

DNA methylation differences between cord and adult white blood cells reflect postnatal immune cell maturation

Corresponding Author: Dr Michael Kobor

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

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

This study makes important contributions to the field. Understanding dynamic changes in immune cell development from birth to adulthood can inform analysis of high dimensional DNAm data in cord blood and inform investigations aimed at understanding how cell type proportions can impact function. The authors have identified that there is greater variation DNA methylation in adult vs cord immune cells, which is more evident in lymphoid vs myeloid cells. Also, epigenetic age of B cells and monocytes was lower than other cell types from the same individual in cord blood, but not adult blood. In addition, the authors have created a shiny app for R, publicly available. It is important to note, as the authors do, that interpretation of these data are limited by the potential for batch effects between adults and infants, and that differences between cord and adult DNA methylation are attributed to genetic differences.

How many blood samples were included in each cohort, and/or overall? Is this what is in Table 1? Greater detail about cohort characteristics would be helpful for interpretation.

While the minimally adjusted samples are of importance, where differences in characteristics of the initial cohorts that should be adjusted for or at least referenced? For example, obesity or gestational age? Without knowing more about the initial cohorts, it is difficult to appropriately interpret results.

How were the 450K and 850K differences adjusted for/reconciled? Were the 'extra' probes in the 850K simply removed?

The possibility of batch differences mentioned should be adjusted for or otherwise verified as having small effect. Given the large number of differences in sample collection/sorting, etc.. between cord and adult, greater effort should be made to ensure methylation differences are truly biological rather than methodological.

Why was the p value 9×10^{-8} chosen rather than a standard FDR or other adjusted value? Please list q-values or adjusted p values for easier interpretation.

Many studies use multiple gestational age calculators, given the various strengths and weaknesses of the different models. Additional analyses with similar results would strengthen the conclusions.

Only summary data are included, which limits data transparency.

The cutoff change in beta value in supplemental Figure 2 appears to be 10% difference in beta, but the corresponding text reads a 10% difference in DNAm (line 229). Given that beta is calculated a percent change in DNAm on a scale from 1-100, a 10% difference in beta is only a difference of 0.1% DNAm, as listed in the Figure legend. Which is true? Also, the figure legend for Supp Fig 2 says the p value cutoff is 1×10^{-7} , but the text reads 9×10^{-8} , which is correct?

Line 287: which three lists? Adult, cord, and both?

Line 572: there is a space... is the p value 9×10^{-8} or 9×10^{-850} what are the values listed for cell types in Table 1?

Line 79: typo "leel"

Reviewer #3

(Remarks to the Author)

The manuscript from Jones and Konwar accessed, harmonized, and analyzed purified cell type DNA methylation across the lifecourse comparing cord blood cell-specific DNA methylation profiles with adults. The manuscript leverages data from a large set of samples to investigate important biological aspects of leukocyte maturation that have not been possible to assess through individual preceding efforts that collected the original data. Limitations of some of the existing data are appropriately assessed and addressed in the work (e.g. neutrophils / granulocytes). Generally, the work adds content,

context, and value to the understanding of DNA methylation differences between cord blood and adult immune cell types that will benefit many in the field. Importantly, the group brings accessibility to their work through the development and deployment of a user-friendly interactive analysis tool.

Potential sex-specific differential methylation within cell type between cord blood and adult appears not to be included and would add value.

The genomic location analyses of differentially methylated sites would benefit from an enrichment testing approach that determines an odds ratio and confidence interval with adjustment for probe type. In addition, such an analysis could include additional annotation file genomic regions such as enhancer sites. Ideally, such an analysis also would leverage existing data from (at least in adults) other epigenomic marks like histone modifications.

Reviewer #4

(Remarks to the Author)

In the manuscript of Jones and colleagues, the authors investigate DNA methylation changes between lymphoid and myeloid cell types of cord blood and adult blood. They specifically detect changes in the lymphoid cell lineages and attribute those changes to the antigen-dependent maturation of those cells throughout the lifespan. They further also investigate changes in the epigenetic ages of those cell types.

The manuscript uses six published datasets to compare adult blood samples with cord blood samples and gives a solid and nice general overview of the DNA methylation changes that occur between those two age groups. The authors also clearly discuss the limitations of many of their analyses, which makes the study transparent for the reader. It would be interesting to further analyse if the observed DNAm differences might really be due to the different maturation status between adult and cord blood samples, as claimed by the authors.

