Summary and General discussion
The general objectives of this thesis were to explore genetic and phenotypic aspects of cancer predisposition in children. In the current chapter, the main findings of the studies described in the six previous chapters will be summarized and discussed. The strengths and limitations of the studies will be discussed and general conclusions will be formulated. The general discussion concludes with recommendations for future studies and clinical practice within the field of tumor predisposition syndromes.

Case illustration tumor predisposition syndrome in childhood cancer

The spectrum of tumor predisposition syndromes in childhood cancer is too diverse to describe them all. To illustrate the clinical problem and potential difficulties in relating a certain tumor to a known tumor predisposition syndrome, Chapter 2 describes a patient with molecularly proven PTEN Hamartoma Tumor Syndrome and Gorham-Stout phenomenon. PTEN hamartoma tumor syndrome (PHTS) is a group of syndromes caused by mutations in PTEN.1-3 Gorham-Stout phenomenon (GSP) is a rare condition characterized by proliferation of vascular structures in bones, resulting in progressive osteolysis.4 Here we present a one-year-old boy with PHTS and GSP.

We describe the clinical and family history, molecular and pathology examination. Gorham-Stout phenomenon has not been reported before in a patient with a constitutional mutation in the PTEN gene. Also, our report is unique in being the first report of a proven germline mutation (i.e. PTEN, c.517 C>T (p. Arg173Cys)) in a patient with Gorham-Stout phenomenon (GSP). Up to this date, this mutation is the only genetic defect possibly involved in the etiology of Gorham-Stout phenomenon which is plausible given the known function of PTEN in angiogenic signalling. Analysis of the lymphatic malformation tissue revealed no loss of heterozygosity (LOH) nor a second, somatic PTEN mutation of the remaining wild type allele. The germline p.Arg173Cys mutation was also present in the mother and the propositus’ younger sister and brother, who have not shown GSP thus far. Further molecular work-up showed a heterozygous variant c.2180C>T (p.Ala727Val) FLT4 in the lymphatic malformation tissue, which was also present in the germline of mother and two sibs.

We hypothesize that the PTEN mutation was the first of two or more steps in the relentless growth of lymphatic malformation in the propositus. Additional (epi)genetic, either somatic or germline, or environmental factor(s) might have led to the severity of the phenotype in the propositus. Further studies of other genes, especially in the PI3K-Akt pathway and performed in affected tissue may explain this. Currently, involvement of a panel of genes, known to be concerned in lymphatic vessel formation is tested in DNA of the lymphatic malformation tissue.
Part I. Genetic aspects of tumor predisposition syndromes in childhood cancer

In Part I of this thesis we explored genetic aspects of tumor predisposition syndromes, more specific, we studied potential structural genomic variants (inversions, translocations, microdeletions and/or microduplications) in patients showing specific patterns of co-occurring morphological abnormalities, indicative of new tumor predisposition syndromes\(^5\), \(^6\). Therefore, in Chapter 3 our aim was as follows:

1. to identify the structural genomic variants that might underlie the four patterns of co-occurring morphological abnormalities

We identified an inherited inversion of chromosome 15, inv(15)(q25q26) in a proband from the asymmetric lower limbs (LLA)-pattern. Evaluation of the genes at the breakpoints made it unlikely that these explained the phenotype and tumor in this patient. We identified an inherited duplication involving BCL9 in a proband diagnosed with Burkitt lymphoma. An inherited duplication involving PCM1 was identified in a proband diagnosed with pre-B-ALL. Both probands showed the epicanthal folds (EF)-pattern of morphological abnormalities. An inherited deletion involving TRA@ was identified in two probands from the blepharophimosis (BP)-pattern diagnosed with rhabdomyosarcoma and pre-B-ALL respectively.

In all present cases, the specific copy number variant (CNV) event was inherited from one of the parents. Finding de novo CNV events would make the relation between the events and the phenotype including malignancy strong. However, finding inherited CNV events does not necessarily exclude them as being causative. Various publications have discussed mechanisms explaining this phenomenon\(^7\), \(^8\). Several syndromes have shown that not all carriers of the genetic defect exhibit the full phenotype ("incomplete penetrance"). Also, there’s the phenomenon of variable expressivity, the extent to which a genotype exhibits its phenotype expression. Both incomplete penetrance and variable expression can be explained by modifier genes, genes that alter the expression of other genes. These modifier genes can affect the threshold for trait expression, leading to a larger or smaller proportion of individuals affected by a certain event and thus affect penetrance. Also, modifier genes can affect the range of phenotypes associated with a certain event and thus lead to variable expressivity. Other explanations for incomplete phenotypes in parents carrying the same genomic variant could be allelic variation or complex environmental and genetic interaction\(^9\). Furthermore, sometimes a mutation is detectable only in other (tumor) tissues (mosaicism). Well-known examples are Proteus syndrome\(^10\) and Cornelia de Lange syndrome\(^11\) but it has also been described in segmental NF1\(^12\) and NF2\(^13\). Also, in our study we have not investigated epigenetic events such as methylation. Methylation differences between a proband and parent could explain for differences in phenotype like is the case in Beckwith-
Wiedemann syndrome\textsuperscript{14}. When studying methylation in the probands, it is important to realize that different methylation patterns can be present in various tissues. Translating this to oncogenesis, the CNV can be a susceptibility locus; having this CNV event makes that particular individual more susceptible for developing cancer, but still other factors are needed to actually develop a malignancy. We suggest the identified CNV events to be susceptibility loci.

