Epigenetics

Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function

--Manuscript Draft--

Manuscript Number:	KEPI-2021-0060R1
Full Title:	Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function
Article Type:	Research Article
Manuscript Classifications:	DNA Methylation
Abstract:	DNA methylation (DNAm) in mammals is mostly examined within the context of CpG dinucleotides. Non-CpG DNAm is also widespread across the human genome, but the functional relevance, tissue-specific disposition, and inter-individual variability has not been widely studied. Our aim was to examine non-CpG DNAm in the wider methylome across multiple tissues from the same individuals to better understand non-CpG DNAm distribution within different tissues and individuals, and in relation to known genomic regulatory features. DNA methylation in umbilical cord and cord blood at birth, and peripheral venous blood at age 12-13 years from twenty individuals from the Southampton Women's Survey cohort was assessed by Agilent SureSelect methyl-seq. Hierarchical cluster analysis (HCA) was performed on CpG and non-CpG sites, and stratified by specific cytosine environment. Analysis of tissue and inter-individual variation was then conducted in a second dataset of twelve samples: eight muscle tissue, and four aliquots of cord blood pooled from two individuals. HCA using methylated non-CpG sites showed different clustering patterns specific to the three base pair triplicate (CNN) sequence. Analysis of CAC sites with non-zero methylation showed that samples clustered first by tissue type, then by individual (as observed for CpG methylation), while analysis using non-zero methylation at CAT sites showed samples grouped predominantly by individual. These clustering patterns were validated in an independent dataset using cord blood and muscle tissue. This research suggests that CAC methylation can have tissue-specific patterns, and that individual effects, either genetic or unmeasured environmental factors, can influence CAT methylation.
Author Comments:	Thank you for considering our research. The response to reviewer's comments has also been uploaded as a word document that contains helpful formatting and figures.
Order of Authors Secondary Information:	
Response to Reviewers:	Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function – response to reviewer's comments We thank the reviewer for their comments and suggested improvements to the manuscript. We have addressed the points they have raised, answering them below and modifying the manuscript accordingly. The response to reviewer's comments has also been uploaded as a word document that contains helpful formatting and figures. Reviewer 1: The Authors submitted a very interesting manuscript about the poorly understood and poorly studied topic of the so-called non-CpG methylation. Their conclusions were that some structural motifs, with cytosines involved in the non-CpG methylation, have tissue-specific and individual-specific patterns, which suggests a functional role. The methods seem appropriate and the conclusions supported by results. I have the following suggestions/requests. 1) Although the Agilent SureSelect platform is well known (and a reference is quoted), I think the readers can benefit of a synthetic description in the text. In particular, the following aspects should be briefly clarified: libraries preparation and enrichment, hybridization and capture, bisulfite conversion, PCR amplification of bisulfite-treated libraries, sequencing, analysis. A flowchart (as supplementary figure) may be useful.

Response 1: Thank you for this helpful suggestion; we have added a flowchart to the Supplementary Figures (Supplementary Figure S1) demonstrating a summary of the pipeline from SureSelect library preparation right through to analysis. We have also incorporated into this the suggestion contained in the reviewers 4th comment (which mentioned we could include an example of the matrices involved in creating the dendrograms). We agree this summary, and the inclusion of the generalised methylation and distance matrices, will aid readers' understanding of the SureSelect methodology and analysis process. (Supplementary Figure S1 is mentioned in the Methods section on line #151-152).

2) Particular attention should be paid to the quantification of DNA methylation of each C moiety among a non-CpG element. When the Authors use the term non-zero methylation, what do they really intend? Please, define/specify: a) what is the non-zero methylation; b) how the methylation of each C was quantified and expressed; c) if quantitative differences between the same elements in different tissues and cells were measured and highlighted (for example, there is a brief mention about the median methylation between 3.4% and 8.0%, but only in a cumulative way); d) in particular, if there are different levels of methylation of CAC and CAT elements, between different tissues and cells. I specify that I am speaking about the % methylation of each C and not about the % of the number of methylated C. In synthesis, the Authors should make more clear how they were able to measure not only the number (in %) of methylated sites but also the amount (in %) of methylation for each site.
Possibly, showing the amount of methylation in a less cumulative way may be useful.

Response 2a-b: Comments 2a and 2b follow on nicely from each other, so shall be answered together:
Non-zero methylation is when the % methylation at a particular cytosine is not zero, i.e. methylation at that site is greater than zero. More specifically, this means that (per sample) out of all the n sequencing reads for a particular a cytosine, at least one of the reads was methylated. See the example figure (can be found in the uploaded word document version of response to reviewer's comments) and accompanying explanation:

In the diagram there are n reads at the reference 'GCT' cytosine in question, k of those reads at this particular loci were methylated, so the % methylation (or more precisely, proportion) is k/n. If k>0, then the cytosine would be considered non-zero methylated for that particular sample.

The 671,751 non-zero methylated non-CpG sites frequently referred to in this study are cytosines for which k>0 in all of the 60 samples from the discovery dataset i.e. zero was not a methylation value ascribed to any of the 60 samples at any of these 671,751 'non-zero' methylation sites. In matrix form: the methylation matrix (dimension: 60 x 671,751) did not contain any zeros.
We have updated the Methods section to contain an explanation of how the methylation level for each cytosine is calculated (line #145-147) and clarified what we mean by non-zero methylated sites (line #160-161).

Response 2c-d: Comments 2c and 2d both pertain to whether quantitative differences between elements in different tissues were measured and particularly in relation to CAC and CAT methylation, and the reviewer suggests showing the methylation data in a less cumulative way. As such, a joint response will also be given for 2c-d.
The reviewer raises an interesting point. There were differences between the methylation levels in CAC and CAT sites. Given the amount of methylation data contained in just CAC (n=141,674) and CAT (n=68,866) sites, we have quantified these methylation levels using medians and 5th-95th percentiles. To present the data in a less cumulative way, we have added these statistics separately for each individual, broken down by the three tissue types, and also by subset of non-CpG methylation type for CAC and CAT sites. A table containing this information has been added as a Supplementary Table (Supplementary Table S4) and mentioned in the Results (line #191-193).

Supplementary Table S4 shows that, within each of the three tissue types in discovery data, for each individual median methylation at non-zero methylated CAC sites was consistently higher than median methylation at non-zero methylated CAT sites. More precisely, these differences in median methylation within the same sample were 0.6 - 0.8 % methylation higher in CAC sites compared to CAT sites for cord blood tissue and also for 12 year peripheral blood. However, although in the same direction, for umbilical cord these differences were larger, with CAC – CAT median methylation differences 0.9 - 1.5 % higher in CAC sites compared to CAT sites.

The reviewer also asked for clarification on how we calculated the number of methylated cytosines in addition to the % methylation at each cytosine. A detailed description of how methylation % of each cytosine was calculated is provided in Response 2a-b (number of methylated reads / total number of reads). Once this calculation had been performed for each cytosine (in each sample), we were able to count how many non-CpG sites had methylation values greater than zero (non-zero) in all 60 samples in the discovery data set; this left 671,751 non-CpG sites. This is how we calculated the methylation level at each cytosine, and then used that to count the number of non-CpG sites with methylation >0 for all 60 samples in the discovery data set.
In summary, to address the reviewer's suggestions in comment 2 we have:
-clarified and added an explanation of how the methylation level of each cytosine in each sample was calculated (using the number of methylated reads / total number of reads) to the methods section (changes on lines #145-147 & #160-161), and
-added Supplementary Table S4 containing the median methylation level of each individual by tissue type, further broken down by subset of non-CpG methylation context: CAC and CAT methylation (mentioned on line #191-193).

3) Correlated to the previous point, a possible way to quantify the percentage of methylation of each C by NGS is to use the number of reads (as for other quantification issues in NGS, for example deletions); discussing this possibility also in relation to the choice of a read-depth of 30X (instead of, for example, 50X) could be useful.

Response 3: As the reviewer highlights, this question in part relates to the previous point (comment 2). The response to the previous comment (2a-b) demonstrates how the methylation level at each cytosine was quantified for each sample, and this was indeed done using the number of reads (#methylated reads / #total reads). An explanation of this calculation method has been added to the Methods (line #145-147). In addition, the reason that 30x read-depth was selected as a cut-off was because the literature [1] demonstrated that performance in reproducibility of MC-seq data improved minimally past x30, and this was consistent with our data. We have added an explanation of this to the methods section and referenced (line #149-151).

4) It could be also useful to show, in supplementary material, an example of the original data matrix (I suppose n specimens x m cytosines) used for the production of the distance matrix (I suppose n x n), in turn used for dendrograms.

Response 4: As detailed in the response to comment 1, we have included generalised examples of the methylation matrix (s x c) and distance matrix (s x s) into the analysis flowchart as Supplementary Figure S1 (where s = number of samples, and c= number of cytosines). We hope that this will aid the readers' understanding. (Supplementary Figure S1 is mentioned in the Methods section on line #151-152).

5) An explanation about the reason why the analysis of structural non-CpG elements was limited to trinucleotides (and not extended, for example, to elements spanning 4, 5 or more nucleotides) should be reported.

Response 5: We have re-worded the section in the Introduction with regards to the

context in which non-CpG methylation is reported in the literature (line #69-74) and updated the limitations of the Discussion (line #347-351).
Initial analysis of non-CpG methylation in plants examining sequence preferences for methylation in different base contexts found that non-CpG methylation preferentially occurred at CHG and CHH sites with sequence context outside of these bases less conserved [2]. Work in human tissues has also focused upon trinucleotides (CHG/CHH), finding non-CpG methylation in this context in embryonic stem cells [3] with CAH the most common form identified [4], with some studies suggesting that CAC is the predominant form observed in vertebrates [5, 6]. Studies also suggest that the second base in the triplicate is more important for methylation than the third base, in part due to binding affinities of DNA methyltransferases [4, 7]. Overall, these prior studies led us to focus our work on trinucleotides in both the CHG/CHH context, though we agree that analysis of non-CpG methylation in mammals is still in its nascency, has been explored in relatively few tissues in humans, and there is some data to suggest that non-CpG methylation occurs within other contexts, where trinucleotides could be viewed as part of a longer recognition sequence in some situations [5, 8]. However, this was outside the scope of this study and we have updated the limitations section to discuss non-CpG methylation outside of a CHG/CHH context.

6) Non-CpG methylation has been often associated to active demethylation. I think this link should be highlighted in Introduction and/or Discussion (possibly with quotations).

Response 6: Active demethylation of non-CpG methylation has now been referred to in the Introduction, in conjunction with the answer to (7) below (line #80-85).

