DNAmFitAge

Research Paper

DNAmFitAge: biological age indicator incorporating physical fitness

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ABSTRACT

Physical fitness is a well-known correlate of health and the aging process and DNA methylation (DNAm) data can capture aging via epigenetic clocks. However, current epigenetic clocks did not yet use measures of mobility, strength, lung, or endurance fitness in their construction. We develop blood-based DNAm biomarkers for fitness parameters gait speed (walking speed), maximum handgrip strength, forced expiratory volume in one second (FEV1), and maximal oxygen uptake (VO2max) which have modest correlation with fitness parameters in five large-scale validation datasets (average r between 0.16–0.48). We then use these DNAm fitness parameter biomarkers with DNAmGrimAge, a DNAm mortality risk estimate, to construct DNAmFitAge,

a new biological age indicator that incorporates physical fitness. DNAmFitAge is associated with low-intermediate physical activity levels across validation datasets (p = 6.4E-13), and younger/fitter DNAmFitAge corresponds to stronger DNAm fitness parameters in both males and females. DNAmFitAge is lower (p = 0.046) and DNAmVO2max is higher (p = 0.023) in male body builders compared to controls. Physically fit people have a younger DNAmFitAge and experience better age-related outcomes: lower mortality risk (p = 7.2E-51), coronary heart disease risk (p = 2.6E-8), and increased disease-free status (p = 1.1E-7). These new DNAm biomarkers provide researchers a new method to incorporate physical fitness into epigenetic clocks.

INTRODUCTION

Physical fitness declines with aging and is well known to correlate to health [1]. This decline is evident in reduced function in specific organs, like lungs [2], and in performance tests of strength [3] or aerobic capacity [4]. The rate of this decline varies between individuals [5, 6], and those who preserve physical fitness as they age are at lower risk for a range of diseases and tend to live longer lives [6–8]. At the molecular level, changes in fitness and related indices of functional capacity correlate with changes in molecular signs of decline thought to reflect underlying biological processes of aging [9]. Measures of fitness may therefore provide a new window into biological aging [10]. However, direct measurement of fitness parameters can be challenging, requiring in-person data collection by trained personnel with specialized equipment [11]. Furthermore, fitness measurements are not possible for studies with remote data collection or those conducted with stored biospecimens. To enable such studies to quantify fitness, we developed blood based DNAm biomarkers of fitness parameters spanning mobility, strength, lung function, and cardiovascular fitness and use these to construct a novel indicator of fitness-based biological age, DNAmFitAge.

Three lines of evidence support a focus on DNAm to develop biomarkers of fitness and aging-related changes in fitness. First, aging is reflected in DNAm changes; hundreds of thousands of CpG sites across the genome change methylation states as organisms grow older, enabling construction of high-precision algorithms to predict age [12, 13]. These are collectively known as epigenetic clocks, and a large body of literature demonstrates these clocks are associated with human mortality risk [14, 15], various age-related conditions [15–17], and are reflective of one's biological age [14, 17]. Second, prediction of aging-related morbidity, disability, and mortality by DNAm biomarkers is enhanced by the incorporation of physiological data, like smoking pack years and white blood cell counts, into prediction algorithms [14, 15, 18]. This suggests utility in including physical fitness in DNAm biomarkers, however, current DNAm biomarkers do not use fitness parameters in their construction. Third, there is emerging evidence that epigenetic clocks are sensitive to lifestyle factors [19], individual differences in fitness parameters are reflected in DNAm data [20, 21], and blood DNAm differs between athletes and controls [22]. Therefore, a growing body of evidence suggests blood DNAm carries information related to physical fitness, but it was unknown if fitness parameters could be estimated using blood DNAm levels.