A strength of this study was that these are patients from a unique, clinically well-defined cohort of patients. The phenotypes of these patients have been extensively described because all patients underwent a standardized extensive body surface examination focused on morphology and tumor predisposition syndromes in childhood cancer and their morphological examinations had been re-evaluated. Also, family histories of the patients were complete and updated. This makes it easier to relate possible genomic variants to the clinical phenotype.

However, a limitation in this study was that in the majority of cases, no tumor material was available to further elaborate the relation between the identified CNV events, the malignancy and morphological abnormalities in the proband. Also, our approach using SNP array to detect deletions and duplications and conventional cytogenetics to identify large inversions and translocations has limitations in detecting structural variations such as small insertions or inversions and point mutations. In part of the patients, there could be a polygenic cause of the childhood malignancy and morphological abnormalities. We think the additional value of Next-Generation Sequencing (NGS) rests in the identification of second genomic variants that could not be detected in the current approach.

Recently, a whole-exome study was started in a selection of probands showing one of the patterns of co-occurring morphological abnormalities, in whom we had not identified a structural genomic variant using our approach as described in Chapter 3. However, current results and validation of these analyses are pending so they could not be included as a separate chapter.

**Part II. Phenotypic aspects of tumor predisposition syndromes in childhood cancer**

In Part II of this thesis we explored phenotypic aspects of tumor predisposition syndromes. In Chapter 4 and 5 our aim was as follows:

2. to further delineate the morphological abnormalities in patients from the patterns of co-occurring morphological abnormalities suggestive for new tumor predisposition syndromes, using 3D photography and determine the added value of 3D photography in characterization of facial morphology
Originally, in our 3D analyses, a mixed UK-Dutch control group was used in our 3D analyses. In order to test whether the use of a mixed UK-Dutch control group was justified or not, in Chapter 4 we used 3D photography to assess differences in face shape between individuals from the UK and the Netherlands. We showed statistically significant face shape differences between these two European Caucasian populations of close phylogenetic and geographic proximity. We visualized the average face shape differences between the Dutch and UK cohorts in dynamic morphs and signature heat maps. Furthermore, their statistical significance was quantified using both conventional anthropometry and state of the art dense surface modelling techniques.

Our results demonstrated significant differences between Dutch and UK face shape. Firstly, Dutch women have significantly longer and broader faces compared to UK women. Also, their palpebral fissure length and nasal width are significantly greater, their nasal ridge length and upper face proportion are significantly reduced and their nares are significantly more anteverted. In particular, the nasal differences from UK women show that the nose in Dutch women is more likely to be shorter and more retroussé. Dutch men did not have significantly longer faces than UK men, despite their greater height. Their nasal ridge length relative to face length is significantly shorter. Also, relative to face length, they have longer palpebral fissures than UK men.

It is remarkable that Dutch and UK females show significant differences for nearly every measure, whereas for Dutch and UK males few measures show significant difference. This could be explained by sexual dimorphism, which would mean that overall, Dutch and UK females differ more from each other than males do. Facial and cranial sexual dimorphism has been observed in many human populations. One could argue that a limitation of the study is that the study group (consisting of both medical/scientific professionals and family members) has a biased composition and that this could have led to the differences in facial morphology we find between UK and Dutch populations. However, in both populations both medical/scientific professionals and family members covering a range of social backgrounds were recruited. Also, the proportion of professionals to family members and the age ranges in both ethnic groups were comparable. Furthermore, we considered normalized mean difference of professionals from family members within ethnic groups. We also normalized the mean of the UK family members against UK medical/scientific professionals and detected no significant difference. We normalized Dutch family members against Dutch medical/scientific professionals producing minimal difference around the lips (especially the lower lips) and zygoma region. Because neither of these comparisons shows any nasal bias, this reconfirms the differences we find between our Dutch and UK subgroups as both realistic and generalizable.

A strength of the study is that we use an objective measure for visualization and quantification of differences in facial morphology. The two study populations were comparable in composition and age and as we discussed earlier, it was shown that the
differences between the populations were not influenced by biased composition of the study group.

Face shape differences are an important determinant of phenotype variation in humans. Craniofacial development is a complex process determined by genetic regulation and genetic variants influence facial morphology in the general population\textsuperscript{17, 18}. Thus, face shape difference between populations reflects underlying genetic differences. Other studies have shown that genetic variants influence normal facial variation. This should be taken into account in genotype-phenotype studies and we recommend that in those studies reference groups be established in the same population as the individuals who form the subject of the study.

In Chapter 5, the added value of 3D shape analysis to characterize facial morphology of childhood cancer patients from the four distinct patterns of morphological abnormalities was investigated. Primarily, we showed in an objective and quantitative manner that the overall facial dysmorphism of the individuals who had cancer as a child was significantly greater than that of the controls. We also demonstrated a similar disparity for a more localized malar region, confirming that the malar region is more dysmorphic in patients compared to controls. In addition, we confirmed that the patient group had significantly greater facial asymmetry than the controls. Each of these results made use of surface shape differences not detectable by simple linear or angular characteristics as might be used in analyses based on measures captured manually or derived from landmarks annotating 2D photographic images.

Therefore, we conclude that 3D morphometric analysis of a relatively small heterogeneous patient group can further delineate face shape differences from typically developing individuals that are too subtle or geometrically complex to identify or quantify objectively with conventional clinical and anthropometric approaches.

