nature portfolio

Peer Review File

Population-based genomic detection of childhood cancer predisposition using newborn dried blood spots

Corresponding Author: Professor Lisa Diller

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is an awesome paper – many congratulations. It reports important work, it is of high quality, and should be published. I have two minor / pedantic points to raise:

1) It would be useful to have a table that lists for each gene what the frequency of pathogenic mutations is in the comparison cohort (including the internal control).

2) May I suggest to changes to the writing:

Sentence: “Cancer predisposition syndromes, characterized by deleterious germline variants in cancer-associated genes, are present in at least 10% of children with malignancy.”
CHANGE TO >>> “Cancer predisposition syndromes, caused by pathogenic germline (or rarely post-zygotic) mutations in certain cancer-associated genes, are present in at least 10% of children with malignancy.”

Sentence: “Genomic newborn screening could identify these at-risk children, who could be subsequently monitored using syndrome-specific cancer surveillance protocols.”
CHANGE TO >>> “Genomic newborn screening could identify these at-risk children, which may potentially translate into patient benefits through syndrome-specific cancer surveillance protocols.”

Reviewer #2

(Remarks to the Author)
Thank you very much for the opportunity to review this very well written manuscript.

SUMMARY
This manuscript describes a population-based study in Michigan using dried blood spots from neonates to identify children with cancer predisposition syndromes (CPS). The study included 1948 children with a solid malignancy by age 8 years. Of those, 132 carried a pathogenic or likely pathogenic (P/LP) variant in one of 11 genes associated with childhood-onset malignancies. The study calculated that 1 in 27’000 newborn children harbor a P/ LP CPS variant and a malignancy before age 8 years. There was a strong gene-tumor specificity. Two patients had malignancies not expected considering the described tumor spectrum.

As comparison group, 38’448 infants from four published population-based newborn screening studies were used. Two children were identified with P/ LP variants. Both patients were found to harbor lesions compatible with the tumor-spectrum associated with the identified CPS gene variants. Compared with published variant prevalence data, P/ LP variants in the selected 11 CPS were very rare or absent in the general population. The authors conclude that newborn genomic CPS screening might be beneficial for selected CPS genes.

I congratulate the authors for the nicely drafted manuscript. The study aggregated a large dataset and showed various important findings. Dried blood spots were a good source of DNA for targeted gene sequencing in >99% of children in a systematic collection of archived samples. Use of dried blood spots in CPS identification has been used before but not on

such a large number of patients. The number of identified children with a CPS matched what can be expected from the literature and the tumor spectrum was according to the published associations in most cases.

General Comments

I have general major comments:
1. The comparison of the findings in children with malignancies versus a large control population is important and does not show up in the abstract. I would add a sentence highlighting your control population with the rarity of P/ LP variants in the selected genes there.
2. Could you please justify the choice of age 8 years for cutoff for malignancy development?
3. Could you also justify the choice to omit germ cell tumors? While I understand the omission of hematological malignancies (though debatable for diagnosis with MDS as an “early stage” of disease), the exclusion of germ cell tumors seems less obvious to me. The exclusion of skin cancers is likely due to an incomplete reporting in the registry but could also be addressed in a sentence.
4. Please justify your choice of autosomal dominant CPS genes (and the omission of autosomal recessive ones).

Specific Comments

Abstract:
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--> Please add “8 years” everywhere to make clear at what age the cutoff for malignancy development was set.
6. L.28: “6.8% of cases (n=132) carried germline variants.”
--> Please add: “pathogenic/ likely pathogenic (P/ LP) germline variants”.

Introduction:
7. L.58: “but would require infeasibly large sample sizes and extended follow-up to prospectively study the impact of the inclusion of cancer genes on outcomes.”
--> Please rephrase and omit “infeasibly” and rather state that “very large populations” will be needed. History has told us that increasingly large populations get sequenced and we might be able to look at such large cohorts one day.
8. L. 43: Consider adding a statement that clinical screening is recommended and quite successful but is often not done in clinical practice and thus many remain undetected in clinical practice (see e.g. https://doi.org/10.1038/s41431-026-02040-x)

Methods:
9. L.125: “well babies”
--> Do “unwell babies” not get DBS collected? Most countries include all children, with sick or very premature ones being resampled after a certain delay. Please adapt or explain which children get excluded. I assume that all (also children who later developed malignancies) were included.

