Epigenetic Clock Testing
An accurate, low cost biomarker of aging

JAMES WATSON, MD, FACS
PLASTIC & RECONSTRUCTIVE SURGERY
THOUSAND OAKS, CA
CLINICAL FACULTY,
UCLA DIVISION OF PLASTIC SURGERY
Aging “Clocks”

Q: Why do we need a new “Clock” for measuring aging?

A: The old Clocks are not very accurate “age time keepers”

Examples of other “clocks”: DNA damage markers, Leukocyte telomere length testing (both average LTLT and % short LTLT tests), biomarkers of Cellular senescence (Ex: H2AX staining, β-galactosidase staining, p16INK4A staining)
What is Epigenetics?

**Simple Definition:** Molecular ways that regulate gene expression besides the classical Watson-Crick transcription factor method of gene regulation.
Q: What is DNA Methylation?
A: It is part of “Epigenetics”
Q: How many DNA methylation (CpG) sites are there in the Human Genome?

Answer: About 28 million CpG sites in the human genome. Only a fraction of these undergo age-related increases in DNA methylation or demethylation.

**Age-related Differential DNA methylation** (definition): The DNA methylation sites that lose or gain a methyl group with aging. These sites can be found with computer programs that “data mine” existing databases of human methylomes.
Which DNA Bases get Methylated?

- Only Cytosine (C) DNA bases are methylated
- 3 main methylating enzymes in humans – DNMT1, DNMT3A, and DNMT3B (2 minor ones)
- Methyl groups come from folate cycle (SAM), which activates DNMT enzymes
- SAH is the byproduct of DNA methylation, which inhibits DNMT enzymes

Clinical Correlation – Folate and B12 deficiencies prevent normal DNA methylation from occurring, but excess folate and B12 does not “stop” aging (DNA methylation is a tightly controlled, “site-specific” process)
What is a “CpG”? 

- A “CpG” is a cytosine, next to a guanine on the 5’ DNA strand. CpG also called a “Cytosine-phosphate-Guanine dinucleotide”
- Only 1-1.5% of the human genome is made up of CpGs
- Certain areas of the human genome have more than 1.5% CpG content, such as promoters in CpG islands (18%) and 5’UTRs, and repetitive DNA (Ex: Alu repeats – 3%)
Q: What is the difference between DNA Methylation and DNA Demethylation?

DNA Methylation *

DNA Demethylation *

Both DNA Methylation and Demethylation occur with aging at specific locations in all genomes

* DNA methylation/demethylation only occurs on Cytosine DNA bases next to an adjacent Guanine DNA base
How DNA Methylation and DNA Demethylation alters gene expression

Methylation: MBD

Demethylation: MBD

Transcriptional Repression

Transcriptional Activity
DNA Methylation “silences” genes by creating a binding site for methyl binding proteins to attach to the methylated cytosines at promoters. This prevents transcription factors from “turning on” gene expression.

**Key Terms:**
- **DNMT** - DNA methyltransferase
- **SAM** – S-adenosylmethionine
- **SAH** – S-adenosylhomocysteine
- **TF** – Transcription factor
- **MBP** – Methyl binding protein
How DNA Methylation Silences Genes

**Transcription factor** – a protein that binds to DNA to “turn on” gene expression

**Promoter** – the beginning of a gene DNA sequence where the transcription factor binds

**DNA Methylation** of CpG sites at promoter prevents the gene from being transcribed
Q: What DNA is normally hypermethylated?
A: Repetitive DNA (aka junk DNA)

Types of Repetitive DNA (aka Interspersed repeats, Transposable elements, TEs retrotransposons, etc.)

1. **LINE** – long interspersed nuclear elements
2. **SINE** – short interspersed nuclear elements
3. **LTR** – Long terminal repeats
Clinical Significance of DNA Methylation in Age Management (aka DNAm Clock Testing)

Using computer algorithms and database mining of DNA methylomes, scientists have identified specific CpGs that either increase or decrease as a function of aging. Making a so-called “clock” of these site-specific CpGs can predict age very accurately.