Common facial differences between patients and controls could not be identified and a high dispersal rate, reflecting lack of similarity clustering of the patient group, provided supporting evidence. Similarly, when considering patients on their own, no intra-pattern similarities or inter-pattern differences were evident in the signatures or the signature graphs. We demonstrated significantly greater dysmorphism of the malar region in the patients compared to controls using shape signature graphs. In terms of intra-pattern analysis of anterior-posterior malar shape difference, the LLA pattern subgroup produced a lower dispersion rate suggesting some commonality of form for malar flattening or prominence.

Finally, we identified increased facial asymmetry in patients compared to controls. Patients who had undergone radiotherapy or surgery in the facial region were excluded from these analyses as this might have influenced potential asymmetry. Therefore, the increased facial asymmetry in patients is more likely to reflect ‘intrinsic asymmetry’ rather than asymmetry caused by external effects. Asymmetry occurs frequently in genetic conditions associated with a malignancy. Asymmetry is a well-known feature in several genetic conditions.
associated with (malignant) tumors such as Bannayan-Riley-Ruvalcaba syndrome (BRRS, OMIM: 153480) \(^{19}\) and Beckwith-Wiedemann Syndrome (BWS, OMIM: 13060) \(^{20}\). It should be noted that in our current patient population no established syndromes such as BRRS or BWS were diagnosed. Some potential for inter- and intra-pattern analysis of facial asymmetry was identified for particular parts of the face, e.g. supra-orbit and mandible, and may assist in future determination of face shape differences and even alternative patterns of dysmorphism.

The four patterns of morphological abnormalities consist of abnormalities located both in the face and in other body parts. As 3D scanning only captures the face, it is impossible to evaluate the complete patterns this way. 3D face images were only available for patients showing one of the four new patterns of morphological abnormalities suggestive of new tumor predisposition syndromes. Therefore, it was impossible to repeat the grouping of the patients using signature graphs for the complete cohort (1,073 childhood cancer patients) described by Merks et al. \(^{5}\), blinded from the statistical patterns of co-occurring morphological abnormalities. The relatively small number of individuals of the various patterns hampers statistical analyses and prevents subgroup analyses. Another overall limitation of our analysis could be that, in computing face signatures, the age- and sex-matched controls selected are according to a matching running mean age, which could be biased by lack of coverage of certain ages.

Despite these limitations, the 3D face image capture and subsequent analysis has shown significant added value. We conclude that dense surface modelling techniques expand the possibilities for physicians (clinical geneticists, plastic surgeons etc.) to describe and characterize the phenotype of an individual or group of individuals and allow objective comparisons to age- and sex-matched controls.

Chapter 6 reviews the clinical and molecular aspects of syndromes associated with brain tumors in children, thereby facilitating recognition of syndromes in children with a brain tumor and early diagnostics of brain tumors in children with syndromes. (Brain) tumors usually occur in aged individuals. If a brain tumor occurs in a child there is a possible genetic susceptibility for this. Such genetic susceptibilities often show other signs and symptoms. We describe all major brain tumor types occurring in children and the various syndromes that have been reported to be associated with these tumors. Also, we summarized the most frequent tumor predisposition syndromes with a high chance to develop a brain tumor. We provided short descriptions restricted to major presentations and co-occurring brain tumor of each of the entities. Also we discussed pitfalls in clinical and molecular diagnosis, and the consequences of diagnosing a hereditary disorder for family members.
Part III. Screening for tumor predisposition syndromes in childhood cancer

In part III (Chapter 7) of this thesis, our aim was

3. to develop a screening instrument for known tumor predisposition syndromes in childhood cancer patients

Identification of tumor predisposition syndromes in patients who have cancer in childhood is paramount for optimal care. This screening instrument aims to identify childhood cancer patients with tumor predisposition syndromes, thereby facilitating physicians caring for children with cancer. The complete screening instrument consists of a standardized series of pictures and a screening form for manifestations not visible in the pictures. We describe the development of such a screening form based on an international two-stage Delphi process. Through the Delphi process, 49 manifestations were found to contribute to the diagnosis of a tumor predisposition syndrome and were included in the screening form. The pilot validation study showed that patients suspect of having a tumor predisposition syndrome were recognized. Excellent correlation for indications of patient’s referral between the screening instrument and the reference standard (personal evaluation by an available evidence on prevalence and experienced clinical geneticist) was found.

Because available evidence on prevalence and recognition of tumor predisposition patients with childhood cancer is scarce\(^5,21\), evidence-based guidelines or international consensus do not exist. The value of the expert opinions elicited in the Delphi process, reflects and values the clinical consensus of these international opinion leaders in their field. One of the strengths of the Delphi method and therefore this study, is that it offers the opportunity to obtain expert opinions without potential influence of group dynamics on individual opinions. Because we chose to give feedback on the scores anonymously, experts could see their own opinion in relation to that of other experts, making it a safe, non-judgmental environment for sharing one’s opinion. Because the time frame of response was defined and standardized, this assessment gave a good reflection of the current opinion in the field of expertise at a given time point, making it more reliable.

All expert opinions were weighed equally, based on the general assumption that experts participating in a Delphi process are equal in experience and knowledge. However, this may not have been the case, especially if their fields of expertise were very specialized, as in our study. For our expert panel, we chose international clinical geneticists who functioned as opinion leaders in both dysmorphology and clinical cancer genetics, or are leaders in one of the two with good experience in the other field. The number of qualified experts was relatively small, which restrained the number of experts in our panel. The small size of the expert panel could be considered as a potential limitation in the current study. However we feel that, considering the small field of expertise, the expert panel optimally represented
international expert opinions. Also, other Delphi studies with similar sized expert panels have shown to yield reliable results.\textsuperscript{22, 23} One might feel that the fact that we have not added criteria for referral indications for each tumor type and family history on our screening form is a potential limitation of current study. However, to establish reliable algorithms for the large range of tumor predisposition syndromes and the accompanying clinical spectrum with respect to tumor type and family history separate, extensive studies are needed and this was outside the scope of our current study. We have decided to leave this consideration to the expertise of the senior clinical geneticists evaluating the results of the instrument.

