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Age-associated DNA methylation changes in immune genes, histone modifiers and chromatin remodeling factors within 5 years after birth in human blood leukocytes

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Abstract

Background: Age-related changes in DNA methylation occurring in blood leukocytes during early childhood may reflect epigenetic maturation. We hypothesized that some of these changes involve gene networks of critical relevance in leukocyte biology and conducted a prospective study to elucidate the dynamics of DNA methylation. Serial blood samples were collected at 3, 6, 12, 24, 36, 48 and 60 months after birth in ten healthy girls born in Finland and participating in the Type 1 Diabetes Prediction and Prevention Study. DNA methylation was measured using the HumanMethylation450 BeadChip.

Results: After filtering for the presence of polymorphisms and cell-lineage-specific signatures, 794 CpG sites showed significant DNA methylation differences as a function of age in all children (41.6% age-methylated and 58.4% age-demethylated, Bonferroni-corrected P value <0.01). Age-methylated CpGs were more frequently located in gene bodies and within +5 to +50 kilobases (kb) of transcription start sites (TSS) and enriched in developmental, neuronal and plasma membrane genes. Age-demethylated CpGs were associated to promoters and DNase-I hypersensitivity sites, located within −5 to +5 kb of the nearest TSS and enriched in genes related to immunity, antigen presentation, the polycomb-group protein complex and cytoplasm.

Conclusions: This study reveals that susceptibility loci for complex inflammatory diseases (for example, IRF5, NOD2, and PTGER4) and genes encoding histone modifiers and chromatin remodeling factors (for example, HDAC4, KDM2A, KDM2B, JARID2, ARID3A, and SMARCD3) undergo DNA methylation changes in leukocytes during early childhood. These results open new perspectives to understand leukocyte maturation and provide a catalogue of CpG sites that may need to be corrected for age effects when performing DNA methylation studies in children.

Keywords: Age-modified CpG, Childhood, DNA methylation, Genes, Leukocytes, Longitudinal

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Background
Methylation of cytosines to 5-methylcytosines in the context of CpG dinucleotides is an important epigenetic modification that regulates gene expression and cell-specific functions. Some DNA methylation signatures are maintained during mitosis and contribute to the so-called 'epigenetic memory', which determine cell lineage. Other DNA methylation patterns are very dynamic, change during lifetime and mediate several physiological events such as cell differentiation, cell maturation and tissue-specific gene expression [1,2]. From early developmental stages through senescence, CpG sites are methylated by DNA methyltransferases (DNMT3a/DNMT3b and DNMT1) [3] and demethylated either passively or by active mechanisms implicating 5-hydroxymethylation, ten-eleven translocator (TET) proteins and thymidine glycosidases [4,5]. Studies in diverse human tissues have demonstrated that DNA methylation levels are modified as a function of age [6-10]. Indeed, it is possible to predict the age of a tissue based on its methylation signatures on a broad number of CpG sites [6,9,11-13]. Most studies investigating age-associated DNA methylation changes have been performed in adults and from the perspective of cell senescence, longevity, cancer, stem cell functions and chronological age [12,14-19]. Still, few studies have documented the dynamics of DNA methylation during early childhood [20-23].

It is known that increasing age leads to genome-wide demethylation in transposable repetitive elements (including Alu and L1) as well as in gene coding regions [19,24,25]. Increasing age is also associated to increased methylation of certain CpGs in specific gene families, CpG islands [26], polycomb (PcG) target genes [27] and promoters with bivalent chromatin domains [28]. Age-associated changes in DNA methylation have been implicated in tumour development and certain chronic diseases [29]. The recognition of age-modified CpG sites in infants is essential to identify genes that might be epigenetically modified during this period of life and, if disturbed, might contribute to the susceptibility to complex inflammatory diseases in childhood. The identification of age-modified CpG sites during early childhood is also important, because early exposure to environmental factors such as pollutants and pesticides might alter the methylation levels of inflammatory genes and these signatures may be sustained during years, possibly predisposing to disease [30,31]. The aims of this study were the following: 1) to identify CpG sites with longitudinal changes in DNA methylation levels within 3 to 60 months after birth in healthy children and 2) to annotate the genomic distribution and functional relationships of age-modified CpG sites during early childhood. The present study provides a catalogue of 794 age-modified CpG sites that robustly reflect the changes in DNA methylation levels that occur in human blood leukocytes within 3 to 60 months after birth. Notably, we found that the genomic location of age-modified CpG sites differs depending whether the CpGs become age methylated or age demethylated. The functional annotation of the genes containing age-modified loci indicated that methylation changes related to age may not be due only to a stochastic DNA methylation drift but rather correspond to a programme with potential functional relevance in leukocyte biology during this period of life.

Results
We analysed the longitudinal changes in DNA methylation in a total of 60 samples at 3, 6, 12, 24, 36, 48 and 60 months after birth, using serial DNA samples extracted from peripheral blood leukocytes of ten healthy girls participating in the Finnish Type 1 Diabetes Prediction and Prevention Study (DIPP) (Table 1). DNA methylation levels were measured in 485,577 CpG sites distributed in 99% of the annotated RefSeq genes using the HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) [32]. DNA methylation levels were log2 transformed to M values and then statistically evaluated using limma [33]. A single procedure consisting of two steps was used to infer the association between age and DNA methylation. In the first step, a linear model was used considering the age and the individual (repeated samples from the same person); the study of the variance was performed but no list of differentially methylated probes was generated. Then, the information on the variance was utilized as prior for the second step of the analysis, which consisted of a moderated t-test carried out comparing the DNA methylation in samples at 3 months vs the samples at 60 months. We found 853 CpG sites with significant differential methylation due to age (Bonferroni-corrected P value <0.01). Of these, 476 CpGs were exclusively affected by age and 377 CpGs were affected by both age and individual (Figure 1A). Since single nucleotide polymorphisms (SNPs) in the probe sequence may affect methylation measurements, all age-modified CpG sites containing a SNP within the probe with a minor allele frequency (MAF) above 0.01 in the Finnish population were filtered out (n = 48). Moreover, to avoid the confounding effects of CpG sites that are differentially methylated among leukocyte populations due to cell lineage (cell specific), the 853 age-modified CpG sites were contrasted against a list of 2,228 CpG sites with significant differential DNA methylation in sorted leukocytes [34], which serve as cell-type classifiers. Eleven age-modified CpG sites were found in this list and therefore excluded. After these filtering steps, 794 age-modified CpG sites remained for further analyses (330 age-methylated sites and 464 age-demethylated sites) (Figure 1B). The detailed list of age-modified CpG sites and fold changes of M values and P values is found in Additional file 1.