I have the following specific comments:

- 1) For differential methylation analysis the authors did not use adjusted p-values. They chose however to use a very small unadjusted p-value. It would be interesting to see if their findings can be recapitulated by using p-values adjusted for multiple testing since such algorithms can prevent both to get either false positives or false negatives.
- 2) It would be interesting to see if the differentially methylated CpGs are somehow associated with genes that may have a functional impact for example on the maturation of lymphoid cells.
- 3) The authors investigated if differentially methylated CpGs were positioned in co-methylated regions and were able to find some co-methylated regions both in cord and adult blood samples. It seems that some of them are even quite large. It would be interesting to know what kind of regions got co-methylated and if these could have functional impact.
- 4) The authors discuss that the lymphoid cells in adult blood are more variable. It would be interesting to see which CpGs between those lymphoid cells are causing the high variability and if those CpGs could have functional impact on maturation to further support the author's claim.
- 5) In this regard it might also be interesting to compare datasets of stimulated and unstimulated T and B cells to see if the stimulation and differentiation of the cells really induces DNA methylation changes and if those might be in part recapitulated between adult and cord blood samples.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Reviewer #3

(Remarks to the Author)

The authors have addressed my comments.

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We would like to express our sincere thanks to the reviewers and editor for their constructive comments, as well as their positive feedback on our work, we have made the requested revisions, as outlined below.

Reviewer 2

This study makes important contributions to the field. Understanding dynamic changes in immune cell development from birth to adulthood can inform analysis of high dimensional DNAm data in cord blood and inform investigations aimed at understanding how cell type proportions can impact function. The authors have identified that there is greater variation DNA methylation in adult vs cord immune cells, which is more evident in lymphoid vs myeloid cells. Also, epigenetic age of B cells and monocytes was lower than other cell types from the same individual in cord blood, but not adult blood. In addition, the authors have created a shiny app for R, publicly available. It is important to note, as the authors do, that interpretation of these data are limited by the potential for batch effects between adults and infants, and that differences between cord and adult DNA methylation are attributed to genetic differences.

How many blood samples were included in each cohort, and/or overall? Is this what is in Table 1? Greater detail about cohort characteristics would be helpful for interpretation.

While the minimally adjusted samples are of importance, where differences in characteristics of the initial cohorts that should be adjusted for or at least referenced? For example, obesity or gestational age? Without knowing more about the initial cohorts, it is difficult to appropriately interpret results.

Response: We appreciate the Reviewer's recognition of our work and its impact. Thank you for your time to review our manuscript.

We certainly agree with the insightful suggestion from the Reviewer to include some more details on the cohort characteristics. To clarify, Table 1 does indeed include information on the number of samples from each data set. While each data set retrieved from GEO has previously been published, we have now incorporated the Reviewer's request for additional clarity and cohort information and added some necessary details on each cohort in Table 1. These include method of cell separation, mean age in years, mean gestational age in weeks, sex information, delivery method and any relevant information on known disease/conditions.

How were the 450K and 850K differences adjusted for/reconciled? Were the 'extra' probes in the 850K simply removed?

Response:

This is indeed an excellent question given that our study included both 450K and 850K GEO datasets. To address this, we used a R package to combine the two arrays which evidently drops the "extra" probes present on the 850k array. To include this important information, we have modified our methods as follows:



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Finally, `combineArrays()` in R `minfi` package⁴⁷ was used to combine the adult and cord blood datasets, leaving only the 404,779 CpGs present on both the 450k and EPIC arrays for preprocessing.

The possibility of batch differences mentioned should be adjusted for or otherwise verified as having small effect. Given the large number of differences in sample collection/sorting, etc.. between cord and adult, greater effort should be made to ensure methylation differences are truly biological rather than methodological.

Response:

We appreciate the Reviewer's concern and astute insight into the fabric of our data, clearly highlighting the importance of delineating biological from methodological differences wherever possible and taking into account the limitations of using combined publicly available data. As recommended, we have added information to Table 1 to describe the specific methodological and phenotypic differences between the datasets used to ensure readers can evaluate the differences with all the information given. Since few variables overlapped across studies, it was not possible to take them into account during our analysis, but we sincerely hope that adding the data to Table 1 will increase transparency of interpretation. We also note that aside from batch variables as discussed below, there were no other obvious variables which differed between cord and adult blood. We have modified the results as follows to delineate this point:

"All data sets were generated from sorted blood, either obtained by flow cytometry (De Goede, Gervin, and Reinius) or magnetic sorting (Bakulski, Lin, and Salas). Further, the data used here were a mixture of samples either measured on the 450K (Bakulski, De Goede, and Gervin) or the 850K platform (Lin and Salas). The methodological differences in array type and sample collection methods occurred in both cord and adult data sets and so we do not expect these differences will severely impact our comparisons".