We performed a pilot validation study in an independent series of ten pediatric cancer patients and compared the clinical judgment and rationale for referral by two independent clinical geneticists using the instrument, to those of a third clinical geneticist who evaluated the patients in routine consultations. In the pilot validation study, the clinical judgements of the two clinical geneticists using the screening instrument were very similar despite their different specialisation (dysmorphology vs oncogenetics). A limitation of this pilot validation was that it was not a randomly selected patient cohort; the cohort in this clinical validation study consisted of both patients suspect as well as patients unsuspected for having a tumor predisposition syndrome which could give rise to selection bias. In our pilot validation study, a genetic counsellor (genetic nurse) filled in the screening form; we do not expect a significant difference if the form were filled in by a pediatric oncologist or other physician involved in the care of childhood cancer patients, although this was not explicitly tested.

A limitation of the pilot validation was the small size of the validation cohort; we recognize that the validation cohort was too small to support clinical implementation of the instrument. The optimal validation of the instrument would be a comparative prospective study in a cohort of newly diagnosed pediatric oncology patients: referrals and reasons for referral using the screening instrument could then be compared to the routine consultation done by an experienced clinical geneticist (‘reference standard’). However, the low incidence of tumor predisposition syndromes requires the recruitment of large numbers of patients for a considerable period of time to perform a study that potentially will yield useful results. This approach was not suitable and feasible for an initial, short-term validation. Such a prospective study, in which the feasibility and validity of the final screening instrument will be assessed, is needed to determine the diagnostic value of the instrument and is planned at present (see ‘Recommendations for further studies’).

However, we showed that geneticists using the screening instrument recognized those childhood cancer patients that might have a tumor predisposition syndrome when compared to the reference standard. Therefore, we conclude that the Delphi process performed by international specialists with a function as opinion leaders in their field of expertise, has led to a screening instrument for childhood cancer patients. Our initial validation showed that
this could be a promising instrument, however, a prospective validation study is needed before it can be implemented in clinical practice.

**Recommendations for further studies**

Several findings in this thesis lead to new research questions and thus have implications for future studies. Further studies on genetic aspects, phenotypic aspects and screening for tumor predisposition syndromes will be discussed separately.

**Further studies on genetic aspects of tumor predisposition syndromes**

In this thesis, we described the structural genome variants identified in patients showing one of the four patterns of morphological abnormalities. Our current approach was confined to using SNP array to detect (micro)deletions and (micro)duplications and conventional cytogenetics to identify large inversions and translocations. That approach has limitations in detecting structural variations such as small insertions or inversions and point mutations. These changes at the level of individual nucleotides can be detected by DNA sequence analysis. Direct sequence techniques, in which single genes or gene panels are studied, are labour-intensive. Over the last five years, genome-wide sequencing using next-generation sequencing techniques has proven to be an efficient and effective alternative for discovering new genes involved in diseases.

In part of the patients, there could be a polygenic cause of the childhood malignancy and morphological abnormalities. We think the additional value of Next-Generation Sequencing (NGS) rests in the identification of second genomic variants that could not be detected in the current approach. However, more detail comes with a price: namely the presence of individual variants (both inherited or de novo) which may not be related to the syndrome of the proband. NGS techniques such as exome sequencing might also identify variations in the probands who were found negative for variations in the current approach. Therefore recently, a whole-exome study was started in a selection of probands showing one of the patterns of co-occurring morphological abnormalities. In our exome study, we selected 6 patients from the (LLA, EF and BP) patterns of morphological abnormalities in whom we had not identified a structural genomic variant using our approach as described in Chapter 3. These patients either had a strong phenotype or showed phenotypic overlap with another patient within the same pattern of morphological abnormalities. For 4/6 patients also germline DNA of parents was available so that we can perform a trio-analysis in these 4 patients. Because the majority of tumor predisposition syndromes show autosomal dominant inheritance, this scenario will be tested to begin with. Firstly, we will focus on de novo variants, i.e. rare variants that are confined to the proband since the family histories of these probands are not highly suspicious for an inherited tumor predisposition syndrome. However, as we discussed in Chapter 3, finding inherited variants does not necessarily
exclude them as being causative due to incomplete penetrance, variable expressivity and/or involvement of modifier genes. As for the CNV events, a variant could also act as a risk factor, increasing susceptibility for developing a malignancy.

Secondly, we will look at variants in genes related to the phenotype of the patient (in terms of tumor type and/or morphological abnormalities). Lastly, we will investigate shared variants between probands from the same pattern of morphological abnormalities. However, taking into account the small number of patients per morphological pattern, will make it unlikely to identify a shared variant that will explain the phenotype in probands from the same morphological pattern. When finding a variant in a candidate gene that could explain for the morphological abnormalities and/or the development of a malignancy, one would want to confirm the involvement of the gene and/or variant in an independent series of patients with the same tumor and/or same pattern of morphological abnormalities. Particularly finding patients that meet the criteria for the patterns of morphological abnormalities would take considerable time, because the current cohort was a rather small and specific selection of patients (n= 49) from a large cohort of childhood cancer patients (n= 1073).