Age-modified CpG sites were found in all autosomes with frequencies that correlated with the distribution of
probes in the assay \((r = 0.86, P < 0.0001, \text{Figure 1C})\) except for the X chromosome which had only one age-modified CpG site in the 5’ UTR of the gene encoding claudin 2 (chrX: 106161451, \(P_{\text{Bonf}} = 3.34 \times 10^{-6}\)). Considering that this chromosome contains 11,232 of all tested probes (2.3%), our finding reproduces previous observations suggesting that the X chromosome is ‘reluctant’ to methylation changes over time [20,22]. Furthermore, age-modified CpG sites were most frequently located in RNA coding genes than in intergenic regions. There were no deviations from the expected proportions according to the distribution of probes in the 450 K assay between age-methylated and age-demethylated sites (Figure 1D).

The effects of age on the DNA methylation levels of these sites were supported by the identification of genes having at least two age-modified CpG sites (range two to six sites) spanning over stretches of sequence from few base pairs (bp) up to kilobases (mean 19.7 ± 51.1 kb). If at least two CpG sites showed the same methylation trend in a given loci, they configure an age-modified region. Nowadays, the length of a differentially methylated region or the number of CpG sites that they should contain is debated; therefore in the present study, we adopted this more global definition to consider a broader sequence length and the tendency of the age effects. Genes containing age-demethylated regions are presented in Table 2, and genes containing age-demethylated regions are presented in Table 3. Further support on these findings was suggested by the detection of age-modified CpG sites in genes belonging to the same families but encoded on separate chromosomes, for instance the homeobox cluster A on chromosome 7p15.2 (HOXA3 and HOXA10) and the homeobox cluster B on chromosome 17q21.3 (HOXB6) (Additional files 1 and 2).

Since age-modified CpG sites were detected in whole blood, we further investigated their cell-type specific annotations according to the Illumina manifest. First, none of the 794 age-modified CpG sites was annotated to cancer-specific DMR (c-DMR). However, 12 age-modified CpG sites were annotated to cancer-specific DMR (c-DMR) and 62 CpG sites to reprogramming-specific DMRs (r-DMR) [35]. Based on the regulatory feature group, 15.8% of the 794 age-modified CpG sites was annotated to cancer-specific DMR (c-DMR) and 62 CpG sites to reprogramming-specific DMRs (r-DMR) [35].

Table 1 Descriptive information on the study individuals \((n = 10)\)

| Child number | Date of birth | HLA-DR-DQ haplotype | Risk class \(a\) | Mode of delivery | Maternal smoking during pregnancy | Age at end of exclusive breast-feeding (months) | Age at end of total breast-feeding (months) | Samples (time points) included in the analysis after QC |
|--------------|---------------|----------------------|----------------ler class | Mode of delivery | Maternal smoking during pregnancy | Age at end of exclusive breast-feeding (months) | Age at end of total breast-feeding (months) | Samples (time points) included in the analysis after QC |
| 1            | 2000.03.21    | DRB1*04:01-DQB1*03:02/DRB1*04:04-DQB1*03:02 | 3 | Caesarean section | No | <3 | 3 m, 12 m, 24 m, 36 m, 48 m, 60 m |
| 2            | 2000.04.10    | DRB1*04:04-DQB1*03:02/(DR1/10)-DQB1*05:01 | 3 | Caesarean section | No | 2.2 | 3, 5 | 24 m, 60 m |
| 3            | 2002.04.18    | DRB1*04:04-DQB1*03:02/(DR1/10)-DQB1*05:01 | 3 | Vaginal         | No | <5 | 7 to 11 | 3 m, 6 m, 12 m, 24 m, 48 m, 60 m |
| 4            | 2002.05.16    | DRB1*04:04-DQB1*03:02/(DR7)-DQA1*02:01-DQB1*03:03 | 1 | Vaginal         | Yes | 0.2 | 10.5 | 3 m, 6 m, 12 m, 24 m, 48 m, 60 m |
| 5            | 2002.08.04    | DRB1*04:01-DQB1*03:02/DRB1*04:04-DQB1*03:02 | 3 | Vaginal         | No | 5.0 | 10.0 | 3 m, 6 m, 12 m, 24 m, 36 m, 48 m, 60 m |
| 6            | 2002.08.21    | DRB1*04:01-DQB1*03:02/(DR9)-DQA1*03-DQB1*03:03 | 3 | Vaginal         | No | <3 | 13 to 17 | 3 m, 12 m, 24 m, 48 m, 60 m |
| 7            | 2002.10.04    | DRB1*04:01-DQB1*03:02/(DR8)-DQB1*04 | 3 | Vaginal         | No | 3.0 | 8.0 | 3 m, 6 m, 12 m, 24 m, 36 m, 48 m, 60 m |
| 8            | 2002.10.29    | DRB1*04:01-DQB1*03:02/(DR1/10)-DQB1*05:01 | 3 | Caesarean section | No | <3 | 8 to 11 | 3 m, 6 m, 12 m, 24 m, 36 m, 48 m, 60 m |
| 9            | 2002.11.20    | DRB1*04:03-DQB1*03:02/(DR13)-DQB1*06:01 | 0 | Vaginal         | No | 5.5 | 9.0 | 3 m, 6 m, 12 m, 24 m, 36 m, 48 m, 60 m |
| 10           | 2002.11.21    | DRB1*04:04-DQB1*03:02/(DR1/10)-DQB1*05:01 | 3 | Vaginal         | No | 2.0 | 2.7 | 3 m, 6 m, 12 m, 24 m, 36 m, 48 m, 60 m |

\(a\)Risk for T1D classified in five classes from decreased risk (0) to strongly increased risk (4) as presented in Hekkala et al [50].

HLA = human leukocyte antigen; m = months.
result of differences in cell composition. In contrast, 7.4% of all the age-modified CpG sites had a difference of at least two units in M value between the mononuclear fraction and the granulocyte fraction (Figure 1E), suggesting that methylation at those age-modified CpG sites is much variable between mononuclear cells and granulocytes, and therefore they are more susceptible to be affected by cell heterogeneity.

The genomic distribution of age-modified CpG sites

The chromosomal distribution of the age-modified CpG sites according to their Bonferroni-corrected P value ($p_{\text{bonf}}$) is presented in Figure 2A. Genes containing the most significant age-modified CpG sites in peripheral blood leukocytes within 5 years after birth are annotated in the figure ($p_{\text{bonf}}$ below $6.5 \times 10^{-8}$). The Illumina identifier is presented for three age-methylated CpG sites without any transcripts.
### Table 2 Age-methylated regions within 3 to 60 months after birth in blood leukocytes

