

Epigenetic aging waves:	1
Artificial intelligence detects clustering of switch points in DNA	2
methylation rate in defined sex-dependent age periods	3
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Abstract 23

Background: Aging is linked to hypermethylation of CpG sites on promoters and enhancers, 24
along with loss of methylation in intergenic zones. That such changes are not necessarily a 25
continuous process is exemplified by the extensive changes in DNA methylation during 26
development with another significant time of change during adolescence. However, the 27
relation between age and DNA methylation during adult life has not been systematically 28
evaluated. In particular, potential changes in methylation trends in the same CpGs over the 29
years that may occur with aging remain largely unexplored. 30

Methods: Here we set out to determine the average trends by age of the CpG sites represented 31
in the Illumina 450 platform, based on data from 2143 subjects of the age range of 20 to 80 32
years, compiled from 24 different cohorts. Using several mathematical procedures, we 33
initially separated stationary probes from probes whose methylation changes with age. Among 34
the latter, representing ~20% of the probes, we then focused on the identification of CpG sites 35
with switch points, i.e., a point where a stable trend of change in the age-averaged methylation 36
is replaced by another linear trend. 37

Results: Using several mathematical modeling steps, we generated a machine learning model 38
that identified 5175 CpG sites with switch points in age-related changes in the trend of 39
methylation over the years. Switch points reflect acceleration, deceleration or change of 40
direction of the alteration of methylation with age. The 5175 switch points were limited to 41
2813 genes in three waves, 80% of which were identical in men and women. A medium-size 42
wave was seen in the early forties, succeeded by a dominant wave as of the late fifties, lasting 43
up to 8 years each. Waves appeared ~4-5 years earlier in men. No switch points were detected 44
on CpGs mapped to the X chromosome. 45

Conclusion: In non-stationary CpG sites, concomitant switch points in age related changes in 46
methylations can be seen in a defined group of sites and genes, which cluster in 3 age- and 47
sex- specific waves. 48

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Introduction

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Age-related changes in DNA methylation have been recognized for more than four decades 67
(1–3). In brief, global DNA methylation declines from the onset of adulthood to advanced age 68
(4–7), but this trend is comprised of two opposing vectors: CpG sites with overall low DNA 69
methylation, such as promoter-associated CpG islands, tend to increase methylation with age, 70
whereas hypermethylated DNA zones, such as intergenic non-island CpGs tend to lose 71
methylation with age. The resultant overall decrease in global DNA methylation with age 72
reflects the fact that CpGs residing outside of CpG islands and tend to be hypermethylated 73
outnumber the CpG sites in the CpG islands, whose methylation level rises with the passage 74
of time (8-10). This also leads to a gradual shift in DNA methylation levels toward the mean 75
with increasing age (8–12). Age-related changes in DNA methylation tend to occur 76
preferentially at CpG island shores and shelves and enhancers (13). That certain CpG sites are 77
closely and linearly related to age has been utilized by Horvath et al (4,5), Hannum (11), and 78
Weidner et al (12) to formulate the concept of age/time related epigenetic clocks, according 79
to which an epigenetic age, in years, can be mathematically derived from specific CpG sites 80
such that it approximately parallels the chronological age. Shahal et al. (14) subsequently 81
deconvoluted the Horvath's epigenetic clock to its components, showing significant inter- 82
individual variability. Furthermore, a large body of work now links upward drifting from the 83
epigenetic age, which reflects accelerated epigenetic aging, to earlier mortality, decreased 84
healthy longevity and a number of diseases, such as breast cancer (15–21). Since most 85
epigenome-wide DNA methylation data is now based on large platforms identifying the level 86
of methylation in predefined CpG sites, it is of interest that Florath et al (7) who analyzed 87
>480000 CpG sites in whole blood DNA of a population-based cohort study aged between 50 88
and 75 years, found only 162 CpG sites with the high Spearman correlation coefficients 89
($R > 0.6$) between DNA methylation and age. 90

Whereas these and likely other CpG sites selected to comprise the basis for "epigenetic clocks" 91
apparently serve as excellent aging markers, or may even be causatively linked to the aging 92
process, they are obviously too few to account for the large age-related changes seen in DNA 93
methylation. Because many reports revealed the linear relation to age and its link to health 94
conditions, we sought, in the present report, to explore the overall pattern/s of changes in DNA 95
methylation as a function of age. However, non-linear changes in DNA methylation with age 96
have been previously reported in humans (22) and canids (23). Extreme examples of large 97
changes in DNA methylation are known to occur in specific ages in early life: after birth, 98
average DNA methylation levels increase in blood throughout the first year of life (24,25). 99
Likewise, during adolescent transition changes in DNA methylation were observed in more 100
than 15000 CpGs, many of which were associated with genes relevant to cell growth and 101
immune system development (26). 102

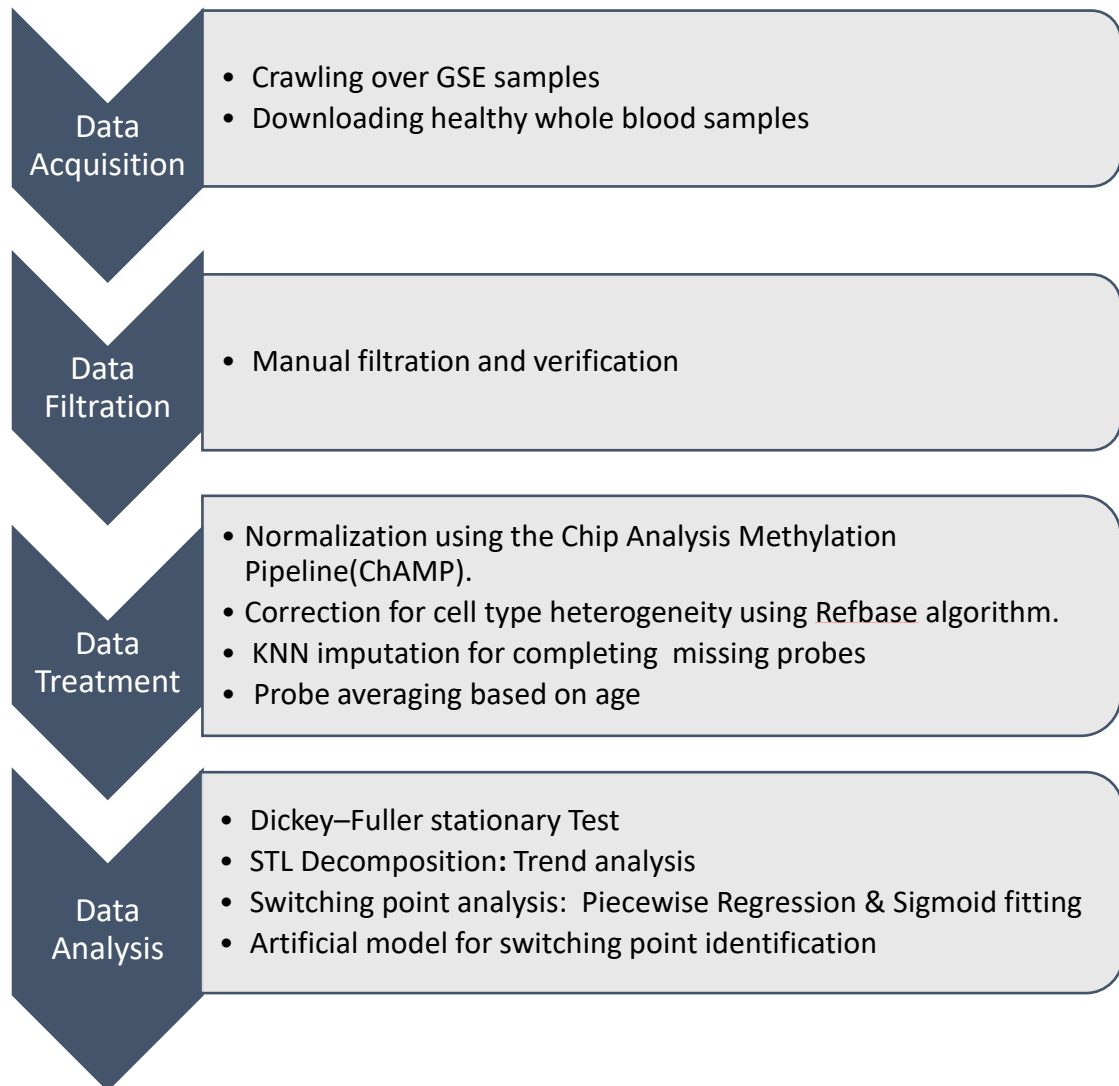
In the present study we pursued the following aims: 1) To identify non-stationary CpG 103
sites, i.e., all CpG methylation sites that undergo detectable age-related changes between the 104
age of 20 and 80 years. 2) To identify CpG sites with age-related switch point (SP), i.e., CpG 105
sites whose methylation fraction changes in a curvilinear manner such that changes in DNA 106
methylation are either accelerated, decelerated or switch their trend direction at a certain age. 107
3) To investigate the possibility that acceleration or deceleration in methylation cluster around 108
defined age-related zones (e.g., perimenopause). To this aim we used a compiled data base 109
constructed of 24 different published cohorts who all used the Illumina 450K human DNA 110
methylation platform. 111

