

**Title: Molecular aging of human liver: an epigenetic/transcriptomic signature**

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### **List of abbreviations**

DMPs: Differentially methylated positions

MDS: Multidimensional scaling

DNAmAge: DNA methylation age

DMRs: Differentially methylated regions

BMI: Body Mass Index

DE: Differentially expressed

GSEA: Gene Set Enrichment Analysis

EMT: Epithelial–mesenchymal transition

## **Abstract**

The feasibility of liver transplantation from old healthy donors suggests that this organ is able to preserve its functionality during aging. To explore the biological basis of this phenomenon, we characterized the epigenetic profile of liver biopsies collected from 45 healthy liver donors ranging from 13 to 90 years old using the Infinium HumanMethylation450 BeadChip. The analysis indicates that a large remodeling in DNA methylation patterns occurs, with 8823 age-associated differentially methylated CpG probes. Notably, these age-associated changes tended to level off after the age of 60, as confirmed by Horvath's clock. Using stringent selection criteria we further identified a DNA methylation signature of aging liver including 75 genomic regions. We demonstrated that this signature is specific for liver compared to other tissues and that it is able to detect biological age-acceleration effects associated with obesity. Finally we combined DNA methylation measurements with available expression data. Although the intersection between the two omic characterizations was low, both approaches suggested a previously unappreciated role of epithelial-mesenchymal transition and Wnt signaling pathways in the aging of human liver.

## **Keywords**

DNA methylation; Epigenetic clock; Epithelial-mesenchymal transition

## Introduction

The liver is a highly sophisticated metabolic factory. It performs a vast array of biochemical functions necessary to maintain whole-body homeostasis and it is pivotal in integrating and elaborating signals that originate from peripheral tissues. Still, details on how and to what extent liver physiology is affected during aging is an open question in basic biological research. Compared to other organs, liver has long been known to be unique in terms of extended functionality. Despite some age-related changes in morphology and function, liver appears to preserve its functionality at older ages much better than other tissues <sup>1</sup>, with important implications in translational research, including the selection of donors in liver transplants. Indeed, available data suggest that transplants from older donors have duration and success rates comparable to those from young donors <sup>2,3</sup>. Collectively, these evidences suggest that liver aging rate is decelerated compared to other organs.

In this frame, we recently reported that the proteasomes' function and the microRNA expression in liver biopsies from healthy liver donors ranging from 12 to 92 years are largely preserved at least until the age of 60 <sup>4,5</sup>.

To deepen and complement these findings, and on the basis of recent literature <sup>6-8</sup>, we here investigate genome-wide DNA methylation in liver biopsies from 45 healthy donors with age ranging from 13 to 90 years. In fact, DNA methylation is the epigenetic change that received most attention in the study of human aging, due to the large remodeling that it undergoes in lifespan <sup>9</sup> and to its ability to detect acceleration/deceleration effects in the biological age of an individual <sup>10,11</sup>. Finally, to get further insight into the molecular pathways altered during liver aging, we corroborated the epigenetic analysis with the matched characterization of gene expression changes.

## Methods

### *Samples*

Liver biopsies were selected from 45 heart-beating and brain dead donors (age range 13-90 years), as described in Capri et al. 2016<sup>4</sup>, following approval of the local ethical committee (code: 44/2008/Tess) and obtainment of written informed consent. No donor organs were obtained from executed prisoners or other institutionalized persons. Donors' death causes are described in Capri et al. 2016<sup>4</sup>; no significant differences in bilirubin, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyl transferase between donors younger than 70 years and older than 70 years were observed.

Human hepatocytes were derived from an independent set of 22 patients undergoing either partial or total hepatectomy and from 10 organ donors (Supplementary Table 1). Cells were isolated as reported in<sup>12</sup>, centrifuged at 80g for 6 minutes and frozen as dry cell pellets for long-term storage at -80° C.

DNA methylation datasets used to investigate DNA methylation in healthy tissues other than liver are reported in Supplementary Data (Supplementary Table 2). Two datasets (GSE61256 and GSE48325) were used to assess the effects of obesity on age-dependent DNA methylation changes. GSE61256 includes 79 liver samples from patients undergoing liver biopsy for assessment of liver histology or for suspected non-alcoholic fatty liver disease and from normal controls to exclude liver malignancy during oncological surgery<sup>13</sup>. GSE48325<sup>14</sup> includes 85 liver samples from 62 patients (17 normal controls, 17 healthy obese, 3 obese without clinical characterization, 10 with non-alcoholic fatty liver disease and 15 patients with non-alcoholic steatohepatitis). Liver biopsies follow-up available for 23 individuals were discarded from our analysis.

### *Genome-wide DNA methylation analysis.*

Genomic DNA was extracted from 45 liver biopsies (27 males and 18 females, age range 13-90 years) using the Qiagen kit QiAmp mini Kit®, following manufacturer's instructions. DNA was bisulfite-converted using the EZDNA Methylation-Gold Kit (Zymo Research) and analysed on the Infinium HumanMethylation450 BeadChip (Illumina) following manufacturer's instructions. Arrays

were scanned by HiScan (Illumina). Data processing and analysis are described in Supplementary Data. DNA methylation data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE107039 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107039>), which includes also expression data (see below).