| Gene symbol | Gene name | Function | Locus | Number of CpGs | Illumina ID | Region length (bp) |
|-------------|-----------|----------|-------|----------------|-------------|-------------------|
| TTC22       | Tetratricopeptide repeat domain 22 | Mediate protein-protein interactions and chaperone activity | 1p32.3 | 3 | cg24550149, cg15645660, cg11949335 | 454 |
| SPEG        | SPEG complex locus | Myocyte cytoskeletal development and marker of differentiated vascular smooth cells | 2q35 | 3 | cg14074251, cg26530345, cg02557933 | 51,924 |
| SNED1       | Sushi, nidogen and EGF-like domains 1 | Membrane-bound signalling molecule; hormonal regulation | 2q37.3 | 5 | cg02532017, cg07644939, cg25241559, cg19075225, cg17053285 | 34,671 |
| TRIM7       | Tripartite motif containing 7 | Ubiquitin protein ligase; Initiation of glycogen synthesis | 5q35.3 | 2 | cg17279652, cg26600753 | 170 |
| DDR1        | Discoidin domain receptor tyrosine kinase 1 | Regulation of cell growth, differentiation and metabolism; cell communication with environment | 6p21.3 | 3 | cg16215084, cg00934322, cg09965419 | 238 |
| TNXB        | Tenascin XB | Anti-adhesive effect; matrix maturation during wound healing | 6p21.3 | 3 | cg19071976, cg16662408, cg02657865 | 51,088 |
| MAD1L1      | MAD1 mitotic arrest deficient-like 1 | Mitotic spindle-assembly checkpoint; cell cycle control and tumour suppression | 7p22 | 6 | cg13700912, cg06555468, cg19513987, cg16026522, cg09174162, cg00963171 | 182,950 |
| UPP1        | Uridine phosphorylase 1 | Phosphorolysis of uridine to free bases and ribose-1-phosphate | 7p12.3 | 3 | cg14983135, cg10317717, cg21484940 | 170 |
| ZNFS03      | Zinc finger protein 503 | TF; transcriptional regulation; neural precursor cell proliferation | 10q22.2 | 2 | cg13997975, cg03487027 | 553 |
| DGKZ        | Diacylglycerol kinase, zeta | Kinase; regulate diacylglycerol levels in intracellular signal transduction | 11p11.2 | 2 | cg18098017, cg09802018 | 3,940 |
| B4GALNT1    | Beta-1,4-N-acetyl-galactosaminyl transferase 1 | Biosynthesis of G(M2) and G(D2) glycosphingolipids | 12q13.3 | 2 | cg09932758, cg25663970 | 1,936 |
| BTBD11      | BTB (POZ) domain containing 11 | Transcription cofactor; Protein heterodimerization activity (?) | 12q23.3 | 3 | cg27567561, cg13935577, cg01478234 | 566 |
| TEP1        | Testis, prostate and placenta expressed | Unknown | 16q21 | 2 | cg12499872, cg00491255 | 91 |
| CNTNAP1     | Contactin-associated protein 1 | Recruitment and activation of intracellular signalling pathways in neurons | 17q21 | 2 | cg16308533, cg11629889 | 39 |
| TBCD        | Tubulin folding cofactor D | Folding of beta-tubulin | 17q25.3 | 2 | cg16555866, cg0063986 | 35,310 |
mapped to their position (intergenic), including the most significant age-modified CpG at chr. 22:28074071 (cg16331674, $P_{\text{bonf}} = 8.1 \times 10^{-11}$). The majority of the top significant age-methylated CpG sites were also homogeneously methylated in sorted peripheral blood leukocytes from healthy adults (showed with an asterisk in Figure 2A). Furthermore, we found that many of the top significant age-modified CpG sites were embedded into age-modified regions (see Figure 2A, Tables 2 and 3). Examples of the time trends for age effects on DNA methylation in methylated and demethylated sites are presented in Figure 2B. Overall, the kinetics of the DNA methylation changes over time differed according to each site. Some CpGs were initially unmethylated (M value below −1) and became methylated (M value above 1) while other CpGs had M values above 1 that further increased over time (Figure 2B).

Since the majority of age-modified CpG sites were associated to a known transcript (Figure 1D) and their location can provide insights on their putative biological relevance, we analysed the genomic distribution of the 794 age-modified CpG sites according to their proximity to a CpG island and other genomic regulatory features like DNAse I hypersensitivity sites (DHSs) and enhancers. The annotation to be inside a CpG island was significantly over-represented in age-methylated CpG sites (20.9%) compared to age-demethylated sites (12.9%) ($\chi^2 = 8.44, P = 0.003$), Figure 3A. There were no differences in the distribution of age-modified CpG sites with regard to CpG island shores (39.6% vs 33.6%, $P = 0.08$) or the ‘open sea’ (37.9% vs. 33.6%, $P = 0.21$) (Figure 3A). Regarding the connection of age-modified CpG sites with regulatory features, age-demethylated CpG sites were more frequently found in DHS (26.7% vs 14.5%, $\chi^2 = 12.4, P = 0.0004$) and promoter-associated regions (29.7% vs 33% $\chi^2 = 88.2, P < 0.00001$) than in age-demethylated sites (Figure 3B). There were no differences in the distribution of age-modified CpG sites within enhancers or known differentially methylated regions (DMRs, Figure 3B).

**Differential TSS relationship between age-methylated and age-demethylated sites**

We then investigated the distribution of age-modified CpG sites according to their position within the gene structure. Provided that any given CpG site can be annotated to a gene in more than one accession number (for instance, in case of isoforms or anti-sense transcripts), all locations associated to an age-modified CpG (TSS1500, TSS200, 5′UTR, 1st exon, gene body, 3′UTR and intergenic) were included in the analysis. We found that age-methylated CpG sites were over-represented in the gene body compared to age-demethylated CpG sites (52.5% vs 34.9%, $\chi^2 = 39.8, P < 0.0001$), and age-demethylated CpG sites were more frequently annotated within 1,500 bp of the transcriptional start site (TSS) compared to age-demethylated sites (22.4% vs 8.93%, $\chi^2 = 41.3, P < 0.0001$), Figure 3C. To obtain further insights on their relationship with promoter regions, we calculated the position (upstream or downstream) and distance of each site to its nearest TSS. The distribution binned by the absolute distance revealed that about half of the age-demethylated CpG sites spanned within 0 to 5 kilobases (kb) of a TSS compared to age-demethylated CpG sites (51.7% vs 32.1%, $\chi^2 = 30.1, P = 0.0001$). Conversely, age-methylated CpG sites were more frequently annotated from 5 to 50 kb of a TSS (42.1% vs 32.3%, $\chi^2 = 7.0, P = 0.004$) and from 50 to 500 kb (27.7% vs 15.9%, $\chi^2 = 11.5, P = 0.0007$), Figure 3D. We also found differences in the proportions regarding directionality to the TSS (upstream/downstream): age-demethylated sites were more frequent within −5 to +5 kb and age-demethylated sites within +5 to +50 kb downstream of the TSS (Figure 3E).