Methods 112

We extracted data from published databases and performed several analytical procedures 113
resulting in the development of an artificial model that can identify the CpGs with a SP 114

(epigenetic switch point; ESP) in their mean methylation fraction between the ages of 20 and 115

80 years. The entire process is described in Fig1. 116



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Figure 1: A schematic overview of the data extraction and analytical procedures used for the 118

identification of ESP. 119

Data acquisition and processing 120

1. *Crawling over GSE samples:* We used an extensive automatic web crawler to search for 121

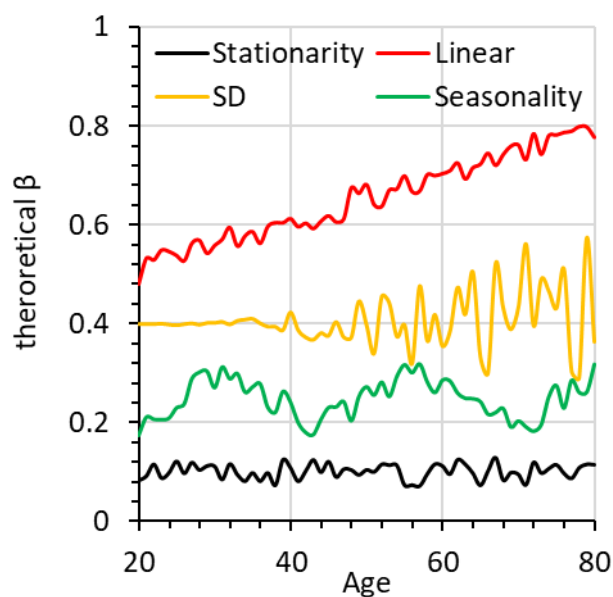
all available repositories that match our query for whole blood and healthy subjects at the 122

age range of 20–80 years (14) from the Gene Expression Omnibus (GEO) Data sets	123
repository.	124
2. <i>Downloading healthy whole blood samples</i> : All relevant β values and idat values were	125
downloaded, Samples with idat values were converted to β values using champ package	126
(27)	127
	128
3. <i>Data Filtration and verification</i> : All samples were manually inspected. Samples from	129
datasets that were not labeled as healthy and contained case-control groups were manually	130
examined to ensure that control samples were indeed healthy. The entire process yielded	131
a total of 2221 samples from 24 different data sets, which were then subjected to further	132
analysis	133
4. <i>Data treatment</i> : The β values of all filtered samples were normalized using BMIQ code	134
“champ.norm” implemented at Champ package (28).	135
5. <i>Correction for cell type heterogeneity</i> : We corrected for cell type heterogeneity using	136
"champ.refbase" implemented at Champ package (29).	137
6. <i>K-nearest neighbors' algorithm [KNN] for completing 47 missing probes</i> : To maximize	138
the preservation of CpG sites and remove poor-quality samples across the different	139
datasets we used a k-nearest neighbors' algorithm (KNN) based approach. To infer the	140
degree of relative similarity between the different samples based on their CpG site β	141
values, the sample-linked gender, age and the percentage of missing CpG sites were	142
entered. In the process of searching for the nearest neighbors sharing the same metadata	143
(age, gender, similar β values) and emplacing the missing β values with the values of the	144
closest neighbor, 47 samples could be thus adjusted and were retained. However, 78 other	145
samples for which no nearest neighbor were removed from our dataset. The entire process	146
yielded a total of 2221 samples from 24 different data sets, which were then subjected to	147

further analysis (table 1S). Of the resulting 2143 samples, 1119 were females and 1024 148
males, with an age distribution presented in figure S1, supplementary file S1. 149

7. *Probe averaging based on age:* Probes missing in more than 1% of our dataset were 150
filtered out, leaving 341,247 probes. The 2143 samples were tabulated by age and the 151
average β for each CpG site was calculated for each age (in years, between 20-80). This 152
data structure allowed us to remove noise and converge to age-specific trends. Moreover, 153
it reduced the data spread from 2143 columns for each of the 341,247 probs to 61 154
columns, a ~35-fold condensation which allowed more extensive calculations. Hence, we 155
ended up handling a data set of 61 columns, one column for each year of age (20-80 years) 156
with a mean β value for each CpG site in each of the cells. This data representation not 157
only reduced the size of the data analyzed but also can be viewed as a signal sampled with 158
a frequency of 1 year, which allows the usage of a vast variety of tools, algorithms and 159
mathematical models derived from the branches of time series analysis to uncover hidden 160
aging patterns that may appear as methylation β values. An example as to how such a 161
signal of a CpG site looks before and after the averaging can be seen in Figure 3A, B, E, 162
F. 163

8. *Dickey–Fuller stationary Test*: Since we strived to focus on CpG sites that undergo significant change over the age years of 20-80, we applied the augmented Dickey–Fuller test, which afforded the identification of stationary probes, i.e., probes that do not change significantly ($p < 0.05$) in terms of their means, standard deviations (SD) as well as seasonal (cyclic, sinus-like) behavior. This is conceptually depicted in Figure 2, in which 4 hypothetical probes are presented. The black curve represents a stationary probe which undergoes no significant change in the beta value or SD. Such hypothetical signal probes can be statistically described as discrete variables with normal distribution and a given mean and SD. Since there is no significant change across the years, in either the beta value or the SD, these probes were not further considered for evaluation in the present report.



On the other hand, the red orange and green curves are non-stationary signals representing change in beta value, SD, or seasonality behavior (sine function-like behavior), respectively, over time. In all, this analysis revealed 69,275 "non-stationary" CpG sites.

Figure2: Four hypothetical CpG signals: in black, a stationary signal; the red, orange and green curves exemplify three different non-stationary signals: a signal with an ascending change in beta value over time in red; in orange, a signal showing a change in SD value over time, and in green a signal with seasonal behavior, i.e., a sine like behavior.