#### *Gene-targeted DNA methylation analysis*

DNA was extracted using the AllPrep Mini Kit (Illumina) following manufacturer's instructions. Site specific DNA methylation analysis was performed by the EpiTYPER Assay. Briefly, 500 ng DNA were converted via bisulphite treatment using EZ-96 DNA Methylation Kit (Zymo Research Corporation). Ten ng bisulphite-converted DNA were amplified using bisulphite-specific primers for ELOVL2, ZIC1, ZIC1\_Shore, MACROD1, CCNJ and CYP1B1 (Supplementary Table 3) using a step-down amplification routine as indicated in <sup>15</sup> (Supplementary Table 4). Methylation data analysis was performed using the R *stats* package.

#### *Gene expression analysis*

Total RNA (including microRNAs) was extracted from 33 liver biopsies belonging to the same collection analysed for genome-wide DNA methylation <sup>4</sup> (11 females and 22 males, age range 13-90 years) using the mirVana microRNA Isolation Kit (Ambion), following manufacturer's instructions. Of these 33 biopsies, 26 had also been analysed for DNA methylation profiles. The HG-U133 plus 2.0 GeneChip (Affymetrix) platform was used to evaluate gene-expression, as already described <sup>4</sup>. Analysis of gene expression data is described in Supplementary Data.

## Results

### *Identification of age-associated changes in liver DNA methylation*

We used the Illumina Infinium 450k microarray to characterize DNA methylation of liver tissues collected from 45 individuals ranging from 13 to 90 years of age.

We run two types of analyses (See Methods Section). The first identified 8823 differentially methylated positions (DMPs, Figure 1A and Supplementary File 1). Most of these probes (5772) were positively associated with age. The overlap between our results and those recently published<sup>8</sup> (3518/8823 probes) is reported in Supplementary File 1 and commented in Supplementary Data. The analysis of the chromatin states of the genomic regions harbouring age-DMPs (Supplementary Data and Supplementary Figure 1) showed an enrichment of hyper-age-DMPs in bivalent chromatin domains, consistently with previous reports on other tissues<sup>16</sup>.

Multidimensional scaling (MDS) on the list of 8823 differentially methylated positions (age-DMPs) showed a clear separation of the samples along the first dimension (Figure 1B), linearly correlated with chronological age until 60 years of age, while a trend to a plateau was observed at older ages (Figure 1C). The same trend was confirmed when we estimated DNA methylation age (DNAmAge) of our samples using Horvath's epigenetic clock<sup>17</sup> (Figure 1D), suggesting a slower epigenetic aging rate of liver after 60 years of age.

As a second type of analysis, we focused on the regions in which multiple adjacent CpG sites show age-dependent changes (age-dependent differentially methylated regions, age-DMRs; see Materials and Methods) owing to their more interpretable biological meaning compared to DMPs<sup>18</sup>. We identified a signature of 75 age-DMRs, including 687 probes mapping on 89 genes, all positively associated with aging (Figure 2A, Supplementary Files 2 and 3). Unsurprisingly, the top-ranking differentially methylated region mapped in the CpG island of gene *ELOVL2*, already well documented and known to undergo age-associated hypermethylation in several tissues<sup>19,20</sup>. MDS analysis on the 75 age-DMRs signature gave results similar to those achieved using the list of age-DMPs, with a clear association of MDS1 with chronological age until 60 years and a levelling off after this threshold (Supplementary Figure 2).

### *Validation of the DNA methylation signature on isolated hepatocytes*

Although hepatocytes are the predominant cell types in human liver, we cannot exclude *a priori* that the observed epigenetic alterations in aging liver are in fact related to changes in cell types abundances in the tissue. Therefore, we evaluated age-dependent DNA methylation of 6 randomly selected age-DMRs (*ELOVL2* island, *ZIC1* island, *ZIC1* Shore, *MACROD1* island, *CCNJ* island and *CYP11B1* island) in an existing collection of hepatocytes isolated from liver tissues in an independent cohort of 32 subjects (age range: 0-82 years). Pearson correlation between DNA methylation and age was significant (p-value <0.05) for multiple CpG sites within each of the 6 regions analysed (Figure 2B, Supplementary Files 4 and 5) indicating that, at least for these DMRs, the observed epigenetic changes occur in hepatocytes.

### *Tissue-specificity of the DNA methylation signature*

Subsequently, we investigated whether the signature identified in liver could show similar age-dependent trends in other human tissues. We used 14 publicly available datasets where DNA methylation is measured in healthy tissues (Supplementary Data) from subjects of different ages. An independent liver dataset [GSE61258, considering only subjects with a body mass index (BMI)<25] was included in this analysis in order to verify the reproducibility of the age-DMRs identified in our study. For each of the 687 probes included in the 75 age-DMRs, we considered the slope values of the linear regression between DNA methylation and age in each tissue. Hierarchical clustering using these tissue-specific slope values (Supplementary Data) showed that the two liver datasets form a distinct group compared to the other tissues, suggesting that the age-dependent DNA methylation signature that we identified is liver-specific (Figure 3A).

