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Author's Response to the Commentary by Raitoharju E *et al*. on "Epigenome-wide analysis across the development span of pediatric acute lymphoblastic leukemia: backtracking to birth"

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We thank Drs. Raitoharju and Marttila for their thoughtful commentary [1] on our recent article by Ghantous A et al., [2] and are pleased to have the opportunity to address the important points they raised. In our study, we charted the epigenomic landscape throughout the trajectory of pediatric pre-B acute lymphoblastic leukemia (B-ALL) —from *in utero* through birth, diagnosis, remission, and relapse — demonstrating a "proof-of-concept" that epigenetic precursors of the disease can be detectable at birth and may serve as biomarkers for early detection and prognosis and/or as targets for therapeutic intervention [2].

The commentary raised two major arguments:

1. The first argument was that "the observed methylation difference at birth is due to an uneven distribution of imprinted and non-methylated individuals among the cases and controls, with all individuals presenting normative physiological *VTRNA2-1* methylation levels". As the commentary and our article describe, the methylation levels of *VTRNA2-1* in the general population exhibit a bimodal distribution, clustering primarily into two groups: approximately ¾ of individuals show ~ 50% methylation consistent with imprinting, while the remaining ¼ exhibit low or absent methylation. Our results have shown that *VTRNA2-1* was

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hypermethylated at birth in nested cases relative to controls, with sex dependency. We agree that this altered methylation could be due to an uneven mix of individuals with different imprinting statuses between cases and controls. This distribution may represent biologically meaningful variation with potential relevance to disease susceptibility, rather than a purely statistical artefact. The authors correctly note that only 9% of the MoBa cases show low methylation and that this is substantially lower than the ~ 25% expected proportion that was observed in the MoBa cohort as a whole or reported in population cohorts. We validated this observation by reanalysing the MoBa data after categorizing individuals by *VTRNA2-1* methylation levels using criteria similar to those applied by Raitoharju et al., [1]: low methylation (median methylation < 20%), intermediate methylation (40% ≤ median methylation ≤ 60%) and high methylation (median methylation > 60%). Indeed, the low methylation fraction was significantly under-represented in the cases as compared to the controls ($p = 4.06 \times 10^{-2}$) (Fig. 1). We also concur with the authors that a low methylation status may confer protection against the risk of pediatric leukemia. This aligns with the hypothetical model proposed in our article, in which low *VTRNA2-1* methylation allows for a wider gene expression gradient (Fig. 2E in Ghantous A et al. [2]). Such flexibility may permit a 'drop' in *VTRNA2-1* expression, serving as a critical mechanism to activate the PKR cell death pathway, which eliminates pre-cancerous cells through the "tumor surveillance" model. These results will need to be interpreted with caution given the relatively small sample sizes of nested cases and controls in MoBa. As the MoBa and other few large birth cohorts available globally continue to mature and accumulate more cases over time, the validation of these findings may become feasible.

While methylation levels at birth are largely consistent with the reported bimodal distribution (with potentially uneven mixes between nested cases and controls), it is not fully accurate to state that this bimodal pattern is retained at diagnosis. In fact, a substantial proportion of cases at diagnosis fall into a distinct high methylation category (i.e., exceeding the 60% imprinting threshold), which was entirely absent in both types of control samples—non-tumorigenic tissues and tumor tissues collected at remission ($p = 1.58 \times 10^{-2}$ and $p = 1.6 \times 10^{-3}$, respectively) (Fig. 2). In both control groups, only the ~¾ 50%-imprinted and ~¼ lowly methylated categories were observed, as expected in the general population. This suggests the presence of a physiologically distinct *VTRNA2-1* methylation profile in leukemic tissues compared to controls, potentially supporting the leukemic