9. *STL Decomposition / Trend analysis: Seasonal and Trend using Loess decomposition* 188

(STL) was used to reduce noise (30). This mathematical method allows the splitting of 189

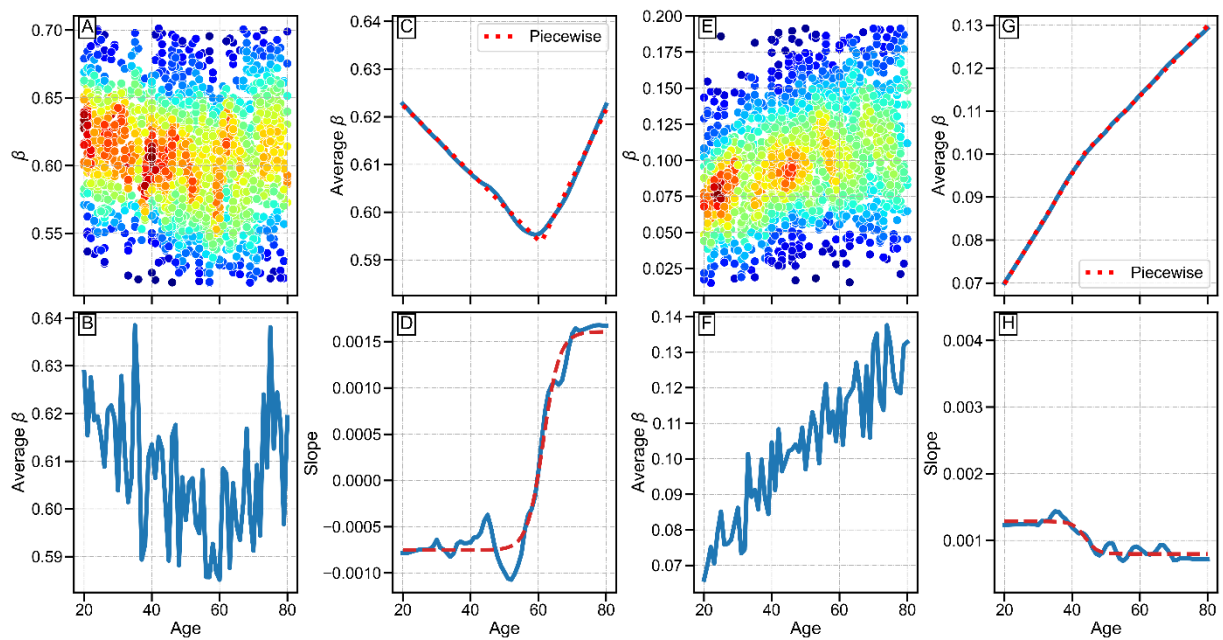
the CpG signal over time into three components: trend, seasonal and a remainder (noise) 190

component. The average methylation curve between 20 to 80 years before and after STL 191

process can be seen for probes cg03037684 and cg16803083 mapped to ESR1 gene and 192

to a region that was not annotated, respectively in figure 3B and C, and in figure 3 F and 193

G, respectively. 194



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Figure 3: Mean change in β values of two CpG sites between the ages of 20 to 80 years. A- 196

D and E-H represent 2 CpG sites with a change in mean methylation fraction with age, one 197

with (cg03037684; mapped to ESR1; curvilinear change) and the other without an ESP 198

(cg16803083; linear change), respectively. (A, E) the original data of the β values of 2143 199

individuals between 20 to 80 years old, (B, F) Average β values of all individuals (presented 200

in A, E, respectively) at the same age with one year resolution, (C, G) An STL analysis of 201

the average values presented in B and F (blue line), a piecewise model fitting to the STL 202

data is presented as two linear red dashed lines, and (D, H) The first derivative of the 203

averaged data following SLT, yields a sigmoid shape curve in D and a close to horizontal 204

shaped curve in H (blue lines). The dashed red line shows the close fitting of a sigmoid to
the STL average data. Color code in A and E is for the density of the number of individuals
for each beta value and for each age (per one year interval; red for most dense and blue for
list dense).

10. *Switch point analysis-Piecewise Regression & Sigmoid fitting*: The previous filtering
eliminated CpG sites showing no significant change with age. We next identified CpG
sites with ESP, i.e., CpG sites having a definable behavior until a certain age, but
showing a change in behavior as of a specific age and on as demonstrated in figure 3A-D.
To this aim we combined two methods which generated parameters for the construction
of an artificial model that we subsequently used for the identification and selection of
ESPs.

A. *Piecewise regression analysis*, which is based on fitting two linear curves to the
average methylation values at each year of age between 20 to 80 years (red dashed
lines in figure 3C and 3G) as formulated in equation 1, where $(\alpha_1, \alpha_2, \beta_1, \beta_2)$ are the
intercepts and slopes of each of the linear curve, respectively, calculated based on the
least squares, and S_p is the switch point.

Equation 1:

$$Piecewise(age_i) = \begin{cases} \alpha_1 + \beta_1 * (age_i - 20), 20 < age_i \leq S_p \\ \alpha_2 + \beta_2 * (age_i - S_p), S_p < age_i \leq 80 \end{cases}$$

The age of switch point is where the two fitted linear curves meet.

B. *Sigmoid fitting analysis*, which is based on fitting a sigmoid curve to the first
derivative of the average β values, between 20 to 80 years, with 1 year intervals for

each of our non- stationary CpGs sites. In Figure 3D and 3H the first derivatives of 229
average β values are presented as blue lines and the fitting to a sigmoid as dashed red 230
lines, as described in equation 2, where x is the age, λ the middle of the sigmoid and 231
S,B, and K are scaling parameters (supplementary file S1). 232

$$\text{Equation 2: } F(x) = \frac{S}{(1+e^{-K*(x-\lambda)})+B} \quad 234$$

The least squares method was used to calculate the optimal parameters (S, B, K, λ). 235
The age of SP, then, is the inflection point of the sigmoid curve (indicated by the 236
vertical grey dashed arrow in Figure 3D). 237
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The piecewise analysis provides a very ideal behavior of a particular time point where the 239
linearity of a beta values of a CpG site changes. Mathematically it is equivalent to a single 240
point in which the slope changes and can be defined as discontinuous for its first derivative 241
(Figure 4A). In population studies, a switch point will not be seen insentiently at a certain 242
age but will rather take place gradually over several years. Therefore, a sigmoid curve, 243
fitted to the first derivative of our non- stationary, STL-processed average β values between 244
20 to 80 years, may better describe the gradual emergence of a SP, as illustrated in figure 245
4B). According to this analysis, the behavior of ESPs can be also characterized by a 246
bilinear course having the first slope of methylation loss/ gain rate up to a certain age range 247
and a second different slope from that age range onwards, as described in figure 3C. This 248
is depicted by the two green arrows, where the vertical green lines denote the start and the 249
end of an ESP period. 250
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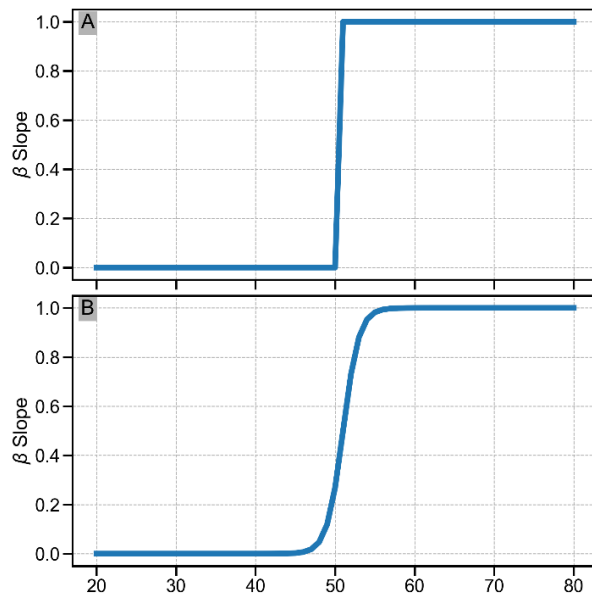


Figure 4: illustration of the first derivative of β values of a SP CpG site from 20 to 80 years. (A) an instantaneous SP, (B) a gradual, more realistic SP.