A deeper analysis (Figure 3B; Supplementary Files 6 and 7) indicates that although in some regions trends in age-association are similar between liver and several tissues (for example, *ELOVL2* island or *OTUD7A* island), other regions harbour a completely liver-specific age-association (for example, *SRCINI* or *MAP6DI* islands). In particular, we observed that for some regions liver and mesenchymal stem cells display opposite trends in age-associations. In most of the cases (*MIB2*, *MACROD1* and *FAM18A* islands) the same CpG probes were positively (liver) and negatively (mesenchymal stem cells) associated with age, while in the *B3GALT4* island the 5' CpG probes showed positive age-association in liver and no association in mesenchymal stem cells, while the 3' CpG were negatively associated with age in mesenchymal stem cells but not age-associate in liver.

#### *Validation of the liver aging signature in obese subjects*

To assess the ability of our physiological aging signature to capture pathological conditions of the liver we revisited the liver Infinium 450k datasets used by Horvath (GSE61258 and GSE48325, see Materials and Methods) where the authors demonstrated that human livers from obese subjects have higher DNAmAge values than livers from non-obese subjects<sup>13</sup>. We therefore evaluated whether the DNA methylation status of our shortlist of age-DMRs was also affected by BMI using the same liver Infinium 450k datasets used by Horvath (GSE61256 and GSE48325, see Materials and Methods). For each CpG probe in the 75 age-DMRs signature, we calculated the association with BMI, correcting for age. Eighty probes, mapping in 25 DMRs, resulted significantly associated with BMI in both datasets (p-value <0.01; Supplementary File 8, Supplementary File 9 and Figure 4) and in all cases lean subjects (BMI<25) tended to have lower DNA methylation levels, for the same age, compared to obese subjects. Given that all the 75 DMRs are hypermethylated with age, this result indicates that the liver aging signature that we identified is able to detect acceleration effects in the epigenetic age of liver from obese subjects.

### *Identification of age-associated changes in liver mRNA expression*

To gain insight into the molecular activities altered over liver aging we selected a different and more appropriate type of omic technology and associated analysis, i.e. transcriptomics and functional analysis. Pearson correlation between expression signal and age was computed for each probe along with p-values corrected for multiple hypothesis testing (Supplementary File 10), leading to 56 probes significantly differentially expressed (age-DE; adjusted p-value < 0.001), with a high prevalence of probes positively associated with the age of the donor (47 out 56; Figure 5A). MDS was computed on the expression values of the selected 56 age-DE probes, returning well separated groups along the first component (Figure 5B) which, differently from DNA methylation data, presented an almost linear association with chronological age (Figure 5C).

Gene Set Enrichment Analysis was performed on gene expression data to gain insight into the biological processes involved in aging liver. We identified several gene sets significantly enriched at a False Discovery Rate of 0.05 (Supplementary Table 5). Genes with a positive correlation with age were enriched in allograft rejection, interferon gamma response, inflammatory response, epithelial mesenchymal transition (EMT) and myogenesis, while genes with a negative correlation with age were enriched in metabolic processes.

### *Multi-omic analysis*

As it is now well recognized, the joint analysis of multiple omics allows the identification of properties of a system that are not visible when exploring single layers<sup>21</sup> and this has specifically been shown for the exploration of phenomena as diverse as cellular transdifferentiation and response to vaccination<sup>21</sup>. In this context, we searched for the genes that displayed age-associated changes in both DNA methylation (using the list of 8823 age-DMPs) and mRNA expression (using the list of 56 age-DE genes) and identified 11 genes: *FZD2*, *ICMT*, *KCNIP4*, *LGALS4*, *PTGDS*, *SDK1*, *SORCS2*, *TDRD10*, *TSPYL5*, *VASH1*, *ZIC1*. For each of these genes, we calculated Pearson correlation between DNA methylation values of the probes in the age-DMPs list and gene

expression values of the probes in the list of age-DE (Supplementary File 11). Pearson correlation was significant (nominal p-value <0.01) for 3 genes: *ZIC1*, *TSPYL5* and *FZD2*. The vast majority of the age-DMPs probes mapping on gene *ZIC1* were positively correlated with the signal from the 3 age-DE Affymetrix probes mapping on the same gene. For gene *TSPYL5*, a negative correlation between Affymetrix probe 213122\_at and both Infinium cg04917181 and cg00032205 probes, located on a CpG island could be identified; for gene *FZD2*, a positive correlation was found between Affymetrix probe 210220\_at and Infinium probe cg01684881, located on a CpG island.

## Discussion

In the present study, we explored the genome-wide epigenomic and transcriptional molecular changes that accompany aging in human liver.

Our data highlight for the first time that age-associated epigenetic changes level off after 60 years of age in human liver. This trend is confirmed, although less strikingly, when the DNAmAge is calculated using Horvath's epigenetic clock. This is expected as Horvath's clock is optimized to be a multi-tissue predictor of age, while our analysis is specifically focused on liver and therefore it is more likely to detect the specific epigenetic remodelling that occurs in this tissue during aging. Although quantified for the first time, our finding is in agreement with experimental evidences showing that healthy liver undergoes a successful aging, preserving liver's functionality at older ages<sup>1,4,5</sup>. The integration of DNA methylation data with results from further studies on cell morphology, cellular functions, proteomic and enzymatic profiles will provide a more complete picture of the biological basis of successful aging in liver.