A limitation in our current approaches is that we have do not have possession of tumor material for most probands. Ideally, exome sequencing of the DNA from both the proband, parents and the tumor would have to be performed. This way involvement of the variant in the tumor and germline of the proband can be studied. In tumor predisposition syndromes, a variant will be present in both the germline and tumor of the proband. Comparing the tumor genome with the germline genome of the proband, also creates the opportunity to identify somatic alterations in the tumor. Currently, in the St. Jude Children’s Research Hospital- Washington University ‘Pediatric Cancer Genome Project’ (PCGP) such an approach is chosen. In the PCGP, the genomes of 600 pediatric tumors and their matched germline samples of the probands (thus 1200 total genomes) are studied by whole-genome sequencing. The PCGP aims to identify somatic mutations that underlie the major subtypes of pediatric cancer. With the identification of driving mutations in pediatric cancer, they hope to catalyze research in pediatric malignancies, improving diagnosis, monitoring and targeted therapy. Although the project is still in process, several important findings have emerged from initial studies. So far, the PCGP has facilitated significant insights in the genetics of T-cell precursor acute lymphoblastic leukemia, diffuse intrinsic pontine gliomas, non-brainstem glioblastomas, neuroblastoma and retinoblastoma. In the course of time, the PCGP will also make all data publicly available which will be a wonderful source for many research groups working on childhood cancer, tumor predisposition syndromes or cancer in general.

There is one important limitation in the approach of the PCGP and that is, that as far as we know, unfortunately no morphological data of the probands is collected in a structured and uniform way. This is a missed opportunity, because this would have been an ideal situation to relate genotype-phenotype relations in childhood cancer. With the increasing amount of tumors being sequenced leading to increased insight into tumor genetics, it is conceivable
that personalized therapy will become possible for tumor predisposition syndromes, also in children. However, gathering morphological information in a standardized, structural manner in parallel to gathering genetic information of the proband and tumor should not be neglected; the morphology of a proband can be directive in interpreting genetic results as phenotype is more than tumor type alone.

Further studies on phenotypic aspects of tumor predisposition syndromes

As we discussed in Chapter 5, because only a 3D picture of the face was captured, it was impossible to evaluate the complete patterns of morphological abnormalities. Also, these 3D face images were only available for patients showing one of the four patterns of morphological abnormalities. Therefore, it was impossible to repeat the grouping of the patients using signature graphs for the complete cohort (1,073 childhood cancer patients) described by Merks et al. 5, blinded from the statistical patterns of co-occurring morphological abnormalities. Currently, we are planning a study in which 3D scanning will be applied in a large, prospective, nation-wide study of newly diagnosed childhood cancer patients. Together with our recently developed tool to recognize individuals with a high chance to have a tumor predisposition syndrome 35, we foresee to recognize larger subgroups of similar morphological abnormalities within pediatric cancer patients. This will allow further characterization of the faces and facilitate subsequent molecular analyses. Also, such an approach could expand our knowledge on the spectrum of morphological abnormalities in childhood cancer patients.

In tumor predisposition syndromes, the genetic defect underlying the clinical syndrome also predisposes the patient to develop cancer. To study the genetic landscape of tumor predisposition syndromes, understanding the complexity of genotype-phenotype relations is vital. Several groups have shown that phenotypic overlap implies genetic overlap 36-40. In this view, genetic diseases are considered as being ‘modular’, i.e. in genetically heterogeneous diseases such as Fanconi-anemia (OMIM: 603467, 614083, 227646, 614082, 609053, 614087, 600901, 609054, 613390, 615272, 227645, 613951) or Bardet-Biedl syndrome (OMIM: 209900), the various genes involved work in a single functional or biological module 41. One gene can act in various pathways; genes acting in the same pathway can result in related phenotypes and disorders sharing manifestations in phenotype often also share their tumor type as well. This concept is illustrated in Figure 1, with Wilms tumor used as an example.

Over the past few years, progress has been made in understanding the ‘human phenome’. The human phenome is defined as the set of phenotypes that result from sequence variation in the human genome 37. The fact that genetic diseases can be clustered in functional or biological modules based on their phenotypic similarities which indeed reflect biological relationships of the genes involved, also creates the possibilities to use phenotypic similarity to predict the involvement of apparently unrelated genes. Moreover, the same
bioinformatics analyses can be used to make predictions about new genes that share functional or biological properties \(^{36, 41}\).

In parallel to the bioinformatics tools that exist to cluster on pheno- and/or genotype, efforts have been made to come to a uniform annotation for the human phenotype and human hereditary diseases. One important initiative is the Human Phenotype Ontology (HPO), that aimed to cover all phenotypic abnormalities that are commonly encountered in human monogenic diseases, as well as annotation of clinical entries in the Online Mendelian Inheritance in Man (OMIM) database\(^{42}\). Currently, the HPO contains 10088 classes (terms) with 13326 subclass relationships between those classes. The HPO contains three different, independent subontologies that cover the mode of inheritance, onset and clinical course.