7) There are at least 3 articles pointing to non-CpG methylation that should be quoted and discussed, for example as follows:
- Fuso A. et al., Early demethylation of non-CpG, CpC-rich, elements in the myogenin 5'-flanking region: a priming effect on the spreading of active demethylation? Cell Cycle (2010) 9(19):3965-3976. In particular for: the role of non-CpG methylation in muscle; the statistical analysis of DNA methylation by cluster analysis; the selection of specific short non-CpG (CpC-rich) elements with possible functional roles.
- Monti N. et al. CpG and non-CpG Presenilin1 methylation pattern in course of neurodevelopment and neurodegeneration is associated with gene expression in human and murine brain. Epigenetics (2020) 15(8):781-799. In particular for: the role of non-CpG methylation in brain, neurodevelopment and neuropathology; DNA methylation in peripheral blood as biomarker.
- Lucarelli M. et al. Active demethylation of non-CpG moieties in animals: a neglected research area. International Journal of Molecular Sciences (2019) 20(24):6272. In particular for the underrepresentation of non-CpG methylation in DNA methylation studies (taking into account also dynamics of active demethylation).

Response 7: These studies have been added as supporting literature in the Introduction, providing further evidence of the links between non-CpG methylation and disease, as well as the active and targeted demethylation of non-CpG methylation as evidence of a functional role in genomic epigenetic regulation (line #80-85).

8) When referring to placental blood or peripheral venous blood the Authors should specify which cell type is the source of the DNA (although obvious).

Response 8: The methods section has been updated to include the specific cell types contained within cord blood (B cells, granulocytes, monocytes, natural killer cells, nucleated red blood cells, and CD4 & CD8 T cells) and peripheral venous blood (B cells, neutrophils, monocytes, natural killer cells, and CD4 & CD8 T cells) (Line #106-109).

9) In Figure S2 the terms "5' UTR", "Downstream" and "Promoter" should be better

	defined with the aim of clarifying the structural and positional differences between these analyzed zones. Response 9: The annotations for genomic regions in this work were sourced from the UCSC hg19 genome assembly based on the knownGene track and applied using the ChIPseeker package in R. The regions are defined as: •Promoter - A region 2kbp upstream and 500bp downstream of transcriptional start sites. •3’ / 5’UTR - Messenger RNA sequences that are untranslated and lie three prime/five prime to sequences which are translated. •Downstream - A region 1-300bp downstream of the gene end. The definitions of these regions have been added to the explanation for Supplementary Figure S3 (previously known as Supplementary Figure S2). References 1.Teh, A.L., et al., Comparison of Methyl-capture Sequencing vs. Infinium 450K methylation array for methylome analysis in clinical samples. Epigenetics, 2016. 11(1): p. 36-48. 2.Cokus, S.J., et al., Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. Nature, 2008. 452(7184): p. 215-9. 3.Lister, R., et al., Human DNA methylomes at base resolution show widespread epigenomic differences. Nature, 2009. 462(7271): p. 315-22. 4.Laurent, L., et al., Dynamic changes in the human methylome during differentiation. Genome Res, 2010. 20(3): p. 320-31. 5.Guo, W., et al., Characterizing the strand-specific distribution of non-CpG methylation in human pluripotent cells. Nucleic Acids Res, 2014. 42(5): p. 3009-16. 6.de Mendoza, A., et al., The emergence of the brain non-CpG methylation system in vertebrates. Nat Ecol Evol, 2021. 5(3): p. 369-378. 7.Wienholz, B.L., et al., DNMT3L modulates significant and distinct flanking sequence preference for DNA methylation by DNMT3A and DNMT3B in vivo. PLoS Genet, 2010. 6(9): p. e1001106. 8.Fuso, A., et al., Early demethylation of non-CpG, CpC-rich, elements in the myogenin 5'-flanking region: a priming effect on the spreading of active demethylation. Cell Cycle, 2010. 9(19): p. 3965-76

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Abstract

DNA methylation (DNAm) in mammals is mostly examined within the context of CpG dinucleotides. Non-CpG DNAm is also widespread across the human genome, but the functional relevance, tissue-specific disposition, and inter-individual variability has not been widely studied. Our aim was to examine non-CpG DNAm in the wider methylome across multiple tissues from the same individuals to better understand non-CpG DNAm distribution within different tissues and individuals, and in relation to known genomic regulatory features.

DNA methylation in umbilical cord and cord blood at birth, and peripheral venous blood at age 12-13 years from twenty individuals from the Southampton Women's Survey cohort was assessed by Agilent SureSelect methyl-seq. Hierarchical cluster analysis (HCA) was performed on CpG and non-CpG sites, and stratified by specific cytosine environment. Analysis of tissue and inter-individual variation was then conducted in a second dataset of twelve samples: eight muscle tissue, and four aliquots of cord blood pooled from two individuals.

HCA using methylated non-CpG sites showed different clustering patterns specific to the three base pair triplicate (CNN) sequence. Analysis of CAC sites with non-zero methylation showed that samples clustered first by tissue type, then by individual (as observed for CpG methylation), while analysis using non-zero methylation at CAT sites showed samples grouped predominantly by individual. These clustering patterns were validated in an independent dataset using cord blood and muscle tissue.

This research suggests that CAC methylation can have tissue-specific patterns, and that individual effects, either genetic or unmeasured environmental factors, can influence CAT methylation.

Introduction

Epigenetics, the study of changes in gene expression that occur without alterations in the nucleotide sequence, plays a fundamental role in regulating the accessibility of DNA to the transcriptional machinery, and the regulation of tissue-specific gene expression, as well as genomic imprinting and X chromosome inactivation in early development [1]. DNA methylation (DNAm) is a widely studied epigenetic modification, and is normally examined in the context of a CG dinucleotide (CpG), where the cytosine base can be modified at the 5th carbon position by the addition of a methyl (CH_3) group. DNAm in the CpG context has a well-established role in genomic regulation and control of gene expression, with evidence that altered CpG methylation may link early-life environmental exposures with later non-communicable diseases, such as cardiovascular disease or obesity [2, 3]. Animal models have shown how different environmental factors such as diet, exercise and stress can affect DNA methylation and gene expression [4-8].

DNA methylation outside the CpG context has been far less extensively studied, yet may be far more prevalent within the methylome; there are approximately 28 million CpG dinucleotides in the human genome, but in excess of 556 million cytosines in a non-CpG context (USCS, hg19). For example, in human and mouse central nervous system neurons, ~2-6% of non-CpG sites are methylated, with a mode of ~20-25% methylation across those sites, whereas methylation levels at methylated CpG sites are typically ~60-90% [9, 10]. However CpG dinucleotides are relatively underrepresented in the genome [11], leading to far greater numbers of non-CpG sites, with non-CpG methylation representing up to half of all the methylation present; a study by Woodcock et al. suggests that, in DNA from human spleen, up to 54.5% of all methylation present is in a non-CpG context [12]. Levels of non-CpG methylation may depend highly on tissue type, with higher levels of specific non-CpG methylation reported in neurones and human embryonic stem cells, but present at much lower levels in other tissue types [9, 10, 13-15]. Studies in plants have found that non-CpG methylation predominantly occurs at base-pair triplicates [16, 17], while studies in vertebrates examining non-CpG methylation have identified both symmetric CHG (H=A, C, or T) [18] and asymmetric CHH methylation patterns [14, 19], at conserved positions in the genome [20, 21], with the sequence flanking the cytosine position potentially modulating DNA methyltransferase 3A (DNMT3A)/DNMT3B binding, in conjunction with DNMT3L [22].

The role of non-CpG DNAm within the genome is unclear, and DNAm in different contexts may perform specific or overlapping functions. Non-CpG methylation has been linked to disease status such as in type 2 diabetes where increases in non-CpG methylation within the promoter of Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) were associated with impaired glucose tolerance. Moreover, PGC-1α non-CpG methylation was increased by free fatty acids, suggesting potential environmental modulation of the methylation status of these sites [23]. Non-CpG methylation has also been implicated in Alzheimer's disease (AD), where non-CpG methylation patterns within the promoter of *Presenilin1* in human brain tissue were inversely correlated with expression in AD samples [24], suggesting active demethylation of non-CpG methylation, epigenetically regulating gene expression.

Active demethylation of non-CpG methylation has been reported in other contexts [25], but remains understudied [26].

Differences in levels of non-CpG DNAm between human samples could be the result of stochastic change in the epigenome, but there are two main alternative explanations as to why non-CpG DNAm levels may differ in human samples. (1) Differences may exist between individuals: resulting from either environmental factors during development or an individual's genetic sequence, (2) non-CpG DNAm patterns may differ by tissue type due to developmental programming during cell differentiation. If differences in non-CpG DNAm are not just purely due to random processes then similarities in the patterns of non-CpG DNAm within tissue types or within individuals may be expected.

To investigate whether individual or tissue-specific factors may influence non-CpG methylation, here we have examined both CpG and non-CpG methylation in placental cord and cord blood at birth, as well as peripheral venous blood collected at 12-13 years in a group of individuals (n=20) from the Southampton Women's survey (SWS) cohort. Methylation data was captured using the Agilent SureSelectXT Human Methyl-Seq (SureSelect platform). Hierarchical clustering analysis (HCA) was applied to the CpG and non-CpG data to observe the clustering patterns. We then validated our findings

Methods

Discovery data

In the discovery dataset, a total of 60 samples from the Southampton Women's Survey (SWS) cohort, a UK prospective cohort study in which women were recruited before conception of the child [27], were interrogated by Agilent SureSelectXT Human Methyl-Seq capture and sequencing method (SureSelect). These 60 samples consisted of 20 individuals each analysed across three tissue types: cord blood, umbilical cord, and 12-13 year peripheral blood. Cord Blood consists of B cells, granulocytes, monocytes, natural killer cells, nucleated red blood cells, and CD4 & CD8 T cells, and peripheral venous blood contains B cells, neutrophils, monocytes, natural killer cells, and CD4 & CD8 T cells. Individuals were selected on the basis of having DNA from all three tissues available in sufficient quantities (1µg), were split equally by sex (10 males/10 females), and five individuals from each quarter of the % fat distribution from DXA measurements taken at age 8-9 years.

Validation data

For the validation dataset, twelve samples were interrogated by Agilent SureSelect in total, across five individuals. Muscle tissue samples from four males aged between 73-79 years from the Hertfordshire Sarcopenia Study (HSSe), a UK based cohort study [28], were assayed in duplicate (1A/B, 2A/B, 3A/B, 4A/B), and one cord blood sample (pooled from two individuals, both male) from the SWS was assayed as two duplicate pairs (5A/B and 5C/D). Agreement in methylation levels between duplicates using a subset of 671,751 non-zero methylated non-CpG sites was assessed showing on average 93.4% of sites agreeing to within 10% methylation, reproducibility statistics and sample DNA quantities can be seen in Supplementary Table S1.