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--> This can be shortened to “36”
22. Table 1: PTCH1/ SUFU: Consider adding the term “Gorlin syndrome” to make that term findable when people search for

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23. Table 2: Can you clarify why the last year bracket (2011-2020) is longer than the other but has less patients included? Is the proportion of participation in dried blood spot collection different in different years? If so, comment in the results/ discussion section.
24. Table 2: “other CNS”
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25. Table 2: Staging, treatment outcome: This part is confusing to me as different variables are put together. Particularly “Regional or metastatic disease at presentation” does not make sense to me, what are the other if not regional OR metastatic?
--> Please adjust this part of the table making clear categories and ideally divide Staging from treatment and outcome.
26. Figure 2: Please make clear that the y axis is different in the left and right part of the figure lowering the 30% mark a bit and adding a visual cue like “// 100%” on top.

Supplement:
27. I suggest adding the title and the authors on the first page of the supplements. Additionally, a footnote or header on all subsequent pages might help identifying the supplement as part of this manuscript.
28. Figure S1: Please add the total number of entries in the Michigan Cancer Registry (born 1987-2020), also add the number of DBS overall (born 1987-2020) to understand the base populations better.
29. Figure S1: “Other brain tumors”, subdivide in HGG, LGG, other CNS if possible.
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31. Supplementary Table S1a: “Variants Classified as Pathogenic/Likely Pathogenic in Germline DNA from Children with Solid Tumor or Brain Tumors at age <8”
--> Please adjust: “with Solid Tumors or Brain Tumors at age <8 years”
32. Supplementary Table S1b: “Large Deletions”
--> Please provide a more meaningful title that explains the content more thoroughly.

Reviewer #3

(Remarks to the Author)
Diller and colleagues present a targeted sequencing study of rare genetic variants affecting pediatric cancers. They show that such variants can be detected using approaches apparently feasible to apply at scale, and would identify a non-trivial minority of emergent childhood cancer cases. The approach is innovative and the results persuasive, albeit with the caveats discussed by the authors. I think the paper will be significant, and is clearly one component of a series of papers that should influence and impact emerging infant sequencing projects.

I was asked to review this paper from a statistical genetic perspective, and am not an expert in cancer genomics per se. Nonetheless, I have only fairly minor comments, which I list in order below.

1. Abstract, lines 25-27: I found the phrasing here confusing, making it sound like only children with later cancers had blood spots archived. The phrasing from the main text (page 4, lines 67-69) was clearer and should be used instead.
2. Abstract, line 30: as above - I found this confusing, the line from the main text (page 8, lines 155-156) is clearer and should be substituted.
3. Methods, page 5, lines 86-87: the reason for excluding of other cancers should be provided – this is partly done in the discussion (page 12, lines 257-258), but would be more useful here.
4. Methods, page 5, line 88: please clarify if age was assessed at diagnosis or at some other time point.
5. Methods, page 6, line 103: please can the authors comment on whether the level of sequencing depth and coverage they achieve is likely to be feasible at scale in infant screening programmes, given the necessity to sequence other targets (presumably at similar depth)?
6. Methods, page 6, line 109-110: please clarify how (i.e. by what criteria) variant pathogenicity was classified?
7. Methods, page 6, line 117: please include a summary of variant detection and quality control in the main text, including software and quality control parameters for variant inclusion. Given the multi-ethnicity dataset, to what extent can ethnicity / ancestry differences account for the effects observed?
8. Results, page 8, line 160: given multiple statistical tests used, the statistical test should be included alongside all p-values.
9. Discussion, page 11, line 223-224: the statement that a 1/27000 detection rate is consistent with or higher than diseases currently included in screening is an important one for the impact of this work. This needs to be supported with explicit, cited examples of the detection rate of existing screening.
10. Discussion, page 12, lines 248-250: the caveat that clinical data was not available is an important one, and one that the authors should expand on. Can the authors discuss to what extent the 1/27000 children identified via their screening could be detected by other means (as well as the nuances of that, such as stage of detection etc.) That is, would full information on these children notably attenuate the discussed detection rate?
11. Figure 3: there is a potential participant disclosure issue here. The figure is in effect a cross-tabulation of multiple sources of information, including small categories such as Hispanic ethnicity and cancer type. As such, there are a few places where an individual has a unique set of characteristics (for example, I think there is only one living Hispanic child with a retinoblastoma in the dataset). Given the spatial and temporal locality of the dataset, it is feasible that information about specific individuals could be inferred from this figure. The authors should consider whether the value of this figure is great enough to justify the risk of disclosure, or otherwise make this figure less disclosive (for example, by collapsing

categories).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
No further comments. Excellent work.

Reviewer #2

(Remarks to the Author)
Thank you for addressing the reviewers' comments in a very nice way. I have no further comments.

Reviewer #3

(Remarks to the Author)
I am satisfied by the authors' responses – all of my comments have been addressed.