It is still a matter of debate whether age-associated changes in DNA methylation are biologically relevant.

| Table 2 Age-methylated regions within 3 to 60 months after birth in blood leukocytes (Continued) |
|-----------------------------------------------|
| **NFIX** | Nuclear factor I/X CCAAT-binding transcription factor | Transcription factor (TF) | 19p13.3 | 4 | cg06458248 |
|         | | | | | cg27392771 |
|         | | | | | cg01634146 |
|         | | | | | cg10767662 |
| **LRFN1** | Leucine-rich repeat and fibronectin type III domain containing 1 | Promotes neurite outgrowth in hippocampal neurons. Regulates and maintain excitatory synapses | 19q13.2 | 2 | cg26910511 |
|         | | | | | cg25156118 |
| **TMC2** | Transmembrane channel-like 2 | Ion channel; expression in the inner ear suggests that it may be crucial for normal auditory function | 20p13 | 3 | cg12233487 |
|         | | | | | cg23648082 |
|         | | | | | cg03243506 |
| **CLDNS** | Claudin 5 | Claudin (physical barrier to solutes); membrane protein and tight junctions | 22q11.21 | 2 | cg0463638 |
|         | | | | | cg14553765 |

It is still a matter of debate whether age-associated changes in DNA methylation are biologically relevant.
| Gene symbol | Gene name                                                                 | Function                                                                                   | Locus               | Number of CpGs | Illumina ID                      | Region length (bp) |
|------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|---------------------|----------------|----------------------------------|-------------------|
| PRDM16     | PR domain containing 16                                                  | TF; zinc finger transcription factor (KRAB box)                                            | 1p36.23-p33         | 4              | cg17001566, cg12436196, cg01418153, cg03254465 | 249,737           |
| CITED4     | Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 | Transcriptional co-activator; CBP and p300 binding; co-activator of AP2                   | 1p34.2              | 2              | cg08719289, cg10705800            | 42                |
| ATOH8      | Atonal homolog 8                                                         | TF, DNA binding, transcriptional regulation; nuclease                                      | 2p11.2              | 3              | cg05318142, cg08079596, cg05584950 | 13,349            |
| HDAC4      | Histone deacetylase 4                                                   | Histone deacetylase; reductase; transcriptional repression when tethered to a promoter    | 2q37.3              | 3              | cg05870586, cg15058210, cg05903736 | 362               |
| CLEC3B     | C-type lectin domain family 3, member B (tetranectin)                    | Extracellular matrix structural protein                                                   | 3p22-p21.3          | 3              | cg02396676, cg22505962, cg0617855 | 224               |
| B3GALT4    | UDP-Gal: betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4        | Glycosyltransferase; synthesis of type 1 carbohydrate chains. Biosynthesis of ganglioseries glycolipid. | 6p21.3              | 2              | cg17103217, cg06362282            | 172               |
| NFE2L3     | Nuclear factor (erytroid-derived 2)-like 3                              | TF; binding of antioxidant response elements in target genes.                            | 7p15.2              | 2              | cg14684457, cg1036999            | 143               |
| CUX1       | Cut-like homeobox 1                                                      | TF; DNA binding protein. Regulate gene expression, morphogenesis, differentiation and cell cycle progression | 7q22.1              | 3              | cg10692693, cg05910443, cg03310593 | 82                |
| NACC2      | NACC family member 2, BEN and BTB (POZ) domain containing                | Histone deacetylase                                                                      | 9q34.3              | 2              | cg14147151, cg14126392            | 37,942            |
| BLOC1S2    | Biogenesis of lysosomal organelles complex-1, subunit 2                  | Dehydrogenase; formation of lysosome-related organelles                                   | 10q24.31            | 2              | cg26610808, cg15298486            | 5                 |
| HCCA2      | YY1-associated protein 1                                                | TF                                                                                       | 11q22               | 3              | cg01469847, cg20973931, cg12007048 | 13,771            |
| ADRBK1     | Adrenergic, beta, receptor kinase 1                                      | Phosphorylation of beta-2-adrenergic receptor                                            | 11q13.1             | 2              | cg13924996, cg11436362            | 100               |
| SHANK2     | SH3 and multiple ankyrin repeat domains 2                               | Molecular scaffold in the postsynaptic density                                            | 11q13.2             | 2              | cg11155924, cg27643147            | 68,036            |
| PSTPIP1    | Proline-serine-threonine phosphatase interacting protein 1              | CD2 binding protein; CD2-triggered T cell activation; membrane trafficking regulatory protein | 15q24.3             | 2              | cg26227523, cg21322248            | 1,804             |
| GPRC5C     | G protein-coupled receptor, family C, group S, member C                 | G-protein coupled receptor; cellular effects of retinoic acid (?)                        | 17q25               | 3              | cg12776171, cg26663490, cg16120833 | 157               |
| MGAT5B     | Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl glucosaminyltransferase, isoenzyme B | Synthesis of complex cell surface N-glycans                                             | 17q25.2             | 2              | cg23838005, cg05514299            | 66                |

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We evaluated which biological processes, cellular components and molecular functions were related to genes containing age-modified CpG sites (Additional file 3) and if there were known interactions between the age-modified loci. Induced network analysis using the combined list of age-methylated and age-demethylated loci revealed that several of these genes were known to interact within protein–protein complexes or biochemical reactions (Figure 4). The over-representation analyses were also performed with separated lists as an attempt to dichotomize relevant biological functions that might be specific to age-methylated and age-demethylated loci, and these results are explained below.

**Genes containing age-methylated CpG sites code for products involved in development, cell adhesion and the plasma membrane**

Gene ontology (GO) analysis revealed that age-methylated loci were significantly over-represented in the biological processes of development and morphogenesis of anatomical structures (Figure 5A and Additional file 4). We also found that genes having age-methylated CpGs were over-represented in neuronal-related functions (Figure 4A). The GO annotations of neuron part (GO:00077458, 20 genes), axon part (GO:0033267, seven genes) and neuron projection (GO:0043005, 17 genes) were the most significant in the enrichment based on cell components (Additional file 4). The over-representation of age-methylated loci within neuronal genes was also supported by the enrichment in the biological processes of transmission of nerve impulse (GO:0019226, 18 genes) and neuron precursor cell proliferation (GO:0061351, five genes), Figure 5A and Additional file 4. Another two highly significant annotations for age-methylated loci included the plasma membrane (GO:0005886, 62 genes) and cell adhesion (GO:0007155, 20 genes), Figure 5A.