DNA extraction

DNA from muscle biopsy samples was stored and extracted as described previously [29]. For umbilical cord, a 5–10 cm segment was cut from the mid portion of each cord, immediately following delivery, flushed with saline to remove fetal blood, flash-frozen in liquid nitrogen and stored at −80 °C until required for DNA isolation. Peripheral blood was stored at -80 °C until further processing. Genomic DNA from peripheral blood was extracted using QIAamp DNA Mini Kit (Qiagen, UK), following the manufacturers recommendations. Genomic DNA was prepared from umbilical cord, umbilical cord blood, and muscle tissue by a standard high salt method [30].

Agilent SureSelect Methyl-Seq data

Methyl-seq data was generated by the Centre for Genomic Research (CGR) at the University of Liverpool using the Agilent SureSelect platform [31]. Both the discovery data and the validation data were cleaned, processed and analysed using the same procedure detailed below. The data arrived as FASTQ files, trimming of adapters was performed using Cutadapt v1.2.1 with the option -O 3, so the 3' end of any reads which matched the adapter sequence for 3 base pairs or more were trimmed [32], and a minimum window quality score 20, using Sickle v1.2 [33]. Reads<10bp removed. The unmasked

human genome was downloaded from UCSC, and the genome hash table was built using Extended Randomised Numerical Aligner (ERNE) create [34]. The alignment against the genome was performed using ERNE-BS5 2 [35]. Unprocessed data contained paired-end reads, and singleton reads. Singleton reads result from one read of a pair failing the Sickle quality control. The singlet files contained sequences whose pair had been removed due to poor sequence quality or adapter contamination. SureSelect data in the discovery dataset and the validation dataset produced similar summary statistics. Paired reads aligned uniquely to the genome at a greater rate (88.8% & 85.0%) than singleton reads (66.1% & 59.3%), and singleton reads were negligible in number (0.76 & 1.37 million reads) compared with paired end reads (85.7 & 99.4 million reads) for discovery and validation datasets respectively. As a result of this, singleton reads were not included in any analysis. For each sample methylation calls, calculated by the number of methylated reads / total number of reads at each cytosine, were made using ERNE-METH 2 [35]. This provided the methylation level for each cytosine for each sample. Options '--annotations-bismark' and '--annotations-erne' were used during the methylation calling process to provide detailed cytosine context. Previous studies have demonstrated that reproducibility improvements are minimal beyond 30x read-depth [36], therefore a minimum read-depth of 30x was used for all downstream analyses. A flowchart summarising the steps from SureSelect library preparation to statistical analysis is shown in Supplementary Figure S1.

Statistical analysis

Data manipulation and summary statistics were created using Stata (version 15.0 & 16.0) and unix bash commands, and hierarchical cluster analysis was performed in R (version 3.5.1 & 3.6.1) using the 'hclust' command with complete linkage method and Euclidean distance as the metric to measure dissimilarity. Other linkage methods were also tested, with similar results ('average linkage method' and 'weighted pair group method with arithmetic mean'). For hierarchical cluster analysis on non-CpG methylation, non-CpG sites were restricted to 671,751 sites where methylation was >0 for all 60 samples, i.e. all 20 individuals across all three tissue types had non-zero methylation values at these sites; these 671,751 non-CpG sites are frequently referred to as 'non-zero' methylation sites.

Ethics

The HSSe received ethical approval from the Hertfordshire Research Ethics Committee. In the SWS, the recruitment of women, follow-up through pregnancy, follow-up of the children, and sample collection/analysis were carried out under Institutional Review Board approval (Southampton and SW Hampshire Research Ethics Committee) with written informed consent. In both studies clinical investigations were conducted according to the principles expressed in the 1964 Declaration of Helsinki.

Results

To understand tissue and individual differences in non-CpG methylation, DNA samples from 20 individuals in three tissue types (umbilical cord, cord blood, and peripheral blood) were interrogated for non-CpG (and CpG) methylation using Agilent SureSelect. Table 1 shows the number of sites for the CpG and non-CpG sites in the discovery dataset with over 30 fold read-depth, split by tissue type and those sites covered in all 60 samples. In the discovery dataset ~2.52 million CpG sites (>30x read-depth) were captured in at least one of the 60 samples, and similarly ~2.58 million in the validation dataset. When considering the number of CpG sites with over 30 reads across all 60 samples in the discovery dataset, the number reduced to 1,222,537 CpG sites. Over 17.6 million non-CpG sites (>30x read-depth) were captured in at least one of the 60 samples in the discovery dataset (and ~17.7 million in the validation dataset). The number of non-CpG sites with non-zero methylation in all of the 60 samples was 671,751, with a median methylation between 3.4% – 8.0% (median and 5^{th}-95^{th} percentile of methylation for each sample are shown in Supplementary Table S2a). Of the 671,751 non-CpG sites that were non-zero methylated in the discovery dataset, 667,922 (99.4%) were covered with over 30 reads across all 12 samples in the validation dataset, and 586,435 of those were also non-zero methylated (Supplementary Table S3).

Median methylation levels for the 671,751 non-CpG sites (identified in discovery dataset) were between 6.3-7.2% for samples in the validation data (Supplementary Table S2(b) and Supplementary Figure S2). The distribution of non-CpG and CpG methylation in relation to genomic features was examined in the validation dataset (Supplementary Figure S3), finding a higher % of non-CpG sites vs CpG sites located within introns (37.7% vs. 28.9%) and a lower % in promoters (30.1% vs. 38.4%). These differences were slightly larger when comparing CpG sites specifically to CAC or CAT sites that were non-zero methylated (Supplementary Figure S3). Non-zero methylated CAC and CAT sites showed very similar distributions across genomic features (Supplementary Figure S3), but median methylation levels for CAC sites were consistently higher than at CAT sites (Supplementary Table S4). Promoter regions were defined as 2kbp upstream and 500bp downstream of transcriptional start sites.

Discovery data: Hierarchical cluster analysis of CpG and non-CpG methylation

Hierarchical cluster analysis (HCA) was performed on DNA methylation patterns in umbilical cord, cord blood and peripheral blood samples to investigate tissue-specific methylation patterns, and the relationship between inter- and intra-individual methylation. CpG methylation analysis was carried out on 1,222,537 sites, for which a minimum read depth of 30-fold across all 60 samples was available (Figure 1). DNAm at CpG sites was found to separate first by tissue type, with cord blood and peripheral blood samples from the same individual clustering together, disparate from a cluster of umbilical cord samples.

To determine whether non-CpG methylated sites would cluster samples similarly to CpG sites, hierarchical cluster analysis was applied to non-CpG DNAm. Data was available for ~9.8 million non-

CpG sites for which a minimum read depth of 30-fold was met across all 60 samples. This data contained a large proportion of unmethylated sites, so the dataset was limited to 'commonly methylated' non-CpG sites that had greater than 30 reads and non-zero methylation levels across all three tissue types in the twenty individuals in the study, identifying 671,751 non-zero methylated non-CpG sites across all 60 samples for use in further analysis. Hierarchical clustering revealed differences in the way that samples were clustered: Of the 60 samples, 10 samples clustered by tissue type – umbilical cord samples from 10 different individuals clustering together; 21 samples grouped by individual, with all 3 tissue samples (cord blood, umbilical cord, and peripheral blood) clustered together for 7 individuals; and 26 samples grouped into pairs of tissue, with cord blood and peripheral blood clustered together for 13 individuals – leaving 3 outlying samples (Supplementary Figure S4).

Sequence context of non-CpG methylated sites influences inter-tissue and inter-individual hierarchal clustering

Analysing all non-zero non-CpG sites together combines cytosines from a range of different underlying sequence contexts, which may obscure specific patterns in their DNA methylation profiles, and it has been previously suggested that the cytosine sequence context (CHG and CHH, in the 5' to 3' direction, where H=A, C, or T) may have an influence on methylation patterns in mammals [37]. Non-CpG methylation was therefore separated into 12 different cytosine contexts and analysed separately: CTG, CAG, CCG, CTT, CAT, CCT, CTA, CAA, CCA, CTC, CAC, and CCC (Supplementary Table S3). Hierarchical cluster analysis revealed clear differences in clustering patterns depending on the adjacent DNA sequence of the non-CpG sites. Dendrograms for non-zero methylated CAC (n=141,674), CTC (n=27,559), and CAT (n=68,866) sites are shown in Figures 2(a-c). For the other nine non-CpG cytosine specific contexts, samples still showed some clustering by tissue or individual, but displayed less distinct clustering patterns and are shown in Supplementary Figure S5(a-i).

For cytosines in CAC or CTC context, samples clustered by tissue type with DNA samples from cord blood and peripheral blood clustering together in each individual, but separately from umbilical cord tissue (Figures 2a-b). Samples were then paired by individuals within the cluster of peripheral and cord blood, with cytosines in CAC context pairing all 20 individuals, and 19 of 20 individuals pairing using CTC sites. In cluster analysis using DNAm occurring at CAT sites, samples grouped predominately by individual, with DNA samples from cord tissue, cord blood and peripheral blood forming triads by individuals (Figure 2c). Non-zero methylated CAT sites showed a different pattern of clustering (separation by individuals), compared with using non-zero methylation data exclusively from CAC or CTC sites (tissue separation).

Given these observations that clustering patterns are affected by different cytosine contexts of non-CpG DNAm, we next examined different cytosine contexts for CpG methylation to determine whether clustering of samples varied between CGA, CGC, CGG, or CGT methylation. Differences were seen between the four analyses, whereby CGT and CGG methylation sites were able to separate out first by umbilical cord tissue, and then successfully cluster all remaining peripheral and cord blood samples by pairing individuals. CGC and CGA sites were similar to CGT and CGG sites, but within the 40 samples

of peripheral and cord blood, not all samples were clustered by individuals (Supplementary Figure S6(a-d)).

Different cytosine contexts in non-CpG methylation cluster analysis – validation data

Having seen that using non-zero non-CpG methylation sites could generate different clustering patterns depending on the genomic sequence adjacent to the cytosines, we wanted to examine whether this phenomenon could be validated in an independent dataset. In order to test not only whether this pattern would occur in a different set of individuals, but also in a tissue type not analysed in the discovery data, HCA was conducted in a dataset of twelve samples consisting of four individuals in duplicate using muscle tissue, and cord blood data from a pooled DNA sample carried out in two duplicate pairs. Dendrograms were created using the subset of 671,751 non-CpG sites that were non-zero methylated in the discovery dataset, provided these sites had over 30 reads in the validation dataset. Figure 3 shows dendrograms for three different cytosine contexts: CAC, CTC, and CAT. This shows that methylation at these CAC sites clustered samples by tissue type first, then individuals within muscle tissue (as with the discovery dataset), whereas methylation at these CAT sites clustered samples by individuals, with no initial separation of muscle from cord blood samples.