Here, we develop blood DNAm biomarkers of four fitness parameters: gait speed (walking speed), maximum handgrip strength, forced expiratory volume in 1 second (FEV1; an index of lung function), and maximal oxygen uptake (VO2max; a measure of cardiorespiratory fitness). These parameters were chosen because handgrip strength and VO2max provide insight into the two main categories of fitness: strength and endurance [23], and gait speed and FEV1 provide insight into fitness-related organ function: mobility and lung function [8, 24]. Furthermore, each parameter is commonly measured and known to be associated with aging, mortality, and disease [8, 24–26]. We then use these DNAm fitness biomarkers to develop the novel DNAm fitness-related biological age indicator, DNAmFitAge, which quantifies the relationship between physical fitness and biological aging processes. This novel measure incorporates mortality risk with strength, mobility, and cardiovascular fitness using blood DNAm biomarkers. Our newly constructed DNAm biomarkers and DNAmFitAge provide researchers and physicians a new method to incorporate physical fitness into epigenetic clocks and emphasizes the effect lifestyle has on the aging methylome.

RESULTS

DNAm fitness parameter biomarker models

The DNAm fitness parameter biomarkers built with blood DNA methylation had modest correlation with direct fitness parameters. Average correlations across validation datasets ranged from 0.16–0.48 (Figure 1, Table 1). DNAmGripmax in males and females had moderate correlations in validation datasets but do not perform well in CALERIE. We hypothesize this may

be due in part from the stringent enrollment criteria: free of chronic disease, non-obese, and relatively young, which yields less variation in fitness measures. Correlation of DNAmVO2max to FEV in LBC1921 and LBC1936 was weak within each sex, likely caused by the small age range in each cohort; however varying correlations between FEV and VO2max have also been described in literature [27–29]. Reported correlations between VO2max and FEV vary from 0 to 0.5, likely because VO2max is a measure of cardiovascular health whereas FEV is a measure of lung volume. Correlation of DNAmVO2max to VO2max in CALERIE, the one validation dataset with the same direct fitness parameter, has good correlation overall and within sex (overall R = 0.55, female R = 0.19, male R = 0.47).