11. *Artificial model for the identification of switch points:* To filter out poor quality and/or noisy

probes, we constructed a mathematical model resulting in a collection of parameters which were used to distinguish between valid and non-valid SP CpGs. The model was based on an in person, CpG by CpG, visual inspection of randomly selected 1500 CpG sites and their features (as detailed below) out of the entire non-stationary 69,275 CpG sites population. During this procedure each screened CpG was labelled as having/not having a SP. ESP sites were identified as such only if any of the following exclusion criteria was present: a) the first derivative of the curve of the average β values as a function of age, did not fit a sigmoid curve, as indicated, for example, by the horizontal, first derivative curve derived from a linear methylation gain with age (figure 3H); b) derived STL was incompatible with the original data curve, c) the STL suggested the existence of a SP, but the actual curve could be fitted into an alternative pattern; d) a SP point generated by an abrupt and large change in beta values, which is likely fortuitous secondary to technicalities as may be suggested from the illustration in figure 4A. The piecewise regression and sigmoid models were used to derive 19 parameters (table 2S in supplementary). Based on in person visual inspection of the 1500 CpG sites, a decision

tree model was trained and evaluated with an average F1 Score of 0.89 on 5 fold cross 277
validation. The decision tree model was then used to examine all 69,275 non-stationary 278
probes in order to distinguish valid ESP probes from invalid probes. Eventually, 5175 279
probes that passed this filtration process were identified as ESPs. 280

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Pathway enrichment analysis for ESP genes

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Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (31) was performed 283
using DAVID (32) based on the 2,813 genes mapped to the 5175 ESPs. Pathways with 284
Benjamini < 0.05 were considered as significantly enriched. 285

Presence of ESP in genes associated with aging and longevity

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We compiled a list of aging and longevity genes by assembling genes from four databases that 287
are gathered in The Human Ageing Genomic Resources 288
(HAGR) <https://genomics.senescence.info/> (33): 1) GenAge: Database of Aging-Related 289
Genes, which includes a curated database of over 300 genes related to human aging and a 290
database of over 2000 aging- and longevity-associated genes in model organisms (34,35), 2) 291
GenDR: Database of Dietary Restriction-Related Genes based on genetic manipulation 292
experiments and gene expression profiling (36,37), 3) LongevityMap: a database of human 293
genetic variants associated with longevity of over 2000 genes, some were found to be essential 294
and some had no relation to longevity, 4) CellAge: Database of Cell Senescence Genes (38). 295
Only genes that were found in either human, mouse or human cultured cells, were included in 296
our list. From the LongevityMap database, we only included the genes that were found 297
essential to longevity and deleted the non-essential ones. Finally, we deleted duplications and 298
came up with a list of 973 resource genes related to aging and longevity. Venny 2.0.2 (39) 299
was used to check for common ESP genes and aging/ longevity related genes. 300

Results 301

Stationary vs. non-stationary CpG sites: change in DNA methylation level in relation 302

to age and the identification of CpG sites with an ESP behavior 303

We analyzed the trends of the average methylation level (β values), calculated for each year 304
of age at each age point, from the age of 20 to the age of 80 years and found that the vast 305
majority of the ~340K CpG sites (n=341,247), eventually available for analysis, were 306
stationary whereas 69,275 were found to be "non- stationary" (Materials and methods). We 307
next examined the behavior of the non-stationary probe population and selected these with a 308
clear ESP. The age of ESPs was the time at which acceleration/deceleration of DNA 309
methylation gain/ loss rate occurred or the time at which a constant loss/ gain changed to a 310
constant gain/ loss in methylation, respectably. In all, we identified a total of 5175 ESPs, of 311
which 5113 were present in men and 4067 for women. Most were common for both sexes 312
(supplementary file 2). 313

ESP patterns 315

DNA methylation trends with ESPs over the age range of 20 to 80 years appeared at several 316
different patterns. First, the overall trend of methylation change either rose or declined with 317
age (green arrows in the positive and negative overall trends in figure 5A). The overall trend 318
in the fraction of methylation for each of these CpGs followed one of five sub- SP patterns, 319
based on the relative relation between the first and second slopes of methylation change rate, 320
before and after the ESP period (figure 5A). There were more ESPs with an overall positive 321
methylation than negative methylation trend, especially in men. Pattern III, characterized by 322
a decrease in methylation up to a certain age followed by an increase in methylation from the 323
age period of ESP onwards, was the most dominant ESP pattern for both men and women, in 324

both overall trends (2465 and 1290 for positive and negative overall trends, respectively out 325
of 5113 men ESPs and 2168 and 1676 for positive and negative overall trends, respectively, 326
out of 4067 women ESPs). A sizable number of 1002 ESPs, showed acceleration in DNA 327
methylation increase rate with age (pattern V), and this was seen exclusively in men. 328

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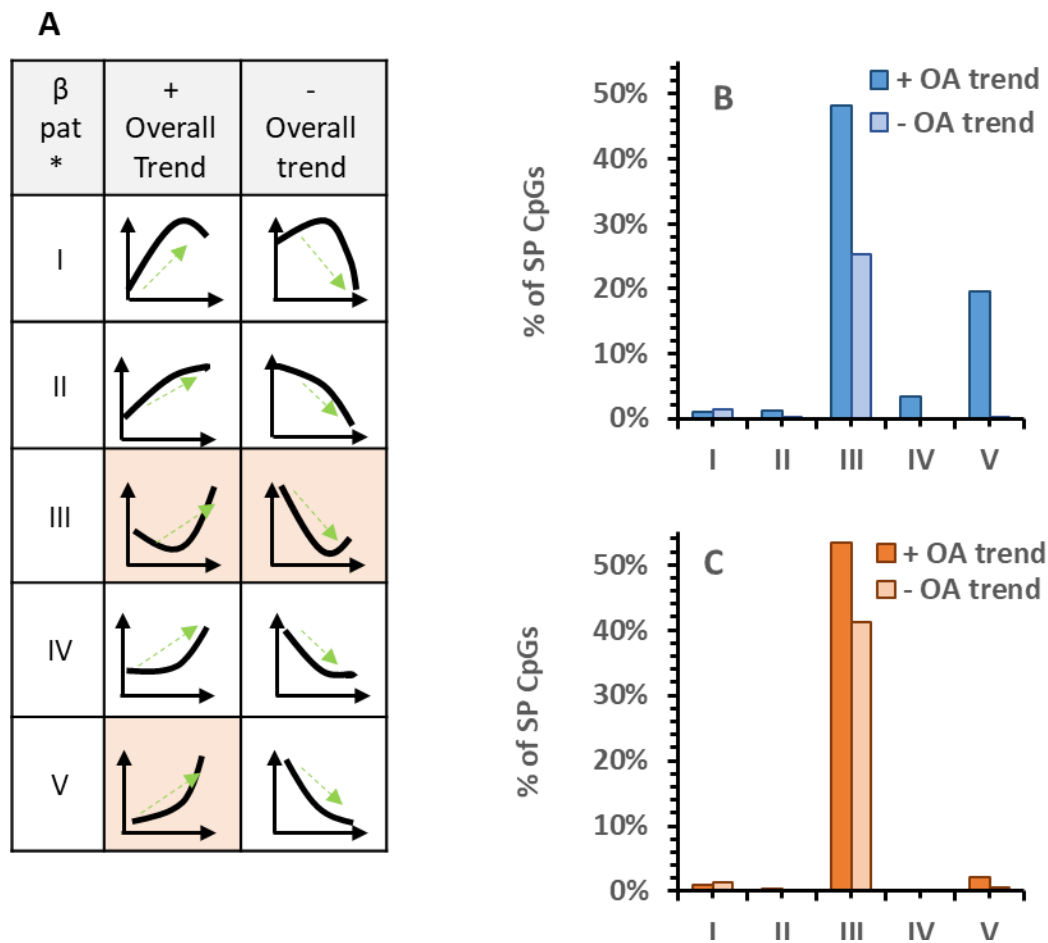