Discussion

Little is known to date on the functional significance of non CpG methylation. In this study we examined non-CpG DNAm across multiple tissues from the same individuals to better understand differences in tissue specificity and inter-individual variability of non CpG methylation. We found that hierarchical cluster analysis, using DNAm data from non-CpG sites in cord blood, peripheral blood, and umbilical cord clustered samples by individual and/or separated certain tissue types. If measured non-CpG DNAm were purely the result of randomness in the epigenome, the expected result from our hierarchical clustering would be samples clustering at random or not at all. This demonstrates that non-CpG methylation is not just occurring randomly in the genome, but that non-CpG methylation patterns can differ by tissue type and that these differences may in part be driven by an individual's genomic or environmental exposures.

In addition to this, non-CpG methylation in certain genomic contexts (e.g. CAC) separated samples by tissue type, grouping samples from different individuals into an umbilical cord cluster, and then grouping cord blood and peripheral blood together from each individual. A similar pattern was observed when analysing CpG sites. However, using non-zero DNAm at CAT sites, predominately all three samples from an individual clustered together (17 of 20 individuals clustered in their triplicates) rather than separating by tissue type; suggesting that some non-CpG DNAm sites are more tissue-specific and others more susceptible to individual effects. Using the subset of methylated non-CpG sites identified from the discovery analysis phase, the concept of cytosine sequence context driving tissue or individual based clustering (for CAC and CAT, respectively) was validated in an independent dataset using cord blood and muscle tissue – a tissue type which had not been used in the discovery data. This suggests that the subset of non-zero methylated non-CpG DNAm sites identified here may have relevance across several tissue types and a broad spectrum of people.

The distribution of non-CpG and CpG sites differed in relation to genomic features, especially within intronic and promoter regions. It is also worth noting that even though non-zero methylated CAC and CAT sites clustered samples differently (by tissue or by individual, respectively), these sites show very similar distributions across genomic features. This suggests that the differences in tissue/individual clustering patterns using methylated CAC and CAT sites may be due to varying levels of methylation across these sites, rather than their distribution in relation to genomic features. Where samples cluster separately by tissue type using non-zero methylated CAC sites, cord and peripheral blood were found to cluster together, indicating a similar methylation profile within these tissue types. This is suggestive of lineage specific non-CpG methylation patterns that have potentially been maintained from a common precursor cell type.

Existing literature on non-CpG methylation is very limited, focusing mainly on stem cells and brain tissue. In addition, studies on non-CpG methylation are mostly limited to two base pair sequence context in the 5' to 3' direction (CpA, CpC, or CpT) [38-40]. Here, we present evidence that a three base pair cytosine sequence context can display either tissue-specific methylation (CAC), individual-specific

297 methylation patterns (CAT), or show no clear clustering by tissue or individual (CAA and CAG). This
298 suggests that restricting analysis of non-CpG methylation data to a two base pair context may be
299 grouping together disparate methylation patterns (e.g. CpA = CAT, CAC, CAA and CAG), and therefore
300 concealing important differences connected to the third base in the triplicate.

301 CpG sites are symmetrical, whereby there is a Cytosine and Guanine on the complementary strand in
302 the 3' to 5' direction, and if methylated, CpG methylation generally occurs on both strands (reciprocal
303 methylation). As a result, CpG methylation can be maintained during cell replication by DNA
304 methyltransferase 1 (DNMT1) [41]. However, it has been shown that some CpG sites are hemi-
305 methylated, and that CpG hemi-methylation can be inherited over several cell divisions, suggesting
306 that, although most hemi-methylated CpG sites become fully methylated during cell divisions, hemi-
307 methylation in some CpG sites may be a stable epigenetic state [42]. CHH sites, such as CAC, are not
308 symmetrical and so any methylation occurring at CHH sites is also hemi-methylated. As samples in our
309 study maintained a tissue/individual specific signature using only subgroups of CHH methylation, this
310 suggests there may exist some form of active maintenance of methylation for non-symmetrical non-
311 CpG sites too.

312 In the discovery data, samples 3 and 4 from umbilical cord clustered separately from all other samples
313 when using non-CpG data. These samples displayed noticeably higher non-CpG methylation values
314 than any other samples, but the reason for such deviation is not known. Interestingly, these individuals
315 did not cluster separately when using CpG data, or when using non-CpG data from cord blood or
316 peripheral blood; it is only non-CpG sites from umbilical cord samples for these individuals that differed
317 in methylation. Umbilical cord is a heterogeneous mixture of tissues types [43], so it is possible that
318 more of a particular tissue type that contains higher levels of non-CpG methylation was present in the
319 aliquot of umbilical cord tissue used for these two individuals. Another explanation could be potential
320 unknown environmental factors, but this would imply that those factors only affected non-CpG DNAm
321 specifically in umbilical cord tissue samples, and not any other measured methylation.

322 One of the strengths of this study is the increased coverage of the methylome provided by Agilent
323 SureSelect data compared with more widely used methods such as the Infinium 850K EPIC array,
324 which only covers ~850,000 methylation sites and is focused on CpG sites. Our SureSelect Methyl-seq
325 dataset contains methylation data on ~2.52 million CpG sites (>30x read-depth), or 1,222,537 CpG
326 sites when selecting CpG sites with over 30 reads across all 60 samples in the discovery dataset;
327 coverage of non-CpG sites was ~17.6 million (>30x read-depth) or 671,751 sites when selecting non-
328 zero percent methylated sites with over 30 reads across all 60 samples in the discovery dataset.

329 A further strength of this study is the multiple different tissue types for each individual, thus allowing for
330 comparisons across tissue types and individuals. Having access to two independent cohorts with
331 SureSelect data on CpG and non-CpG data was also advantageous and made it possible for us to
332 validate our findings from the discovery data. One more novel aspect of this study is the tissue types
333 used, which are not commonly examined for their non-CpG methylation status: cord blood, umbilical

cord, muscle and peripheral blood samples - tissue samples that are generally quite accessible to researchers.

The examination of CGA, CGC, CGG, and CGT sites suggests that, in contrast to observations of cytosine contexts of non-CpG sites, the cytosine context of CpG sites may have less of an effect on the methylation values, and that the clear differences between tissue and individual-driven clustering seen in cytosine sequence contexts may be unique to non-CpG methylation.

One of the limitations of this study is the sequence-based nature of the SureSelect assay, meaning that in a separate SureSelect assay, not all the sites identified in this study will be guaranteed to meet the minimum read-depth cut-off of >30-fold that we used. This would make it difficult for other researchers to replicate our observations using exactly the same non-CpG sites as us. However, we saw 99.4% of non-zero methylated non-CpG sites in our discovery dataset in our validation dataset (with over 30-reads). Even if the subset of non-CpG sites identified by other researchers does not overlap exactly with those used in this study, one option could be to use a subset of the non-CpG sites identified here. In the analysis here, we have presented data in relation to non-CpG methylation in the context of trinucleotides (CHG and CHH sites) as non-CpG methylation in this context has been the most widely reported [14, 19-21]; there is some evidence that suggests additional nucleotides outside of CHG and CHH sites may also play a role in determining methylation [14, 22], but this was outside the scope of our study.

In terms of measurement error on SureSelect platform, Teh et al. [36] have previously shown that, using a 30x read-depth coverage and 1µg of DNA, 71% of probes agreed to within an absolute difference of 5% methylation with the replicate sample, and this increased to 91% agreeing within 10% methylation. We see a very similar level of agreement at methylated non-CpG sites (validation data shown in Supplementary Table S1) with an overall average of 93.4% of data agreeing to within 10% methylation. Median methylation levels for the 671,751 non-CpG sites (identified in discovery dataset) are between 6.3-7.2% in the validation data and measurement error on the array is not negligible compared with this. However, despite the impact of possible measurement error from the array and relatively low levels of methylation across non-CpG DNAm sites compared with CpG sites, we still saw our data clustering in meaningful ways when restricted to only non-zero methylated non-CpG sites.

The process outlined in this paper identified a subset of non-CpG sites that are commonly methylated across 20 individuals and in each of their three tissue types. The results were validated in an independent dataset including the use of previously unused tissue types; this suggests that there may exist a subset of non-CpG sites that are commonly methylated within the population and also across multiple tissue types. Therefore, similar to our approach of using previously untested tissue type in our validation data, other researchers may be able to examine these same non-CpG sites without necessarily having similar cohort or similar tissue types to those seen in this study.

Although the functionality of non-CpG methylation has not been comprehensively explained in mammals, it is clear that non-CpG methylation profiles can be used to differentiate between tissue types

and between individuals. In addition, certain subsets of non-CpG methylation sites are better able to differentiate between tissue types, while others are able to more easily differentiate between individuals. More research is needed to gain insight as to why data from some non-CpG contexts cluster by individuals and others principally by tissue type, and also what functional significance these, or any other, non-CpG sites may have in development of health and disease.

Ethics statement: The HSSe received ethical approval from the Hertfordshire Research Ethics Committee. In the SWS, the recruitment of women, follow-up through pregnancy, follow-up of the children, and sample collection/analysis were carried out under Institutional Review Board approval (Southampton and SW Hampshire Research Ethics Committee) with written informed consent. In both studies clinical investigations were conducted according to the principles expressed in the 1964 Declaration of Helsinki.

Funding: BHF Programme Grant PG/14/33/30827. KMG is supported by the UK Medical Research Council (MC_UU_12011/4), the National Institute for Health Research (NIHR Senior Investigator (NF-SI-0515-10042), NIHR Southampton 1000DaysPlus Global Nutrition Research Group (17/63/154) and NIHR Southampton Biomedical Research Centre (IS-BRC-1215-20004)), the European Union (Erasmus+ Programme ImpENSA 598488-EPP-1-2018-1-DE-EPPKA2-CBHE-JP), the US National Institute On Aging of the National Institutes of Health (Award No. U24AG047867) and the UK ESRC and BBSRC (Award No. ES/M00919X/1).

Conflicts of interest: KMG has received reimbursement for speaking at conferences sponsored by companies selling nutritional products, and are part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone. PT, RM, MHewitt, EA, CC, KMG, KAL, MHanson, SJB are part of academic research programmes that have received research funding from Abbott Nutrition, Nestec and Danone.