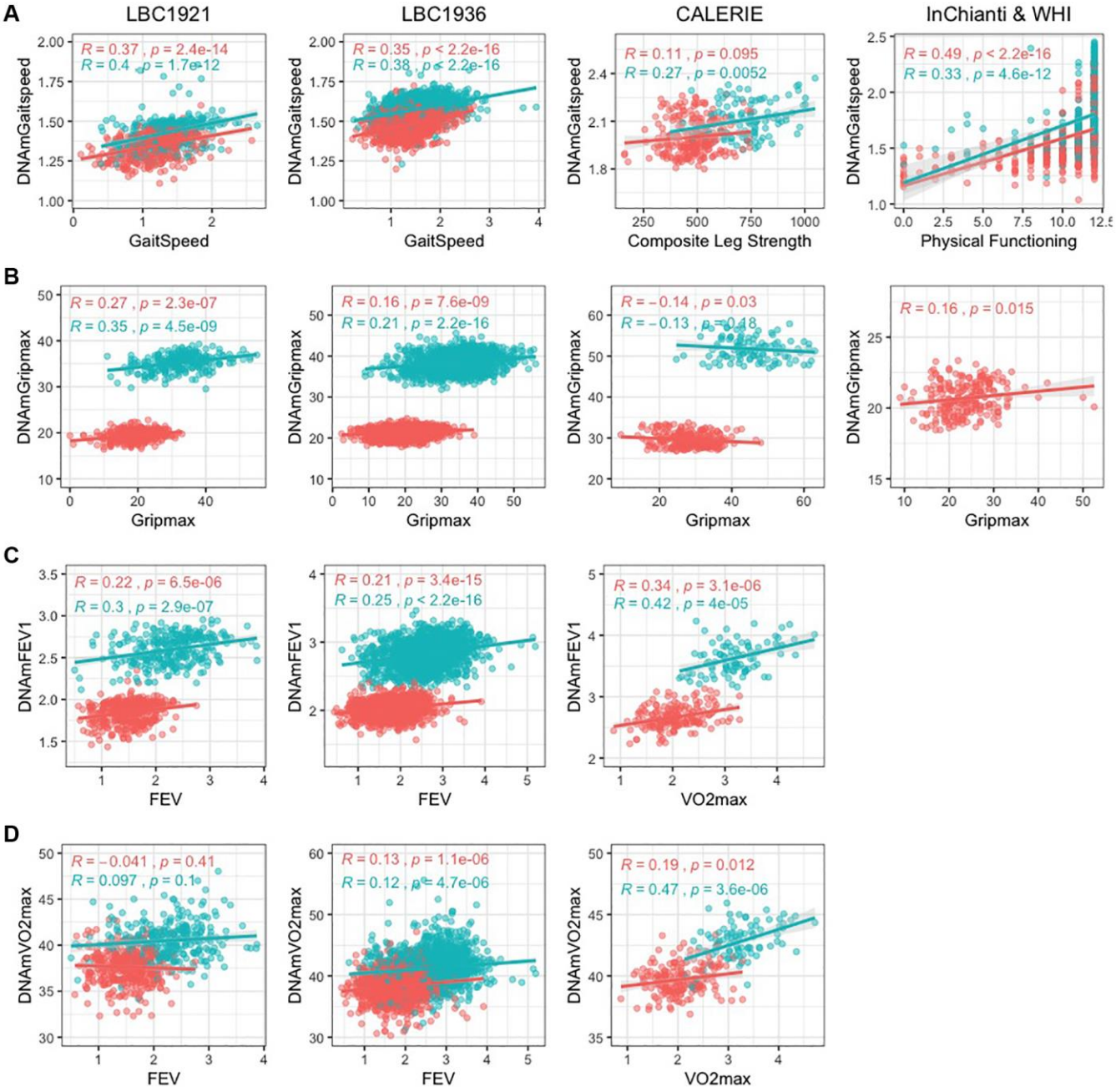

Figure 1. Scatterplots of DNAm fitness biomarker models versus true values in test datasets. Pink indicates females, and blue indicates males. When original variables were unavailable, best alternative variables are plotted against the DNAm fitness estimates. Each panel corresponds to the performance of one DNAm-based model built with chronological age across test datasets displayed with Pearson correlation and *p*-values. (**A**) DNAmGaitspeed with performance in InChianti dataset displayed, (**B**) DNAmGripmax with performance in WHI dataset, (**C**) DNAmFEV1, (**D**) DNAmVO2max. (**A–C**) (DNAmGaitspeed, DNAmGripmax, and DNAmFEV1) were built in each sex separately while (**D**) (DNAmVO2max) was built in both sexes jointly.

Table 1. DNAm fitness parameter biomarker Pearson correlation.

DNAm biomarker	CpG	Age in model	Sex	FHS + BLSA	Budapest	LBC 1921	LBC 1936	CALERIE	InChianti	WHI	Average test R
Gait speed	42	Y	Females	*0.61*	0.61	0.37	0.34	0.11*	0.49+	0.15+	0.34
	26	Y	Males	*0.43*	0.59	0.40	0.38	0.27*	0.33+		0.39
	53	N	Females	*0.56*	0.56'	0.17	0.17	0.095*	0.43+	0.12+	0.26
	59	N	Males	*0.60*	0.53'	0.23	0.21	0.26*	0.34+		0.31
Gripmax	52	Y	Females	*0.66*	0.54	0.27	0.16	−0.14		0.16	0.20
	52	Y	Males	*0.68*	0.50	0.35	0.19	−0.089			0.24
	91	N	Females	*0.66*	0.52	0.22	0.10	−0.16		0.12	0.16
	93	N	Males	*0.66*	0.43	0.21	0.14	−0.078			0.18
FEV1	77	Y	Females	*0.59*	0.50v	0.21^	0.20^	0.34			0.31
	73	Y	Males	*0.63*	0.30v	0.30^	0.25^	0.42			0.32
VO2max	40	Y	Both	0.52$	*0.70*	0.43^	0.40^	0.55			0.48

Superscripts indicate correlation is with the closest fitness parameter available: 'Jumpmax, *Composite Leg Strength, +Physical Functioning, ^FEV, $FEV1, vVO2max. Gray italics denote training dataset.