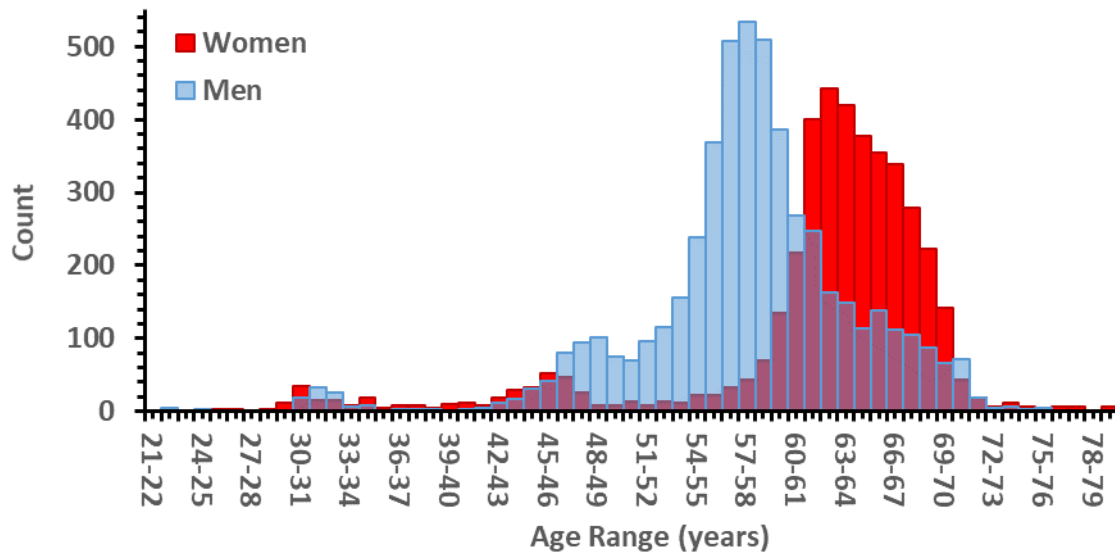
Figure 5: (A) a schematic view of the possible methylation SP patterns with age, for positive 330
(+) and negative (-) overall trends (OA). Shown is the frequency of the different SP patterns 331
in men (blue) (B) and women (orange) (C). SP CpG sites, for both positive and negative 332
overall trends (green arrows in A), where 100% is the total SP CpG sites for each gender. 333

There was a relative even distribution of the mean beta value of the CpG probes with ESPs 334
between 0.3 to 1, in CpGs with both negative and positive overall slop, for both men and 335

women (supplementary file 1, figure S2). This is in complete contrast to a typical beta value 336
distribution of the total 450K probes presenting CpGs in which most of the probes have either 337
a beta value close to 0.2 or to 0.9 (28). This means that compared to the total CpGs population, 338
a higher fraction ESPs have beta values around 0.5, which may imply a more dynamic 339
behavior between the methylated and de- methylated state. It is also possible that more ESPs 340
are mapped to imprinted genes. None of the CpGs resided on the X chromosome and hence, 341
the higher beta values in probes with ESP are not due to x- inactivation. 342

The age at which the epigenetic waves occur: relation to sex 343

Each ESP occurs at a definable age. Figure 6 demonstrates the frequencies of SP ages for all 344
ESPs, separately for men and women (5113 and 4067 ESPs for men and women, respectively). 345
The earliest and smallest (n=115, 98; M/F, respectively) wave of ESPs lays between the ages 346
of 30 to 33 years for both genders. A second wave, which includes several hundred ESPs 347
(n=765, 445; M/F, respectively) is seen between the ages of 45-51 years and 42-48 years for 348
men and women, respectively. This ESP age period for women begins slightly earlier than 349
that of men. Finally, the largest and dominant wave of SPs is discernible between the ages of 350
54- 62 years and 60- 69 years in men and women (n=4234, 3524; M/F respectively), 351
respectively. Hence, most SP tend to appear later in women than in men. Indeed, 2 out of the 352
3 waves, and collectively, the vast majority of ESPs take place ~six years and end ~seven 353
years later in women compared to men). 354



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Figure 6: Sexual dimorphism in DNA methylation switch points as a function of age: Bimodal sex related distribution. The amount of CpGs with a switch point at a specific age, at 1 year intervals.

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Most SP CpG are common for women and men

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Figure 7A is a Venn diagram showing that most ESPs are common for men and women (77.4%) while 21.4% ESPs are unique for men and only 1.5% are unique for women. We next constructed a Venn diagram for the 2,813 genes mapped to the ESPs (Figure 7B). In parallel to the ESPs, most genes with ESPs (81.4%) are common for men and women, 17.7% are unique for men and 0.9% are unique for women. This may imply that the aging process of men and women is mostly linked to similar biological pathways, with a clear difference in the age of onset. The full list of 5175 ESPs, their beta values, overall trends, first and second slopes (before and after the ESP period), age at the specific ESP, their mapped genes and their location in relation to their related gene, is listed in supplementary file S2. Since more than one CpG could be mapped for the same gene and since some CpGs were not mapped to a specific gene, the number of genes with ESPs is smaller than that of SP CpG sites.

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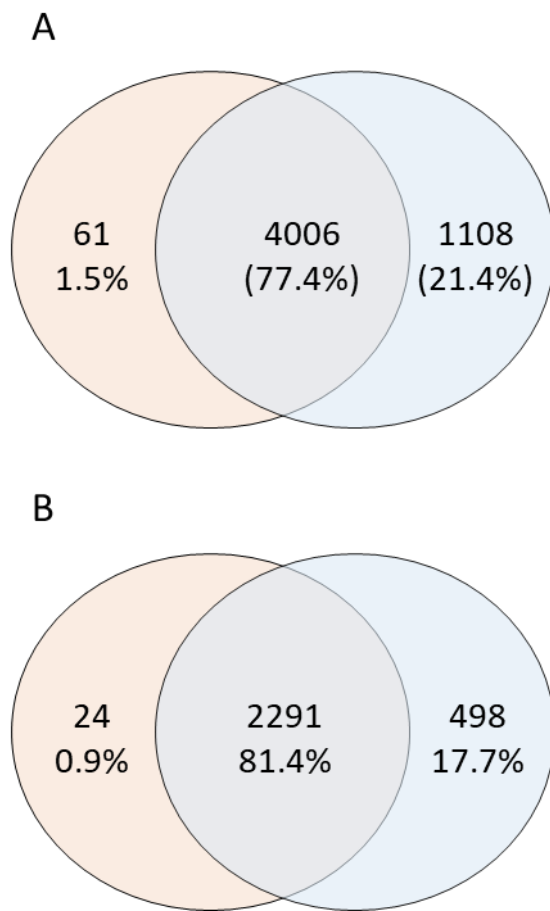


Figure 7: Venn diagram indicating the number of common and sex-specific ESPs (A) and genes related to the SP CpG sites (B) for men (blue) and women (Orange).

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ESP gene enriched for defined biological pathways

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To examine the potential links of genes with ESP to aging, we conducted biological pathways enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Fifty-nine pathways were enriched from the ESP gene list with a Benjamini threshold below 0.05 and a fold enrichment of around 2 (Supplementary file 2, Table S4). These pathways were predominantly related to the endocrine system (23%), cancer (13%), neurological communication (10%), cardiovascular system, immune system, cellular focal adhesions and additional pathways that serve multiple biological functions. Table 1 presents a sample of 9 pathways with prominent information regarding their relation to aging. The

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complete list of age-related pathways which involve genes with ESPs, with the associated ESP 393

genes for each pathway can be found in Supplementary file 2, Table S4. 394

Table 1: Age related significantly enriched Kyoto Encyclopedia of Genes and Genomes 395

(KEGG) biological pathways in the SP genes list 396

Pathway	# of Genes	Fold Enr*	Benjamini	Age related changes
hsa04261: Adrenergic signaling in cardiomyocytes	47	2.3	1.5E-05	Decline in heart performance and myocardial remodeling ability; decrease in cardiomyocytes survival ability, which involves reduced response to adrenergic signaling
hsa04360: Axon guidance	52	2.1	2.3E-05	Dysregulation of axonal mRNA localization and local protein translation
hsa04713: Circadian entrainment	32	2.4	1.3E-04	Increasingly dysregulated with age; disruption of circadian entrainment accelerates aging
hsa04725: Cholinergic synapse	34	2.2	3.2E-04	Cholinergic synapse function declines in the brain and neuromuscular junction.
hsa04611: Platelet activation	36	2.1	3.6E-04	Increases with age → increased cardiovascular thromboembolic clinical and subclinical events; noncanonical platelet effects accelerate neurological diseases including Alzheimer's disease, possibly by increased secretion of beta-amyloid.
hsa04911: Insulin secretion	27	2.3	1.0E-03	Aging beta cells display lower insulin secretory capacity in response to cellular and circulating cues

hsa04915: Estrogen signaling pathway	36	1.9	2.8E-03	Beside traditional tissue targets for estrogens (bone and cardiovascular system), decline in estrogen activity unfavorably affect the preservation of brain connectivity, synapse structure and function
hsa04930: Type II diabetes mellitus	15	2.4	1.9E-02	Aging is modulated by metabolic dysregulation of carbohydrate, fat and protein metabolism, overlapping with or culminating type 2 diabetes
hsa04211: Longevity regulating pathway	22	1.8	4.4E-02	Normal insulin response, caloric restriction, maintenance of autophagic flow and sufficient mitochondrial biogenesis and controlling inflammation and oxidative stress, improve survival and longevity and retard aging

*Fold enrichment; # = number

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ESP Genes known to be associated with aging

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We next searched for recognized associations between aging or longevity with ESP genes.