Acknowledgements

The authors acknowledge the use of the IRIDIS High Performance Computing Facility, and associated support services at the University of Southampton, in the completion of this work.

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Tables and Figures

All tables and figures are property of the author.

Table 1: Summary of CpG and Non-CpG sites with over 30 reads by tissue type in discovery dataset. Summary tables with number of CpG and Non-CpG sites with over 30 reads by tissue type: **a)** in at least one individual **b)** in all individuals and **c)** having non-zero methylation in all individuals.

Figure 1: **Hierarchical cluster analysis on CpG sites in discovery dataset.** Analysis carried out on 1,222,537 CpG sites with >30x read-depth in each of the 60 samples. Cluster dendrograms shows separation of umbilical cord tissue (green), and remaining samples grouped by pairs (cyan) of individuals' cord blood (CB) and 12-13 year peripheral blood (peripheral) samples.

Figure 2 (a-c): Three dendrograms of non-CpG methylation sites in discovery dataset. Hierarchical cluster analysis was carried out using methylation sites with >30x read-depth across all 60 samples with at least one methylated read (non-zero methylated sites). Where all three tissues from an individual clustered together in triplicates (magenta), pairs of peripheral blood and cord blood samples grouped by individual (cyan), umbilical cord samples not clustering by individual (green). UC = umbilical cord sample, CB = cord blood sample, peripheral= 12-13yr peripheral blood. Restricted to three separate cytosine sequence contexts: **(a)** CAC sites (n=141,674), **(b)** CTC sites (n=27,559), and **(c)** CAT sites (n=68,866).

Figure 3 (a-c): Dendrogram of non-CpG methylation in validation dataset. Muscle tissue samples (yellow) were assayed in four individuals in duplicate (1A/B, 2A/B, 3A/B, 4A/B), and one cord blood sample (red) from the SWS was assayed in quadruplicate (5A/B/C/D) (pooled from two individuals). Hierarchical cluster analysis was carried out on samples from validation dataset using non-CpG sites (>30x read-depth) overlapping with 671,751 non-zero non-CpG sites from discovery dataset, restricted to **(a)** 140,188 CAC sites, **(b)** 68,468 CAT sites, and **(c)** 27,469 CTC sites.

Table 1

a) Number of sites with over 30 reads in at least one individual

Environment	12-13 year peripheral blood	Cord blood	Umbilical cord	Across all 60 samples
CpG	2,448,736	2,397,488	2,418,723	2,518,311
Non-CpG	17,292,430	17,038,713	17,218,661	17,603,383

b) Number of sites with over 30 reads in *all* 20 individuals

	Overlap			
Environment	12-13 year peripheral blood	Cord blood	Umbilical cord	All 60 samples
CpG	1,334,219	1,433,637	1,397,930	1,222,537
Non-CpG	10,843,753	11,321,097	10,940,859	9,779,206

c) Number of sites with over 30 reads and non-zero methylation values in all 20 individuals

	Overlap of non-zero methylation			
Environment	12-13 year peripheral blood	Cord blood	Umbilical cord	All 60 samples
CpG	1,026,360	1,098,718	1,075,501	862,472
Non-CpG	1,657,153	1,923,066	2,633,428	671,751

Figure 1

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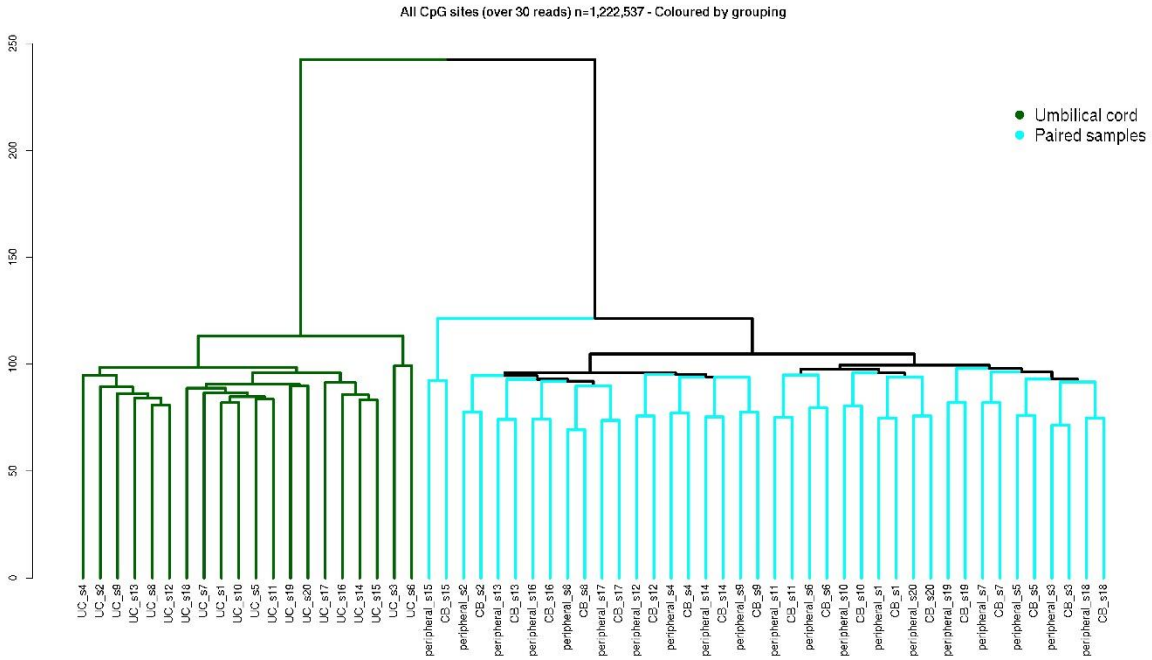

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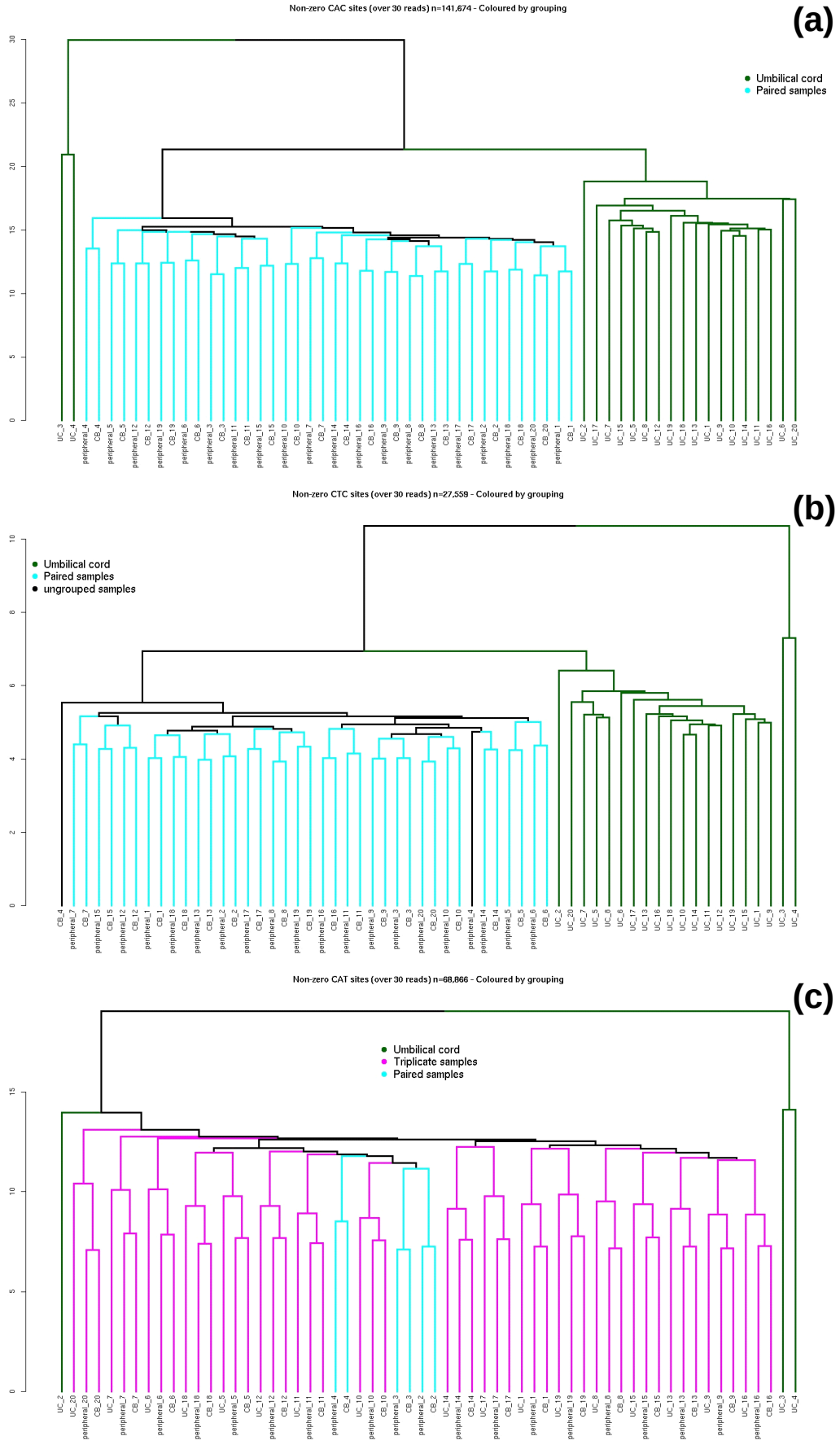