In the context of batch effects in DNA methylation studies, the common sources are associated with study center/production facility, chip and plate. Although correcting for chip-related batch effects would have been ideal, we were not positioned to account for chip due to limitations in the available data. Extracting varying subsets of data from publicly available GEO datasets resulted in an unbalanced distribution of samples across the chips and ComBat, while being the most commonly used tool to correct for batch effects, has unfortunately been shown to introduce false signal to the data in such unbalanced designs (PMIDs 32605541, 25352862, and 29616078). Moreover, for several chips, the sample sizes were fewer than three, which further hindered the use of ComBat for batch correction. We were also not able to correct for plate, since no studies had both adult and cord blood samples, meaning plate was confounded with one of our main variables of interest. However, to address in the best possible way the reviewer's comments given these limitations, we have now even more thoroughly examined our data for potential plate effects and have added a Supplementary figure as shown below with an alternative visualization from Figure 2. This approach demonstrates that cells clustered by cell type regardless of which plate they came from, which is now represented by the shape of the point in the figure, and described in the modified results as follows:

"It is important to note that this PC may reflect both age-associated and batch differences between the samples, though given that the cord samples represent four different batches, and the adult samples

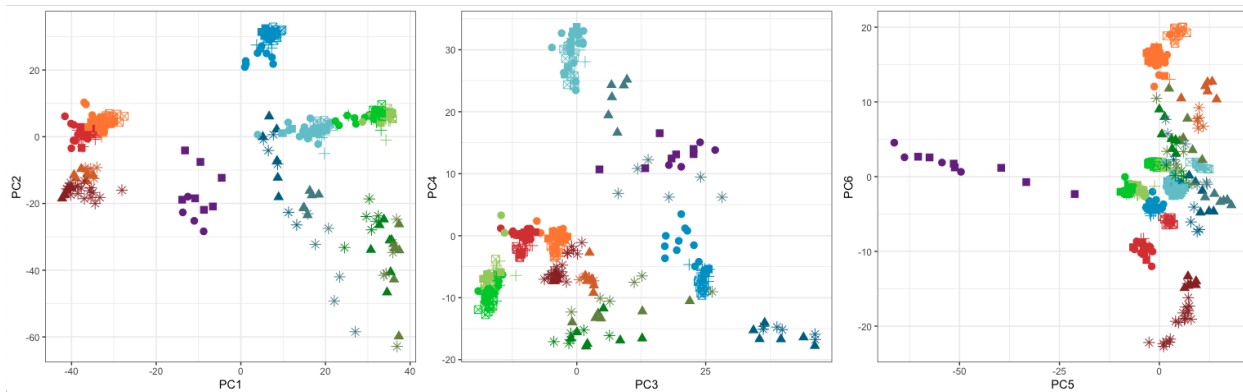


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two batches, the batch effects appeared to be smaller than the differences between cord and adult blood. When we specifically visualized the effect of batch by color coding the samples based on batch, we still observed clustering by cell type with no apparent influence of batch effects (Supplementary Figure 2).”.



Finally, we also incorporated a stringent statistical p-value cut-off (9×10^{-8}), recommended for studies on the larger EPIC/850K array which we adopted in our 450K data to ensure more stringency in our study, and applied an additional biological filter of a high effect size ($\Delta\beta > 0.10/10\%$) in part to deal with the residual batch effects. Thus, while we are unable to completely eliminate the effects of batch due to the limitations of working with public data from a variety of sources, we are confident that our findings minimize the effects of batch as much as possible. To clarify this issue in the manuscript, we have added the following to the discussion:

“We were unable to correct for batch effects using tools such as ComBat, as either the batch variables were confounded by age, and/or the distribution of samples across the chips were unbalanced. This imbalance was an unavoidable consequence of extracting varying subsets of samples from publicly available GEO datasets. Although it was not possible to specifically remove these effects, we thoroughly examined our data for potential batch influences. Plotting the top principal components and color-coding the samples by batch/study showed no substantial batch effects. Additionally, we used a stringent p value cut-off (9×10^{-8}), recommended for studies on the larger EPIC/850K array which we adopted in our 450K data and a high effect size ($\Delta\beta > 0.10/10\%$) cut off to enrich our findings for biological effects, ensure greater stringency and minimize the residual influence of batch. Importantly, since there is no expectation that batch effects would be cell-type specific, the comparisons between cell types should be equally affected by batch effects, and thus not bias the interpretation of the findings”.

