2 | 3.84 |
| BCORL1 | Xq26 | 22 | 3 | 3.84 |
| MYCN | 2p24 | 22 | 2 | 3.69 |
| COL6A3 | 2q37 | 19 | 2 | 3.23 |
| MLLT1 | 19p13 | 19 | 1 | 3.07 |
| NF1 | 17q11 | 17 | 2 | 2.92 |
| SIX2 | 2p21 | 17 | 1 | 2.76 |
| BCOR | Xp11 | 14 | 3 | 2.61 |
| DICER1 | 14q32 | 12 | 4 | 2.46 |
| NONO | Xq13 | 12 | 1 | 2.00 |
| ARID1A | 1p36.11 | 11 | 1 | 1.84 |
| MAX | 14q23 | 11 | 0 | 1.69 |
| MAP3K4 | 6q26 | 9 | 2 | 1.69 |
| ASXL1 | 20q11 | 8 | 3 | 1.69 |
| BRD7 | 16q12 | 8 | 2 | 1.54 |
| XPO5 | 6p21 | 8 | 2 | 1.54 |
| FGFR1 | 8p11 | 6 | 3 | 1.38 |
| CHD4 | 12p13 | 6 | 2 | 1.23 |
| HDAC4 | 2q37 | 6 | 2 | 1.23 |
| PALB2 | 16p12 | 3 | 5 | 1.23 |
| CHEK2 | 22q12 | 6 | 2 | 1.23 |
| ACTB | 7p22 | 3 | 4 | 1.08 |
| CREBBP | 16p13 | 6 | 0 | 0.92 |
| EP300 | 22q13 | 4 | 2 | 0.92 |
| RLIM | Xq13 | 3 | 2 | 0.77 |
| NF2 | 22q12 | 2 | 1 | 0.46 |
| KRAS | 12p12 | 2 | 1 | 0.46 |
| SALL1 | 16q12 | 1 | 1 | 0.31 |
| TERT | 5p15 | 1 | 1 | 0.31 |

Data are from Gadd et al.[30] Whole-exome or whole-genome sequencing was performed in a discovery set of 117 high-risk Wilms tumours. High-risk Wilms-tumours were defined as favourable-histology Wilms tumours (FHWT, n = 78) that relapsed and diffuse anaplastic Wilms tumours (DAWT, n = 39). High-quality variants were selected for target capture in a validation set of 533 FHWT and 118 DAWT samples, and recurrent variants are provided in the table.

Table2: Syndromes and Non-syndromic constitutional genetic and epigenetic alterations associated with Wilms tumour

| Syndrome | Inheritance | Gene and/or **[Au:OK?]** Locus | Associated Features | Wilms Risk | Reference |
|---|---|---|---|---|---|
| WAGR Syndrome | Sporadic | 11p13 deletion including *WT1* and *PAX6* | - Aniridia<br>- Genitourinary malformations<br>- Developmental delay<br>- Renal Failure | ~50% | 112,113,252 |
| Denys-Drash Syndrome | Sporadic | *WT1* (mostly exons 8 or 9) | - Renal Failure<br>- Genitourinary malformations | ~30% (missense) ~80% (truncating) | 114,253 |
| Frasier Syndrome | Sporadic | *WT1* (intron 9 donor splice site) | - Genitourinary malformations<br>- Gonadoblastoma | ~5% | 36 |
| Beckwith-Wiedemann Syndrome | Sporadic (with exceptions) | 1) Loss of imprinting at 11p15.5<br>2) Uniparental disomy of 11p15.5<br>3) *CDKN1C* point mutations<br>4) Microdeletions or duplications at 11p15.5 | - Overgrowth<br>- Hemihyperplasia<br>- Macrosomia<br>- Organomegaly<br>- Abdominal wall defects<br>- Macroglossia<br>- Neonatal hypoglycemia<br>- Hemangiomas<br>- Hepatoblastoma<br>- Other childhood tumours | 5% - 30% | 116,254 |
| Isolated Hemihyperplasia | Sporadic | 25% have loss of imprinting 11p15 | - Hemihyperplasia | 10% | 111,255 |
| Perlman Syndrome | Sporadic | *DIS3L2* | - Overgrowth<br>- Organomegaly<br>- Developmental delay | ~60% of infants surviving past | 256,257 |

| | | | | | |
|---|---|---|---|---|---|
| | | | - Perinatal demise | neonatal period | |
| Simpson-Golabi-Behmel Syndrome | X-linked | *GPC3* | - Overgrowth<br>- Macroglossia<br>- Organomegaly<br>- Embryonal tumours<br>- Variable developmental delay | ~5% | [258] |
| PIK3CA-related overgrowth syndromes | Sporadic | *PIK3CA* | - Lipomatous overgrowth<br>- Vascular malformations<br>- Epidermal nevi<br>- Skeletal abnormalities | 3% | [259] |
| Sotos Syndrome | Sporadic | *NSD1* | - Overgrowth<br>- Macrocephaly<br>- Developmental delay | 1% - 2% | [260] |
| Li-Fraumeni Syndrome | Autosomal Dominant | *TP53* | - Multiple tumours | Case Reports | [261] |
| FANCD2 Fanconi Anemia | Autosomal Recessive | *FANCD2* | - Pancytopenia<br>- Short stature<br>- Radial anomalies<br>- Multiple tumours | | [262] |
| Bloom Syndrome | Autosomal Recessive | *BLM* | - Low birth weight<br>- Facial rash<br>- Hypogonadism<br>- Multiple tumours | Case Reports | [263,264] |
| Mosaic Variegated Aneuploidy | Autosomal Recessive | *TRIP13, BUB1B* | - Developmental Delay<br>- Constitutional Aneuploidy<br>- IUGR<br>- Microcephaly | High risk – unclear exact risk | [265-267] |