The decrease in the epigenetic aging rate after 60 years is evident using both the list of 8823 age-DMPs and the shorter list including 687 probes mapping on 75 age-DMRs. In addition to the robustness emerging from confirming the results with two different approaches, the latter signature is defined using a region-centric approach<sup>18</sup>, more likely to guarantee biologically interpretable

results compared to a single-probe analysis. We demonstrated that the 75 age-DMRs list is: i) validated in hepatocytes, ii) liver specific and iii) able to detect epigenetic age acceleration effects associated with obesity.

With respect to the first feature, the signature on hepatocytes was confirmed on 6 regions tested by gene-targeted analysis of DNA methylation in a collection of hepatocytes isolated from subjects at different ages.

The second feature was assessed by testing and confirming the 75 age-DMRs signature in an independent liver dataset including tissues from non-obese subjects (GSE61256). In the same analysis, we demonstrated that the signature is liver-specific compared to other tissues, suggesting that the peculiar aging process characteristic of this tissue has a specific epigenetic landscape. This refines on the work of Steve Horvath who has shown how methylation changes are a ubiquitous phenomenon in human tissues <sup>17</sup>, with however limited emphasis on the tissue specificity of epigenetic remodelling during aging. Bysani et al. demonstrated that a large fraction of age-associated DNA methylation changes in liver were reflected also in blood, while the overlap with age-associated changes in pancreatic islets and adipose tissue was smaller <sup>8</sup>. Our work complements these findings, as we were able to identify a liver-specific epigenetic signature of aging, in line with the apparently lower aging rate of liver compared to other body districts.

We then evaluated the effect of BMI on our epigenetic signature of aging liver. Eighty probes, mapping in 25 age-DMRs analysed, were hypermethylated, for the same age, in overweight or obese subjects compared to lean subjects in two independent datasets. This result indicates that, similarly to Horvath's epigenetic clock, specific components of our signature are able to capture the already known age acceleration effects in liver from obese subjects. Furthermore, literature search indicates that several of the genes we identified are hypermethylated in hepatocellular carcinoma (*ZIC1*, *FOXD3*) <sup>22,23</sup> or in other cancer types (*NEFM*, *KCNG3*, *PRDM14*, *FOXD3*, *CELSR3*, *HEYL*) <sup>24-30</sup>, suggesting a role of these genes in liver homeostasis.

Finally, we investigated gene expression changes in our cohort and correlated them with DNA methylation variations. Differently from the epigenetic signature of liver aging, we observed a linear change in gene expression from young to old subjects. This confirms the different nature and informativity of omic screens, and emphasizes the necessity to integrate them to achieve higher levels of understanding of the underlying molecular events. In particular, although transcriptomics cannot capture the aging deceleration typical of liver, it allows to explore functional alterations thanks to the ample and well validated number of tools designed for enrichment analysis. Accordingly, Gene Set Enrichment Analysis highlighted several biological processes associated to liver aging. Genes up-regulated with aging are enriched in inflammatory response and in EMT. While alterations in immune and inflammatory responses have been described also in murine models of aging liver <sup>31</sup> and are in accordance with the accrual of chronic inflammation during aging (inflammaging) <sup>32</sup>, a possible role of EMT in liver aging has not been investigated so far, probably because the contribution of hepatocytes to EMT in liver fibrosis is still a controversial issue <sup>33</sup>. The role of EMT in kidney fibrosis is increasingly accepted <sup>34</sup> and it is noteworthy that caloric restriction alleviates age-related EMT in kidneys from aged mice <sup>35</sup>.

We noted that a number of genes in the 75 age-DMRs signature is involved in the Wnt pathway (*ZIC1*, *NEFM*, *FOXD3*, *MIR155HG*, *CELS3*, *HEYL*) <sup>25,36-39</sup> and in the regulation of the epithelial-to-mesenchymal transition (*PRDM14*) <sup>39</sup>. A role of Wnt signalling in aging has been recently proposed, prompted from the observation that it is downregulated in cellular senescence <sup>40</sup>. Age-dependent variations in the expression of Wnt pathway genes could affect tissue homeostasis and fibrosis <sup>41</sup>. Although the results from our DNA methylation analysis are not conclusive, as they do not allow to clearly infer an activation/reduction of Wnt signalling in the aging of human liver, they sustain previous evidences and, to the best of our knowledge, for the first time suggest an epigenetic regulation of this pathway in aging. Further experiments should investigate EMT-related changes in expression and epigenetic profiles during liver aging.

The integration of the two levels of information (DNA methylation and expression) highlighted 3 genes sharing differential methylation and expression: *ZIC1*, *TSPYL5* and *FZD2*.