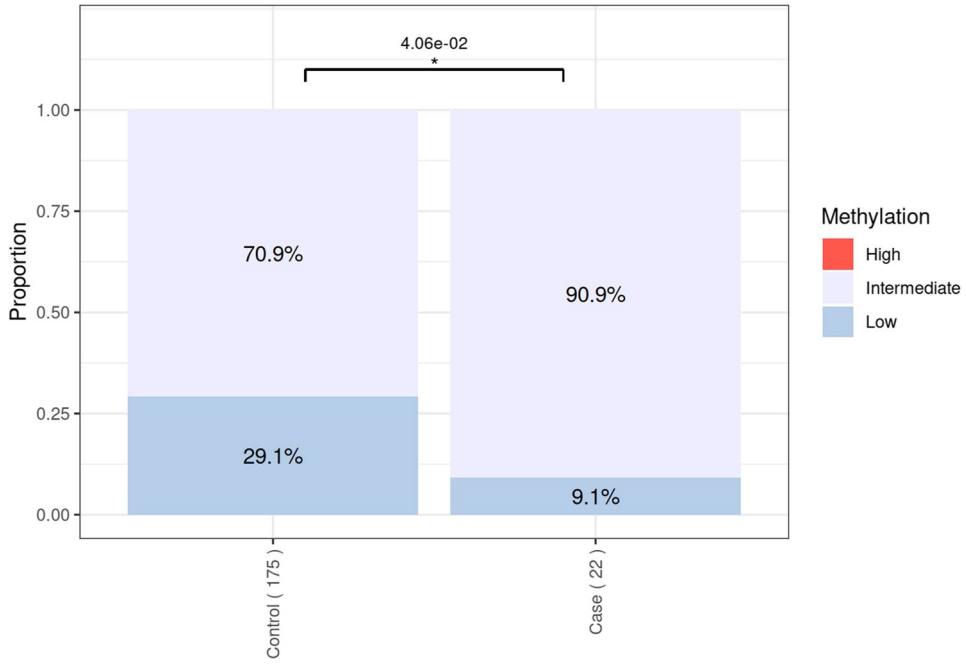

Fig. 1 Proportion of individuals showing high, intermediate or low *VTRNA2-1* methylation levels in cord blood samples taken from the MoBa cohort. The median methylation (beta value) across the 16 VTRNA2-1 CpGs was used an indicator of the methylation category for each individual: low methylation (median beta value < 0.2), intermediate methylation (0.4 ≤ median beta value ≤ 0.6) and high methylation (median beta value > 0.6). The relatively few samples exhibiting a median methylation level in the range of 0.2 to < 0.4 were omitted from the analysis. The Proportion test was performed to compare the proportions of samples with high, intermediate or low methylation between cases and controls

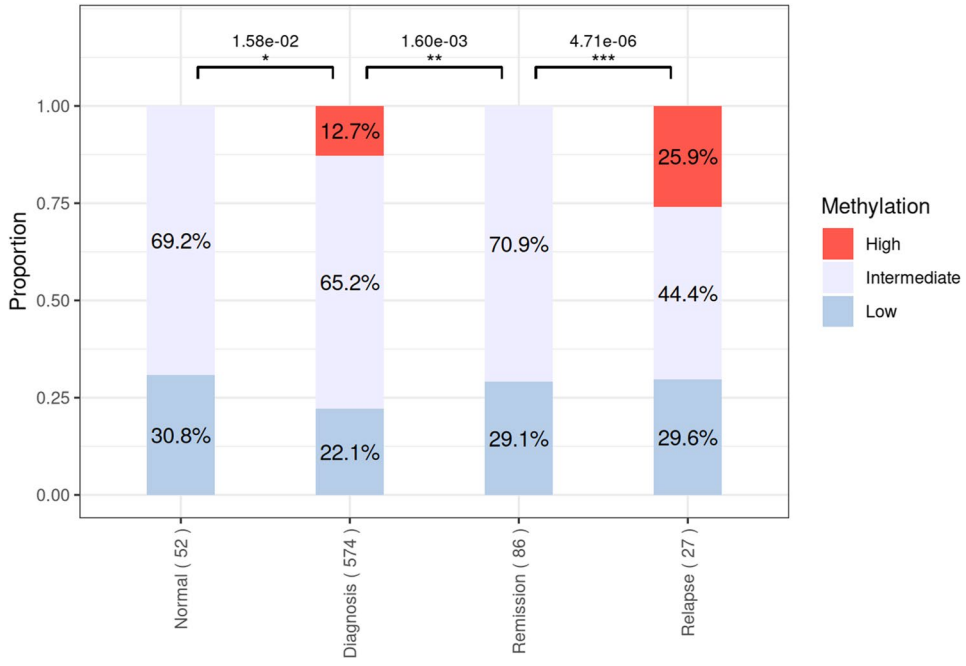

Fig. 2 Proportion of individuals showing high, intermediate or low *VTRNA2-1* methylation levels in peripheral blood and bone marrow samples taken from 4 groups of the NOPHO cohort: Normal (sorted B-cells from bone marrow of fetuses and from peripheral blood) and tumor samples taken at diagnosis, remission and relapse. The median methylation (beta value) across the 16 VTRNA2-1 CpGs was used an indicator of the methylation category for each individual: low methylation (median beta value < 0.2), intermediate methylation (0.4 ≤ median beta value ≤ 0.6) and high methylation (median beta value > 0.6). The relatively few samples exhibiting a median methylation level in the range of 0.2 to < 0.4 were omitted from the analysis. The Proportion test was performed to compare the proportions of samples with high, intermediate or low methylation in tumor compared to normal and remission samples and in remission compared to relapse samples

phenotype. Notably, this high methylation category re-emerges significantly at relapse ($p = 4.71 \times 10^{-6}$ and $p = 4.79 \times 10^{-4}$, when compared to normal and remission samples, respectively) (Fig. 2). The high methylation pattern at diagnosis and relapse could be explained by the clonal expansion of hypermethylated alleles during leukemogenic progression. This result is in line with a tumor suppressor role of *VTRNA2-1*, which has been documented in several types of cancer [3]. These interpretations are further supported by the observation that the high methylation *VTRNA2-1* cases return to the normal ~ 50% imprinting level upon remission in matched patient samples (Fig. 2B in Ghantous A et al., [2] and Fig. 1B in Raitoharju E et al., [1]).