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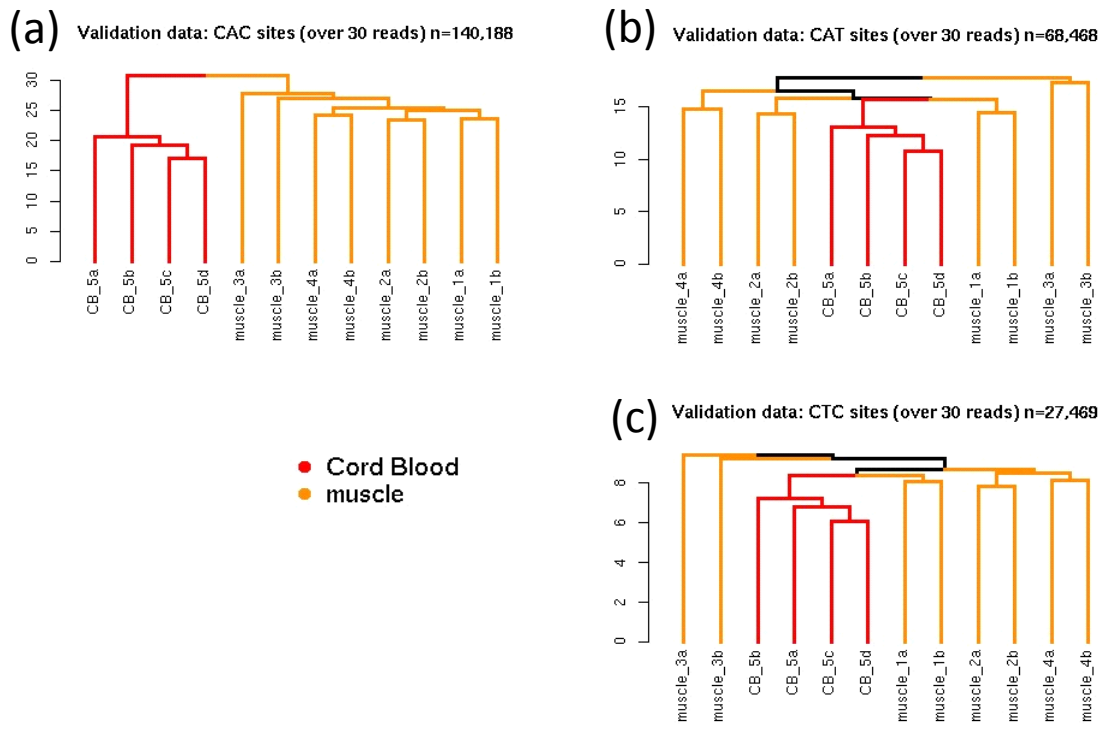

Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function.

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Abstract

DNA methylation (DNAm) in mammals is mostly examined within the context of CpG dinucleotides. Non-CpG DNAm is also widespread across the human genome, but the functional relevance, tissue-specific disposition, and inter-individual variability has not been widely studied. Our aim was to examine non-CpG DNAm in the wider methylome across multiple tissues from the same individuals to better understand non-CpG DNAm distribution within different tissues and individuals, and in relation to known genomic regulatory features.

DNA methylation in umbilical cord and cord blood at birth, and peripheral venous blood at age 12-13 years from twenty individuals from the Southampton Women's Survey cohort was assessed by Agilent SureSelect methyl-seq. Hierarchical cluster analysis (HCA) was performed on CpG and non-CpG sites, and stratified by specific cytosine environment. Analysis of tissue and inter-individual variation was then conducted in a second dataset of twelve samples: eight muscle tissue, and four aliquots of cord blood pooled from two individuals.

HCA using methylated non-CpG sites showed different clustering patterns specific to the three base pair triplicate (CNN) sequence. Analysis of CAC sites with non-zero methylation showed that samples clustered first by tissue type, then by individual (as observed for CpG methylation), while analysis using non-zero methylation at CAT sites showed samples grouped predominantly by individual. These clustering patterns were validated in an independent dataset using cord blood and muscle tissue.

This research suggests that CAC methylation can have tissue-specific patterns, and that individual effects, either genetic or unmeasured environmental factors, can influence CAT methylation.

Keywords: DNA methylation; Non-CpG; methylation; CpG; CHH; CHG; CAT; CAC; CNN; Hierarchical clustering analysis; HCA; cluster; tissue-specific; individual-specific; methylation patterns; human; umbilical cord; umbilical cord blood; muscle; peripheral blood; comparison

Introduction

Epigenetics, the study of changes in gene expression that occur without alterations in the nucleotide sequence, plays a fundamental role in regulating the accessibility of DNA to the transcriptional machinery, and the regulation of tissue-specific gene expression, as well as genomic imprinting and X chromosome inactivation in early development [1]. DNA methylation (DNAm) is a widely studied epigenetic modification, and is normally examined in the context of a CG dinucleotide (CpG), where the cytosine base can be modified at the 5th carbon position by the addition of a methyl (CH_3) group. DNAm in the CpG context has a well-established role in genomic regulation and control of gene expression, with evidence that altered CpG methylation may link early-life environmental exposures with later non-communicable diseases, such as cardiovascular disease or obesity [2, 3]. Animal models have shown how different environmental factors such as diet, exercise and stress can affect DNA methylation and gene expression [4-8].

DNA methylation outside the CpG context has been far less extensively studied, yet may be far more prevalent within the methylome; there are approximately 28 million CpG dinucleotides in the human genome, but in excess of 556 million cytosines in a non-CpG context (USCS, hg19). For example, in human and mouse central nervous system neurons, ~2-6% of non-CpG sites are methylated, with a mode of ~20-25% methylation across those sites, whereas methylation levels at methylated CpG sites are typically ~60-90% [9, 10]. However CpG dinucleotides are relatively underrepresented in the genome [11], leading to far greater numbers of non-CpG sites, with non-CpG methylation representing up to half of all the methylation present; a study by Woodcock et al. suggests that, in DNA from human spleen, up to 54.5% of all methylation present is in a non-CpG context [12]. Levels of non-CpG methylation may depend highly on tissue type, with higher levels of specific non-CpG methylation reported in neurones and human embryonic stem cells, but present at much lower levels in other tissue types [9, 10, 13-15]. Studies in plants have found that non-CpG methylation predominantly occurs at base-pair triplicates [16, 17], while studies in vertebrates examining non-CpG methylation have identified both symmetric CHG (H=A, C, or T) [18] and asymmetric CHH methylation patterns [14, 19], at conserved positions in the genome [20, 21], with the sequence flanking the cytosine position potentially modulating DNA methyltransferase 3A (DNMT3A)/DNMT3B binding, in conjunction with DNMT3L [22].

The role of non-CpG DNAm within the genome is unclear, and DNAm in different contexts may perform specific or overlapping functions. Non-CpG methylation has been linked to disease status such as in type 2 diabetes where increases in non-CpG methylation within the promoter of Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) were associated with impaired glucose tolerance. Moreover, PGC-1α non-CpG methylation was increased by free fatty acids, suggesting potential environmental modulation of the methylation status of these sites [23]. Non-CpG methylation has also been implicated in Alzheimer's disease (AD), where non-CpG methylation patterns within the promoter of *Presenilin1* in human brain tissue were inversely correlated with expression in AD samples [24], suggesting active demethylation of non-CpG methylation, epigenetically regulating gene expression.

Active demethylation of non-CpG methylation has been reported in other contexts [25], but remains understudied [26].

Differences in levels of non-CpG DNAm between human samples could be the result of stochastic change in the epigenome, but there are two main alternative explanations as to why non-CpG DNAm levels may differ in human samples. (1) Differences may exist between individuals: resulting from either environmental factors during development or an individual's genetic sequence, (2) non-CpG DNAm patterns may differ by tissue type due to developmental programming during cell differentiation. If differences in non-CpG DNAm are not just purely due to random processes then similarities in the patterns of non-CpG DNAm within tissue types or within individuals may be expected.

To investigate whether individual or tissue-specific factors may influence non-CpG methylation, here we have examined both CpG and non-CpG methylation in placental cord and cord blood at birth, as well as peripheral venous blood collected at 12-13 years in a group of individuals (n=20) from the Southampton Women's survey (SWS) cohort. Methylation data was captured using the Agilent SureSelectXT Human Methyl-Seq (SureSelect platform). Hierarchical clustering analysis (HCA) was applied to the CpG and non-CpG data to observe the clustering patterns. We then validated our findings

Methods

Discovery data

In the discovery dataset, a total of 60 samples from the Southampton Women's Survey (SWS) cohort, a UK prospective cohort study in which women were recruited before conception of the child [27], were interrogated by Agilent SureSelectXT Human Methyl-Seq capture and sequencing method (SureSelect). These 60 samples consisted of 20 individuals each analysed across three tissue types: cord blood, umbilical cord, and 12-13 year peripheral blood. Cord Blood consists of B cells, granulocytes, monocytes, natural killer cells, nucleated red blood cells, and CD4 & CD8 T cells, and peripheral venous blood contains B cells, neutrophils, monocytes, natural killer cells, and CD4 & CD8 T cells. Individuals were selected on the basis of having DNA from all three tissues available in sufficient quantities (1µg), were split equally by sex (10 males/10 females), and five individuals from each quarter of the % fat distribution from DXA measurements taken at age 8-9 years.

Validation data

For the validation dataset, twelve samples were interrogated by Agilent SureSelect in total, across five individuals. Muscle tissue samples from four males aged between 73-79 years from the Hertfordshire Sarcopenia Study (HSSe), a UK based cohort study [28], were assayed in duplicate (1A/B, 2A/B, 3A/B, 4A/B), and one cord blood sample (pooled from two individuals, both male) from the SWS was assayed as two duplicate pairs (5A/B and 5C/D). Agreement in methylation levels between duplicates using a subset of 671,751 non-zero methylated non-CpG sites was assessed showing on average 93.4% of sites agreeing to within 10% methylation, reproducibility statistics and sample DNA quantities can be seen in Supplementary Table S1.

DNA extraction

DNA from muscle biopsy samples was stored and extracted as described previously [29]. For umbilical cord, a 5–10 cm segment was cut from the mid portion of each cord, immediately following delivery, flushed with saline to remove fetal blood, flash-frozen in liquid nitrogen and stored at −80 °C until required for DNA isolation. Peripheral blood was stored at -80 °C until further processing. Genomic DNA from peripheral blood was extracted using QIAamp DNA Mini Kit (Qiagen, UK), following the manufacturers recommendations. Genomic DNA was prepared from umbilical cord, umbilical cord blood, and muscle tissue by a standard high salt method [30].

Agilent SureSelect Methyl-Seq data

Methyl-seq data was generated by the Centre for Genomic Research (CGR) at the University of Liverpool using the Agilent SureSelect platform [31]. Both the discovery data and the validation data were cleaned, processed and analysed using the same procedure detailed below. The data arrived as FASTQ files, trimming of adapters was performed using Cutadapt v1.2.1 with the option -O 3, so the 3' end of any reads which matched the adapter sequence for 3 base pairs or more were trimmed [32], and a minimum window quality score 20, using Sickle v1.2 [33]. Reads<10bp removed. The unmasked

human genome was downloaded from UCSC, and the genome hash table was built using Extended Randomised Numerical Aligner (ERNE) create [34]. The alignment against the genome was performed using ERNE-BS5 2 [35]. Unprocessed data contained paired-end reads, and singleton reads. Singleton reads result from one read of a pair failing the Sickle quality control. The singlet files contained sequences whose pair had been removed due to poor sequence quality or adapter contamination. SureSelect data in the discovery dataset and the validation dataset produced similar summary statistics. Paired reads aligned uniquely to the genome at a greater rate (88.8% & 85.0%) than singleton reads (66.1% & 59.3%), and singleton reads were negligible in number (0.76 & 1.37 million reads) compared with paired end reads (85.7 & 99.4 million reads) for discovery and validation datasets respectively. As a result of this, singleton reads were not included in any analysis. For each sample methylation calls, calculated by the number of methylated reads / total number of reads at each cytosine, were made using ERNE-METH 2 [35]. This provided the methylation level for each cytosine for each sample. Options '--annotations-bismark' and '--annotations-erne' were used during the methylation calling process to provide detailed cytosine context. Previous studies have demonstrated that reproducibility improvements are minimal beyond 30x read-depth [36], therefore a minimum read-depth of 30x was used for all downstream analyses. A flowchart summarising the steps from SureSelect library preparation to statistical analysis is shown in Supplementary Figure S1.