- DNA Methylation increases with aging at certain sites (millions of CpG sites)
- DNA Methylation decreases with aging at other sites (millions of CpG sites)
Epigenetic Clock Testing
An accurate, low cost biomarker of aging

Older Less Accurate

“Aging Clocks”

- **Blood Leukocyte Telomere Length (LTL)**
  - Measures Ave. LTL or % short LTLs
  - Correlation w/age is poor:
    \[ r = -0.51 \text{ in females} \]
    \[ r = -0.55 \text{ in males} \]

- **DNA Damage Bioarkers**
  - H2AX antibody staining
  - DNA metabolites – 8-OH-dG
  - Free radical damage biomarkers
    - HNE – lipid peroxidation product
    - MDA – malondialdehyde
    - Isoprostanates
    - Reactive aldehydes
  - None correlate with chronological age
Older Less Accurate
“Aging Clocks”

- **Cellular Senescence Biomarkers**
  - β-Galactosidase or P16INK4 antibody staining
  - Correlation w/chronological age is poor
    - \( r = 0.56 \) only in T cells
    - No correlation w/age in other WBCS

- **Microsatellite mutations**
  - Correlates with # of cell divisions
  - Does NOT correlate with chronological age

---

Young VSMC
Aged VSMC

\( p16^{INK4a} \)
Key Point of Lecture: DNA Methylation Testing is 3X more accurate than WBC Telomere Length Testing (average and % short)
The Horvath DNAm Clock

- **2012: Horvath** - described 1st “DNA methylation clock” in 2012 from 14,000 human methylome datasets

- **CpG sites selection** - via computer algorithm based on “elastic net regularization”

- **353 CpG sites** – “selected” to make a “DNAm Clock”
  - 193 CpGs were hypermethylated with aging
  - 160 were hypomethylated with aging

- **Conclusion**: Differential DNA methylation at these 353 CpG specific sites predicted chronological age with extremely high correlation:
  - Training data: $R = 0.97$
  - Test data: $R = 0.96$
  - 3rd party testing: $R = 0.98$ *

DNA Methylation Clockmakers:

- **110 CpG Clock** – Horvath, 2013
  Developed using blood & tissue samples (> 30 sites)
  Originally used the Illumina 29,369 CpG chip
  Chronological age prediction: ?

- **353 CpG Clock** – Horvath, 2013
  Developed using same algorithm as 110 CpG clock
  Validated using the Illumina 450K CpG chip data
  Chronological age prediction: +/- 3.6 yrs

- **3 CpG Clock** – Weidner, etc., 2014
  Developed using only blood samples
  Originally used Illumina 485K CpG chip data
  Chronological age prediction: +/- 4.5 years

- **73 CpG Clock** – Hannum, 2013
  Developed using only whole blood samples
  Originally Used Illumina 485K CpG chip
  Chronological age prediction: +/- 3.6 years

- **102 CpG Clock** – Hannum, 2014
  Developed using only whole blood samples
  Used Illumina 485K CpG chip
  Chronological age prediction:
DNAm Clock Comparisons:

**Hovath (353 CpG) vs Hannum (102 CpG)**

**Part II**

- **Finding #3**: Horvath and Hannum clocks showed the same age acceleration in stroke patients
- **Finding #4**: Horvath and Hannum clocks showed equal correlation: chronological vs DNAm age and “Inter-clock correlation”
  
<table>
<thead>
<tr>
<th>DNAm Clock</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horvath</td>
<td>$r = 0.93$</td>
</tr>
<tr>
<td>Hannum</td>
<td>$r = 0.93$</td>
</tr>
<tr>
<td>Horvath vs Hannum</td>
<td>$r = 0.94$</td>
</tr>
</tbody>
</table>
- **Finding #5**: $\Delta$ age was greater with Hannum DNAm clock in all 4 models

<table>
<thead>
<tr>
<th>Clock</th>
<th>Odds Ratio (OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hannum</td>
<td>1.13-1.14</td>
</tr>
<tr>
<td>Horvath</td>
<td>1.05-1.07</td>
</tr>
</tbody>
</table>