The DNAm biomarkers improve estimation of fitness parameters beyond what is explained through age and sex in many validation datasets (Supplementary Table 1). Table 1 shows between 26 and 93 CpG loci were selected through LASSO to estimate each fitness parameter. Without age as a covariate in the DNAm biomarker estimates, more CpG loci were needed to achieve similar precision- between 53 and 93. DNAmVO2max model includes several CpG loci on the X chromosome, likely capturing sex effects. Interestingly, DNAmGaitspeed and DNAmGripmax built without chronological age have lower correlation with true fitness parameters compared to the models built with chronological age, however these biomarkers explain more additional variation in fitness parameters compared to the age-included versions. This suggests the DNAm biomarkers capture different information than age and sex for understanding fitness parameters. R code to calculate DNAm fitness biomarkers is available in our GitHub repository at https://github.com/kristenmcgreevy/DNAmFitAge.

DNAm fitness biomarkers in age-related conditions

All DNAm fitness biomarkers are individually predictive of mortality and disease-free status, and some are predictive of type 2 diabetes status and number of comorbidities in the validation datasets. After controlling for age and sex, higher (or more fit) values of DNAmGaitspeed without age (p = 1.1E-10), DNAmGripmax without age (p = 2.6E-9), DNAmFEV1 (p = 2.2E-20), and DNAmVO2max (p = 0.003) are associated with decreasing mortality risk (Supplementary Figure 1). For example, on average, every 1 kg stronger DNAmGripmax is has an associated 5% decrease in mortality risk compared to a person of the same age and sex (hazard ratio = 0.95, confidence interval = [0.93, 0.96]). DNAmGaitspeed and DNAmFEV1 are both predictive of type 2 diabetes status (p = 0.0013, p = 0.0032) and number of comorbidities (p = 0.0004, p = 4E-12). Stronger values of any DNAm fitness biomarkers are associated with disease-free status. Relationship of each DNAm fitness biomarker with time-to-death, type 2 diabetes, number of comorbidities, and disease-free status after adjusting for age and sex are displayed in Supplementary Figure 1. Relationship of DNAm biomarkers to physical activity are explored alongside DNAmFitAge below.

DNAmFitAge

DNAmFitAge provides an estimate of biological age, and FitAgeAcceleration is a measure of epigenetic age acceleration. DNAmFitAge had strong correlation to chronological age in validation datasets. The average Pearson r between DNAmFitAge and chronological age across validation datasets was 0.77 (Figure 2), and the lower correlation in LBC1921 (r = 0.38) and LBC1936 (r = 0.68) can be attributed to the small age range they cover. LBC1921 ages ranged from 77 to 90 and LBC1936 ages ranged from 67 to 80. The average r excluding LBC cohorts was 0.92. The DNAm fitness biomarkers contribute 319 unique CpG loci to construct DNAmFitAge, and the contribution among DNAm fitness biomarkers were very similar in males (13.9–17.9%) with slightly more variation in females (10.4–22.4%). DNAmGripmax, DNAmVO2max, and DNAmGaitspeed contributed around 50% to estimating DNAmFitAge in each sex, and DNAmGrimAge contributed the remaining 50% (Table 2A). In addition, each validation dataset had low median absolute deviation (median of the absolute difference from chronological age to biological age) ranging from 2.3 to 4.9 years (Supplementary Table 2). Reproducibility

across a wide span of ages (21 in CALERIE to 100 in InChianti) demonstrate DNAmFitAge's calibration across a wide adult age range.

Applying each DNAmFitAge model to the opposite sex shows strong correlation with age but with substantial over and underestimation of age in females and males, respectively (Supplementary Table 2, panels B–H Supplementary Figure 2). Over and under estimation are explained by universal differences in male and female fitness parameters. Females tend to have lower fitness parameters compared to males. Hence males were predicted to be younger than they are using the female DNAmFitAge model because larger values of DNAmGaitSpeed, DNAmGripmax, or DNAmVO2max indicates stronger (or more physically fit) females.