Statistical analysis

Data manipulation and summary statistics were created using Stata (version 15.0 & 16.0) and unix bash commands, and hierarchical cluster analysis was performed in R (version 3.5.1 & 3.6.1) using the 'hclust' command with complete linkage method and Euclidean distance as the metric to measure dissimilarity. Other linkage methods were also tested, with similar results ('average linkage method' and 'weighted pair group method with arithmetic mean'). For hierarchical cluster analysis on non-CpG methylation, non-CpG sites were restricted to 671,751 sites where methylation was >0 for all 60 samples, i.e. all 20 individuals across all three tissue types had non-zero methylation values at these sites; these 671,751 non-CpG sites are frequently referred to as 'non-zero' methylation sites.

Ethics

The HSSe received ethical approval from the Hertfordshire Research Ethics Committee. In the SWS, the recruitment of women, follow-up through pregnancy, follow-up of the children, and sample collection/analysis were carried out under Institutional Review Board approval (Southampton and SW Hampshire Research Ethics Committee) with written informed consent. In both studies clinical investigations were conducted according to the principles expressed in the 1964 Declaration of Helsinki.

Results

To understand tissue and individual differences in non-CpG methylation, DNA samples from 20 individuals in three tissue types (umbilical cord, cord blood, and peripheral blood) were interrogated for non-CpG (and CpG) methylation using Agilent SureSelect. Table 1 shows the number of sites for the CpG and non-CpG sites in the discovery dataset with over 30 fold read-depth, split by tissue type and those sites covered in all 60 samples. In the discovery dataset ~2.52 million CpG sites (>30x read-depth) were captured in at least one of the 60 samples, and similarly ~2.58 million in the validation dataset. When considering the number of CpG sites with over 30 reads across all 60 samples in the discovery dataset, the number reduced to 1,222,537 CpG sites. Over 17.6 million non-CpG sites (>30x read-depth) were captured in at least one of the 60 samples in the discovery dataset (and ~17.7 million in the validation dataset). The number of non-CpG sites with non-zero methylation in all of the 60 samples was 671,751, with a median methylation between 3.4% – 8.0% (median and 5^{th}-95^{th} percentile of methylation for each sample are shown in Supplementary Table S2a). Of the 671,751 non-CpG sites that were non-zero methylated in the discovery dataset, 667,922 (99.4%) were covered with over 30 reads across all 12 samples in the validation dataset, and 586,435 of those were also non-zero methylated (Supplementary Table S3).

Median methylation levels for the 671,751 non-CpG sites (identified in discovery dataset) were between 6.3-7.2% for samples in the validation data (Supplementary Table S2(b) and Supplementary Figure S2). The distribution of non-CpG and CpG methylation in relation to genomic features was examined in the validation dataset (Supplementary Figure S3), finding a higher % of non-CpG sites vs CpG sites located within introns (37.7% vs. 28.9%) and a lower % in promoters (30.1% vs. 38.4%). These differences were slightly larger when comparing CpG sites specifically to CAC or CAT sites that were non-zero methylated (Supplementary Figure S3). Non-zero methylated CAC and CAT sites showed very similar distributions across genomic features (Supplementary Figure S3), but median methylation levels for CAC sites were consistently higher than at CAT sites (Supplementary Table S4). Promoter regions were defined as 2kbp upstream and 500bp downstream of transcriptional start sites.

Discovery data: Hierarchical cluster analysis of CpG and non-CpG methylation

Hierarchical cluster analysis (HCA) was performed on DNA methylation patterns in umbilical cord, cord blood and peripheral blood samples to investigate tissue-specific methylation patterns, and the relationship between inter- and intra-individual methylation. CpG methylation analysis was carried out on 1,222,537 sites, for which a minimum read depth of 30-fold across all 60 samples was available (Figure 1). DNAm at CpG sites was found to separate first by tissue type, with cord blood and peripheral blood samples from the same individual clustering together, disparate from a cluster of umbilical cord samples.

To determine whether non-CpG methylated sites would cluster samples similarly to CpG sites, hierarchical cluster analysis was applied to non-CpG DNAm. Data was available for ~9.8 million non-

CpG sites for which a minimum read depth of 30-fold was met across all 60 samples. This data contained a large proportion of unmethylated sites, so the dataset was limited to 'commonly methylated' non-CpG sites that had greater than 30 reads and non-zero methylation levels across all three tissue types in the twenty individuals in the study, identifying 671,751 non-zero methylated non-CpG sites across all 60 samples for use in further analysis. Hierarchical clustering revealed differences in the way that samples were clustered: Of the 60 samples, 10 samples clustered by tissue type – umbilical cord samples from 10 different individuals clustering together; 21 samples grouped by individual, with all 3 tissue samples (cord blood, umbilical cord, and peripheral blood) clustered together for 7 individuals; and 26 samples grouped into pairs of tissue, with cord blood and peripheral blood clustered together for 13 individuals – leaving 3 outlying samples (Supplementary Figure S4).

Sequence context of non-CpG methylated sites influences inter-tissue and inter-individual hierarchal clustering

Analysing all non-zero non-CpG sites together combines cytosines from a range of different underlying sequence contexts, which may obscure specific patterns in their DNA methylation profiles, and it has been previously suggested that the cytosine sequence context (CHG and CHH, in the 5' to 3' direction, where H=A, C, or T) may have an influence on methylation patterns in mammals [37]. Non-CpG methylation was therefore separated into 12 different cytosine contexts and analysed separately: CTG, CAG, CCG, CTT, CAT, CCT, CTA, CAA, CCA, CTC, CAC, and CCC (Supplementary Table S3). Hierarchical cluster analysis revealed clear differences in clustering patterns depending on the adjacent DNA sequence of the non-CpG sites. Dendrograms for non-zero methylated CAC (n=141,674), CTC (n=27,559), and CAT (n=68,866) sites are shown in Figures 2(a-c). For the other nine non-CpG cytosine specific contexts, samples still showed some clustering by tissue or individual, but displayed less distinct clustering patterns and are shown in Supplementary Figure S5(a-i).

For cytosines in CAC or CTC context, samples clustered by tissue type with DNA samples from cord blood and peripheral blood clustering together in each individual, but separately from umbilical cord tissue (Figures 2a-b). Samples were then paired by individuals within the cluster of peripheral and cord blood, with cytosines in CAC context pairing all 20 individuals, and 19 of 20 individuals pairing using CTC sites. In cluster analysis using DNAm occurring at CAT sites, samples grouped predominately by individual, with DNA samples from cord tissue, cord blood and peripheral blood forming triads by individuals (Figure 2c). Non-zero methylated CAT sites showed a different pattern of clustering (separation by individuals), compared with using non-zero methylation data exclusively from CAC or CTC sites (tissue separation).

Given these observations that clustering patterns are affected by different cytosine contexts of non-CpG DNAm, we next examined different cytosine contexts for CpG methylation to determine whether clustering of samples varied between CGA, CGC, CGG, or CGT methylation. Differences were seen between the four analyses, whereby CGT and CGG methylation sites were able to separate out first by umbilical cord tissue, and then successfully cluster all remaining peripheral and cord blood samples by pairing individuals. CGC and CGA sites were similar to CGT and CGG sites, but within the 40 samples

of peripheral and cord blood, not all samples were clustered by individuals (Supplementary Figure S6(a-d)).

Different cytosine contexts in non-CpG methylation cluster analysis – validation data

Having seen that using non-zero non-CpG methylation sites could generate different clustering patterns depending on the genomic sequence adjacent to the cytosines, we wanted to examine whether this phenomenon could be validated in an independent dataset. In order to test not only whether this pattern would occur in a different set of individuals, but also in a tissue type not analysed in the discovery data, HCA was conducted in a dataset of twelve samples consisting of four individuals in duplicate using muscle tissue, and cord blood data from a pooled DNA sample carried out in two duplicate pairs. Dendrograms were created using the subset of 671,751 non-CpG sites that were non-zero methylated in the discovery dataset, provided these sites had over 30 reads in the validation dataset. Figure 3 shows dendrograms for three different cytosine contexts: CAC, CTC, and CAT. This shows that methylation at these CAC sites clustered samples by tissue type first, then individuals within muscle tissue (as with the discovery dataset), whereas methylation at these CAT sites clustered samples by individuals, with no initial separation of muscle from cord blood samples.

Discussion

Little is known to date on the functional significance of non CpG methylation. In this study we examined non-CpG DNAm across multiple tissues from the same individuals to better understand differences in tissue specificity and inter-individual variability of non CpG methylation. We found that hierarchical cluster analysis, using DNAm data from non-CpG sites in cord blood, peripheral blood, and umbilical cord clustered samples by individual and/or separated certain tissue types. If measured non-CpG DNAm were purely the result of randomness in the epigenome, the expected result from our hierarchical clustering would be samples clustering at random or not at all. This demonstrates that non-CpG methylation is not just occurring randomly in the genome, but that non-CpG methylation patterns can differ by tissue type and that these differences may in part be driven by an individual's genomic or environmental exposures.

In addition to this, non-CpG methylation in certain genomic contexts (e.g. CAC) separated samples by tissue type, grouping samples from different individuals into an umbilical cord cluster, and then grouping cord blood and peripheral blood together from each individual. A similar pattern was observed when analysing CpG sites. However, using non-zero DNAm at CAT sites, predominately all three samples from an individual clustered together (17 of 20 individuals clustered in their triplicates) rather than separating by tissue type; suggesting that some non-CpG DNAm sites are more tissue-specific and others more susceptible to individual effects. Using the subset of methylated non-CpG sites identified from the discovery analysis phase, the concept of cytosine sequence context driving tissue or individual based clustering (for CAC and CAT, respectively) was validated in an independent dataset using cord blood and muscle tissue – a tissue type which had not been used in the discovery data. This suggests that the subset of non-zero methylated non-CpG DNAm sites identified here may have relevance across several tissue types and a broad spectrum of people.

