Mentor: Dr. Joseph McClay, Department of Pharmacology and Health Outcomes
Student: Ashley Victor, Center for Biological Data Science, Bioinformatics major

Title: The Investigation of DNA Methylation Differences between Mice Blood and Liver Datasets to Understand Epigenetics Effects with Age

Abstract
Drug metabolism declines with age and older adults are at greater risk of adverse drug reactions. The research described here aimed to understand the similarities between age-related DNA methylation patterns at drug-metabolizing genes in mice blood and liver DNA. Our goal is to evaluate the potential for blood DNA methylation states to serve as biomarkers of drug metabolism gene activity in the liver. The mice blood and liver CpG data were obtained from National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) datasets and analyzed using gene-matching algorithms on RStudio and the GeneOverlap package. It was hypothesized that there would be significant overlap between age-related changes in the mice blood and liver genes. There was a small but significant overlap between the different tissues with a p-value of 2.1e-13. We found that the following drug-metabolizing genes changed with age in both tissue types: *Abca8a*, *Gstp1*, *Gstp2*, *Abca1*, and *Abca4*.

Background:

1.1 Aging and Drug Metabolism

Older individuals experience decreased functioning of hepatic metabolism, and this condition exacerbates the risk for adverse drug reactions (ADRs) for patients with chronic conditions and patients who are taking multiple medications. Approximately 40% of individuals 65 years or older use more than five medications, a term known as polypharmacy (Slone Epidemiology Center 2006). Older adults are seven times more likely than younger individuals to have ADRs (Budnitz et al. 2006). However, there are no good methods for predicting adverse drug reactions in older patients.

The normal aging process induces changes to the epigenome. Prior epigenome-wide association studies (EWAS) in human blood have revealed DNA methylation changes at several genes encoding drug metabolism enzymes with age (Hannum et al. 2013). Moreover, work in laboratory rodents has shown that epigenetic changes with age are associated with reduced gene and protein expression of drug metabolizing enzymes and this leads to slower drug metabolism (Kronfol, 2020) . Therefore, given that age-related epigenetic changes may slow rates of hepatic drug metabolism, developing epigenetic biomarkers could provide a way to personalize drug dosing regimens for older individuals.

1.2 Epigenetic Changes with Age

DNA methylation is an epigenetic mark that regulates gene expression by the transfer of a methyl group to cytosines in CpG regions. These epigenetic marks are induced by a multitude of factors, including aging. With age, particular genomic regions experience hypomethylation, the loss of methyl groups from the cytosines, or hypermethylation, the gain of methyl groups from the cytosines. Horvath et. al (2013) pinpointed 353 CpG sites that experienced methylation modifications with age and developed the concept of an Epigenetic Clock, which is a linear regression model that predicts aging effects. For our purposes, it is important to study DNA methylation changes in hepatic cells given that most drug metabolism happens in the liver. Studies such as Bacalini et. al aimed to investigate how liver physiology is impacted during

aging, specifically the human epigenome (Bacalini et al., 2019). Pinpointing specific genomic regions that exhibit hyper- or hypomethylation (Bacalini et al., 2019) is important because these epigenetic states drive gene expression, with hypermethylation typically leading to reduced gene activity and hypomethylation typically leading to increased gene activity.

1.3 Pharmacy Applications & Personalized Medicine

Epigenetic aging could affect expression of drug metabolism enzymes in the liver and could be a potential reason why there are increased ADRs in older adults. Pharmacogenetics is the field of study where genetic variation is related to drug-related outcomes, efficacy or ADRs. Pharmacoepigenetics is a new field where epigenetic biomarkers are used to predict drug response. Therefore, it is our goal to evaluate if age-related epigenetic changes at genes involved in drug metabolism could be used as biomarkers to decide dose in older adults.

1.4 Objective of the Investigation

Liver is the main organ in drug metabolism . However, we cannot biopsy the liver as part of a routine biomarker test so we need to use peripheral tissues. The objective of the investigation is to determine the extent of overlap in terms of the number of CpG markers that significantly change with age that are common to blood and liver of mice as proof of concept before moving to human studies. There are tissue specific differences in methylation patterns, but there is some evidence that methylation changes with age correlate across tissues (Horvath, 2013). In the current study, we will use publicly available datasets from genome-wide association studies of mice blood and liver to answer this question.