Reference DNAm fitness biomarker values corresponding to fit DNAmFitAge compared to unfit DNAmFitAge within age and sex categories are provided in Table 2B. "Fit" corresponds to biological age being 5 years younger than expected (FitAgeAcceleration ≤ -5), and "unfit" corresponds to biological age being 5 years older than expected. Average differences (Fit – Unfit) across all age and sex categories are 0.2 m/s faster DNAmGaitSpeed, 5.1 kg stronger DNAmGripmax, and 2.0 mL/kg/min better DNAmVO2max. Overall, higher or more physically fit values of DNAmGaitspeed, DNAmGripmax, or DNAmVO2max correspond to younger estimated biological ages in males and females.

FitAgeAcceleration in age-related conditions

We find that the age-adjusted version of FitAge, FitAgeAcceleration, is a significant predictor of mortality risk (all cause mortality), coronary heart disease, and other age-related conditions. Cox Proportional Hazard models demonstrated FitAgeAcceleration is a strong predictor for time-to-death (p = 7.2E-51) and time-to-coronary heart disease (p = 2.6E-8). FitAgeAcceleration had an overall hazard ratio of 1.07 (1.06, 1.08) (Figure 3). Thus, a FitAgeAcceleration value of 10 years was associated

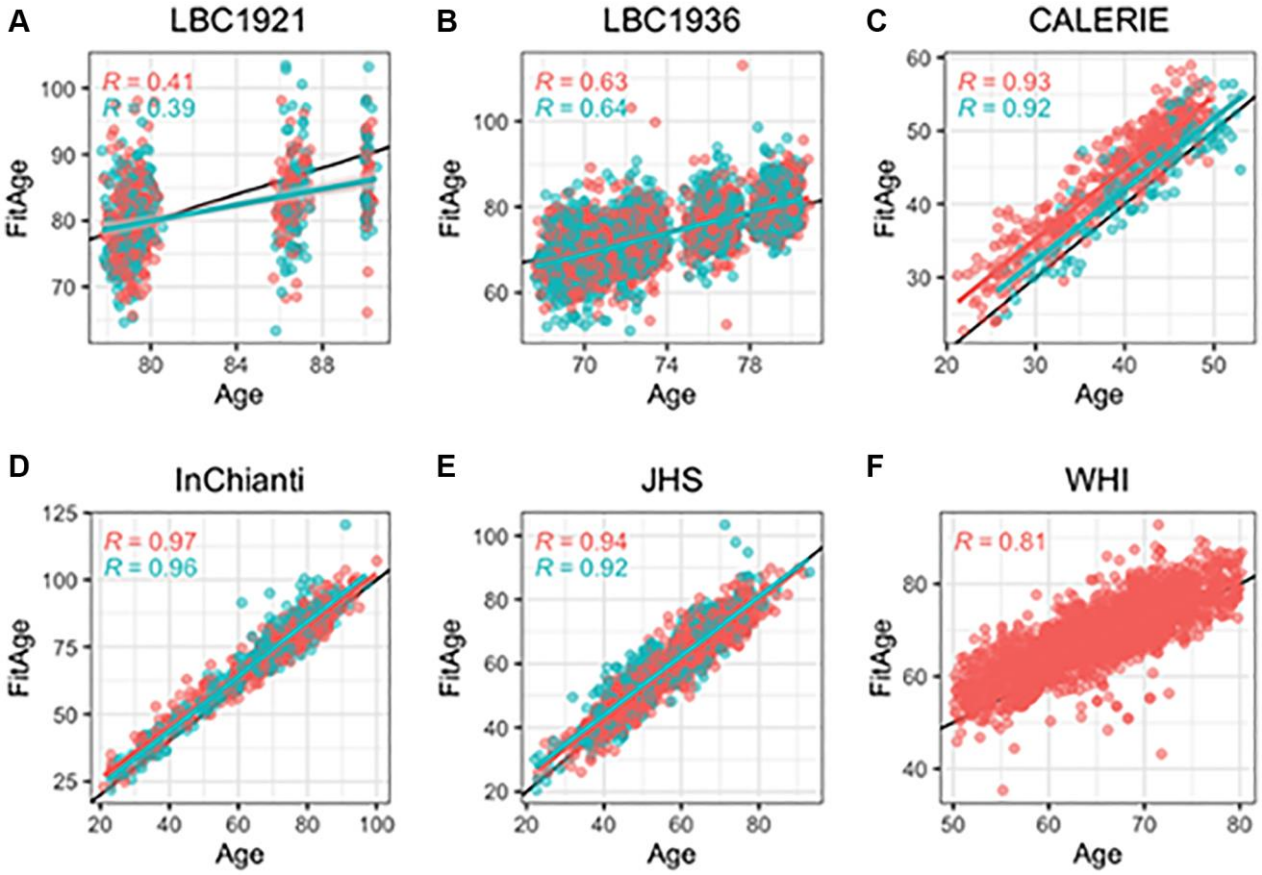