Why was the p value 9×10^{-8} chosen rather than a standard FDR or other adjusted value? Please list q-values or adjusted p values for easier interpretation.

Response:

We thank the Reviewer for the excellent question regarding the choice of p-value versus a standard FDR or other adjusted statistical values. The stringent p-value cut-off used in the current study is based on the recommended guidelines published in PMID 31088362. The researchers recommend a p



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$< 9 \times 10^{-8}$ to adequately control for false positives in DNA methylation studies. While the recommended p-value was in the context of EPIC/850K array DNA methylation studies, we used the threshold in our 450K compiled dataset for ensuring even greater stringency in our analyses.

This p-value cut-off is a more stringent parameter compared to a standard False Discovery Rate (FDR) approach. For example, we identified 54570 CpGs in CD8T cell type that were differentially methylated between cord blood and adult blood samples, using a $p < 9 \times 10^{-8}$ and effect size of delta beta $> 10\%$. The highest p-value among the 54570 CpGs, 8.97×10^{-8} , was equivalent to an adjusted p-value/FDR of 3.93×10^{-7} . A similar pattern, where our chosen p-value is smaller than the adjusted FDR value, was observed in the other cell types too. We now provide Supplementary tables for each cell type which include the t-statistic, p-values, adjusted FDR values and the delta betas for the differentially methylated CpGs, so that readers have access to both the unadjusted and FDR adjusted p values.

The relevance and importance of the chosen p-value has been now added in the discussion as follows:

“Additionally, we used a stringent p value cut-off (9×10^{-8}), recommended for studies on the larger EPIC/850K array which we adopted in our 450K data and a high effect size ($\Delta\beta > 0.10/10\%$) cut-off to enrich our findings for biological effects, ensure greater stringency and minimize the residual influence of batch”.

Many studies use multiple gestational age calculators, given the various strengths and weaknesses of the different models. Additional analyses with similar results would strengthen the conclusions.

Response:

The Reviewer raises an interesting point, and rightfully points out that existing tools differ in their strength and weaknesses. To address this issue, we have modified Figure 5 to now include the two epigenetic clocks designed for the 450k array in cord blood (the Bohlin and Knight clocks) and three chronological clocks designed for the 450k array in adult blood (the original Horvath pan-tissue clock, the Hannum clock, and the SkinBlood clock). Accordingly, we have also modified the results as follows:

“We opted for the two-cord blood gestational epigenetic clocks, specifically designed for samples from the 450K array, the Knight and Bohlin clocks, and three commonly used first generation adult epigenetic clocks, the Horvath pan-tissue clock, the Hannum clock, and the SkinBlood clock. Two cord blood data sets and one adult blood data set had reported chronological ages, and we calculated epigenetic age acceleration in weeks of gestational age for cord blood, or years of chronological age for adult blood as the residuals extracted from a linear model of predicted epigenetic age regressed on reported chronological age (Figure 5). The other two cord blood data sets and one adult data set did not have known ages, but as the samples were matched across cell types, we were able to still assess mean differences in epigenetic age (Figure 5, bottom). Overall, epigenetic age and epigenetic age acceleration showed similar patterns across data sets, though the patterns differed between cord blood and adult samples and between the clocks. In cord blood, for both epigenetic age and epigenetic age acceleration using the the Bohlin clock, monocytes were epigenetically oldest, followed by B cells, while NK, CD4T, CD8T, granulocytes and nRBC were relatively younger in terms of epigenetic age (p value $< 2 \times 10^{-16}$). For the Knight clock, monocytes were also epigenetically oldest, followed by