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We extracted ESP genes from known aging- related genes, which were derived from the

400

GenAge, GenDR, LongevityMap and CellAge databases compiled in The Human Ageing

401

Genomic Resources (HAGR) (33). Of these databases, we identified 149 genes with ESP (for

402

the full list see supplementary file S3, Table S5): 76 genes were from the GenAge, 37 from

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CellAge, 33 from LongevityMap and 28 from GenDR. A number of genes appeared in more

404

than one database. Hence, 5.3% of all ESP genes were related to aging, not significantly

405

different from the fraction (4.9%) of ageing-related genes derived from these databases (973

406

genes) out of the total number of genes in the human genome (19,969 genes). While not

407

enriched, then, the list of aging/longevity related genes with ESPs includes a number of genes

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whose relation to aging is of interest, which are compiled in Table 2. Among the multiple

409

genes with ESPs bearing a particularly significant impact on aging, some, such as ADCY5, 410
APOB, adiponectin, FOXO3, IGF1R, mTORC2, and TXNIP are amongst the most 411
extensively studied in this contest. The overall methylation trend with age, the mean β value 412
and the ESP age of the prominent age- related genes in table 2 are presented for men. The full 413
list of 149 ESP genes linked to aging and longevity with parameters for both genders is 414
provided in tables S5-S7, supplementary file S3. The tables in the supplementary file includes, 415
apart from the parameters presented in table 2, the ESP pattern for each gene and the delta 416
between the first and second calculated slopes (before and after the ESP period). 417

Table 2: Prominent aging and longevity related genes associated with SP CpG sites 418

SP-aging gene Symbol	SP- Aging genes	UCSC probe	RefGene Group	Overall slope trend	β	SP age
ADCY5	Adenylate Cyclase 5	cg25661931	1stExon	+	0.97	61
ADIPOQ	Adiponectin, C1Q And Collagen Domain Containing	cg10681525	Body	+	0.71	55
ADRA1B	Adrenoceptor Alpha 1B	cg09444685	Body	-	0.68	64
AGER	Advanced Glycosylation End-Product Specific Receptor	cg10996463	Body	-	0.87	43
		cg11105830	Body	+	0.81	45
		cg18139800	Body	+	0.92	58
ALOX15B	Arachidonate 15-Lipoxygenase Type B	cg15799267	5'UTR	-	0.34	55
APOB	Apolipoprotein B	cg13287979	Body	+	0.83	55
		cg03350299	TSS200	+	0.43	56
		cg25071744	TSS200	+	0.52	57
		cg23949611	Body	-	0.83	59
ATG7	Autophagy Related 7	cg01796438	TSS1500	+	0.42	55
		cg11277834	5'UTR	+	0.69	61
BCL2	BCL2 Apoptosis Regulator	cg08223235	Body	+	0.31	57
BNIP3	BCL2 Interacting Protein 3	cg15390444	Body	+	0.69	55
CLDN1	Claudin 1	cg03601836	Body	-	0.87	67
CREBBP	CREB Binding Protein	cg04141008	Body	+	0.72	55
CSNK1E	Casein Kinase 1 Epsilon	cg22494725	Body	-	0.63	65
DUSP1	Dual Specificity Phosphatase 1	cg12333707	TSS1500	+	0.50	60
EGFR	Epidermal Growth Factor Receptor	cg05246100	Body	-	0.72	59
		cg16488565	Body	+	0.55	57
		cg16751451	TSS1500	+	0.51	60

ERCC1	ERCC Excision Repair 1, Endonuclease Non-Catalytic Subunit	cg23347323	3'UTR	+	0.73	55
ESR1	Estrogen Receptor 1	cg03037684	3'UTR	-	0.61	58
ETS1	ETS Proto-Oncogene 1, Transcription Factor	cg01900413	Body	+	0.25	62
		cg25068347	Body	+	0.77	66
		cg21695395	Body	+	0.60	67
ETS2	ETS Proto-Oncogene 2, Transcription Factor	cg15892280	5'UTR	-	0.43	55
FOXO3	Forkhead Box O3	cg08792630	Body	+	0.37	63
HDAC4	Histone Deacetylase 4	cg14653043	Body	+	0.93	48
		cg24401044	Body	-	0.97	51
		cg01771737	Body	-	0.79	52
		cg04438064	Body	-	0.49	53
		cg05758467	Body	+	0.45	57
		cg15002163	Body	+	0.61	61
		cg11550064	Body	+	0.61	62
HIPK2	Homeodomain Interacting Protein Kinase 2	cg03520802	Body	-	0.54	53
		cg00995324	Body	+	0.92	61
		cg23646343	Body	+	0.25	68
HK3	Hexokinase 3	cg19791262	TSS1500	+	0.73	51
		cg14709481	5'UTR	+	0.68	56
IGF1R	Insulin Like Growth Factor 1 Receptor	cg08920032	Body	-	0.49	58
IGF2R	Insulin Like Growth Factor 2 Receptor	cg21774926	TSS1500	+	0.37	59

IGFBP2	Insulin Like Growth Factor Binding Protein 2	cg20366479	Body	+	0.56	44
INSR	Insulin Receptor	cg23075968	Body	+	0.39	61
MGST1	Microsomal Glutathione S-Transferase 1	cg22494907	5'UTR	-	0.91	61
MTOR	Mechanistic Target Of Rapamycin Kinase	cg10315903	TSS1500	+	0.65	62
NCOR2	Nuclear Receptor Corepressor 2	cg13146040	5'UTR	+	0.88	46
		cg03821418	Body	+	0.54	52
		cg07241090	Body	+	0.35	56
		cg22820108	5'UTR	-	0.40	57
		cg17825194	Body	-	0.34	57
		cg22700848	5'UTR	+	0.38	58
		cg17187521	5'UTR	+	0.40	60
		cg17387577	Body	+	0.33	69
NEIL1	Nei Like DNA Glycosylase 1	cg02426940	Body	+	0.82	56
NEK6	NIMA Related Kinase 6	cg13958199	TSS1500	+	0.61	53
		cg14082739	5'UTR	+	0.84	49
NFE2L1	NFE2 Like BZIP Transcription Factor 1	cg27568306	Body	+	0.91	53
NGF	Nerve Growth Factor	cg17750109	5'UTR	-	0.80	49
		cg02987481	TSS1500	+	0.43	59
NOS1	Nitric Oxide Synthase 1	cg21006686	TSS1500	+	0.70	57
		cg02500231	5'UTR	+	0.25	71
NOS3	Nitric Oxide Synthase 3	cg00571021	Body	-	0.00	0
NOTCH3	Notch Receptor 3	cg08529654	Body	+	0.41	60
PAX4	Paired Box 4	cg11975652	3'UTR	+	0.68	49

PIK3CD	Phosphatidylinositol-4,5-	cg23251761	5'UTR	-	0.73	49
	Bisphosphate 3-Kinase Catalytic Subunit Delta	cg19267205	Body	+	0.41	56
SLC6A6	Solute Carrier Family 6 Member 6	cg07573937	5'UTR	-	0.51	31
SLC9A3R2	SLC9A3 Regulator 2	cg09462956	Body	+	0.56	56
TNFSF13	TNF Superfamily Member 13	cg13358186	5'UTR	-	0.00	0
TP53	Tumor Protein P53	cg07760161	5'UTR	+	0.67	61
TXNIP	Thioredoxin Interacting Protein	cg19389852	1stExon	+	0.46	60
VWF	Transmembrane Protein 270	cg09117673	TSS200	-	0.79	55
WT1	WW And C2 Domain Containing 1	cg15946571	Body	+	0.94	59

419

Key epigenetic regulators are ESP Genes

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Interestingly, we found that key regulators of DNA methylation have ESPs at related CpG sites (Table 3, and supplementary file 3, Table S6), of which the most prominent ones are listed in Table 3. The earliest mean age of appearance of ESP is seen for TRDMT1 and DNMT1 (47, 49 years, respectively), and the latest age of EPS is notable for TET2 (71 years).