Figure 2. Scatterplots of DNAmFitAge versus age separated by sex. Pink indicates females, and blue indicates males. (**A–F**) Each panel corresponds to the performance of DNAmFitAge in one validation dataset displayed with Pearson correlation to chronological age and corresponding p-values. DNAmFitAge models applied to the same sex it was built in (i.e., DNAmFitAge built for females tested in females and DNAmFitAge built for males tested in males). DNAmFitAge is centered on chronological age with high correlation across all test sets.

Table 2A. DNAmFitAge model weights.

Variable	Female weights	Male weights
DNAmGripmax	0.174	0.179
DNAmGaitSpeed	0.228	0.159
DNAmVO2max	0.104	0.139
DNAmGrimAge	0.493	0.523

Table 2B. Reference DNAm fitness parameter values for fit (FitAge acceleration <= −5 yrs) and unfit (FitAge Acceleration >= +5 yrs) individuals.

	Females							
Age	DNAmGaitspeed		DNAmGripmax		DNAmVO2max		DNAmGrimAge	
	Fit	Unfit	Fit	Unfit	Fit	Unfit	Fit	Unfit
<40	2.1	2.0	34.6	30.5	42.8	40.1	37.1	40.9
40–59	1.9	1.7	31.3	26.9	39.2	37.9	49.2	60.5
60–79	1.7	1.5	28.8	22.4	37.6	36.1	63.2	72.4
80+	1.6	1.3	23.9	19.1	37.0	35.4	74.7	81.8
	Males							
Age	DNAmGaitspeed		DNAmGripmax		DNAmVO2max		DNAmGrimAge	
	Fit	Unfit	Fit	Unfit	Fit	Unfit	Fit	Unfit
<40	2.1	1.8	49.3	43.8	45.1	44.9	34.8	52.9
40–59	1.9	1.7	46.6	42.5	43.9	42.3	47.5	60.1
60–79	1.7	1.5	41.3	36.8	43.1	39.5	68.0	77.9
80+	1.6	1.3	39.3	32.0	41.3	37.7	78.0	86.7