The distribution of non-CpG and CpG sites differed in relation to genomic features, especially within intronic and promoter regions. It is also worth noting that even though non-zero methylated CAC and CAT sites clustered samples differently (by tissue or by individual, respectively), these sites show very similar distributions across genomic features. This suggests that the differences in tissue/individual clustering patterns using methylated CAC and CAT sites may be due to varying levels of methylation across these sites, rather than their distribution in relation to genomic features. Where samples cluster separately by tissue type using non-zero methylated CAC sites, cord and peripheral blood were found to cluster together, indicating a similar methylation profile within these tissue types. This is suggestive of lineage specific non-CpG methylation patterns that have potentially been maintained from a common precursor cell type.

Existing literature on non-CpG methylation is very limited, focusing mainly on stem cells and brain tissue. In addition, studies on non-CpG methylation are mostly limited to two base pair sequence context in the 5' to 3' direction (CpA, CpC, or CpT) [38-40]. Here, we present evidence that a three base pair cytosine sequence context can display either tissue-specific methylation (CAC), individual-specific

methylation patterns (CAT), or show no clear clustering by tissue or individual (CAA and CAG). This suggests that restricting analysis of non-CpG methylation data to a two base pair context may be grouping together disparate methylation patterns (e.g. CpA = CAT, CAC, CAA and CAG), and therefore concealing important differences connected to the third base in the triplicate.

CpG sites are symmetrical, whereby there is a Cytosine and Guanine on the complementary strand in the 3' to 5' direction, and if methylated, CpG methylation generally occurs on both strands (reciprocal methylation). As a result, CpG methylation can be maintained during cell replication by DNA methyltransferase 1 (DNMT1) [41]. However, it has been shown that some CpG sites are hemi-methylated, and that CpG hemi-methylation can be inherited over several cell divisions, suggesting that, although most hemi-methylated CpG sites become fully methylated during cell divisions, hemi-methylation in some CpG sites may be a stable epigenetic state [42]. CHH sites, such as CAC, are not symmetrical and so any methylation occurring at CHH sites is also hemi-methylated. As samples in our study maintained a tissue/individual specific signature using only subgroups of CHH methylation, this suggests there may exist some form of active maintenance of methylation for non-symmetrical non-CpG sites too.

In the discovery data, samples 3 and 4 from umbilical cord clustered separately from all other samples when using non-CpG data. These samples displayed noticeably higher non-CpG methylation values than any other samples, but the reason for such deviation is not known. Interestingly, these individuals did not cluster separately when using CpG data, or when using non-CpG data from cord blood or peripheral blood; it is only non-CpG sites from umbilical cord samples for these individuals that differed in methylation. Umbilical cord is a heterogeneous mixture of tissues types [43], so it is possible that more of a particular tissue type that contains higher levels of non-CpG methylation was present in the aliquot of umbilical cord tissue used for these two individuals. Another explanation could be potential unknown environmental factors, but this would imply that those factors only affected non-CpG DNAm specifically in umbilical cord tissue samples, and not any other measured methylation.

One of the strengths of this study is the increased coverage of the methylome provided by Agilent SureSelect data compared with more widely used methods such as the Infinium 850K EPIC array, which only covers ~850,000 methylation sites and is focused on CpG sites. Our SureSelect Methyl-seq dataset contains methylation data on ~2.52 million CpG sites (>30x read-depth), or 1,222,537 CpG sites when selecting CpG sites with over 30 reads across all 60 samples in the discovery dataset; coverage of non-CpG sites was ~17.6 million (>30x read-depth) or 671,751 sites when selecting non-zero percent methylated sites with over 30 reads across all 60 samples in the discovery dataset.

A further strength of this study is the multiple different tissue types for each individual, thus allowing for comparisons across tissue types and individuals. Having access to two independent cohorts with SureSelect data on CpG and non-CpG data was also advantageous and made it possible for us to validate our findings from the discovery data. One more novel aspect of this study is the tissue types used, which are not commonly examined for their non-CpG methylation status: cord blood, umbilical

cord, muscle and peripheral blood samples - tissue samples that are generally quite accessible to researchers.

The examination of CGA, CGC, CGG, and CGT sites suggests that, in contrast to observations of cytosine contexts of non-CpG sites, the cytosine context of CpG sites may have less of an effect on the methylation values, and that the clear differences between tissue and individual-driven clustering seen in cytosine sequence contexts may be unique to non-CpG methylation.

One of the limitations of this study is the sequence-based nature of the SureSelect assay, meaning that in a separate SureSelect assay, not all the sites identified in this study will be guaranteed to meet the minimum read-depth cut-off of >30-fold that we used. This would make it difficult for other researchers to replicate our observations using exactly the same non-CpG sites as us. However, we saw 99.4% of non-zero methylated non-CpG sites in our discovery dataset in our validation dataset (with over 30-reads). Even if the subset of non-CpG sites identified by other researchers does not overlap exactly with those used in this study, one option could be to use a subset of the non-CpG sites identified here. In the analysis here, we have presented data in relation to non-CpG methylation in the context of trinucleotides (CHG and CHH sites) as non-CpG methylation in this context has been the most widely reported [14, 19-21]; there is some evidence that suggests additional nucleotides outside of CHG and CHH sites may also play a role in determining methylation [14, 22], but this was outside the scope of our study.

In terms of measurement error on SureSelect platform, Teh et al. [36] have previously shown that, using a 30x read-depth coverage and 1µg of DNA, 71% of probes agreed to within an absolute difference of 5% methylation with the replicate sample, and this increased to 91% agreeing within 10% methylation. We see a very similar level of agreement at methylated non-CpG sites (validation data shown in Supplementary Table S1) with an overall average of 93.4% of data agreeing to within 10% methylation. Median methylation levels for the 671,751 non-CpG sites (identified in discovery dataset) are between 6.3-7.2% in the validation data and measurement error on the array is not negligible compared with this. However, despite the impact of possible measurement error from the array and relatively low levels of methylation across non-CpG DNAm sites compared with CpG sites, we still saw our data clustering in meaningful ways when restricted to only non-zero methylated non-CpG sites.

The process outlined in this paper identified a subset of non-CpG sites that are commonly methylated across 20 individuals and in each of their three tissue types. The results were validated in an independent dataset including the use of previously unused tissue types; this suggests that there may exist a subset of non-CpG sites that are commonly methylated within the population and also across multiple tissue types. Therefore, similar to our approach of using previously untested tissue type in our validation data, other researchers may be able to examine these same non-CpG sites without necessarily having similar cohort or similar tissue types to those seen in this study.

Although the functionality of non-CpG methylation has not been comprehensively explained in mammals, it is clear that non-CpG methylation profiles can be used to differentiate between tissue types

and between individuals. In addition, certain subsets of non-CpG methylation sites are better able to differentiate between tissue types, while others are able to more easily differentiate between individuals. More research is needed to gain insight as to why data from some non-CpG contexts cluster by individuals and others principally by tissue type, and also what functional significance these, or any other, non-CpG sites may have in development of health and disease.

Ethics statement: The HSSe received ethical approval from the Hertfordshire Research Ethics Committee. In the SWS, the recruitment of women, follow-up through pregnancy, follow-up of the children, and sample collection/analysis were carried out under Institutional Review Board approval (Southampton and SW Hampshire Research Ethics Committee) with written informed consent. In both studies clinical investigations were conducted according to the principles expressed in the 1964 Declaration of Helsinki.

Funding: BHF Programme Grant PG/14/33/30827. KMG is supported by the UK Medical Research Council (MC_UU_12011/4), the National Institute for Health Research (NIHR Senior Investigator (NF-SI-0515-10042), NIHR Southampton 1000DaysPlus Global Nutrition Research Group (17/63/154) and NIHR Southampton Biomedical Research Centre (IS-BRC-1215-20004)), the European Union (Erasmus+ Programme ImpENSA 598488-EPP-1-2018-1-DE-EPPKA2-CBHE-JP), the US National Institute On Aging of the National Institutes of Health (Award No. U24AG047867) and the UK ESRC and BBSRC (Award No. ES/M00919X/1).

Conflicts of interest: KMG has received reimbursement for speaking at conferences sponsored by companies selling nutritional products, and are part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone. PT, RM, MHewitt, EA, CC, KMG, KAL, MHanson, SJB are part of academic research programmes that have received research funding from Abbott Nutrition, Nestec and Danone.

Acknowledgements

The authors acknowledge the use of the IRIDIS High Performance Computing Facility, and associated support services at the University of Southampton, in the completion of this work.

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Tables and Figures

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Table 1: Summary of CpG and Non-CpG sites with over 30 reads by tissue type in discovery dataset. Summary tables with number of CpG and Non-CpG sites with over 30 reads by tissue type: **a)** in at least one individual **b)** in all individuals and **c)** having non-zero methylation in all individuals.

Figure 1: **Hierarchical cluster analysis on CpG sites in discovery dataset.** Analysis carried out on 1,222,537 CpG sites with >30x read-depth in each of the 60 samples. Cluster dendrograms shows separation of umbilical cord tissue (green), and remaining samples grouped by pairs (cyan) of individuals' cord blood (CB) and 12-13 year peripheral blood (peripheral) samples.

Figure 2 (a-c): Three dendrograms of non-CpG methylation sites in discovery dataset. Hierarchical cluster analysis was carried out using methylation sites with >30x read-depth across all 60 samples with at least one methylated read (non-zero methylated sites). Where all three tissues from an individual clustered together in triplicates (magenta), pairs of peripheral blood and cord blood samples grouped by individual (cyan), umbilical cord samples not clustering by individual (green). UC = umbilical cord sample, CB = cord blood sample, peripheral= 12-13yr peripheral blood. Restricted to three separate cytosine sequence contexts: **(a)** CAC sites (n=141,674), **(b)** CTC sites (n=27,559), and **(c)** CAT sites (n=68,866).

Figure 3 (a-c): Dendrogram of non-CpG methylation in validation dataset. Muscle tissue samples (yellow) were assayed in four individuals in duplicate (1A/B, 2A/B, 3A/B, 4A/B), and one cord blood sample (red) from the SWS was assayed in quadruplicate (5A/B/C/D) (pooled from two individuals). Hierarchical cluster analysis was carried out on samples from validation dataset using non-CpG sites (>30x read-depth) overlapping with 671,751 non-zero non-CpG sites from discovery dataset, restricted to **(a)** 140,188 CAC sites, **(b)** 68,468 CAT sites, and **(c)** 27,469 CTC sites.