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granulocytes whereas B cells were the youngest (p value $< 2 \times 10^{-16}$). The remaining cell types, NK, CD4T, CD8T and nRBCs, showed intermediate predicted gestational ages. B cells and granulocytes displayed the largest differences in both the Bohlin and Knight clock estimates (Figure 5), which were statistically significant for both gestational epigenetic age acceleration (Bohlin $p < 2 \times 10^{-16}$, Knight $p < 2 \times 10^{-16}$) and gestational epigenetic age (Bohlin $p < 2 \times 10^{-16}$, Knight $p < 2 \times 10^{-16}$) in cord blood. Similarly, in adults, epigenetic age acceleration was significantly different between the cell types (Horvath $p = 0.003$, Hannum $p < 3.39 \times 10^{-12}$, SkinBlood $p < 9.79 \times 10^{-9}$), with B cells, granulocytes, and CD8T cells demonstrating the greatest differences between the three adult epigenetic clocks. When examining epigenetic ages between matched samples, only the Hannum epigenetic clock showed specific cell type differences ($p = 0.377$), notably underestimating the epigenetic age of CD8T cells compared to the other cell types. In contrast, both the Horvath pan tissue and the SkinBlood epigenetic clocks did not exhibit differences in epigenetic ages by cell type (Horvath $p = 0.771$, SkinBlood $p = 0.694$),”

Below is the modified Figure 5:

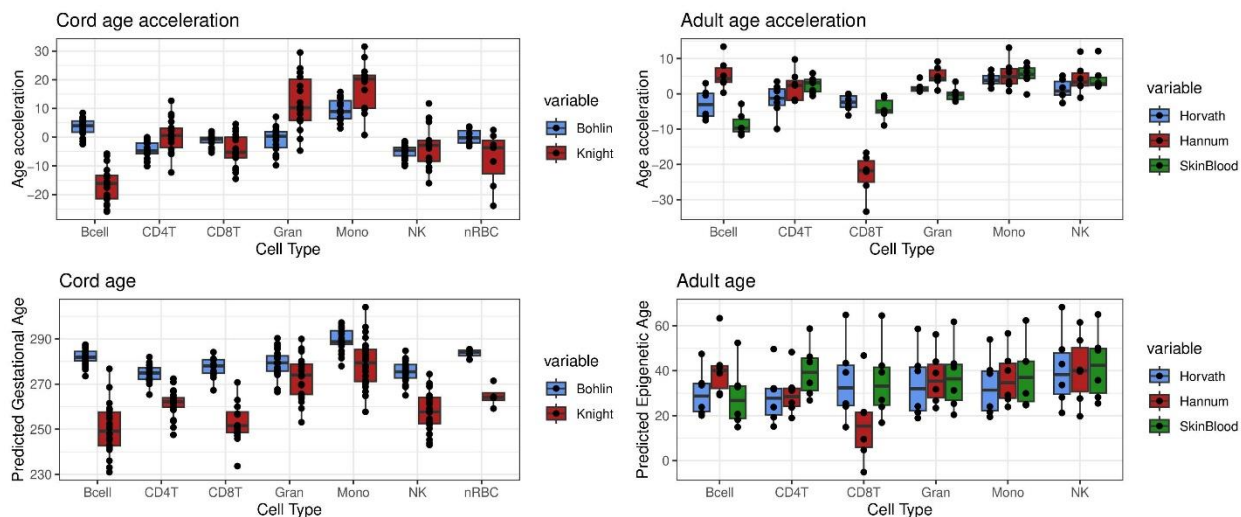


Figure 5. Cord blood cell types demonstrate differences in epigenetic age which are reduced in adult blood. Top: Two cord data sets (de Goede and Gervin, left) and one adult data set (Salas, right) had known gestational or chronological ages, respectively, and thus we calculated epigenetic age acceleration in days (cord) or years (adult) for each sample. Bottom: two cord (Bakulski and Lin, left) and one adult (Reinius, right), did not have chronological ages, but did have matched samples from each participant, and so we compared epigenetic gestational age (cord) or age (adult) across cell types.



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Only summary data are included, which limits data transparency.

Response:

As described above, we have now included Supplementary Table S4-S9 that contain all the EWAS results, showing the effect size, adjusted and unadjusted p value, and t-statistics for all tests performed across all the cell types.

The cutoff change in beta value in supplemental Figure 2 appears to be 10% difference in beta, but the corresponding text reads a 10% difference in DNAm (line 229). Given that beta is calculated a percent change in DNAm on a scale from 1-100, a 10% difference in beta is only a difference of 0.1% DNAm, as listed in the Figure legend. Which is true?