Figure 1: The relation between tumor type, gene, pathway and manifestation illustrated. This figure shows how alterations in different genes can cause one tumor type (Wilms tumor), although alterations in one gene can cause various tumor types; here TP53 was used as an example. Alterations in TP53 can lead to the formation of various tumor types such as adrenocortical carcinoma, choroid plexus carcinoma, colon carcinoma, breast carcinoma and bone or soft tissue sarcoma amongst others. The genes known to be involved in the formation of Wilms tumor act in different pathways; here WT1 was used as an example. WT1 acts in different pathways such as camera-type eye development, meta- and mesonephros development, sex determination and male gonad development amongst others. Furthermore, various manifestations (such as anterior chamber abnormalities, genital or renal malformations and renal dysfunction amongst others) can result from (combinations of) different disturbed pathways. Lastly, different disorders sharing manifestations, such as Denys-Drash syndrome (OMIM 194080), Frasier syndrome (OMIM 136680) and Meacham syndrome (OMIM 608978) sharing renal malformations. Please note that this diagram does not aim to be complete in all existing relationships, but rather illustrates various relationships by specifying one example.
and phenotypic abnormalities of human (genetic) diseases. Thereby the HPO aims to act as a central resource by connecting genomics, phenomics and diseasomics.\textsuperscript{43}

As described in \textbf{Chapter 7}, we identified tumor predisposition syndromes and their manifestations through the Winter-Baraitser Dysmorphology Database (WBDD)\textsuperscript{44} and the textbook “Gorlin’s Syndromes of the Head and Neck”\textsuperscript{45}. Furthermore, we classified the tumors reported in these tumor predisposition syndromes according to the third edition of the International Classification of Childhood Cancer\textsuperscript{46} and included these in a database with the tumor predisposition syndromes, manifestations and genes from the WBDD/Gorlin’s textbook. With this information we tried to answer the following research questions:

Do entities that cluster based on the genes and/or pathways show similar tumor types and manifestations? In other words: is there an overrepresentation of specific tumor types and/or manifestations when clustering based on the genes and/or pathways?

Do entities that cluster based on tumor types show involvement of similar genes and/or pathways and manifestations? In other words: is there an overrepresentation of specific genes and/or pathways and manifestations when clustering on tumor types? In many pathways there are genes that have not been related to clinical recognizable syndromes yet. In addition, can we predict the manifestations of entities without well-defined phenotype when they overlap in tumor type and genes and/or pathways with entities with well-defined phenotypes?

Do entities that cluster based on manifestations show involvement of similar genes and/or pathways and tumor types? In other words: is there an overrepresentation of specific genes and/or pathways when clustering on manifestations? In addition, can we predict tumor types in entities without a known tumor predisposition when they overlap in manifestations and genes and/or pathways of known tumor predisposition syndromes?

We used the HPO equivalence mapping to map the manifestations from the WBDD to the HPO. We used the OMIM ID of the selected tumor predisposition syndromes from the WBDD to link the HPO and to the pathways database ‘the Kyoto Encyclopedia of Genes and Genomes’ (KEGG) http://www.genome.jp/kegg/. By linking the WBDD (with its indexed entities, manifestations and tumors) we could compare these to the manifestations and tumors reported in HPO as well as the diseases and pathways reported in KEGG, planning to study overrepresentation of genes and/or pathways, tumor types and manifestations as described above in the research questions.

However, this approach was hampered by important difficulties. First of all, in the equivalence mapping of the WBDD to HPO, there are sometimes more HPO terms describing one WBDD manifestation. Also, not for every WBDD term there is an equivalent HPO term. This was even more complicated by the fact that the version of the WBDD that HPO used for the equivalence mapping from WBDD to HPO was a later version than the one we used in our database, leading to differences. This made linking both databases more difficult than foreseen.
Secondly, there is considerable overlap in pathways, i.e. many genes are involved in multiple pathways, which makes it difficult to separate pathways from one another. For example, in the KEGG, PTEN hamartoma tumor syndrome (PTEN gene) is not only involved in the mTOR pathway but also in seven other pathways such as MAPK, ErbB and VEGF signalling pathways. This complicates the clustering because there is so much data to cluster on. We tried to circumvent this to select a few well-known pathways involved in development and/or cancer: the mTOR, MAPK, hedgehog, TGFβ, VEGF pathway. However, this of course constrained the data to cluster on but also made it more difficult to identify significant overrepresentations.

Furthermore, like we found discrepancy between the manifestations from the WBDD and the HPO, we also found some discrepancy between the tumors and manifestations from the HPO and the tumor and manifestations that we identified using the WBDD based on the same OMIM ID; this influences the trustworthy of the clustering.

In addition, KEGG reports more entities within one pathway, many of these have several OMIM ID’s and only a minority of these entities are predisposing for tumor development. For example, for the mTOR signaling pathway KEGG reports ‘PTEN hamartoma tumor syndrome (PHTS)’, ‘Peutz-Jeghers syndrome’ and ‘tuberous sclerosis complex (TSC)’, all tumor predisposing conditions as well as ‘polyhydramnios, megalencephaly, and symptomatic epilepsy’ and ‘focal cortical dysplasia of Taylor’, for which tumor predisposition is more difficult to define. One would have to actively select the tumor predisposing entities from the non-tumor predisposing entities, which would mean a labour-intensive and manual (thus easily subject to flaws) work requiring an extensive review of literature for each of the conditions.

Therefore, because of discrepancies between different databases and non-uniform classifications, we have not succeeded in answering the research questions in our current approach. As the HPO constantly tries to improve their linking to molecular biology and disease databases and internationally efforts are being done to define uniform nomenclature for human morphology, we foresee that a comparable effort will be possible in the (near) future.

Further studies on screening for tumor predisposition syndromes

To our knowledge, only one other effort have been done to develop such a screening instrument. Jongmans et al. developed a referral test, to be completed by the pediatrician involved. Based on five criteria, the test determines whether referral for further clinical genetic evaluation is indicated. Currently, the methodology, in- and exclusion criteria which formed the base for the application are not publicly available so that it is difficult to value the application for its quality. In order to make sure that no referrals for possible tumor predisposition syndromes with low incidences are missed, in- and exclusion criteria
should be carefully appreciated. In addition to that, prior to introducing a clinical screening tool into the clinics, validation is essential to ensure no patients are missed for referral.