*ZIC1*, which is also part of the 75 age-DMRs list and which is one of the genes affected by obesity, encodes for a transcription factor belonging to the family of the C2H2-type zinc finger proteins. *ZIC1* plays an important role during development, in particular during neurogenesis<sup>42</sup>, and is has been implicated in liver regeneration<sup>43</sup>. *ZIC1* has been shown to be hypermethylation with aging in peripheral blood mononucleated cells and naïve CD4+ cells<sup>44</sup>. It is interesting to note that in a recent report Sliker et al. demonstrated that *ZIC1* methylation encounters an increase in inter-individual variability with aging in whole blood, and that this is associated to a higher variability in the expression of the gene<sup>45</sup>. On the basis of this results and on our observation that *ZIC1* is hypermethylated in liver tissues from obese subjects, it would be interesting to investigate whether *ZIC1* methylation and/or expression are associated with health status and, more in general, with aging quality.

*TSPYL5* was encodes for the testis-specific Y-like protein 5 and is frequently hypermethylated and silenced in different cancer types, including HCC<sup>46,47</sup>. Furthermore, its methylation is increased in foetal cortex of Down Syndrome, a disease which shows a profound remodeling of DNA methylation patterns<sup>19,48</sup>.

The protein encoded by *FZD2*, Frizzled2, is a Wnt receptor overexpressed in tumors and correlates with markers of EMT<sup>49</sup>. Down-regulation of *FZD2* expression through a short-hairpin RNA suppresses the proliferation of HCC and gastric cancer cells<sup>50</sup>. Although also in this case it is not easy to infer the role of DNA methylation and expression changes of *FZD2* during aging, it is intriguing that the gene is involved in Wnt signaling pathway and EMT.

A possible limitation of our study is that the liver biopsies were collected from brain-dead, heart-beating donors (death by accidents and exclusion of major chronic disease). We cannot exclude that intensive care unit stay could have had an impact on liver transcription, while methylation patterns are likely to be more stable. This observation could also explain, at least in part, the poor correlation

between transcription and methylation that we observed. In any case, the use of livers considered suitable for organ transplantation from brain-dead, heart-beating donors, allowed us to investigate a unique collection of livers from donors of different ages, ranging from very young to very old subjects, that would be otherwise impossible owing to the strict clinical and ethical limitations on biopsies from healthy, non-obese subjects.

In conclusion, in this study we characterized the epigenomic and transcriptomic remodelling that occurs in healthy liver tissues during aging. Epigenomic data indicate a levelling off in the epigenetic aging rate of liver after 60 years, supporting the clinical and molecular observations that liver can preserve its functionality also at older ages. Functional studies are needed to characterize the contribution of the genes that we identified in DNA methylation and expression analysis to verify whether the observed changes are associated with the balance between age-related decline and maintenance of functionality. We suggest that in particular the regulation of EMT and Wnt signalling pathways can play a yet unappreciated role in liver aging.

### **Conflict of interest**

The authors declare that there are no conflicts of interest.

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## Figure legends

Figure 1. Identification of age-DMPs (differentially methylated positions) in human liver. (A) Volcano plot of age-DMPs. The difference between mean DNA methylation values in older (age 71-90 years) and younger individuals (age 13-30 years) is plotted on the x axis, while the nominal p-value for the regression between DNA methylation and age, corrected for sex and batch, is on the y axis ( $-1 \times \log_{10}$  scale). The dotted line corresponds to a BH-corrected p-value of 0.001. Probes with a mean differential methylation of at least 0.15 and 0.30 are highlighted respectively in blue and yellow. (B) MDS plot of methylation values of the 8823 significant age-DMPs. (C) Scatter plot of the first dimension of the MDS against chronological age of the subjects. The blue line corresponds to the LOWESS regression between MDS1 and age. (D) Scatter plot between chronological age (x axis) and DNAmAge (DNA methylation age) (y axis). The cyan line corresponds to the regression between age and DNAmAge in subjects younger than 60 years of age. The blue line corresponds to the LOWESS regression between DNAmAge and age, considering the whole cohort.

Figure 2. Human liver age-DMRs (differentially methylated regions). (A) DNA methylation profiles of the CpG islands located in *ZIC1* and *MACROD1* genes according to Illumina Infinium 450k measurements. The dotted grey lines indicate the regions assessed by EpiTYPER. Mean methylation values and standard deviations are reported for four age classes (0-30 years old; 31-50 years old; 51-70 years old; 71-90 years old). (B) Pearson correlations between age and DNA methylation, measured by EpiTYPER, in the CpG islands located in *ZIC1* and *MACROD1* genes.

Figure 3. Identification of age-DMRs (differentially methylated regions) in human liver (A) Heatmap of the slope values of the regressions between DNA methylation of each CpG probe within the 75 age-DMRs and age in the analysed tissues. CpG probes are ordered according to their genomic localization. (B) For the CpG probes within the 6 regions in *ELOVL2*, *OTUD7A*, *SRCIN1*,