Detailed Response to Reviewers: Please note the abstract has been reworked given word count limitations and suggestions from reviewers. In addition, the Methods Section has moved, given Nature Communications formatting instructions.

Reviewer #1:

This is an awesome paper – many congratulations. It reports important work, it is of high quality, and should be published. I have two minor / pedantic points to raise:

Thank you for your kind words!

1) It would be useful to have a table that lists for each gene what the frequency of pathogenic mutations is in the comparison cohort (including the internal control).

We have created a table which reflects this request, which is shown below and included in the revised Supplement as Supplementary Table 4.

GENE	MICHIGAN CANCER COHORT n (%)*	INTERNAL COMPARISON COHORT n/N (%)**	PUBLISHED NEWBORN COHORTS (n/N) (%)+	GNOMAD P/LP ALLELE FREQUENCY ++
RB1	69 (3.54)	0/1,774 (0)	1/38,448 (0.0026)	5.58×10^{-5}
TP53	24 (1.23)	NA	0/1,000 (0)	2.78×10^{-4}
SMARCB1	8 (0.41)	NA	NR	9.9×10^{-6}
WT1	7 (0.36)	0/1,639 (0)	0/1,000 (0)	3.10×10^{-5}
RET	6 (0.31)	0/1,942 (0)	0/5,000 (0)	9.66×10^{-5}
SUFU	6 (0.31)	NA	NR	9.04×10^{-5}
PTCH1	4 (0.21)	NA	0/1,000 (0)	1.86×10^{-5}
DICER1	4 (0.21)	NA	1/1,000 (0.10)	2.04×10^{-4}
APC	3 (0.15)	1/1,873 (0.053)	NR	2.04×10^{-4}
PHOX2B	1 (0.051)	1/1,498 (0.067)	NR	5.70×10^{-5}
ALK	0 (0)	0/1,498 (0)	NR	1.2×10^{-6}

*Frequencies in the study cancer cohort are calculated as the proportion of the full cohort of 1,948 children with cancer carrying a P/LP variant in each gene.

**Frequencies in the Internal comparison cohorts were defined as the number of infants in the study cohort who did not develop the tumor type typically associated with that gene, as described in Supplementary Table 2; denominators therefore vary by gene.

+Frequencies were derived from published population population-based genomic screening studies; denominators reflect the number of individuals in whom each gene was evaluated.

++gnomAD frequencies are shown as allele frequencies (proportion) based on ClinVar-classified pathogenic/likely pathogenic variants.

P/LP, pathogenic or likely pathogenic, NA, not applicable, NR, not reported

2) May I suggest to changes to the writing:

Sentence: "Cancer predisposition syndromes, characterized by deleterious germline variants in cancer-associated genes, are present in at least 10% of children with malignancy."
CHANGE TO >>> "Cancer predisposition syndromes, caused by pathogenic germline (or rarely post-zygotic) mutations in certain cancer-associated genes, are present in at least 10% of children with malignancy."

Thank you for this suggestion. We have made changes to the MS per your suggestion and to that reflect the possibility of post-zygotic (germline mosaic) variants.

Revised text (Line 40-1):
Cancer predisposition syndromes, caused by deleterious germline (or germline mosaic) variants in cancer-associated genes, are present in at least 10% of children with malignancy.

Sentence: "Genomic newborn screening could identify these at-risk children, who could be subsequently monitored using syndrome-specific cancer surveillance protocols."
CHANGE TO >>> "Genomic newborn screening could identify these at-risk children, which may potentially translate into patient benefits through syndrome-specific cancer surveillance protocols."

Thank you for this suggestion. We have made this change, as suggested (Line 42-3).

Reviewer #2:

Thank you very much for the opportunity to review this very well written manuscript.

SUMMARY
This manuscript describes a population-based study in Michigan using dried blood spots from neonates to identify children with cancer predisposition syndromes (CPS). The study included 1948 children with a solid malignancy by age 8 years. Of those, 132 carried a pathogenic or likely pathogenic (P/LP) variant in one of 11 genes associated with childhood-onset malignancies. The study calculated that 1 in 27'000 newborn children harbor a P/ LP CPS variant and a malignancy before age 8 years. There was a strong gene-tumor specificity. Two patients had malignancies not expected considering the described tumor spectrum.

As comparison group, 38'448 infants from four published population-based newborn screening studies were used. Two children were identified with P/ LP variants. Both patients were found to harbor lesions compatible with the tumor-spectrum associated with the identified CPS gene variants. Compared with published variant prevalence data, P/ LP variants in the selected 11 CPS were very rare or absent in the general population. The authors conclude that newborn genomic CPS screening might be beneficial for selected CPS genes.