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Table 3: Key SP genes involved in de/methylation processes

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SP- Epigenetic gene Symbol	SP- Epigenetic gene name	Probe	UCSC RefGene Group	Overall slope	Mean β	SP age
TET3	Tet Methylcytosine Dioxygenase 3	cg15827185	Body	+	0.90	60

TET2	Tet Methylcytosine Dioxygenase 2	cg12306086	5'UTR	+	0.36	71
DNMT1	DNA Methyltransferase 1	cg23662947	Body	+	0.97	49
DNMT3B	DNA Methyltransferase 3 Beta	cg09835408	5'UTR	+	0.35	56
TRDMT1	TRNA Aspartic Acid Methyltransferase 1	cg23661704	Body	+	0.98	47

426

Estrogenic and androgenic SP genes

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Because most EPS begin to cluster during the fifth decade of life, in which women experience 428
menopausal transition and hormonal changes begin to afflict men as well, and since the 429
findings in Table 1 show that genes with ESPs are enriched with genes which participate in 430
the estrogen signaling pathway, we queried our ESP gene list regarding the presence and 431
potential enrichment with estrogen and androgen related genes. We found 24 genes that are 432
involved in estrogenic signalling pathway: 14 (of 2813 SP genes) are specifically involved in 433
estrogen receptor alpha (ESR1) signalling. The number of genes related estrogen receptor 434
alpha signalling in the entire 19,969 human gene population, is 55. We calculated an 435
enrichment Chi Square P value of 3.2e-08 and a binomial probability of 0.02 for randomly 436
getting 14 or more genes related to estrogen receptor alpha when taking a sample of 2813 437
genes. This indicates that there is an enrichment of estrogen receptor alpha- related signalling 438
genes in our ESP list with 1.8fold enrichment. Additionally, we found 28 genes linked to 439
androgen related pathways. 440

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Discussion

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In the present study we set out to assess the relation between age and DNA methylation level 445
in over 2000 subjects collected from 24 different cohorts, whose age spanned from 20 to 80 446
years. On the average, more than 80% of the CpG sites screened by the Illumina 450K 447
platform which then passed our internal data validation process were found to be stationary 448
over the years. Stationarity does not exclude the possibility of significant intra-individual 449
changes over the years in stationary probes, as the analyzed data is cross-sectional and not 450
based on longitudinal analysis with repeated measurement of methylation of the same probes 451
in the same subjects. Rather, our observations suggest that the majority of the probes included 452
in the 450 k Illumina platform show a stable average methylation fraction between the ages 453
of 20 to 80 years in apparently healthy subjects collected from heterogenous GSE sources. 454

About one fifth of the validated CpG sites probed in the 450K Illumina platform 455
(69275/341247) were not stationary: they underwent, on the average, consistent changes in 456
methylation level over time that can be described as linear/close to linear, curvilinear or 457
displaying changes in the standard deviation of the mean beta values (methylation fraction). 458
Among the latter group, 5175 non-stationary CpG sites changed linearly or in a manner close 459
to linearity over a certain age span, but then changed course at/around certain age zones, thus 460
displaying a switch point in their trend over the ages of 20-80 years, which we termed an 461
epigenetic switch point (EPS). 462

In general, switch points detected in the non-stationary probes can be characterized as follows: 463

1) they are limited to a fraction of the known genes, 2813 of the ~20,000 human genes. 2) 464
they appear in clusters as three apparent age-related waves: a small wave afflicting ~100 CpG 465
sites as of the age of 30 to the age of ~34; a second wave between the ages of 40 to 50 years, 466
composed of ~750 CpG sites which ends 3-4 years earlier in men; and a large wave, including 467

~4500CpG sites, between the ages of 50-68 years. 3) their distribution across each of waves 468
grossly follows a normal Gaussian pattern. 4)~80% of the CpG sites with switch points are 469
shared by women and men; 5) still, there is sexual dimorphism in the distribution of the switch 470
points, such that most of them emerge, peak, decline and vanish earlier in men than in women. 471
Further, in males ~20% of the ESPs are "male-only" CpGs, compared to only 1.5% "female 472
only" ESPs. Concordantly, switch points occur in less CpG sites and genes in women than 473
in men . Lastly, only men show ESPs with acceleration of an increasing trend in the fraction 474
methylation beyond the ESP-specific age (pattern V, Figure 5). 475

The clustering of ESPs in defined CpG sites of a limited number of genes, at defined age zones 476
with sexual dimorphism suggests some form of organization rather than a random occurrence. 477
Random switch points would have displayed a spotty pattern spread all over the platform's 478
probe population, with no predilection for CpG sites, genes, age or sex parameters. 479

What drives the appearance of EPS clusters in successive waves between the ages of 40-70 480
years? Could these waves be somehow related to aging-related processes, even though they 481
begin to decline rather sharply as of the late fifties in men and early sixties in women and then 482
entirely fade just a few years later and do not continue to appear in the eight's decade of life? 483
Certainly, our data cannot provide a fact-based elucidation of this question. However, one 484
cannot escape the consideration of several physiological and clinical changes that precede, 485
succeed or coincide with these waves in contemporary human life: hormonal and metabolic 486
changes and the emergence of cardiovascular morbidities tend to cluster as of the age of 40. 487
In females, menopause does not normally take place until 5 years following the initiation of 488
the second wave, but the menopausal transition is a gradual process lasting 7 up to 14 years, 489
most of which take place before the actual cessation of menses (40). Notably, the list of genes 490
with ESP is enriched with ESR1 (estrogen receptor α)-related genes Further, acceleration in 491

metabolic and cardiovascular disease in women actually takes place in post-menopausal 492
women (41,42). Interestingly, ESP in estrogen/estrogen action related genes are nearly all 493
shared by men and women (supplementary file S3, Table S7). 494

In men, testosterone declines by 0.8% annually as of the age of (43). The decline is much 495
steeper when health status undergoes unfavorable but common impairments (44) with the 496
appearance of overweight, obesity, diabetes and hypertension and cardiovascular events, all 497
of which are on the rise in midlife, particularly after the age of 40 years (40–47). The latter 498
conditions become significant players in females' health, on the average, about a decade later. 499
Table 1 lists some of the biological pathways that are most strongly enriched with ESPs, in 500
terms of statistical strength (Benjamini's test with $p=0.04-0.00015$) and involve processes 501
which are inseparable from the same health trends observed in and as of the fifth decade of 502
life. As examples, we find enrichment with CpG sites with SP in genes linked to adrenergic 503
signaling (hypertension, cardiovascular disease), circadian rhythmicity, cholinergic synapse 504
(obesity, aging, metabolic syndrome), platelet activation (cardiovascular events), insulin 505
secretion (overweight, obesity, diabetes), type II diabetes mellitus and estrogen signaling 506
(menopausal transition). 507

A close look at the list of genes showing ESPs discloses several examples CpG's/genes/groups 508
which are of interest vis-à-vis switch points and /or aging. Histone deacetylase 4 (HDAC4) 509
has 7 CpG sites with ESP (Table 2) and interestingly, estrogen act to retain HDAC4 in the 510
nucleus and thus inhibit hypertrophic gene expression and cardiac hypertrophy (48), a 511
common phenotypic phenomenon of heart aging (49). cg15799267, located on the enhancer 512
of arachidonate 15-lipoxygenase B, an enzyme implicated in atherosclerosis (50,51), has an 513
ESP at the age of 45 in women and 54 in men, after which it is increasingly demethylated with 514
age (Table 5S, supplement file 3S). 515