**In Chapter 7** we describe the development and pilot validation of our screening instrument. We show an excellent correlation for indication for referral of a patient between the screening instrument and the reference standard (personal evaluation by an experienced clinical geneticist). Therefore we conclude that our screening instrument identifies patients who may have a tumor predisposition syndrome and thus have an indication to be referred for further genetic analysis. However, we also recognize that a prospective study, in which the feasibility and validity of the screening instrument will be assessed, is needed to determine the diagnostic value of the instrument. The optimal validation of the instrument would be a comparative prospective study in a cohort of newly diagnosed pediatric oncology patients: referrals and reasons for referral using the screening instrument could then be compared to the routine consultation done by an experienced clinical geneticist (“reference standard”). Because of the low incidence of tumor predisposition syndromes, large numbers of patients would have to be recruited for a considerable period of time. Currently we designed such a study for which its methodology is shown in *Figure 2*.

In the validation study, we aim to assess the clinical validity of the screening instrument in identifying childhood cancer patients with a tumor predisposition syndrome from a

![Figure 2: Graphical representation of methodology in the validation study](image)
non-selected prospective cohort of childhood cancer patients. We hypothesize that the screening instrument will be equivalent/at least as good as current practice in recognizing patients with a tumor predisposition syndrome (non-inferiority) and assume high sensitivity (94%). Validity of the instrument will be expressed as sensitivity and specificity of the instrument.

The primary outcome measurement will be sensitivity (number of true positive patients divided by the sum of the number of true positives patients and false negative patients). True positive patients are defined as patients with a positive screening result in whom presence of a tumor predisposition syndrome was confirmed in routine clinical genetic consultation. False negative patients are defined as patients with a negative result by the screening instrument and in whom a tumor predisposition syndrome was diagnosed. To determine this we will perform a full clinical genetic consultation in a random sample of 20% from the series of patients with a negative screening result.

Secondary outcome measurement will be specificity (number of true negative patients divided by the sum of the number of true negative patients and false positive patients). True negative patients are defined as patients with a negative result by the screening instrument and in whom no tumor predisposition syndrome was diagnosed. To determine this, we will assign a sample of the series of patients with a negative screening result to clinical genetic consultation. False positive patients are defined as patients with a positive screening result in whom no tumor predisposition syndrome was found by the routine clinical genetic consultation. Another secondary outcome measurement includes inter-observer variability of the three clinical geneticists evaluating one patient using the screening instrument, expressed as a Fleiss’ kappa score. Also, individual attribution of the scoring form and (2D and 3D) picture series to the conclusion of a clinical geneticist evaluating a patient using the screening instrument will be a secondary outcome measurement. A last secondary outcome measurement is the difference in costs of the diagnostic process using the instrument compared to current standard clinical genetic care.

We hypothesize that this screening instrument can efficiently select patients who possibly have a tumor predisposition syndrome from a prospective cohort of pediatric cancer patients. We expect a high number of true positive patients and a low number of false negative patients, resulting in a high sensitivity of the screening instrument. In order for a clinical screening instrument to work, a high sensitivity is necessary since a poor sensitivity would mean that patients with a tumor predisposition syndrome are missed. In our sample size calculations we have used a minimum sensitivity of 94%. We expect that the number of false positive patients will be higher, resulting in a lower specificity. This means that potentially more patients will be referred for further evaluation by a clinical geneticist than eventually turn out to have a tumor predisposition syndrome. In a clinical setting it is important that the specificity is not too low which would mean that the screening instrument would not help in selecting patients. In our sample size calculation we tolerated a minimum specificity of 50%.
Based on our previous results, we expect that the opinions of the three clinical geneticists evaluating a single patient based on the screening instrument will be in agreement. Better agreement will result in a higher Fleiss’ kappa score. A Fleiss’ kappa score above 0.61 reflects substantial agreement so we expect Fleiss’ kappa scores of 0.61 or above. In our analyses on the individual contribution of the different parts of the screening instrument (screening form, 2D and/or 3D picture series) to the conclusion of a clinical geneticist most, we expect that the picture series will attribute most to the decision of a clinical geneticist to refer. In our initial validation of the screening instrument we found that “findings in morphological examination” was most often used as reason for referral. This could still be based on the screening form and/or the picture series, because we did not specifically ask for screening form versus picture series’ contribution. We expect that the screening form helps in directing the clinical geneticist’s attention to key giving facts from the family history and manifestations that can be missed on picture series. This will increase recognition of tumor predisposition syndromes, leading to optimal care for childhood cancer patients. We also expect that the cost of the diagnostic process using the screening instrument will be lower than costs in current care, because of efficient use of personnel and more targeted additional genetic testing.

**Recommendations for clinical practice**

The diagnosis of a specific tumor predisposition syndrome in patients with childhood cancer is important and clinically relevant because it affects management. Some syndrome-associated malignancies require specific treatment strategies and some require screening for subsequent malignancies. Also, it may guide care for non-malignancy manifestations; it facilitates recurrence risk assessments and can facilitate pre-symptomatic identification of other relatives at risk for malignancies.

In previous studies, we found a substantial incidence of morphological abnormalities and recognizable clinical genetic syndromes in patients with childhood cancer. Half of these syndromes had not been recognized by the routine caregivers involved, despite expert pediatric care. We and others recommended that all children diagnosed with a malignancy should be assessed by a clinical geneticist or a pediatrician skilled in clinical morphology. A screening instrument such as we developed guarantees that all children with cancer are evaluated for the presence of a tumor predisposition syndrome, where this is not standard practice in the vast majority of pediatric oncology centers in developed countries/Western world.