Notably, the high methylation pattern observed in cases at diagnosis and relapse was not significantly evident at the time of birth (i.e. in neonatal blood samples) (Fig. 1), although a significant increase in average *VTRNA2-1* methylation at birth was consistently observed in nested cases compared to controls across all of the three studies —MoBa, CCLS and MEDC (Fig. 1 in Ghantous A et al. [2]). Given that the DNA methylation readout of the assay utilized herein reflects average levels across a heterogeneous mix of cells within the samples analyzed, small observed effect sizes at the bulk level in neonatal samples may mask substantial alterations occurring in specific cell subpopulations. These subpopulations may clonally expand during leukemogenesis, thereby, amplifying the effect sizes and potentially giving rise to the distinct high-methylation category observed at diagnosis and relapse. Our findings suggest that *VTRNA2-1* hypermethylation may reflect an early epigenetic predisposition that becomes more pronounced as the disease progresses. As single-cell DNA methylation technologies continue to advance, future studies will be better equipped to resolve cell-specific methylation patterns and directly test these hypotheses at higher resolution. Moreover, measuring maternal and paternal methylation can help map more precisely the parent-of-origin effects of imprinted genes and determine whether an observed neonatal methylation alteration is inherited from the parental alleles or acquired *in utero*.

2. The second argument was that Ghantous et al. [2], had suggested that "*VTRNA2-1* methylation could be utilized as a biomarker or a therapy target for B-ALL", although "the polymorphic imprinting in *VTRNA2-1* methylation levels calls into question the validity of *VTRNA2-1* methylation as a biomarker". We would like to clarify that our paper did not conclude that *VTRNA2-1* would serve as a sole or specific biomarker for ALL. Our conclusion, as stated in the

abstract of Ghantous et al., [2], was that we provided "*proof-of-concept* to detect at birth epigenetic precursors of pre-B ALL. These alterations … *can offer* biomarkers for early detection and prognosis as well as actionable targets for therapy". Therefore, our conclusion was intended to refer more broadly to epigenetic markers identifiable through the proof-of-concept approach outlined in our study, rather than apply to *VTRNA2-1* specifically. Any references to this gene within the text were purely illustrative, suggesting its potential as a biomarker rather than asserting a definitive role, as is stated in the manuscript: "Overall, these results *suggest VTRNA2-1* methylation as a marker of pre-B ALL prognosis, including leukemic state". This gene may contribute more effectively to early detection and prognosis when considered not as a standalone biomarker, but as part of a broader panel of biomarkers that future studies may uncover as additional data become available. This was emphasized in the concluding paragraph of our paper : "Future work based on more cases may be able to uncover further molecular precursors of pediatric pre-B ALL etc.".

We sincerely thank Drs. Raitoharju and Marttila for their insightful commentary and hope our clarifications have fully addressed their comments and added further evidence for the broader readership supporting the results and contextual interpretation as presented in Ghantous A et al., [2].

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Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization.

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The authors used ChatGPT (OpenAI, 2025) to refine some of the wording of the manuscript.

Author Contribution
Conceptualization: AG, JLW, and JAM. Data analysis and interpretation: VC, AG, FJN, JLW, JAM. Supervision: AG and JLW. Writing original draft: FJN and AG. Editing or reviewing the manuscript: all authors. All authors approved the final version of the manuscript.

Declarations

Ethics approval and consent to participate
The project was approved by the IARC Ethics Committee. The establishment of MoBa and initial data collection was based on a license from the Norwegian Data Protection Agency and approval from The Regional Committees for Medical and Health Research Ethics. The MoBa cohort is currently regulated by the Norwegian Health Registry Act. The current study was approved by The Regional Committees for Medical and Health Research Ethics and the Norwegian Data Inspectorate (REK number 2011/2551b), and written informed consent was provided by both parents. For CCLS, written informed consent was obtained by the legal representative of all included participants. The California State and University of California IRBs have approved the study. For MEDC, newborn blood spot punches from children later diagnosed with ALL were obtained from archival Guthrie cards under a waiver of consent granted as part of the project Epigenetic determinants of Childhood Cancer Incidence' Human Research Ethics Committee Approval (HRE/16/RCHM/62). The UKCS study used samples from the Newcastle Biobank which holds approvals from the Newcastle and North Tyneside Research Ethics Committee 1 (17/NE/0361). Additional approvals specific to the use of neonatal blood spot samples and linkage of these samples to the Northern Region Young Persons' Malignant Disease Registry were obtained from the Health Research Authority Confidentiality Advisory Group (17CAG0177) and Newcastle and North Tyneside Research Ethics Committee 1 (17/NE/0334), For QcALL, informed consent was obtained from all participating individuals, and the Sainte-Justne UHC Institutional Review Board approved the research protocols, For NOPHO, the study was approved by the Regional Ethical Review Board in Uppsala, Sweden and was conducted according to the guidelines of the Declaration of Helsinki. The patients and/or their guardians provided informed consent.

Competing interests
The authors declare no actual or potential competing financial interests.

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