**Age-demethylated sites were enriched in GO categories of response to diverse stimuli, immune effector processes and the cytoplasm**

Genes containing age-demethylated CpG sites in blood leukocytes were significantly enriched in the biological processes of (1) response to diverse stimuli including microorganisms, chemicals and organic substances; (2) positive regulation of biological process; (3) immune effector process; and (4) cell communication and signalling, Figure 5B. Detailed information on the gene ontology enrichment for age-demethylated loci is presented in Additional file 5. Furthermore, genes harbouring age-demethylated sites were significantly enriched in the cellular components: cytoplasm (GO:0005737, 194 genes), intracellular-membrane-bound organelles (GO:0043231, 191 genes) and the Golgi apparatus (GO:0044431, 22 genes). Altogether, this indicates that demethylation in blood leukocytes within 3 to 60 months after birth is mainly related to the interaction of the cells with the environment and the development of immune effector responses. As shown in Figure 5B, we found that age-demethylated CpGs were enriched in genes of the major histocompatibility protein complex (MHC, chr. 6p21.3), including type I (HLA-B, HLA-C) and type II alleles (HLA-MA, HLA-DPB1) as well as the MHC class I polypeptide-related sequence A (MICA). We also found age-demethylated loci in genes encoding defensins (DEFA4, DEFB132), prostaglandin receptors (PTGER2, PTGER4), members of the tumour necrosis factor superfamily (TNFAIP8L1, TNFRSF8, TNFES1), interleukin 18 binding protein (IL18BP), interferon regulatory factor 5 (IRF5), leukotriene B4 receptor (LTB4R), the CD2 ligand on T cells (CD58) and pattern recognition receptors (NOD2). The longitudinal changes in DNA methylation levels for some CpG sites located in immune genes are presented in Figure 6. GO analysis also revealed that age-demethylated CpG sites were enriched in genes from the PcG protein complex (CBX7, RNF2, KDM2B, JARID2, PHF1), Figure 5B and Additional file 5.

**Age-modified CpG sites spanned over genes encoding chromatin remodelling factors and transcription factors**

Together with the PcG complex, we found age-modified CpG sites in genes encoding histone modifiers and chromatin remodelling factors. These included the lysine-specific ‘K’ histone demethylases with F box domains (KDM2A and KDM2B), AT-rich interaction domains containing proteins (JARID2 and ARID3A), the structure-specific recognition protein 1 (SSRP1), the SP140 nuclear body protein-like

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**Table 3 Age-demethylated regions within 3 to 60 months after birth in blood leukocytes (Continued)**

| Gene   | Description                                      | Chromosome | Start | Length |
|--------|--------------------------------------------------|------------|-------|--------|
| ARID3A | AT-rich interactive domain 3A (BRIGHT-like)      | 19p13.3    | 4     | 722    |
| TEF    | Thyrotrophic embryonic factor                    | 22q13.2    | 2     | 419    |
| TSPO   | Translocator protein (18 kDa) Steroid hormone synthesis | 22q13.31  | 2     | 722    |
| JARID2 |                                                   |            |       |        |
| ARID3A |                                                   |            |       |        |
| PTGER4 |                                                   |            |       |        |
| et al. Clinical Epigenetics                |            |       |        |
Figure 2 Chromosomal distribution and DNA methylation trends of the significant age-modified CpG sites. (A) Dot plot showing the chromosomal distribution of age-methylated CpGs (blue dots) and age-demethylated CpGs (red dots) in relation to the Bonferroni-corrected P value. For methylated genes: TTC22 = tetratricopeptide repeat domain 22; NES = nestin; NGEF = neuronal guanidine nucleotide exchange factor; SNED1 = sushinidogen and EGF-like domains 1; FOXD2 = forkhead box 12; LAG3 = lymphocyte activation gene 3; CRYL1 = crystallin lambda 1; TEPP = testis prostate and placenta expressed; TSC2 = tuberous sclerosis 2; RHBDL3 = rhomboid, veinlet-like 3 (Drosophila); NFIX = nuclear factor I/X; TMC2 = transmembrane channel-like 2; SOX10 = SRY-box 10. For demethylated genes: ATOH8 = atonal homolog 8; CLEC3B = C-type lectin domain family 3, member B, NRG2 = neuregulin 2; PTK7 = protein tyrosine kinase 7; ANKR22 = ankyrin repeat domain 2; RPRK = RPRK-like; NOO2 = nucleotide-binding oligomerization domain containing 2; ARID3A = AT-rich interactive domain 3A; ZMYND8 = zinc finger, MYND-type containing 8; TSPO = translocator protein (18 kDa); CLDN2 = claudin 2. An asterisk next to the gene symbol indicates that the age-modified CpG site has similar DNA methylation levels in sorted blood leukocytes of healthy adults. Genes in bold indicate that the annotated CpG site is embedded in an age-modified region. Detailed information on P values is presented in Additional file 1. (B) Time trends in DNA methylation (M value) for age-methylated sites (blue) and age-demethylated sites (red). M values above 1 represent that the site is methylated, and M values below −1 represent that the site is demethylated. A value of 0 is proportional to a beta value of 0.50. Each line represents a CpG site.
(SP140L) and the gene SMARCD3 involved in the ATP-dependent chromatin remodelling complex (specific of neuronal progenitors). The known interactions for nine age-modified loci involved in chromatin remodelling are presented in Figure 7A. Some of these genes had more than one CpG site modified by age that followed the same trends of age-related changes (Figure 7B and Table 3). The DNA methylation changes over time in six genes annotated as chromatin/DNA binding proteins are presented in Figure 7C.

In addition, we found longitudinal changes in DNA methylation in several genes encoding transcription factors (TFs). A table with the annotation of the TF genes harbouring age-modified CpG sites is presented in Additional file 6. As expected, several CpG sites were found in TFs involved in development such as fork head boxes (FOXI2, FOXK1 and FOXK2), T-boxes (TBX1 and TBX2), ANTP/HOX1 homeoboxes (HOXA10, HOXA3, HOXB6), the SRY-related HMG box (SIX10), ANTP/NKL homeoboxes (VENTX, NXX2) and CUT homeoboxes (CLIX1). Several TFs involved in granulocyte differentiation, B-cell immunity and cytokine response were found containing age-modified CpG sites (Additional file 6). These include the nuclear factor of activated T-cell 4 (NFATC4), the
interferon regulatory factor 5 (IRF5), the transcriptional regulator ERG (ERG), the nuclear hormone receptor RARA and the GATA zinc finger domain TF (GATA2). Induced network analysis using the list of genes having age-modified CpG sites revealed that several of these TF are known to interact with the proteins encoded by other age-modified genes as binary protein-protein interactions and/or biochemical reactions (Figure 4). With few exceptions, CpG sites that were age methylated in DIPP children were found methylated in adult blood, and CpG sites that were age demethylated in DIPP children were found demethylated in adult blood. A comparison of the DNA methylation levels (M values) between the children in this study and adult blood leukocytes is presented in Additional file 7.

Discussion

Here we present a prospective analysis on the dynamics of DNA methylation in peripheral blood leukocytes during early childhood. Our study includes data on seven time points (from 3 to 60 months after birth) from the same ten individuals and reveals that DNA methylation levels are modified as a function of age in at least 794 CpG sites distributed in RNA coding genes as well as intergenic regions (Figure 1D). Several age-modified CpG sites are located within the same gene and spread in regions from few base pairs to kilobases (Tables 2 and 3). Our findings indicate that DNA methylation changes related to age may not only be due to stochastic DNA methylation drift [14,36] but rather correspond to a programme with functional relevance in leukocyte biology. We previously described a group of differentially methylated CpG signatures related to the lineage of sorted blood leukocytes in healthy adults [34]. In the present study, we found CpG methylation signatures that change as a function of age within the first 5 years after birth, independently of the individual. It is worth noting that some genes associated to chronic inflammatory diseases (for example, NOD2, PTGER4, IRF5, ADAM33) contain age-modified CpG sites in blood leukocytes.