Moreover, the use of a clinical valid screening instrument could make the evaluation for tumor predisposition syndromes more efficient. In the coming years, the ‘Prinses Máxima Centrum voor Kinderoncologie’, will be realized. In this national hospital for childhood
cancer patients, care and research will be concentrated. The ‘Prinses Máxima Centrum voor Kinderoncologie’ aims to facilitate care and research of supreme quality, thereby improving care and cure for childhood cancer patients. In the care for childhood cancer patients, the evaluation for the presence of tumor predisposition syndromes should be part of regular care. Therefore ‘Prinses Máxima Centrum voor Kinderoncologie’ would be a logical opportunity and setting to implement such a screening instrument.

Our ameliorated knowledge of the etiology of cancer is increasingly translated into management strategies in tumor predisposition syndromes. Textbooks (such as ‘Management of Genetic Syndromes’ by Cassidy and Allanson) and websites such as Orphanet (http://www.orpha.net/consor/cgi-bin/home.php), the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp) or the guidelines from the Dutch foundation for the detection of hereditary tumors ‘Stichting Opsporing Erfelijke Tumoren’ (STOET) (http://www.stoet.nl/uploads/richtlijnenboekje.pdf) are available that describe the general care for individuals with one of the various syndromes described above. However, a lot of these guidelines focus on management strategies for tumor predisposition syndromes in adults. Currently, in the Netherlands, the Dutch Childhood Oncology Group (DCOG) also known as Dutch foundation for pediatric oncology Stichting Kinderoncologie Nederland (SKION, http://www.skion.nl) has a Task Group pediatric oncogenetics (‘Taakgroep Kinderoncogenetica) in which both clinical geneticists and pediatric oncologists are represented. Their terms of reference include the responsibility to contribute to

1. Clinical genetic care: this comprises the recognition of tumor predisposition syndromes and hereditary cancer in children as well as providing care in counselling, prevention and recommendation for therapy; therefore all childhood cancer patients need to be evaluated for the presence of a tumor predisposition syndrome or hereditary cancer, applying the most recent techniques. This also includes the screening for other, with a syndrome associated abnormalities. The care needs to comprise a good transition of pediatric to adult care. Also the care for family members (possibly) at risk should be safeguarded.

2. Research: the aim is to improve the care for childhood cancer patients and their family members by means of translational and applied research and through fundamental research that contributes to understanding of the pathophysiology and underlying genetic predisposition of pediatric tumors and associated syndromes. Optimal research warrants that from all children both germ-line and tumor DNA is available for research by means of a central biobank.

The recommendations for further research and clinical care hopefully contribute to better recognition and follow-up for childhood cancer patients with tumor predisposition syndromes and their relatives.
Conclusions

Within the broad theme of tumor predisposition in childhood cancer this thesis includes some important findings. There are a few we would like to highlight.

General conclusions

1. The copy number variations detected in patients from the four patterns of morphological abnormalities are likely to be susceptibility loci.
2. In genotype-phenotype correlation studies, reference groups should be established in the same population as the individuals who form the subject of the study.
3. The childhood cancer patients from the four patterns of morphological abnormalities suggesting new tumor predisposition syndromes show more overall facial dysmorphism, more dysmorphism in the malar region and increased facial asymmetry compared to controls.
4. 3D morphometric analysis of a relatively small heterogeneous patient group can further delineate face shape differences that are too subtle or geometrically complex to identify or quantify objectively with conventional clinical and anthropometric approaches.
5. Childhood cancer patients who might have a tumor predisposition syndrome and thus have an indication to be referred for further genetic analysis, can be identified using our screening instrument consisting of a screening form and 2D/3D picture series.

Implications for further studies

1. To detect second genomic events and/or structural variations such as small insertions, inversions and point mutations, next generation sequencing should be performed in the patients from the four patterns of co-occurring morphological abnormalities.
2. When performing exome sequencing in childhood cancer patients, DNA from both the proband, parents and the tumor as well as a standardized morphological examination should be performed.
3. The relationship between between tumor types, genes, pathways and manifestations in tumor predisposition syndromes, should be used to investigate if: a: entities that cluster based on the same genes and/or pathways show similar tumor types and manifestations, b: manifestations in entities without well-defined phenotype can be predicted, based on overlap in tumor types, genes and/or pathways of entities with well-defined phenotypes c: tumor types in entities without a known tumor predisposition can be predicted, based on the overlap in manifestations, genes and/or pathways of known tumor predisposition syndromes.
4. A prospective validation study investigating the feasibility and validity of our screening instrument should be performed before implementation in clinical practice.
Implications for clinical practice

1. All children diagnosed with a malignancy should be assessed by a clinical geneticist or a pediatrician skilled in clinical morphology; a screening instrument could facilitate this in clinical practice, as well make the evaluation for tumor predisposition syndromes more efficient.

2. Next to a routinely and standardized evaluation for the presence of tumor predisposition syndromes, biobanking of germline DNA of the proband and parents as well as tumor DNA, is essential to guarantee advances in research and with that, optimization of care for childhood cancer patients.

3. Although our increased knowledge of the etiology of cancer is increasingly translated into management strategies in tumor predisposition syndromes, most guidelines focus on management strategies for tumor predisposition syndromes in adults. Guidelines for management of tumor predisposition syndromes in children should be defined by a Task Group pediatric oncogenetics (‘Taakgroep Kinderoncogenetica) in which both clinical geneticists and pediatric oncologists are represented.
References