I congratulate the authors for the nicely drafted manuscript. The study aggregated a large dataset and showed various important findings. Dried blood spots were a good source of DNA

for targeted gene sequencing in >99% of children in a systematic collection of archived samples. Use of dried blood spots in CPS identification has been used before but not on such a large number of patients. The number of identified children with a CPS matched what can be expected from the literature and the tumor spectrum was according to the published associations in most cases.

Thank you for your observations and congratulations.

General Comments

I have general major comments:

1. The comparison of the findings in children with malignancies versus a large control population is important and does not show up in the abstract. I would add a sentence highlighting your control population with the rarity of P/ LP variants in the selected genes there.

Thank you for this suggestion. We have added a statement that refers to the comparison groups.

Revised text: (Line 31-32)
… Variant prevalence was 100% in medullary thyroid carcinoma, 40% in retinoblastoma, and 11–30% across five additional diagnoses with strong gene–tumor specificity ($p<0.001$). P/LP variants were rare in comparison datasets from healthy newborns and gnomAD.

2. Could you please justify the choice of age 8 years for cutoff for malignancy development?

We chose eight years as a cutoff for a number of reasons:

1. The study is quite focused, as a primary aim, on developing an approach to newborn screening. Newborn screening is focused on detection of conditions which are actionable (in this case, initiation of surveillance) in the first years of life.
2. We chose 8 years of age, given the age of onset expected in the most of the predisposition syndromes included in our analysis. That is, almost all retinoblastomas, neuroblastomas and Wilms tumors, (as well as Gorlin's associated medulloblastoma and *SMARCB1* related tumors occur before age 8) and surveillance for each one of the children with these cancer predisposition syndromes generally starts in infancy.
3. Limitations in funding (in part) suggested this focus. The chosen genes are associated with very early onset cancers. A full cohort of **all** children with cancer would have increased the sample size, with increased cost, without (we suspect) impacting upon our conclusions regarding CPS screening in newborns (although admittedly would be very interesting).

We have revised the text to briefly describe our reasoning.

Revised text (Line 226-227)

Eight years of age was chosen as a cut-off, given the age of onset expected in the predisposition syndromes included in our analysis and given our focus on the potential of our data to inform newborn screening.

3. Could you also justify the choice to omit germ cell tumors? While I understand the omission of hematological malignancies (though debatable for diagnosis with MDS as an "early stage" of disease), the exclusion of germ cell tumors seems less obvious to me. The exclusion of skin cancers is likely due to an incomplete reporting in the registry but could also be addressed in a sentence.

Thank you for the opportunity to clarify these choices. Because germ cell tumors are infrequently associated with established, high-penetrance cancer predisposition syndromes relevant to early-life screening, we excluded them. We did search the dataset provided from Michigan Cancer Registry for cases of gonadal malignancies classified as Sertoli-Leydig Cell (associated with *DICER1* CPS) occurring before age 8, but there were no cases recorded. The exclusion of skin cancers is, as suggested by the reviewer, because of incomplete reporting as well as the difficulty of diagnosis of melanoma (vs. Spitz nevi) in children. We have added this rationale to the Methods.

Revised text (Line 221-223):
Children with diagnoses of germ cell or skin cancers were excluded, due to the lack of association of malignant germ cell tumors with known autosomal dominant pediatric cancer predisposition syndromes and the difficulty of complete ascertainment and proper categorization of malignant skin cancers in childhood, respectively.

4. Please justify your choice of autosomal dominant CPS genes (and the omission of autosomal recessive ones).

We have clarified this point in the Methods. The study focuses on autosomal dominant cancer predisposition genes in which heterozygous pathogenic variants confer early-onset cancer risk relevant to newborn screening. Autosomal recessive syndromes were not included because cancer risk generally requires biallelic variants, complicating interpretation in a screening setting; in addition (and in contrast to autosomal dominant syndromes) many such conditions present with detectable clinical phenotypes in early life. We have pointed out the lack of inclusion of autosomal recessive cancer predisposition in the revised Discussion:

Revised text (Line 184-185)
Finally, by excluding leukemia predisposition genes and autosomal recessive cancer predisposition syndromes, as well as restricting analysis to only 11 solid tumor genes, we likely underestimated the overall number of genetically at-risk newborns.

Specific Comments

Abstract:
5. L. 27 and throughout the manuscript: "age 8."
--> Please add "8 years" everywhere to make clear at what age the cutoff for malignancy development was set.

This has been done.