Horvath vs Hannum DNAm Clock Data in CVA patients

--- Ischemic stroke survivors (CVA)
--- Healthy age-matched controls
DNA Methylation Clock Comparisons

Hovath (353 CpGs) vs Hannum (73 CpGs) vs Weidner (3 CpGs)

\[ r = 0.98 \]
\[ r = 0.97 \]
\[ r = 0.81 \]
Discoveries made with Horvath’s 353 CpG Methylation Clock

- Human tissue mostly ages at the same $\Delta$DNAm rate exceptions: female breast – $\Delta$ DNAm is faster cerebellum – $\Delta$ DNAm is slower
- **Aging rate accelerates in old age** $\sim$ 40%
  - Horvath’s clock shows this age acceleration
  - Falkner’s formula – age acceleration = 40% in old age
- **Non-human primates** – Horvath’s clock is accurate in non-human primates – Ex: Rhesus monkey.
- **Obesity** – accelerates DNA methylation aging ONLY in the liver (not other tissue). Surgery-induced weight loss doesn’t reverse this epigenetic age acceleration
- **Cancer** – some cancers show age acceleration
  - Cancers with silenced TSGs – no change in $\Delta$ DNAm
  - ER/PR+ breast cancer – acceleration in $\Delta$ DNAm
POSSIBLE CAUSES OF DNAm “CLOCK TICKING”

- Stochastic (random)?
- Oxidative Stress (i.e. free radicals)?
- DNA damage?
- Time-dependent biological events?
  - Day-night cycles (circadian)
  - Molecular “drivers” of circadian cycles within the cell?
- Cellular Inflammation or Senescence?
  - NF-κB activated pathways?
  - JAK-STAT pathways?
Q: Is DNA Methylation "CLOCK TICKING" at the DNAm Clock CpG sites a random event?

A: Absolutely NOT!

**Reason:** Stochastic events follow a bell-shaped curve. CpG Clocks are non-stochastic – they undergo the same differential DNA methylation in all 7.2 billion humans.

**Conclusion:** The consistent, reproducible pattern of DNA Differential methylation at specific CpG sites suggests that aging is a "programmed event", just like the consistent, reproducible pattern of DNA differential methylation that occurs during embryogenesis and fetal life.
How Folic acid, Vit B12 and the SAM/SAH ratio affects DNMT activity

• **S-Adenosyl-L-methionine (SAM)** is the necessary methyl donor (substrate) all of the DNMT enzymes

• **S-Adenosyl-L-homocysteine (SAH)** is the by product of DNA methylation and inhibits DNMTs by “feedback inhibition”

  - **SAM/SAH ratio** determines DNMT enzyme activity rate
  - **SAHH** is a “rate limiting enzyme” in the methionine cycle of DNA methylation
Specific Factors that alter the SAM/SAH ratio and DNMT activity

Factors that Accelerate Epigenetic Aging
• Folic acid and Vit B12 deficiency
• Smoking and air pollution
• Heavy metals – Arsenic, Cadmium
• Alcohol, malnutrition
• Chronic stress, sleep deprivation

Factors that Slow Down Epigenetic Aging
• Caloric restriction
• Methionine restriction
• Caffeic acid, Chlorogenic acid
• EGCG, Curcumin, Genistein
• Fish Oil, Vit D
• Metformin
How **Metformin** Slows Epigenetic Aging and Increases Longevity

- **Metformin** activates SAHH enzyme via an AMPK/Let-7/H19 pathway.
- **SAHH** is a “rate limiting enzyme” in the methionine cycle of DNA methylation.
- **Metformin** – activates SAHH enzyme via an AMPK/Let-7/H19 pathway.
- **Net Effect of Metformin** – less “feedback inhibition” of DNMTs due to build-up of SAH.

**Ref:** Zhong, et.al, Metformin alters DNA methylation genome-wide via the H19/SAHH axis, Oncogene, April, 2017
How Metformin Slows Epigenetic Aging and Increases Longevity

Metformin is an FDA-approved drug that could be used off label as an anti-aging pill.