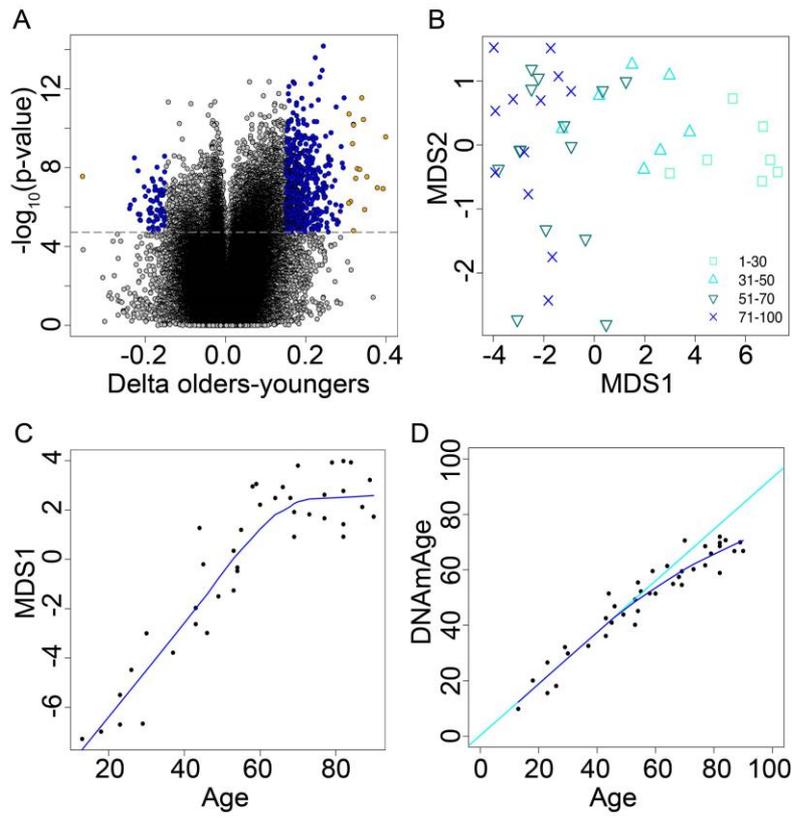
*MAP6D1*, *MIB2* and *B3GALT4* genes, the plots report the slope values of the regression between DNA methylation and age in the analysed tissues. For the sake of clarity, the names of the CpG probes are not reported in the figure, but can be found in Supplementary File 10.

Figure 4. Methylation of cg23449696 within the N-Shore of *ZIC1* in lean and overweight/obese subjects. Black and red lines represent the linear regression between methylation and age in lean and overweight/obese subjects respectively.

Figure 5. Identification of age-DE (differentially expressed) in human liver. (A) Pearson correlation between expression and age for the Affymetrix HG-U133\_Plus\_2 array probes, plotted according to their chromosomal position. (B) MDS (multidimensional scaling) plot of expression values of the 56 significant age-DE. (C) Scatter plot of the first dimension of the MDS against chronological age of the subjects. The blue line corresponds to the LOWESS regression between MDS1 and age.

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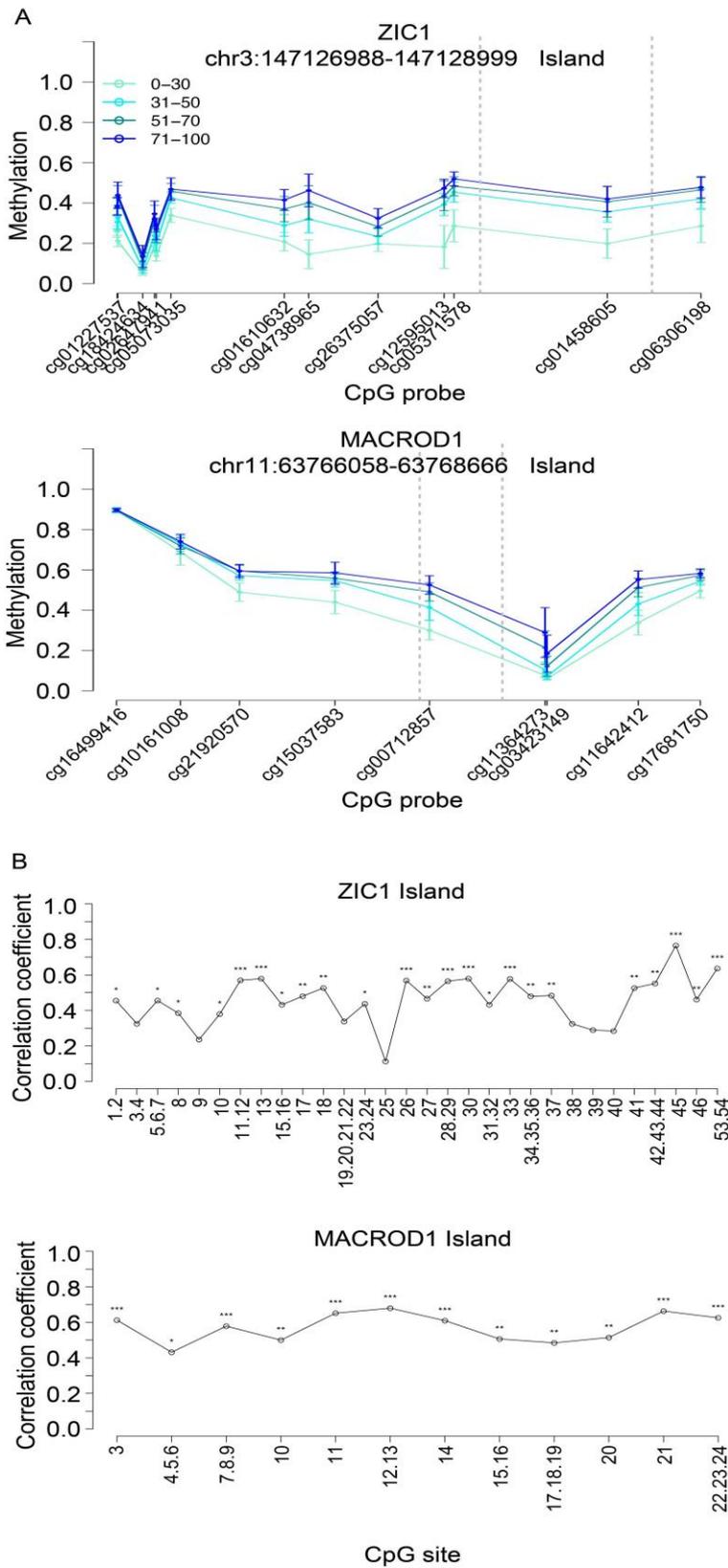
**Figure 1.**