6. L.28: "6.8% of cases (n=132) carried germline variants."
--> Please add: "pathogenic/ likely pathogenic (P/ LP) germline variants".

This has been done.

Introduction:
7. L.58: "but would require infeasibly large sample sizes and extended follow-up to prospectively study the impact of the inclusion of cancer genes on outcomes."

--> Please rephrase and omit "infeasibly" and rather state that "very large populations" will be needed. History has told us that increasingly large populations get sequenced and we might be able to look at such large cohorts one day.

Thank you for this suggestion. This has been done.

Revised text (Line 54):
Prospective genomic newborn screening research is underway worldwide, but would require very large population-based studies and extended follow-up to prospectively study the impact of the inclusion of cancer genes on outcomes.

8. L. 43: Consider adding a statement that clinical screening is recommended and quite successful but is often not done in clinical practice and thus many remain undetected in clinical practice (see e.g. https://doi.org/10.1038/s41431-026-02040-x)

We agree with this important point. We have incorporated this concept into the Discussion to emphasize real-world challenges in implementing surveillance. We have added this important reference.

Revised text (Line 200-202):
Although surveillance is recommended for many cancer predisposition syndromes and can enable earlier tumor detection, implementation into clinical practice is variable, and at-risk infants may remain undiagnosed without health care systems supporting this intervention.

Methods:
9. L.125: "well babies"
--> Do "unwell babies" not get DBS collected? Most countries include all children, with sick or

very premature ones being resampled after a certain delay. Please adapt or explain which children get excluded. I assume that all (also children who later developed malignancies) were included.

We have corrected this.

Results:
10. L. 137: "(N=1948)" can be omitted as stated just before.

This has been done.

11. L. 139: Add the percentage of specimens meeting QC criteria.

Thank you for this suggestion. We have clarified QC criteria in the revised Methods (see below, Reviewer 3, Comment 7) and provided corresponding percentage of specimens meeting QC criteria in the Results section.

Revised text (Line 72-3):

Archived newborn dried blood spot samples were obtained from all subjects, with adequate DNA extracted from 1943 of 1948 (99.7%) samples. All 1943 samples met quality standards for variant and CNV analysis.

12. L.154: "lung/ pleural malignancy" is an unusual term. The malignancy grouping should be explained in the methods section, ideally giving the rationale as well.

We agree that this terminology is unconventional and have clarified this in the Methods. Over the study period spanning more than three decades, pleuropulmonary blastoma has become recognized as the predominant histology underlying primary lung and pleural malignancies in young children and is associated with germline *DICER1* variants. Earlier in the study period, such tumors were often classified more broadly (e.g., as rhabdomyosarcoma or unspecified malignant lung or pleural tumors) and today would be called pleuropulmonary blastoma. To capture these cases consistently across time, we grouped primary malignant tumors of the lung and pleura for analysis.

Revised text (Line 224-226):
Primary malignant tumors of the lung and pleura were analyzed together to account for evolving histopathologic classification over the study period, including recognition of pleuropulmonary blastoma as a distinct entity associated with *DICER1*.

13. L.161: "race/ ethnicity" in the case cohort is described, could this be done for the comparison cohort as well? Are the cohorts comparable?

The comparison cohort studies were conducted in China (Chen et al. 2023), Europe (Boemer et al, 2025), Australia (Lunke, 2025) and the United States (Ziegler, 2025), with the largest cohort from China (see Supplementary Table 2). The lack of comparable race/ethnicity distribution compared to our cohort is emphasized in the revised Discussion.

Revised text:
Although emerging data from large newborn genomic screening studies demonstrate the rarity of pathogenic variants in cancer predisposition genes, these studies employ heterogeneous sequencing platforms, gene panels, variant interpretation approaches, **and study populations with different race and ethnicity**. **Extrapolation from the Michigan birth cohort to populations with different ancestry, demographic structure, and/or potential founder variants should be made cautiously.**

14. L.164: Could you clarify how many retinoblastoma RB1 P/ LP variant carriers had second neoplasms compared to retinoblastoma RB1 non-carriers?

15. L.180: Can you also clarify how many cases with non-RB1 P/ LP variant carriers had second neoplasms compared to no non-RB1 P/ LP carriers?

We have clarified the distribution of second neoplasms for the cohort overall and by *RB1* status among retinoblastoma cases. (We excluded cases in which second cancer data were missing).