Zhong, et.al, Metformin alters DNA methylation genome-wide via the H19/SAHH axis, Oncogene, April, 2017
DNA Methylation Dysregulation in Cancer

“Epigenetic changes in DNA methylation are early events in cancer tumorigenesis and often precede DNA mutations”

Clinical Significance of DNA Methylation in Age Management (aka DNAm Clock Testing)

What’s New and What’s the Future Direction of DNA Methylation Clocks and DNAm testing?
The Horvath DNAm Clock (353 CpG clock) applies to almost all human tissue types across the entire life course.
Exceptions to the Rule: **The Cerebellum**

DNAm Clocks suggest that the cerebellum ages slower than most other tissues => DNAm Age Clock not accurate “time keeper”

---

**Figure 3.** Epigenetic age acceleration in tissues from individual centenarians. (a) Mean DNAm age
New Epigenetic Clocks That are Being Developed: The “DNAm PhenoAge” Clock

- Correlates more with aging “phenotype”, rather than intrinsic aging rate
- Developed mainly for blood methylation data (old clock not as accurate in measuring age with blood samples)
- DNAm PhenoAge clock also applies to other tissues besides blood
- This clock measures differential DNA methylation at 513 CpG sites
DNAm Clocks Can Accurately Predict Risk of Death!

DNA methylation age of blood predicts all-cause mortality in later life

DNAm age estimators in blood predict time to death even after adjusting for other risk factors (Ex: smoking, obesity)
Prediction of Life Span with the new “DNAm PhenoAge” Clock

<table>
<thead>
<tr>
<th>Mortality Cause</th>
<th>Cases</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-Cause</td>
<td>1052</td>
<td>3.8E-49</td>
</tr>
<tr>
<td>Aging-Related</td>
<td>661</td>
<td>4.5E-34</td>
</tr>
<tr>
<td>CVD</td>
<td>272</td>
<td>5.1E-17</td>
</tr>
<tr>
<td>Cancer</td>
<td>265</td>
<td>7.9E-10</td>
</tr>
<tr>
<td>Diabetes</td>
<td>41</td>
<td>1.9E-11</td>
</tr>
<tr>
<td>Lung</td>
<td>53</td>
<td>6.3E-4</td>
</tr>
</tbody>
</table>
Prediction of Morbidity with the new “DNAm PhenoAge” Clock

Morbidity Validation for DNAm PhenoAge

• Higher DNAm PhenoAge is associated with
  – Incident coronary heard disease (P-value=2.43E-10)
  – a decrease in likelihood of being disease-free (P=1.06E-7),
  – a person’s number of coexisting morbidities (P=4.6E-15),
  – an increase in physical functioning problems (P=2.1E-13).
Epigenetic Clock Discovery in 50 Mammals

• Paul G. Allen Foundation has funded a project that will profile 50 different mammals.
A Mammalian Aging Clock for Rodents and other animals will be valuable for pre-clinical studies of anti-aging interventions.

In vitro studies

Anti-aging intervention that resets the epigenetic age of human keratinocytes (Ken Raj).

In vivo studies
An epigenetic aging clock for dogs and wolves

Michael J. Thompson, Bridgett vonHoldt, Steve Horvath, Matteo Pellegrini

Epigenetic estimation of age in humpback whales

ANDREA M. POLANOWSKIL, JOOKE ROBBINS, DAVID CHANDLER and SIMON N. JARMAN

Multi-tissue DNA methylation age predictor in mouse

Thomas M. Stubb\textsuperscript{1}, Marc Jan Bonder\textsuperscript{2}, Anne-Katrien Stark\textsuperscript{3}, Felix Krueger\textsuperscript{4}, BI Ageing Clock Team, Ferdinand von Meyenn\textsuperscript{1}, Oliver Stegle\textsuperscript{2} and Wolf Rek\textsuperscript{1,5,6}

Epigenetic aging signatures in mice livers are slowed by dwarfism, calorie restriction and rapamycin treatment

**Question:** Do we need to measure all of the DNA methylation sites to create the most accurate DNAm “Aging Clock”? (i.e. 28 million CpG sites?)