Methods:

In the current study, liver and blood datasets of mice genes were obtained from GEO databases of NCBI from https://www.ncbi.nlm.nih.gov/ (Table 1). The principal criteria for selecting CpG sites and genes within these datasets was using findings with a False Discovery Rate (FDR) < 0.05 (Benjamini & Hochberg, 1995). From the data, mice and blood CpG sites that showed significant methylation differences were extracted using Excel. The CpG sites were mapped to mice genes through the Stanford GREAT (Genomic Regions of Enrichment Annotation Tool) online tool (Tanigawa, 2022). Relating CpGs to the nearest gene based on position may not be accurate because long-range regulatory effects exist. Therefore, we use GREAT, a tool that outputs all the genes that the CpG site could be associated with, based on functional and regulatory information. To identify genes involved in drug metabolism and transport, the gene lists from mice blood and liver were matched with the Kyoto Encyclopedia of Genes and Genomes (KEGG) drug metabolism and ABC transporters list on RStudio 4.1.3 (Kanehisa & Goto, 2000). The KEGG drug metabolism and ABC transporters list was derived from the following KEGG reference pathways: mmu00982, Drug metabolism - cytochrome P450, mmu00983 (Ko00982), Drug metabolism - other enzymes (Ko00983), and mmu02010, ABC transporters (Ko02010) pathways.

With GREAT gene list output for both the mice blood and liver, a collection of all unique genes was organized in a table. To test the degree of overlap between the mice blood and liver gene lists, the Bioconductor GeneOverlap R package 1.34.0 was used (Shen, 2022). A p-value threshold of less than 0.05 was used to declare significant differences. Using the exhaustive list of mice blood and liver genes, the VennDiagram 1.7.3 R Package was used to create a venn diagram of the overlapping genes (Chen, 2011)

Table 1: List of Blood and Liver Datasets

Accession Number	Contents	Article	Author	Date Downloaded
GSE200527	Mice Blood Tissue	Epigenetic Clocks for Mice Based on Age-Associated Regions That are Conserved Between Mouse Strains and Human	Perez-Correa	8/13/2022
GSE120137	Mice Blood Tissue	A multi-tissue full lifespan epigenetic clock for mice	Horvath, Thompson	8/13/2022
GSE80672	Mice Blood Tissue	Using DNA Methylation Profiling to Evaluate Biological Age and Longevity Interventions	Petkovich	8/24/2022
Supplemental material in the paper PMID: 30858345	Mice Liver Tissue	Remodeling of epigenome and transcriptome landscapes with aging in mice reveals widespread induction of inflammatory responses	Bérénice A. Benayoun`	3/24/2022
GSE199979	Mice Liver Tissue	Genetic loci and metabolic states associated with murine epigenetic aging	Steve Horvath	5/5/2022
GSE137277	Mice Liver Tissue	Body weight and high-fat diet are associated with epigenetic aging in female members of the BXD murine family	Sandoval Sierra	6/22/2022
Supplemental material in the paper https://doi.org/10.1016/j.celrep.2020.108203	Mice Liver Tissue	The Gene-Regulatory Footprint of Aging Highlights Conserved Central Regulators	Maroun Bou Sleiman	6/22/2022

Results:

We first obtained the CpGs in the mouse genome that significantly changed with age in each study. The number of significant CpGs for the following mice liver studies was: Sleiman (X), Sandoval-Sierra (x), Horvath (x), and Benayoun (x). The number of significant genes for the following mice blood studies was: Perez-Correa (x), Petkovich (x), and Horvath (x).