Revised text (Results, subsection on **Germline Variant Detection and Characteristics of P/LP Carriers,** Lines 93-97)

Second cancer data were available from 126/132 (95%) of P/LP variant carriers. Eleven of these 126 carriers (8.7%) developed second cancers, compared to 85/1755 (4.8%) in non-carriers with available data. The odds of second cancer among P/LP variant carriers were 1.88 times that in non-carriers (OR = 1.88 (95% CI (asymptotic): 0.98, 3.62), p (chi-square) = 0.06). Second cancers in carriers occurred in individuals with P/LP variants in *RB1* (n=5), *TP53* (n=4), *APC* (n = 1), and *DICER1* (n = 1).

Revised text (Results, subsection on **RB1 and Retinoblastoma predisposition,** Lines 109-111):

Among individuals with retinoblastoma (excluding 11 with missing data regarding secondary cancers), secondary cancers occurred in 5/64 (7.8%) with germline P/LP *RB1* variants compared with 4/93 (4.3%) without *RB1* variants (OR = 1.89 (95% CI (exact): 0.39, 9.87), p (exact) = 0.488).

Discussion:
16. L.214 & 237: "deleterious variants"
--> Rather use "pathogenic/ likely pathogenic variants" here, as the formal classification of variants adds to the stringency of your analysis.

This has been done.

17. L.234: "often with advanced disease"
--> If I understood correctly, there was no stage difference in CPS P/ LP carriers vs. non

That is correct. We were trying to express that these are not all detected early (as one might expect if these CPS were known/patients are undergoing surveillance). However, to avoid lack of clarity, we have deleted this.

18. L.261: Please add: "… were not evaluated IN the current study".

This has been done.

19. L. 268: Consider adding information on cost-effectiveness of presymptomatic cancer screening.

We have added a further sentence calling for research in cost-effectiveness of presymptomatic cancer screening, commenting that it might vary by syndrome.

Revised text (Line 195-197):
While surveillance strategies for individuals with cancer predisposition syndromes have been shown to enable early tumor detection, their cost-effectiveness likely varies by syndrome, cancer risk, and screening modality, and remains incompletely defined.

20. L. 269ff: Consider adding potential risks of neonatal P/ LP variant detection: Distress to families, screening procedures in young children of which some might never become symptomatic, unnecessary interventions in benign lesions/ unclear imaging findings, etc.

Thanks for this suggestion. This has been added to the Discussion.

Revised text (Lines 197-199):
Potential risks of early identification should also be considered, including psychological distress for families, the burden of surveillance in young children, and the possibility of overdiagnosis or unnecessary interventions arising from indeterminate findings.

Tables & figures:
21. Table 1: In the SMARCB1 row: "36.0"
--> This can be shortened to "36"
This has been done.

22. Table 1: PTCH1/ SUFU: Consider adding the term "Gorlin syndrome" to make that term findable when people search for information.
This has been done.

23. Table 2: Can you clarify why the last year bracket (2011-2020) is longer than the other but has less patients included? Is the proportion of participation in dried blood spot collection different in different years? If so, comment in the results/ discussion section.

Children born in more recent years had shorter follow-up time (<8 years) and had not yet have reached the age at which cancer diagnoses were fully captured. This clarification has been added to the Methods.

Revised text (Lines 226-228):
Cancer outcomes were ascertained through linkage with the cancer registry for cases occurring between 1987 and 2020. As a result, children born in more recent years had shorter follow-up time in which to develop cancers.

24. Table 2: "other CNS"
--> Could this group be further subdivided in HGG, LGG, other CNS?

Grading of tumors was not consistently reported (only malignant tumors are included). However, we are able to subdivide CNS more finely. See revised Table 2.

25. Table 2: Staging, treatment outcome: This part is confusing to me as different variables are put together. Particularly "Regional or metastatic disease at presentation" does not make sense to me, what are the other if not regional OR metastatic?

Thank you for this suggestion. We added a separate section on staging at diagnosis, with all available data. Of note, these categories are cancer registry categories and metastatic disease is defined as distant (as opposed to regional) spread. We have clarified this in the Table.

--> Please adjust this part of the table making clear categories and ideally divide Staging from treatment and outcome.

See revised Table 2 (reformatted to include subsets of CNS tumors and separate out staging from treatment/outcomes).

26. Figure 2: Please make clear that the y axis is different in the left and right part of the figure lowering the 30% mark a bit and adding a visual cue like "// 100%" on top.

This has been done using a dotted line.

Supplement:
27. I suggest adding the title and the authors on the first page of the supplements. Additionally, a footnote or header on all subsequent pages might help identifying the supplement as part of this manuscript.

This has been done.

28. Figure S1: Please add the total number of entries in the Michigan Cancer Registry (born 1987-2020), also add the number of DBS overall (born 1987-2020) to understand the base populations better.

This has been done.