Response:

We apologize for the confusion, our cutoff was indeed 10% difference in DNAm, or 0.1 beta. We have modified the relevant figure caption and text.

Also, the figure legend for Supp Fig 2 says the p value cutoff is 1×10^{-7} , but the text reads 9×10^{-8} , which is correct?

Response:

We thank the Reviewer for pointing out this inconsistency, we confirm the p-value cut-off was 9×10^{-8} and have corrected this in the entire manuscript.

Line 287: which three lists? Adult, cord, and both?

Response:

We apologize for the confusion. We have now modified the sentence to improve clarity which reads as follows:

Given that some of the DNAm differences between cord and adult cell types may reflect age-associated variation potentially captured by epigenetic clocks, we examined the lists of sites that were significantly differentially methylated across all cell types, as well as those specific to the myeloid or lymphoid lineage, for enrichment in the pan-tissue adult epigenetic clock sites.

Line 572: there is a space... is the p value 9×10^{-8} or 9×10^{-850} what are the values listed for cell types in Table 1?

Response:

We appreciate the Reviewer's attention to detail and would like to clarify that this was an issue with the referencing and the p-value used is 9×10^{-8} . We have moved the reference (#50) to the end of the sentence.

Line 79: typo "leel"



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Response:

We appreciate the Reviewer's thoroughness and have fixed the typo from "leel" to "level".

Reviewer 3

The manuscript from Jones and Konwar accessed, harmonized, and analyzed purified cell type DNA methylation across the lifecourse comparing cord blood cell-specific DNA methylation profiles with adults. The manuscript leverages data from a large set of samples to investigate important biological aspects of leukocyte maturation that have not been possible to assess through individual preceding efforts that collected the original data. Limitations of some of the existing data are appropriately assessed and addressed in the work (e.g. neutrophils / granulocytes). Generally, the work adds content, context, and value to the understanding of DNA methylation differences between cord blood and adult immune cell types that will benefit many in the field. Importantly, the group brings accessibility to their work through the development and deployment of a user-friendly interactive analysis tool.

Potential sex-specific differential methylation within cell type between cord blood and adult appears not to be included and would add value.

Response: We truly appreciate the Reviewer's kind words and positive evaluation of our manuscript.

We are in complete agreement with the Reviewer that including sex-specific effects in our analysis would add value to our findings. We were, however, restricted by the constraints of the available GEO datasets. Specifically, we were not positioned to test the sex effects as the two adult datasets had either no or minimal representation of females in the dataset (Reinius et.al had no females, whereas Salas et. al. had 1 female). Due to this, we were not able to directly test sex-specific differential methylation. As an alternate route, we adopted a post-hoc approach by leveraging previously published lists of sex-specific CpGs in cord blood and adult blood and cross-referenced these lists with our findings. Upon cross-referencing, we observed an overlap of only 17.16% of previously identified sex-specific sites with our differentially methylated CpGs. These findings are now incorporated in the discussion (see below):

"Both the immune system and the methylome exhibit sex specific patterns, which may might change during development. Unfortunately, due to the minimal or complete lack of representation of females in the adult blood GEO datasets, we were unable to broadly test for sex-specific effects. However, the uneven number of sexes across our datasets, particularly between the cord and adult blood datasets, raised the possibility of sex being a potential confounding variable in our differential DNA methylation analysis. This then offered us the opportunity to address these issues, using a more targeted approach. Specifically, we leveraged previously published lists of sex-specific autosomal CpGs in cord and adult blood and cross-referencing these lists with the differentially methylated CpGs between cord and adult blood cell types, identified in the current study. We noted an overlap of only 1-7 % between our differentially methylated CpGs and the sex-specific CpGs, depending on the study examined, suggesting that sex had a limited influence on our identified DNAm associations".



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The previous published papers were as follow:

Singmann, P., Shem-Tov, D., Wahl, S., Grallert, H., Fiorito, G., Shin, S.Y., Schramm, K., Wolf, P., Kunze, S., Baran, Y. and Guarrera, S. (2015). Characterization of whole-genome autosomal differences of DNA methylation between men and women. *Epigenetics & chromatin*, 8, pp.1-13.