| | | | | | |
|---|---|---|---|---|---|
| | | | - Other tumours | | |
| DICER1 Syndrome | Autosomal Dominant | *DICER1* | - Multiple tumours | ~1% | [268] |
| Constitutional Mismatch Repair Deficiency | Autosomal Recessive | *MSH6*, *MLH1*, *PMS2*, *MSH2* | - Multiple tumours<br>- Café-au-lait macules | Case Report | [269] |
| Trisomy 18 | Sporadic | Trisomy 18 | - Multiple | ~1% | [270] |
| 9q22.3 Microdeletion | Sporadic | 9q22.3 Microdeletion | - Macrosomia<br>- Craniosynostosis<br>- Basal Cell Carcinoma<br>- Odontogenic Cysts | Case Reports | [271] |
| *REST*-associated Wilms tumour | Autosomal Dominant with Incomplete Penetrance | *REST* | - Non-syndromic | ~2% of Patients without phenotypic features of a Wilms tumour predisposition syndrome **[Au: patients with no syndrome?]** with Wilms tumour | [120] |
| *CTR9*-associated Wilms tumour | Autosomal Dominant | *CTR9* | - Non-syndromic | 3 families reported | [121] |
| *TRIM28*-associated Wilms tumour | Autosomal Dominant | *TRIM28* | - Non-syndromic<br>- Epithelial-predominant tumours | Unknown | [17,18,127] |
| *FBXW7*-associated Wilms tumour | Autosomal Dominant | *FBXW7* | - Non-syndromic | Unknown | [18] |
| *NYNRIN*-associated Wilms tumour | Autosomal Dominant | *NYNRIN* | - Non-syndromic | Unknown | [18] |

WAGR, Wilms tumour, aniridia, genitourinary anomalies and a range of developmental delays

## Legends to figures

### FIGURE 1. Key discoveries in Wilms tumour biology

Timeline describing the key discoveries in Wilms tumour biology research. **[Au: Please provide a brief description of the timeline here.]**

Blue circles: genetic findings; red circles: putative prognostic and prognostic biomarkers; red and dark red circles: a biomarker enters the clinical practice; green circles: kidney development and Wilms tumour; yellow circles: Wilms tumour models; PDX: patient-derived xenografts; the temporal arrow scale is only representative.

### FIGURE 2. Nephrogenic rest

Illustrated is a small nephrogenic rest (arrows), which is an island of undifferentiated and poorly differentiated embryonic renal elements. (Original magnification 20X).

### FIGURE 3. Diffuse anaplasia in Wilms tumour

Montage of representative histology of diffuse anaplasia in Wilms tumour showing giant hyperchromatic nuclei (green arrows) and a giant multipolar cell division (red arrow). It is provided with courtesy by Dr. Kaname Uno.

### FIGURE 4. Blastemal Wilms tumour

The image shows blastema after preoperative chemotherapy with no chemotherapy-induced changes. It is provided with courtesy by Prof. Dr. Gordan Vujanic, chair of the pathology panel of SIOP-RTSG.

**FIGURE 5. Evolutionary trajectories in Wilms tumour**

**(A)** Hypothetical cancer cell phylogeny of Wilms tumour. Stem mutations, exemplified by loss of heterozygosity in chromosome arm 11p, initiate a first clonal expansion of a mother cell population (M) that evolves further through branching evolution into a set of daughter clones (D1-D3) with a set of private mutations, exemplified by 1q gain. Hemizygous mutations in *TP53* (p53+/-) in a daughter clone can initiate further evolution, often leading to parallel loss of the second allele (p53-/-) in distinct populations with anaplastic features (A1, A2). Loss of p53 is in turn coupled to the local emergence of complex chromosomal rearrangements (a type of saltatory evolution), giving rise to a broad repertoire of minor clones (A1a-d, A2a-d). **(B)** The process described in part A can manifest as a distinct set of patterns in the geographic distribution of clones across the anatomical regions of the primary tumour. Subclonal variation implies that different subclones are found in different regions, whereas in clonal coexistence the same daughter clone coexists with a mother or sister clone across different regions. Occasionally, daughter clones can completely overtake an anatomical compartment, manifesting as regional clonal sweeps. Anaplastic regions display a myriad of private clones, a phenomenon referred to as regional evolutionary explosions.

**Short summary**

We describe the history of the efforts to expand knowledge of Wilms tumour biology, genetics, embryonal origin and associated syndromic and familial conditions, and to clinically apply prognostic biomarkers and development of preclinical models.

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