29. Figure S1: "Other brain tumors", subdivide in HGG, LGG, other CNS if possible.

This has been done (see above comment 24).

30. Figure S2: "ACE prediction engine applied to N=1938"
--> This number seems to omit the benign/ likely benign SNV. But the number still doesn't sum up the other variants. Could you clarify this number and adjust if needed?

Thank you for this observation. The ACE prediction engine was applied to all samples with adequate DNA and, as would be expected, there are many more variants than samples. To avoid confusion, we have removed the number of samples from the flow diagram, and we put that in the legend. We added the total number of SNV/indels, and total number of CNVs to flush out the numbers more clearly.

31. Supplementary Table S1a: "Variants Classified as Pathogenic/Likely Pathogenic in Germline DNA from Children with Solid Tumor or Brain Tumors at age <8"
--> Please adjust: "with Solid Tumors or Brain Tumors at age <8 years"

This has been done.

32. Supplementary Table S1b: "Large Deletions"
--> Please provide a more meaningful title that explains the content more thoroughly.

This has been done. The new title is "Large deletions involving >20bp detected in germline DNA from children with solid tumor or brain tumors at age <8 years"

Reviewer #3:

Diller and colleagues present a targeted sequencing study of rare genetic variants affecting pediatric cancers. They show that such variants can be detected using approaches apparently feasible to apply at scale, and would identify a non-trivial minority of emergent childhood cancer cases. The approach is innovative and the results persuasive, albeit with the caveats discussed by the authors. I think the paper will be significant, and is clearly one component of a series of papers that should influence and impact emerging infant sequencing projects.

Thank you for your comments. We agree that, given the emerging newborn sequencing projects, data regarding cancer risk will be important.

I was asked to review this paper from a statistical genetic perspective, and am not an expert in cancer genomics per se. Nonetheless, I have only fairly minor comments, which I list in order below.

1. Abstract, lines 25-27: I found the phrasing here confusing, making it sound like only children with later cancers had blood spots archived. The phrasing from the main text (page 4, lines 67-69) was clearer and should be used instead.

This has been done. Thank you for helping us have a clearer abstract.

2. Abstract, line 30: as above - I found this confusing, the line from the main text (page 8, lines 155-156) is clearer and should be substituted.

This has been done. Again, thank you. Word count limitations in the abstract required a little truncation.

3. Methods, page 5, lines 86-87: the reason for excluding of other cancers should be provided – this is partly done in the discussion (page 12, lines 257-258), but would be more useful here.

See response to reviewer 2, number 3.

4. Methods, page 5, line 88: please clarify if age was assessed at diagnosis or at some other time point.

Thank you for this request for clarification. It is age at diagnosis. We have made this change.

5. Methods, page 6, line 103: please can the authors comment on whether the level of sequencing depth and coverage they achieve is likely to be feasible at scale in infant screening programmes, given the necessity to sequence other targets (presumably at similar depth)?

Currently, about 60 genomic newborn screening pilot studies are underway around the world. Most of these pilots use whole genome sequencing (WGS) with results filtered for interpretation of specific predefined gene list. There are several several pilot studies, particularly in China, that have chosen targeted panels rather than WGS. There may be no actual (rather than pilot) Newborn screening (NBS) programs to have adopted primary next generation sequencing screening, either WGS or targeted WGS is generally at~30× coverage whereas targeted panels typically have much higher coverage. The drawback of WGS, although feasible in funded pilots, is the cost, being much higher than that of targeted panels. Thus, our approach would be more feasible for population-scale screening due to lower cost, not only because the reduced genomic footprint enables substantially higher coverage. We estimate that coverage would remain adequate (50-100X) even if an expansion in the number of panel genes (500-700)was

incorporated to address screening for a broader category of disorders that is being proposed should genomic screening become part of non-pilot public health newborn screening programs.. (No change in Manuscript).

6. Methods, page 6, line 109-110: please clarify how (i.e. by what criteria) variant pathogenicity was classified?

Thank you for this opportunity to clarify that we used criteria from the American College of Medical Genetics and Association of Molecular Pathology. This has been noted in the Methods (Line 256-7).

7. Methods, page 6, line 117:

Please include a summary of variant detection and quality control in the main text, including software and quality control parameters for variant inclusion.

We have added the requested information regarding variant detection and quality control to the Methods (Lines 240-251).