Solomon, O., Huen, K., Yousefi, P., Kippers, L. K., González, J. R., Suderman, M., ... & Holland, N. (2022). Meta-analysis of epigenome-wide association studies in newborns and children show widespread sex differences in blood DNA methylation. *Mutation Research/Reviews in Mutation Research*, 789, 108415.

McCartney, D. L., Zhang, F., Hillary, R. F., Zhang, Q., Stevenson, A. J., Walker, R. M., ... & Marioni, R. E. (2020). An epigenome-wide association study of sex-specific chronological ageing. *Genome medicine*, 12, 1-11.

Grant, O. A., Wang, Y., Kumari, M., Zabet, N. R., & Schalkwyk, L. (2022). Characterising sex differences of autosomal DNA methylation in whole blood using the Illumina EPIC array. *Clinical epigenetics*, 14(1), 62.

Gatev, E., Inkster, A. M., Negri, G. L., Konwar, C., Lussier, A. A., Skakkebaek, A., ... & Aristizabal, M. J. (2021). Autosomal sex-associated co-methylated regions predict biological sex from DNA methylation. *Nucleic Acids Research*, 49(16), 9097-9116.

The genomic location analyses of differentially methylated sites would benefit from an enrichment testing approach that determines an odds ratio and confidence interval with adjustment for probe type. In addition, such an analysis could include additional annotation file genomic regions such as enhancer sites. Ideally, such an analysis also would leverage existing data from (at least in adults) other epigenomic marks like histone modifications.

Response:

We appreciate this insightful suggestion by the Reviewer. In response to this suggestion, we have now used the EpiTools R package for the same and have included this information in the legend of the Supplementary Figure 5.

Further, we also appreciate the Reviewer's excellent suggestion to include additional annotation such as incorporation of enhancers etc. To address this, we employed ChromHMM map pertaining to specific cell type and performed an overrepresentation analysis. This is now included as an additional section in the methods, results and discussion:

Following is the methods section:

"In addition, enrichment analysis of chromatin states, generated using ChromHMM⁵⁷, was performed using the relevant cell type specific ChromHMM maps".

Following is the results section:



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“Considering that chromatin states provide critical insights into the influence of DNAm patterns, we also performed an enrichment analysis of chromatin states underlying our identified CpG associations (Supplementary Tables S16-S21). Differentially methylated CpGs between cord and adult blood cell types, in both lymphoid and myeloid lineage, were enriched in weak repressed polycomb, repressed polycomb, bivalent enhancer, bivalent TSS, active enhancer, and weak enhancer. However, CpGs associated with lymphoid cell types were also enriched in quiescent/low chromatin state and with myeloid cell types showed enrichment in genic enhancers.

Following is the discussion section:

“While our GO analyses revealed enrichment of both distinct and shared pathways in the developmentally age-related CpGs associations, the ChromHMM findings predominantly showed enrichment of common chromatin states underlying the differential DNAm patterns, regardless of cell type lineage. This may suggest that similar chromatin features, underlying the age-associated molecular changes in DNAm, may drive the regulation of key immune responses during development”.

Reviewer 4

In the manuscript of Jones and colleagues, the authors investigate DNA methylation changes between lymphoid and myeloid cell types of cord blood and adult blood. They specifically detect changes in the lymphoid cell lineages and attribute those changes to the antigen-dependent maturation of those cells throughout the lifespan. They further also investigate changes in the epigenetic ages of those cell types.

The manuscript uses six published datasets to compare adult blood samples with cord blood samples and gives a solid and nice general overview of the DNA methylation changes that occur between those two age groups. The authors also clearly discuss the limitations of many of their analyses, which makes the study transparent for the reader.

It would be interesting to further analyse if the observed DNAm differences might really be due to the different maturation status between adult and cord blood samples, as claimed by the authors.

For differential methylation analysis the authors did not use adjusted p-values. They chose however to use a very small unadjusted p-value. It would be interesting to see if their findings can be recapitulated by using p-values adjusted for multiple testing since such algorithms can prevent both to get either false positives or false negatives.

Response: We appreciate the Reviewer’s positive remarks and thoughtful comments.

We thank the Reviewer for highlighting an excellent point regarding the chosen p-value versus other adjusted values. Since this question was previously also noted by Reviewer 2 we are reiterating our response here. The stringent p-value cut-off used in the current study is based on the recommended guidelines published in PMID 31088362. The researchers recommend a $p < 9 \times 10^{-8}$ to adequately control for false positives in DNA methylation studies. While the recommended p-value was in the



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context of EPIC/850K array DNA methylation studies, we used the threshold in our 450K compiled dataset for ensuring greater stringency in our analyses.