Increased DNA methylation is involved in silencing developmental genes [37]. We found that genes with age-methylated CpGs are enriched in biological processes related to embryonic development and cell adhesion, as well as with the plasma membrane (Figure 5A and Additional file 4). Among the most important observations from this study is the differential genomic distribution of age-methylated CpG sites, which are more frequently located within 5 to 50 kb from the TSS and over-represented in gene bodies and intragenic CpG islands (Figure 3). This is very interesting because intragenic methylation can predict gene expression levels, it is crucial in regulating
isoform splicing in neuronal genes [38] and it is over-represented in genes that guide the formation of junctions in the motor neurons [39]. We also found that CpG sites that are age methylated in blood leukocytes are commonly located in genes related to neuronal functions. Several of those (for example, NEGF, SEPT5, PDE2A,) show detectable mRNA expression in brain tissues but not in sorted blood leukocytes (Figure 8A). Besides, some genes related
to immune functions were age methylated (for example, *IL17RD*) reflecting that in human leukocytes, differences in DNA methylation are tightly related with cell differentiation and commitment to lymphoid and myeloid lineages [40].

On the other hand, demethylation in promoter regions is known to facilitate gene expression [41]. Previous studies have shown that age-demethylated sites from birth to the first 2 years are enriched in immune-related genes [22]. Our results replicate these findings and also show that genes harbouring age-demethylated CpGs are enriched in genes related to the response to diverse stimuli including endogenous compounds and organic and chemical substances (Figure 5B and Additional file 5).

Interestingly, age-demethylated CpGs were enriched in genes related to the cytoplasm, the intracellular organelles and the Golgi apparatus. These findings could in part be explained by demethylation of class I and class II MHC molecules as well as by demethylation of at least five enzymes involved in glycosylation pathways that are located in the Golgi apparatus (that is, *B3GALT4*, *GALNT14*, *ST6GAL2*, *FUT7* and *FUT3*). Moreover, we identified CpG sites in genes encoding histone modifiers and chromatin remodelling factors that become demethylated in blood leukocytes by increasing age. The implicated molecules have histone demethylase activity (*JARID2*, *KDM2A* and *KDM2B*) and histone deacetylase activity (*HDAC4*, *NACC2*) (Figure 7). The demethylation
of genes encoding histone demethylases may contribute to the dynamic changes that occur in blood leukocytes during this period of life and may facilitate their maturation towards subpopulations. For instance, global DNA methylation remodelling has been observed in the transition from naïve to memory T cells [42]. In this sense, age-modified loci may participate as functional intermediates in a cascade of events that contribute to leukocyte maturation. Connections to the epigenetic machinery are further suggested by the identification of five age-modified CpG sites in genes encoding microRNAs: three age-methylated sites in MIR219-2, MIR183/MIR96 and MIRLET7A3/MIRLET7B and two age-demethylated sites in MIR10A and MIR574 (Additional file 1).

More studies are needed to investigate which mechanisms direct the methylation machinery to these age-

![Figure 7](https://example.com/figure7.png)

**Figure 7** DNA methylation levels within 3 to 60 months after birth in genes encoding histone modifiers and chromatin remodelers. (A) Protein interactions among genes related to the chromatin remodelling machinery that contain age-modified CpG sites; protein-protein interaction (orange line); biochemical reaction (green line); factors encoded by age-demethylated genes (red) and age-methylated genes (blue). (B) Longitudinal changes in DNA methylation for two CpG sites in the genes encoding for AT-rich interactive domain-containing protein 3A (ARID3A) and the histone deacetylase 4 (HDAC4); each dot represents an individual. (C) Longitudinal changes in DNA methylation for six genes involved in the chromatin remodelling; each dot represents an individual. KDM2A = lysine (K)-specific demethylase 2A; CBX7 = chromobox homolog 7; RNF2 = E3 ubiquitin-protein ligase RING2; SP140L = SP140 nuclear body protein-like; JARID2 = jumonji, AT-rich interactive domain 2; SMARCD3 = SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 3.
modified loci during this time window; and also to elucidate the connection between age-demethylated loci and mRNA expression in blood leukocytes. This study revealed that age-demethylated CpG sites are more frequently located in DHS, in promoters and in close proximity to the TSS (Figure 3), suggesting that these changes in methylation may be biologically relevant at the transcriptional level. We found significant GO categories related to the immune system, and using the FANTOM5 data [43], we observed that some age-demethylated genes are indeed expressed in peripheral blood leukocytes but not in other tissues (for example, PTGER4, Figure 8B and Additional file 8). In agreement with previous studies showing that age-induced differential methylation may occur without
changes in gene expression [44], we found genes with DNA methylation changes over time but without detectable differences in expression (Figure 8B and Additional file 8). Further studies are needed to elucidate which proportion of the age-associated changes in DNA methylation are part of a ‘programme’, how many are stochastic, which ones contribute to differential gene expression and how many are tissue independent or tissue specific.

Previous studies have found age-modified CpG sites that are restricted to certain tissues [45]. However, age-modified CpG sites have been detected in tissues that originate from distinct germ layers, suggesting that tissue-independent changes do occur. For instance, a common age-modified methylation module has been found in whole blood and brain tissue [46]; others have described common age-modified signatures within the whole blood, lung tissue and cervix [27], and studies in adult women revealed age-modified CpG sites in the blood that showed concordant patterns in other non-haematopoietic tissues [7]. Among the reported epigenetic biomarkers of ageing in adult's samples, we validated one age-demethylated CpG site in FHL2 (cg06320277, \( P_{\text{bonf}} = 8.44 \times 10^{-6} \)) but did not detect significant differences for other reported age biomarkers [11,12], suggesting that age-modified loci may differ between children and adults. We also found concordance with 34 age-modified CpG sites that were previously described by Alisch et al., in peripheral blood leukocytes in paediatric populations [20], and 11 differentially methylated CpG sites described by Martino et al., comparing mononuclear cells from cord blood and children age 1 year [22]. Common loci between ours and these studies included TSPO, GAL3ST1, BST2, ASB16, MARK2 and the inner-ear expressed genes OTOS (otospiralin) and TMC2. These common age-modified loci were identified in studies conducted in males [23] and females [22].