Revised text:
Genomic DNA was isolated from a single 3.2 mm dried blood spot (DBS) punch using the Chemagic 360 instrument following the manufacturer's protocol. Isolated DNA was fragmented enzymatically, and enriched by target capture. DNA quality metrics included OD_260/280 and OD_260/230 ratios initially across the first 96 samples, and subsequently using Qubit; fragment size was evaluated using TapeStation PCR performance using library test fragments, and coefficient of variation across replicates. Paired-end (2×150 base-pairs) sequencing was performed using a NovaSeq 6000 Illumina Sequencer (San Diego, CA). Sequencing data were considered acceptable if libraries achieved >250× average coverage and >99% of targets at ≥20× coverage.

Raw sequencing data were aligned to human genome assembly GRCh38 and processed using the Fabric Genomics Sentieon pipeline for SNV and small indel detection. High-confidence variants were retained using standard thresholds, including minimum read depth ≥20×, mapping quality ≥20, and heterozygous allele balance 0.3–0.7, with exceptions reviewed in IGV. CNVs were called using CNVkit with exon-level parameters and required at least one exon with log2 ratio exceeding thresholds and read support, confirmed visually in IGV.

Given the multi-ethnicity dataset, to what extent can ethnicity / ancestry differences account for the effects observed?

Observed variant frequencies in this cancer cohort were not adjusted for ancestry, so any effects due to population structure or bias cannot be fully excluded. We have noted this in the Statistical Methods, and this is limitation is included in the Discussion (Review 2, Comment 13, above).

Revised text (Lines 267-8):

Observed variant frequencies in this cancer cohort were not adjusted for ancestry, so any effects due to population structure or bias cannot be fully excluded.

8. Results, page 8, line 160: given multiple statistical tests used, the statistical test should be included alongside all p-values.

These changes have been made for all p-values in the manuscript.

Revised text (Lines 89-91):
The median age at cancer diagnosis in carriers was 14 months (IQR: 5.0-26.5) compared with 32 months (IQR: 13.0–55.0) in those without detected germline variants (p (Wilcoxon)<0.001).

9. Discussion, page 11, line 223-224: the statement that a 1/27000 detection rate is consistent with or higher than diseases currently included in screening is an important one for the impact of this work. This needs to be supported with explicit, cited examples of the detection rate of existing screening.

Thank you for this suggestion. We have added some context of other diseases included in newborn screening.

Revised text (Lines 154-5):
Overall, this screening would identify 1 in 27,000 newborns with a cancer predisposition syndrome and early onset cancer, a prevalence consistent with, or even higher than, other diseases currently included in newborn screening, **such as Pompe Disease (1/18,000 births), Severe Combined Immunodeficiency (SCID) (1/59,000 births) and Maple Syrup Urine Disease (1/200,000 births).**

10. Discussion, page 12, lines 248-250: the caveat that clinical data was not available is an important one, and one that the authors should expand on. Can the authors discuss to what extent the 1/27000 children identified via their screening could be detected by other means (as well as the nuances of that, such as stage of detection etc.) That is, would full information on these children notably attenuate the discussed detection rate?

Thank you for this interesting question. Of note, our choice of genes in our panel was specifically focused on syndromes that are not, generally speaking, associated with a phenotype. For example, we did not include *NF1*, *AT,* Bloom Syndrome, Fanconi Anemia (syndromes often associated with abnormality in physical exam). In retinoblastoma predisposition and Li-Fraumeni syndrome (associated with TP53 variants), there are no physical exam findings suggesting risk of tumor development and family history of cancer is evident in the minority of cases. Although

screening for red reflex on physical exam is considered a tool for identifying early disease on newborn exam, the sensitivity of this exam is so poor that we do not consider retinoblastoma as reliably detectable based on screening physical exam findings.

We have added to the text of the discussion the caveat that we do not have data regarding clinical evidence of cancer predisposition syndrome.

Revised Text (Lines 175-6):
Second, because clinical data available through the birth and cancer registries are limited, we were unable to assess family history of cancer **nor clinically known cancer predisposition syndromes.**

11. Figure 3: there is a potential participant disclosure issue here. The figure is in effect a cross-tabulation of multiple sources of information, including small categories such as Hispanic ethnicity and cancer type. As such, there are a few places where an individual has a unique set of characteristics (for example, I think there is only one living Hispanic child with a retinoblastoma in the dataset). Given the spatial and temporal locality of the dataset, it is feasible that information about specific individuals could be inferred from this figure. The authors should consider whether the value of this figure is great enough to justify the risk of disclosure, or otherwise make this figure less disclosive (for example, by collapsing categories).

The reviewer brings up an important concern. Michigan's Biotrust for Health/Cancer Registry has worked to help us avoid potential privacy concerns, especially given the rarity of these diseases. Given the small number of cases who were non-white, and the concerns raised by the reviewer (thank you!), we are removing individual race/ethnicity data from this figure. Group data is presented in the Results section.