This p-value cut-off is a more stringent parameter compared to a standard False Discovery Rate (FDR) approach. For example, we identified 54570 CpGs in CD8T cell type that were differentially methylated between cord blood and adult blood samples, using a $p < 9 \times 10^{-8}$ and effect size of delta beta > 10%. The highest p-value among the 54570 CpGs corresponded to a p-value of 8.97×10^{-8} which was equivalent to an adjusted p-value/FDR of 3.93×10^{-7} . A similar pattern, where our chosen p-value is smaller than the adjusted FDR value, was observed in the other cell types too. Further, we now provide Supplementary tables for each cell type which include the t-statistic, p-values, adjusted FDR values and the delta betas for the differentially methylated CpGs

It would be interesting to see if the differentially methylated CpGs are somehow associated with genes that may have a functional impact for example on the maturation of lymphoid cells.

The authors investigated if differentially methylated CpGs were positioned in co-methylated regions and were able to find some co-methylated regions both in cord and adult blood samples. It seems that some of them are even quite large. It would be interesting to know what kind of regions got co-methylated and if these could have functional impact.

Response:

We have taken the liberty to combine the two comments from the reviewer, as they both essentially highlight the same point regarding the need to gain functional insights into the DNA methylation changes between the cord and adult blood cell types.

We concur with the reviewer and have now performed a Gene Ontology (GO) enrichment analysis to gain a biological understanding of the identified molecular changes between cord and adult cell types. This analysis has been included in the methods, results and discussion sections, with Supplementary tables provided:

Following is the methods section:

Enrichment analysis of biological processes for the genes associated with the differentially methylated CpGs between cord blood and adult blood cell types was conducted using the `gometh()` embedded in the R `missMethyl` package.

Following is the results section:

“To further understand the biological processes and underlying function associated with genes annotated to CpGs that showed differential methylation between cord and adult blood cell types, we conducted a Gene Ontology (GO) enrichment analysis using the `missMethyl` R package (Supplementary Table S10-S15). Applying an FDR cut-off of < 0.01 and a minimum of 100 genes per GO term, we first restricted the enrichment analysis to genes associated with differentially methylated CpGs between cord and adult blood cell types specifically within the lymphoid lineage. This revealed enrichment of 181 unique biological processes including regulation of immune effector process, regulation of T cell activation, regulation of chemotaxis, inflammatory response, and calcium ion homeostasis. Similarly, when we focused the GO analysis on the set of genes associated with



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differentially methylated CpGs between cord and adult blood within the myeloid lineage, we found 114 unique biological processes, such as leukocyte differentiation, immune response-activating signal transduction, cellular response to cytokine stimulus, positive regulation of programmed cell death, and antigen receptor-mediated signaling pathway.

Following is the discussion section:

“Given that cord and adult blood cell types are involved in maintaining immune homeostasis, we observed expected enrichment of varying biological processes related to immune activation and regulation across both lymphoid and myeloid DNAm associations. However, processes linked with negative regulation of immune responses, such as apoptotic pathways, were enriched in all the lymphoid lineage cell types. In contrast, processes underlying positive regulation of immune responses such as activation of enzyme activity were overrepresented in both the myeloid cell types, delineating the distinct biological roles of these DNAm associations based on cell type lineage”.

The authors discuss that the lymphoid cells in adult blood are more variable. It would be interesting to see which CpGs between those lymphoid cells are causing the high variability and if those CpGs could have functional impact on maturation to further support the author’s claim.

In this regard it might also be interesting to compare datasets of stimulated and unstimulated T and B cells to see if the stimulation and differentiation of the cells really induces DNA methylation changes and if those might be in part recapitulated between adult and cord blood samples.

Response:

We now provide the lists of variable CpG sites for both cord blood and adult blood cell types in Supplementary Tables (S1-S3). We certainly agree with the reviewer that this would be a fascinating area of investigation. Unfortunately, it is beyond the scope of the current work. Stimulation and maturation are unlikely to show identical DNAm changes, and so the stimulation would have to be done on naive B and T cells from cord blood for this analysis. To the best of our knowledge, such a data set does not yet exist, however, the work we present here do suggest future studies in that area.