**Answer:** The data so far from all species studied suggests not. Only a sampling of 100-1,000 sites need to be measured, but large samples (10,000 to 100,000 CpG sites need to be “data mined” with Computer algorithms to come up with the small number of sites used for creating an “Aging Clock”?)
Epigenetic clock for mice based on only 2956 highly conserved CpGs is similar to clocks based on 96K CpGs

Matteo Pellegrini

Michael Thompson, Richard Davis, Gary Churchill
Why study 50 different animal species?

• A large number of different animal species will be needed
  – for modern phylogenetic comparative approaches
  – for introducing a technological standard that will advance special research communities
    • Evolutionary biologists
    • Veterinarians
    • Animal shelters
    • Conservationists
  
  - To discover why certain species live longer and and others live shorter than expected, based on Longevity Quotients (Ex: Naked mole rat vs laboratory rat – LQ 5 vs 0.5)
Revisiting Leukocyte Telomere Length Testing (LTLT)
Revisiting Leukocyte Telomere Length Testing (LTLT)

- Data: DNAm Age and telomere length were measured on the same samples (data from the Framingham Heart study)
  - LTLT (TRF method): $r = -0.27$
  - DNAm Age: $r = 0.81$

**Message:** DNAm age exhibits a much stronger correlation with age than telomere length
Q: Isn’t Telomere Length testing and DNAm testing measuring the same aspect of aging?

A: No. Epigenetic age acceleration data does not correlate with telomere length in most large scale studies.

- No association of Epigenetic Age and LTLT in the
  1. Women’s Health Initiative
  2. Framingham Heart Study
  3. ESTHER study
  4. Lothian Birth cohort (wave 2)

- Weak positive correlation
  \[ r = 0.08, \ p = 0.016 \] in the Bogalusa study

References:  Chen et al 2017  
            Marioni et al 2016  
            Breitling et al 2016
**Q:** Why Isn’t Leukocyte Telomere Length testing as accurate as DNAm testing for measuring aging?

**A:** Because of genetic variation in the human TERT gene.

GWAS of epigenetic aging rates in blood reveals a critical role for TERT

Lifestyle Factors can Alter Epigenetic Aging Rate

Epigenetic clock analysis of diet, exercise, education, and lifestyle factors

Austin Quach¹, Morgan E. Levine¹, Toshiko Tanaka², Ake T. Lu¹, Brian H. Chen², Luigi Ferrucci², Beate Ritz³, Stefania Bandinelli⁵, Marian L. Neuhouser⁶, Jeannette M. Beasley⁷, Linda Snetselaar⁸, Robert B. Wallace⁸, Philip S. Tsao⁹, Devin Absher¹¹, Themistocles L. Assimes⁹, James D. Stewart¹², Yun Li¹³, Lifang Hou¹⁵, Andrea A. Baccarelli¹⁷, Eric A. Whitsett¹², Steve Horvath¹,¹⁹

Blood methylation data from

- 4,173 postmenopausal female participants from the Women's Health Initiative
- 402 participants from the Italian cohort study,

Authors: Invecchiare nel Chianti
Lifestyle Factors can Alter Epigenetic Aging Rate

- **Extrinsic Epigenetic Aging** – altered by environment

- **Intrinsic Epigenetic Aging** – altered by gene variants (SNPs, heritability)

- Some lifestyle factors alter both Extrinsic Epigenetic Aging and Intrinsic Epigenetic Aging

- Some lifestyle factors alter only Extrinsic Epigenetic Aging

- One lifestyle factor alters only Intrinsic Epigenetic Aging
Lifestyle Factors can Alter Epigenetic Aging Rate

Marginal correlations with epigenetic age acceleration in the WHI. Correlations between select variables and the two measures of epigenetic age acceleration are colored according to their magnitude with positive correlations in red.