Provided that we filtered out cell-type-specific CpG sites from the list of age-modified CpGs and some of the age-modified CpG sites have been previously detected by using fractionated and unfractionated blood, it is less likely that compositional differences in cell counts may have affected these observations. Additional insights about common, non-tissue-specific, age-related methylation signatures were obtained from the identification of 29 CpG sites that were age modified in this study and also found differentially methylated in the buccal epithelium of twins between birth and the age of 18 months [21]. These sites mapped to 21 known genes including ARID3A, KLF9, NOD2, PRKCZ, SOX10, SPEG, TEP9, TRIM7, TTC22 and ZNF710. The gene ARID3A is very interesting because it was found containing four age-demethylated CpG sites in a region of 6.98 kb. This molecule is expressed in leukocytes of myeloid origin and is involved in normal embryogenesis and haematopoiesis. Observed age effects on the DNA methylation levels of ARID3A within the first 2 years of life have also been reported in children with a different genetic background and environmental setting [23], as well as in males [20]. Furthermore, the identification of age-modified CpG sites in several genes related to the formation of organs from the three germinal layers (Additional file 4) suggests that for some loci, the peripheral blood leukocytes remember an age-related programme that is common across different tissues. The results of this study suggest the existence of age-modified loci that are not leukocyte specific but can be detected in blood as a surrogate tissue.

To our knowledge, this is the first time the same individuals have been followed for this number of time points at this early age rendering 60 samples for analysis. The number of age-modified CpGs detected in this study (n = 794) is lower compared to those previously described, reflecting a very stringent statistical model that calculated the variation over many time points and included the individual as covariate. Several factors (gender, lifestyle, environmental exposures, sequence variants in cis,) may influence the dynamics in which a given CpG site is methylated or demethylated during lifetime. We could not rule out that environmental differences like season of birth, maternal smoking, breastfeeding, mode of delivery, infections and/or vaccinations may have introduced sources of variation [47,48]. Nevertheless, we included the parameter related to the individuals in order to attenuate the possible confounding effect coming from the repeated sampling procedure. We think that in combination with assuming additive (and close to linear effects), the model applied here reduced the list of age-modified CpGs to those that have less interindividual variability, some even previously observed. Assuming an additive model in this sense is probably suboptimal but reasonably effective to remove very strong individual’s related effects. It should be mentioned that other analytical strategies such as mixed effects models, which allows a random intercept by individual, are suitable for this type of longitudinal analysis; however, we did not use this approach in this specific study because mixed models with such a big number of probes is computationally expensive and might suffer from the fact that each probe might respond differently from the others.

Another serious limitation of this study is that we measured DNA methylation in unfractionated blood and did not have differential cell counts at the time of sampling to adjust the analysis. In an attempt to remove as much as possible the confounding effects due to differential cell composition, we filtered the list of age-modified CpG sites against those identified as cell-type specific for leukocyte populations. We are aware that filtering age-modified CpG sites in children by the locations having differential methylation in sorted leukocytes in adults is suboptimal, but it is still the best that can be done to date; however, we believe that not considering the locations showing differential methylation in adulthood is not detrimental for this
analysis and is still beneficial as it allows focusing on functionally relevant features. On the other hand, using existing methods for data deconvolution based on the adult cell-specific methylation profiles is risky as this data might not be relevant in children samples with a physiologically different cell composition and, hence, it might produce artefacts. Further studies are needed to address this point properly. A larger prospective study on longitudinal changes in DNA methylation during childhood is now ongoing in our laboratory including both males and females exposed to different lifestyles.

**Conclusions**

This study provides a catalogue of 794 age-modified CpG sites that robustly reflect the changes in DNA methylation levels that occur in human blood leukocytes within 3 to 60 months after birth. Age-methylated CpG sites are significantly over-represented in genes involved in developmental and neuronal-related functions indicating that DNA methylation might play an important role in regulating differentiation and leukocyte-specific functions. On the other hand, genes harbouring age-demethylated sites reflect not only the immunological window in childhood but also suggest that blood leukocytes undergo a programme that allows their interaction with environmental factors and genome remodelling. The fact that methylation in several genes implicated in the pathophysiology of inflammatory diseases is modified during the first years of life opens new perspectives on the role of environmental exposures and strategies for primary prevention. Our results provide valuable information on age-modified loci that can be useful for developing tools to correct for age effects when performing DNA methylation studies in children.

**Methods**

**Study population**

Ten healthy girls were selected from the Type 1 Diabetes Prediction and Prevention Study (DIPP) [49] to conduct a prospective genome-wide methylation analysis during childhood. The children were selected based on the availability of prospective samples, and that all remained healthy and seronegative for the T1D-associated antibodies (ICA, IAA, GADA and IA-2A) by 10 years of age. The DIPP study was launched in 1994 in Finland as a genetic screening programme for type 1 diabetes (T1D) risk alleles in newborn infants from the general population. The children included in this study were born between March 2000 and November 2002 in Tampere, Finland; all followed the Finnish vaccination programme and were carriers of the HLA-DQB1*03:02 allele but lacking DQB1*06:02 allele. The HLA-DR-DQ genotypes of the children as well as genotype-associated risk classes [50] are presented together with demographical characteristics in Table 1. Blood samples were collected during visits to the study centre at 3, 6, 12, 24, 36, 48 and 60 months after birth. Information on the clinical history of autoimmune diseases and exposures to diverse environmental factors (infections, diet, domicile, living habits, vaccinations,) was also collected. This study was conducted in accordance with the ethical principles for medical research stated in the Helsinki Declaration. The ethical committee of the Tampere University Hospital (Tampere, Finland) approved this study. Written informed consent was obtained from the parents of all the participants.

**Blood samples**

Blood samples were taken in sodium citrate tubes and processed within 1 h from venipuncture. Samples were centrifuged at 1,700 g during 10 min at room temperature. After plasma collection, the buffy coat layer was removed to a separate the cryotube and contaminated red blood cells were lysed using osmotic shock in sterile water. The buffy coat containing unfractonated leukocytes was then pelleted by centrifugation, supernatant was removed and cells were suspended in sterile water and pipetted to a separate cryotube. Samples were stored at −80°C until DNA extraction.

**DNA extraction and DNA methylation measurements**

Genomic DNA from peripheral leukocytes was extracted from buffy coats using the FlexiGene kit (QIAGEN, Hilden, Germany, Cat # 51204). DNA samples (n = 70) were diluted at 100 ng/μl in TE buffer (pH 8.0). The mean value for the A260/280 coefficient was 1.90 ± 0.05. DNA samples were diluted at 11 ng/μl, randomized in a 96-well plate and bisulfite treated using the EZ-96 DNA Methylation™ Kit (ZYM0 Research, Irvine, CA, USA, Cat # D5004) according to the manufacturer’s instructions. Six DNA samples with 0%, 50% and 100% methylation (two of each) were included as controls (EpiTect Control DNA, QIAGEN, Cat # 59665 and Cat # 59655). Nine technical duplicates of the study samples were included to evaluate inter-assay correlations. Denatured bisulfite-treated DNA was amplified, fragmented and hybridized onto the HumanMethylation450 BeadChip (Illumina, Cat # WG-314-1003) following manufacturer instructions at the Bioinformatics and Expression Core Facility (BEA, Karolinska Institutet, Stockholm, Sweden). After extension and staining steps, the chips were scanned using the Illumina iScan (Illumina, San Diego). The Infinium methylation data are available in the Gene Expression Omnibus (GEO) database under the accession number GSE62219.