We entered the genomic positions of the significant CpGs into GREAT. This tells us which genes these CpGs may regulate. The total number of genes implicated by the significant CpGs in each of the liver studies was as follows: Slieman (x), Sandoval-Sierra (x), Horvath (x), and Benayoun (x). The number of genes implicated by the mice blood studies was: Perez-Correa (x), Petkovich (x), and Horvath (x). The

Sleiman results were not used for further analysis because too many CpGs were significant and the gene list from GREAT was composed of too many mice liver genes to be specific in the downstream analysis.

Following identification of the genes associated with epigenetic aging in each tissue, we matched our gene lists to known drug metabolism and transport genes from KEGG. The number of drug metabolism and transport genes potentially impacted by epigenetic aging for the following mice liver studies was: Sandoval-Sierra (7), Horvath (14), and Benayoun (19). The number of genes for the following mice blood studies was: Perez-Correa (5), Petkovich (1), and Horvath (2).

Table 2: Exhaustive List of Mice Liver and Blood Genes

Mice Liver Genes	Mice Blood Genes
[1] "Abca8a"	[1] "Abca1"
[1] "Abcb11"	[1] "Abca8a"
[1] "Abcb4"	[1] "Gstp1"
[1] "Abcd2"	[1] "Gstp2"
[1] "Abcd3"	[1] "Uckl1"
[1] "Abcg2"	[1] "Cmpk1"
[1] "Aox3"	[1] "Abca4"
[1] "Ces1c"	[1] "Abcb5"
[1] "Ces2c"	
[1] "Cyp1a2"	
[1] "Gsta1"	
[1] "Gstp1"	
[1] "Gstp2"	
[1] "Tap1"	
[1] "Ugt2b35"	
[1] "Ugt2b36"	
[1] "Ugt2b37"	
[1] "Ugt2b38"	
[1] "Ugt2b5"	
[1] "Abca1"	
[1] "Abca2"	
[1] "Abca3"	
[1] "Abca4"	
[1] "Abcb9"	
[1] "Abcc5"	
[1] "Abcc9"	

[1] "Abcg8"	
[1] "Aox1"	
[1] "Dpyd"	
[1] "Nme6"	
[1] "Uck2"	
[1] "Upp2"	

We then tested to see if there was a significant overlap between the liver and blood genes using the Gene Overlap R package. The input for the Gene Overlap package analysis was two gene lists, the first included all unique genes implicated by any of the three mice blood epigenetic aging studies, while the second included all unique genes implicated by any of the three mice liver epigenetic aging studies (Table 2). Figure 1 shows the code and the output of the Gene Overlap analysis. The amount of significant mice liver and blood genes are 32 and 8, respectively. The intersection size indicated in Figure 1 shows that there are 5 genes that are differentially methylated for both mice blood and liver tissues. The overlapping p-value is 2.1e-13.

```
> library(GeneOverlap)
>  overl <- newGeneOverlap(
+    unique(Mice_Liver_trim),
+    unique(Mice_Blood_trim),
+    genome.size=NULL,
+    spec = c("mm9.gene"))
>
>  overl <- testGeneOverlap(overl)
>  print(overl)
Detailed information about this GeneOverlap object:
listA size=32, e.g. Abca8a Abcb11 Abcb4
listB size=8, e.g. Abca1 Abca8a Gstp1
Intersection size=5, e.g. Abca8a Gstp1 Gstp2
Union size=35, e.g. Abca8a Abcb11 Abcb4
Genome size=23000
# Contingency Table:
      notA inA
notB 22965  27
inB      3   5
Overlapping p-value=2.1e-13
Odds ratio=1353.8
Overlap tested using Fisher's exact test (alternative=greater)
Jaccard Index=0.1
>
```