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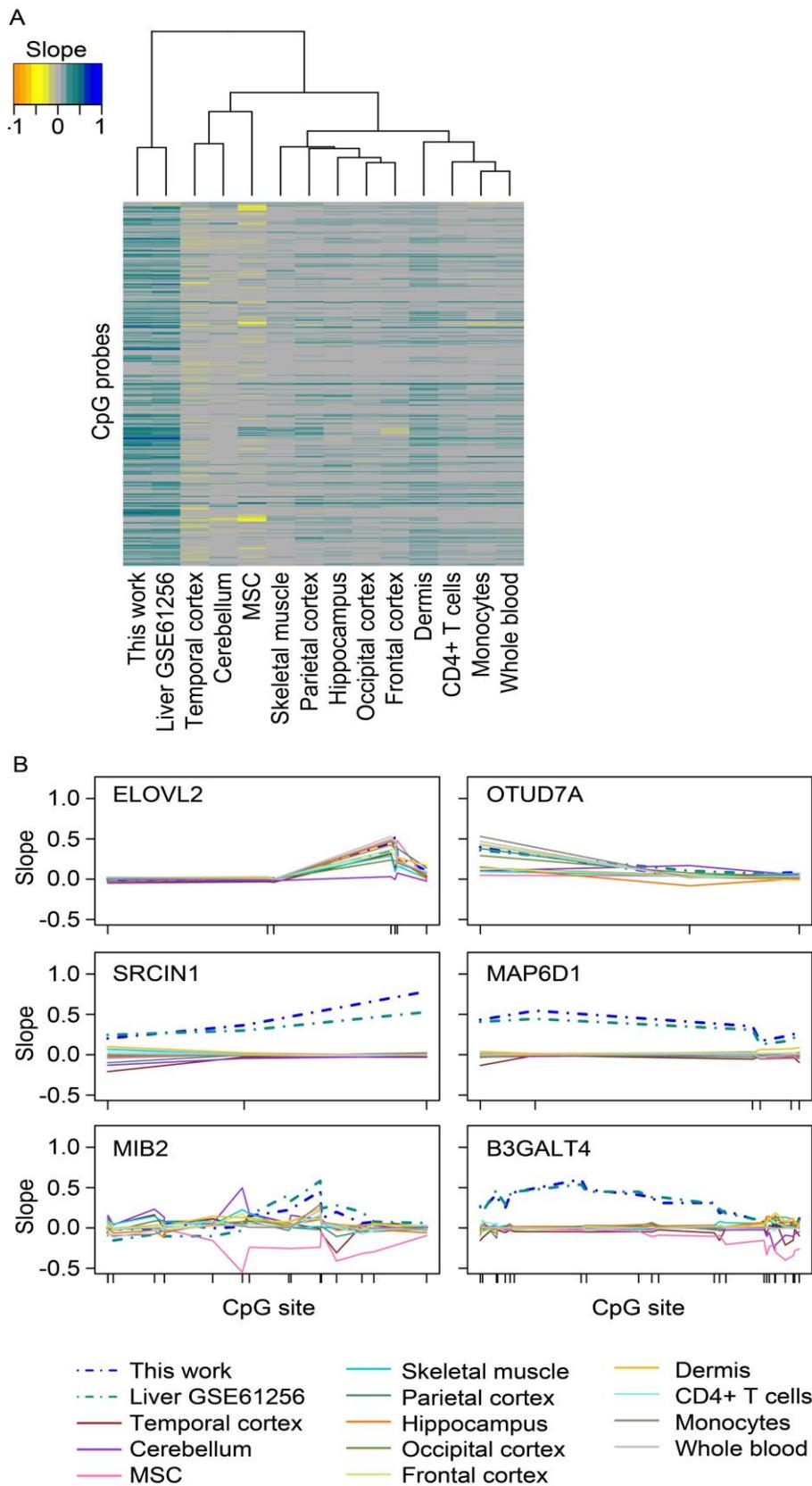
Manuscript