The presence of ESPs on some of the aging/longevity genes is also intriguing. For example, 516
KO of adenylate cyclase 5 (ADCY5), which involves many G-protein coupled receptor 517
signaling, such as the beta-adrenergic receptor signaling, increased the median life span of 518
mice by 30% (52). Not only was lifespan increased, but resistance to cardiac stress rose 519
whereas age- induced cardiomyopathy and reduction in bone density were lessened (52–54). 520
The co-presence of ESPs in the EGFR (epidermal factor receptor), insulin receptor, IGF1R, 521
IG2R, is also notable, as these are network players at the interface of growth control (including 522
cancer growth) and vascular aging with metabolic carbohydrate, fat and protein homeostasis, 523
where both excess activity or improper activation can modulate metabolic disease, bridge 524
insulin resistance to cancer and thus affect survival (55–58). Disruption of the insulin/insulin- 525
like growth factor 1 signaling (IIS) pathway in *Caenorhabditis elegans* was found to double 526
its lifespan (59). At the organ level, IGF1R KO in mice cardiomyocytes attenuates cardiac 527
hypertrophy associated with cardiac aging (60) whereas constitutive activation of IGF1R in 528
vascular smooth muscle cells of old mice accelerates the development of atherosclerosis (61). 529
The IR/IGF-1 signaling cascade also negatively regulates Forkhead Box O3 (FOXO3), a 530
transcription factor with a strong positive impact on aging and age-related phenotypes, 531
operating through enhancement of cellular ability to sustain stress (62). Concordant with this 532
role of FOXO3, natural SNPs in the FOXO3 gene were associated with increased longevity 533
of American men of Japanese ancestry aged ≥ 95 years (61,62). 534

The Mechanistic Target of Rapamycin Kinase (mTOR) is tightly linked to aging biology (65– 535
68). It functions as an intracellular energy sensor and a central regulator of growth, 536
proliferation, metabolism, survival, protein synthesis, apoptosis autophagy and transcription 537
(69). Impressively, rapamycin, an inhibitor of the mTOR complex 1 (mTORC1) has thus far 538
increased lifespan in all model organisms studied (65). The protein complex mTOR2 539
promotes the activation of insulin receptors and insulin-like growth factor 1 receptors and as 540

such, disruption of mTORC2 leads to glucose intolerance, diabetes, lower activity level and 541
immunosuppression (66,70). Its activity is inhibited by insulin/insulin-like growth factor 542
(IGF-1) signaling (IIS) cascade stimulation. Finally, differential methylation in CpG sites 543
related to Thioredoxin Interacting Protein (TXNIP), shown here to have ESPs, were found in 544
Type 2 Diabetes population, commonly, associated with aging (71–73). 545

Table 3 highlights 5 ESPs on genes encoding enzymes which directly affect DNA 546
methylation. DNMT1, DNMT3B are classical writer enzymes responsible for DNA 547
methylations. DNMT1, catalyzes the transfer of a methyl group to a cytosine nucleotide and 548
is mainly responsible for maintaining DNA methylation, which ensures the fidelity of this 549
epigenetic patterns across cell divisions. In contrast, DNA Methyltransferase 3 Beta 550
(DNMT3B) functions in *de novo* methylation. Hence, a change at the ESP for DNMT3B at a 551
mean age of 56 may be consequential. TRDMT1, tRNA aspartic acid methyltransferase 1, 552
also known as DNMT2, can also act as a writer, but is importantly linked to resistance to 553
stress, including oxidative stress, inflammation, salt stress, and cellular senescence (74,75). 554
TET enzymes generally function as 5-mC erasers by the conversion of 5-mC into 5-hmC, an 555
intermediary metabolite that releases the presumed effect of the methylated CpG site. Notably, 556
2 of the three known TET enzymes, TET3 and TET2 are shown to possess ESP age of 71 and 557
60 years, respectively. Hence, some ESPs can be identified in the DNA 558
methylation/demethylation machinery itself, preceding or coinciding with the overall ESPs 559
waves (Table 3). 560

Because these are just few of the >2800 genes with SP, and since ESPs waves encompass a 561
distinct, but broad age ranges, a detailed analysis of the of potential specific potential links 562
between the unfolding clinical unfolding of physiological processes and common diseases in 563
evolution and the epigenetic waves and their individual components comprises a formidable 564
challenge for future work . 565

At the present phase, the conceptualization of the ESPs waves as a potentially significant 566
phenomenon, requires consideration of the fact that whereas aging itself, including epigenetic 567
aging (5–7,14) goes on continuously with advancing years, the ESPs waves apparently begin 568
to wane after the age of ~58 in men and ~63 in women and then completely disappear within 569
the following 5-8 years. This transient pattern of epigenetic waves might indicate that the CpG 570
signals comprising the ESP waves are mechanistically involved in or at the least echo some 571
biological resetting that permits aging to advance, but may also reflect compensatory pro- 572
longevity biosystems activated by signals of aging and senescence. It is also possible, 573
particularly since we analyze age by cross-sectional and not longitudinal tools, that the waning 574
of the ESPs reflects a Darwinian selection, the survival of the fittest: subjects who remained 575
alive to their 8th decade may have not experienced the switch point waves that afflicted non- 576
survivors, thus leaving the 8th decade of life free of ESP waves. While potentially testable, 577
these hypotheses cannot be addressed without in depth longitudinal studies . 578

In conclusion, this is the first report of epigenetic aging waves in the form of clustering of 579
EPS in a fraction of the genes comprising the human genome, in an age and sex specific 580
manner. The main weakness of this report is that it is based on cross sectional analysis of mean 581
methylation level and not on longitudinal observations in the same individuals and therefore 582
does not deal with intra-individual changes in DNA methylation. This novel phenomenon, 583
however, which appears well organized and is linked to several transitional periods in human 584
life, lays the foundations to future quests to understand its underlying triggers as well as health 585
and aging-related consequences. 586

List of abbreviations

 587

ESP: epigenetic switch points 588

HDAC4: Histone deacetylase 4	590
mTOR: Mechanistic Target of Rapamycin Kinase	591
FOXO3: Forkhead Box O3	592
TXNIP: Thioredoxin Interacting Protein	593
ADCY5: adenylate cyclase 5	594
IGFR: insulin growth factor receptor	595
IGF: insulin growth factor	596
IIS: insulin/insulin-like growth factor (IGF-1) signaling	597
DNMT1: DNA methyl transferase 1	598
TRDMT1: tRNA aspartic acid methyltransferase 1, also known as DNMT2,	599
DNMT3B: Methyltransferase 3 Beta	600
TET1/2: Ten-Eleven Translocation 1/2	601
Ethics approval and consent to participate	602
Not applicable	603
Consent for publication	604
Not applicable	605
Availability of data and materials	606
The GEO number of the data sets analyzed during the current study are listed	607
in Additional file 1, table S1.	608
Competing interests	609

The authors declare that they have no competing interests 610

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Author contribution statement 613

N.S. initiated the project. N.S., E.S., T.K and T.S. designed the study. T.K, E.S, T.S, Y.M, 614

and G.S have selected and filtered the data source. T.K and E.S did all the mathematical and 615

statistical calculations. Y.E and M.P gave useful reviews and comments on the paper. N.S, 616

E.S and T.S. wrote the manuscript. All authors gave approval to the final version of the 617

manuscript. 618

619

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Supplementary material: 624

Supplementary file 1: table S1: The GEO number of the data sets analyzed during the current 625

study, table S2: the 19 parameters used in the decision tree model, figure S1: Age distribution 626

of the sample dataset by gender, figure S2: Distribution of β values in type III ESPs. 627

Supplementary file 2: Table 3: The complete list of 5175 ESP, Table 4: The complete list of 628

age-related pathways which involve genes with ESPs. 629

Supplementary file 3: Table 5: The complete list of 149 ESP genes which are aging/ longevity 630

genes. Table 6: DNA methylation process- related ESP. Table 7: Estrogen related ESP. Table 631

8: Androgen related ESP. 632

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