Fig.1: The Output of the Gene Overlap R package

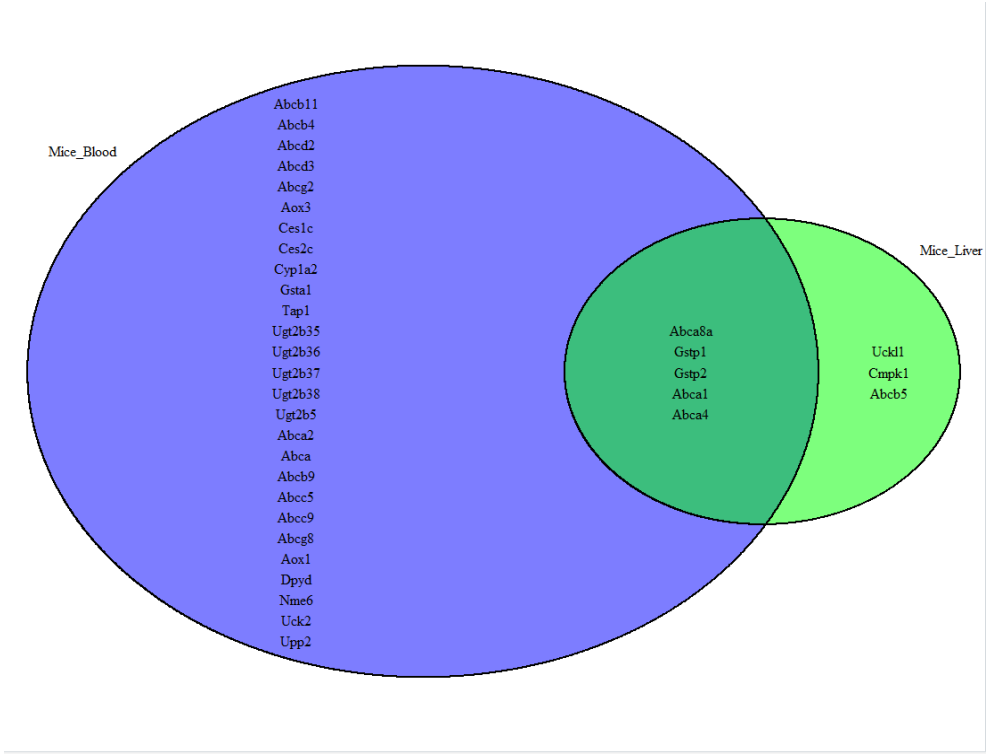

Fig 2: The Output of the GeneOverlap Package showing the overlap of Mice Liver and Blood Genes

3 were abc (ATP binding cassette) transporters and two were GSTP phase II drug metabolizing enzymes. The following drug-metabolizing genes overlap between mice blood and liver: *Abca8a*, *Gstp1*, *Gstp2*, *Abca1*, and *Abca4*. The ABC genes encode for the ABC transporters (ATP-binding cassette). These proteins transport molecules across the extra and intracellular membranes. GSTP1 variant proteins support in xenobiotic metabolism.

Conclusion:

A.Interpretation of Results

The following drug-metabolizing genes overlap between mice blood and liver: *Abca8a*, *Gstp1*, *Gstp2*, *Abca1*, and *Abca4*. The basis of this study is to see how blood genes would serve as an effective indicator of epigenetic aging effects on drug metabolism. Although there are 5 genes that overlap between the mice blood and liver tissues, the overlap is a small fraction relative to all the mice drug-metabolizing genes that showed evidence for epigenetic change in liver. Therefore, further studies need to be done to better understand the correlation between epigenetic effects in blood and liver tissues, or to develop potential biomarkers. One possibility could be to use measures of biological age based on several hundred CpG, known as “epigenetic clocks” (Horvath 2013).

B. Strengths and Limitations

The strengths of the study was that multiple mice liver and datasets were used to identify the extent of gene overlap between the different tissues. Additionally, a comprehensive list of KEGG drug metabolism and ABC transporters list was used to determine how many of the differentially methylated mice blood

and liver genes overlap with these functional classifications. A limitation of the study is that the datasets from the studies utilized mice of different age ranges.

C. Future prospects

It is possible that other peripheral tissues may serve as better indicators of epigenetic aging in the liver. It may be of interest to study epigenetic aging in other peripheral tissues, such as buccal epithelium, which is readily obtained from cheek swabs. If there is a strong overlap between epigenetic aging of drug-metabolizing genes in both the liver and the buccal epithelium, this could be a better tissue to use for aging biomarkers to guide dosing in the elderly.

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