**Figure 2.**



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**Figure 3.**

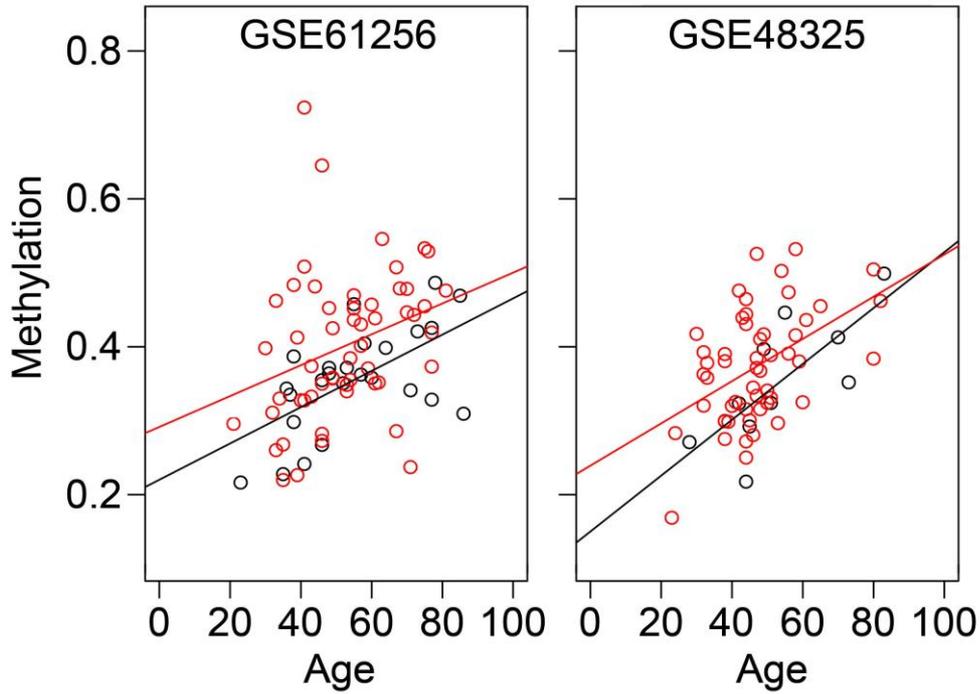


Script

Figure 4.

ZIC1 N-Shore, cg23449696  
chr3:147126988–147128999

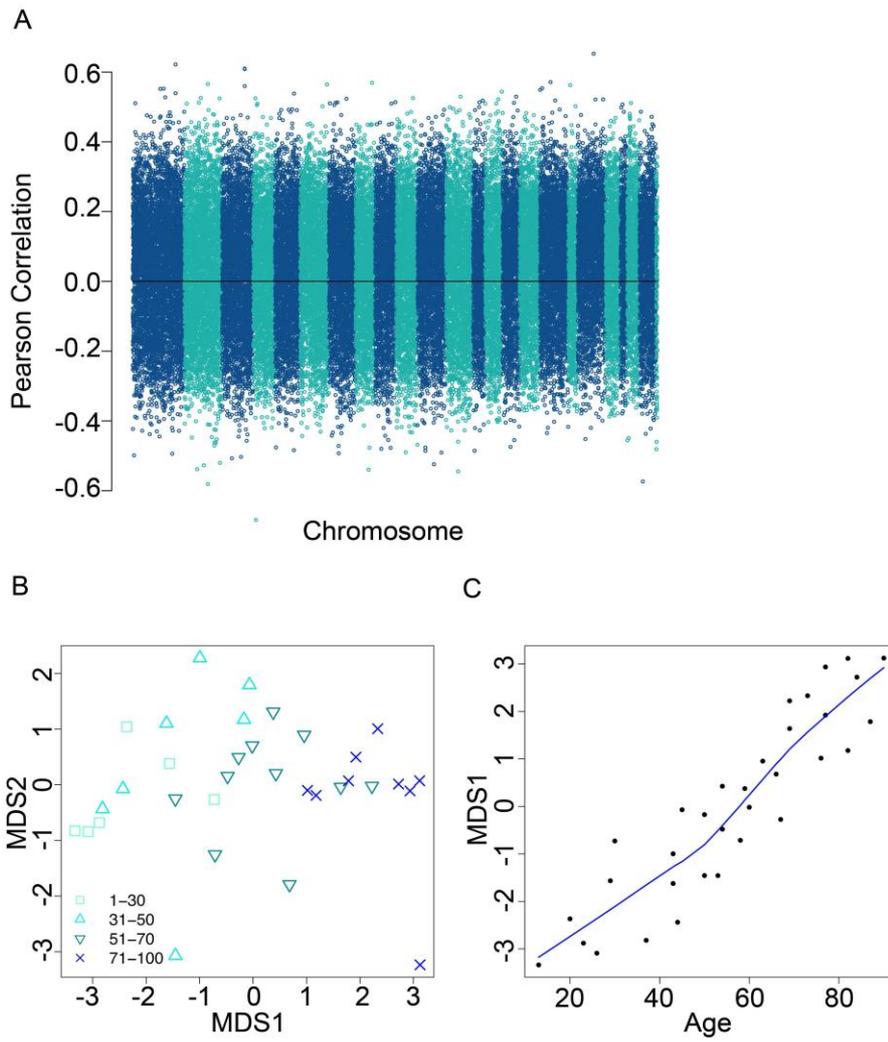
○ BMI < 25  
○ BMI ≥ 25



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Figure 5.



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Script