**Quality control and data normalization**

Image analysis and signal detection were done using the Genome Studio Software. The quality control (QC) included the evaluation of detection P values,
staining, extension, hybridization, bisulfite conversion and specificity. The lumi package was then used for preprocessing and normalization of the data [51]. The QC also included unsupervised hierarchical clustering and principal component analysis (PCA) on sample relationships based on CpG sites. The data was processed exactly as described previously [34] and QC verified as raw data and also after normalization by the quantile method. Based on these analyses, 60 biological samples passed QC and were studied (Table 1). Methylation levels in the 0%, 50% and 100% controls resulted as expected.

### Statistical analysis on differential methylation

DNA methylation levels were log₂ transformed to M values and then statistically evaluated using the limma package [33]. A single procedure consisting of two steps was used to infer the association between age and DNA methylation, which resulted in a unique list of differentially methylated CpG sites. First, a linear model was used considering the age and the individual (repeated samples from the same person); the study of the variance was performed at this step, but no list of differentially methylated probes was generated. The information on the variance was then utilized as prior for the second step of the analysis, which consisted of a moderated t-test to compare the samples between the earliest and the latest time points (that is, 3 months vs 60 months after birth). The magnitude of the change in M values over time is indicated by the logfc: negative values indicate how much a CpG site decreases in methylation with age, while positive values indicate how much a CpG site increases in methylation. The moderated t-statistic is expressed as the column t. The significance level was set at \( P = 0.01 \) after multiple testing correction according to the Bonferroni method (\( p_{\text{conf}} \)).

### Data filtering of differentially methylated CpG sites

Fifty nine of the age-modified CpG sites had a single nucleotide polymorphism (SNP) annotated within less than ten base pairs (bp) from the query site and 99 CpG sites with a SNP annotated within the probe but >10 bp of the query site. The minor allele frequency (MAF) of each SNP within the probe sequence was interrogated in the Finnish population using ENGINEES (Entire Genome Interface for Exploring SNPs) [52], and CpG sites containing a SNP in the probe with MAF above 0.01 were filtered out (\( n = 48 \)). Furthermore, to avoid the confounding effects of CpG sites that are differentially methylated among leukocyte populations (cell-type specific), all age-modified CpG sites were contrasted against a list of 2,228 CpG sites with significant differential DNA methylation in sorted leukocytes [34] that serve as cell-type classifiers. Eleven age-modified CpG sites were found annotated as having significant DNA methylation differences within sorted leukocytes and therefore excluded. Given that all individuals were females, we did not filter out probes based on cross-hybridization [53].

### Genomic distribution and annotation of the features

The distribution of age-modified CpG sites according to their relation to a CpG island, gene structure or regulatory functions (DNAsase I hypersensitivity site, promoter, enhancer or known DMR) was calculated based on the UCSC Genome Browser annotations provided by Illumina. To calculate statistics on the location of age-modified CpG sites (TSS1500, TSS200, 5’UTR, 1st exon, gene body, 3’UTR and intergenic), we included all the annotations connected to a site. The distance of any given CpG site to the nearest TSS was calculated by PeakAnalyzer [54]. The absolute distance and position in relation to single nearest TSS within 1,000 kb was calculated by the Genomic Regions Enrichment of Annotations Tool [55]. The comparisons on the frequency of age-modified CpG sites (age-methylated vs age-demethylated) according to their relation to CpG islands, gene structure or regulatory features (present: yes/no) were performed by using \( \chi^2 \) and Fisher’s exact test. A \( P < 0.05 \) was considered statistically significant.

### Enrichment analyses

Gene ontology analyses were conducted using the DAVID Bioinformatic Resource tool (v 6.7), ConsensusPathDB [56] and WebGesalt (WEB-based GEne SeT AnaLysis Toolkit) [57]. Enrichment significance was determined using the hypergeometric distribution and considered significant if at least five genes of the input list coincide with the gene set of a given gene ontology (GO) category, with a nominal \( P \) value <0.01 and Benjamini-Hochberg \( P \) value <0.05 (\( p_{BH} \)). Visualization of enriched gene ontology terms was done by REVIGO based on semantic similarity-based scatterplots [58]. Annotations on gene families were obtained from PANTHER [59]. Induced network analyses were conducted by ConsensusPathDB to visualize known interactions between the protein products of the genes harbouring age-modified loci [56].

### Additional files

Additional file 1: A catalogue of the age-modified CpG sites identified in this study.

Additional file 2: Gene families containing age-modified CpG sites.

Additional file 3: Gene ontology (GO) analysis using the combined list of genes containing age-methylated and age-demethylated CpG sites.

Additional file 4: Gene ontology (GO) categories significantly enriched in genes containing age-methylated sites.

Additional file 5: Gene ontology (GO) categories significantly enriched in genes containing age-demethylated sites.

Additional file 6: Transcription factor genes harbouring age-modified CpG sites within 3 to 60 months after birth in blood leukocytes.
Additional file 7: DNA methylation levels in age-modified CpGs located on transcription factors and chromatin binding proteins. The histogram represents the M value: blue (methylated) and red (demethylated). A comparison with adult (WB) and sorted leukocytes as described by [34] is included for comparative purposes. PBMC = peripheral blood mononuclear cells. Each individual is represented by a column from 3 months (left) to 60 months (right).

Additional file 8: Gene expression levels for five genes containing age-associated CpG sites across 43 tissues from the FANTOM project [43].

Abbreviations
ARID3A: AT-rich interactive domain-containing protein 3A; CAGE: Cap-analysis of gene expression; CGG: CpG island; DIPP: Type 1 Diabetes Prediction and Prevention Study; G0: gene ontology; HDAC4: histone deacetylase 4; HLA: human leukocyte antigen; IRFs: interferon regulatory factor 5; JARID2: Jumonji, AT-rich interactive domain 2; KDM2A: lysine (K)-specific demethylase 2B; KDM2B: lysine (K)-specific demethylase 2B; MAF: minor allele frequency; MHC: major histocompatibility complex; NO2D: nucleotide-binding oligomerization domain containing 2; Pvalues: P value adjusted by Bonferroni correction; PTF: transcription factor; TSS: transcription start sites.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
NA analysed the data, interpreted the results and wrote the manuscript. LER and CS prepared the samples and experimental designs for 450 K measurements; MW and VF analysed the data. HHo, OV, OS, JT, JJ, MIK and HHY conceived the DIPP study, collected and provided the study samples. AS provided 450 K data on adult sorted blood leukocytes. DG analysed the data and interpreted the results. JK conceived this study, provided general and analytical directions and edited the paper. All authors revised the manuscript for important intellectual content and provided comments. All authors read and approved the final manuscript.

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