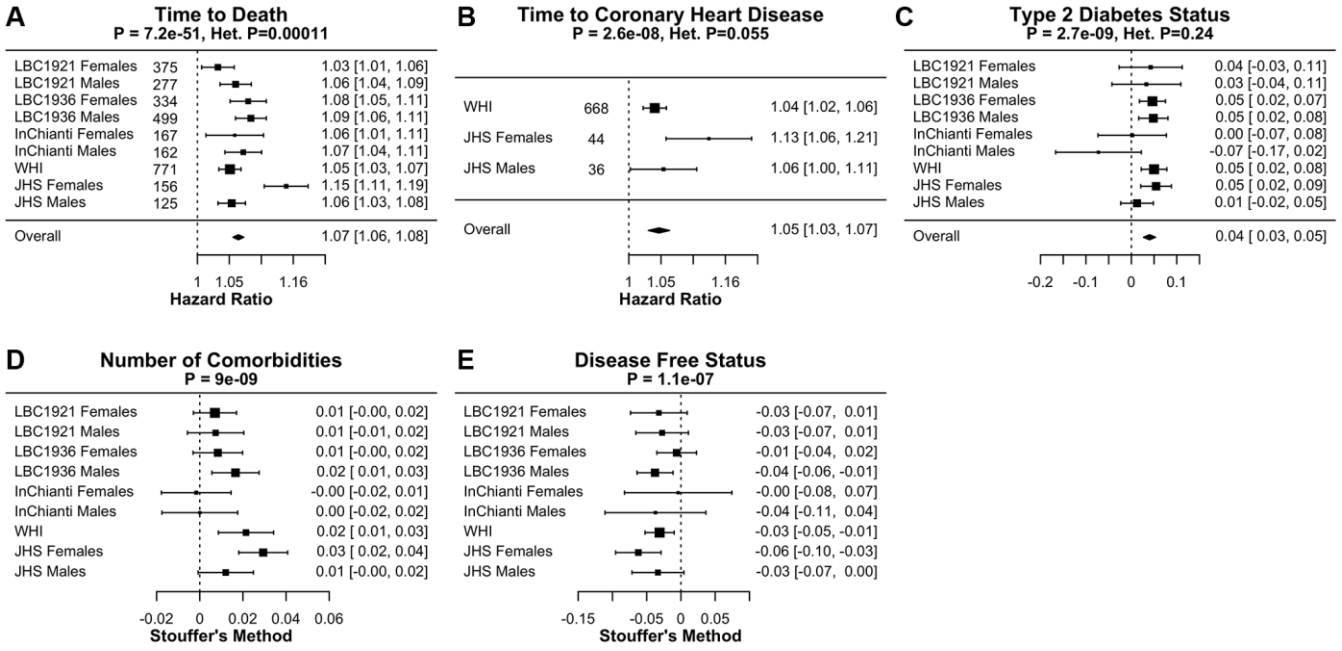

Figure 3. Meta-analysis forest plots for FitAgeAcceleration to age-related conditions adjusted for age and sex. Each panel reports a meta analysis forest plot for combining hazard ratios or regression coefficients across dataset cohorts. (**A**) Time-to-death with number of events, (**B**) time-to-coronary heart disease with number of events, (**C**) type 2 diabetes, (**D**) comorbidity count, and (**E**) disease free status. Meta-analysis *p*-values are displayed in the header of each panel, and test of heterogeneity Cochran *Q* test *p*-value (Het. P) are displayed for fixed effect models. Fixed effects models were used for (**A**–**C**) and Stouffer's method was used for (**D**, **E**).

Table 3. Association of DNAm biomarkers to physical activity and physical functioning in people with low to intermediate activity levels.

Outcome		Females					Males				Meta analysis *p*-value
		LBC 1921	LBC 1936	InChianti	JHS	WHI	LBC 1921	LBC 1936	InChianti	JHS	
DNAmFitAge	coefficient	−0.024	−0.031	−0.095	−0.033	−0.237	0.008	−0.024	−0.041	−0.040	6.37E-13
	p-value	2.3E-04	3.7E-06	0.042	0.046	0.014	0.199	2.0E-05	0.272	0.044	
DNAmGaitSpeed w/ Age	coefficient	−0.51	2.82	8.76	3.07	26.67	−3.31	1.99	4.97	1.78	1.82E-03
	p-value	0.567	0.002	0.084	0.165	0.025	2.4E-04	0.022	0.429	0.672	
DNAmGaitSpeed w/o Age	coefficient	0.87	0.90	1.32	0.40	8.77	−0.10	0.99	2.36	1.49	1.60E-06
	p-value	0.001	0.004	0.536	0.627	0.099	0.725	0.001	0.255	0.293	
DNAmGripmax w/Age	coefficient	0.10	−0.06	0.14	-0.03	0.24	0.00	0.03	0.17	0.02	0.029
	p-value	0.036	0.201	0.635	0.821	0.035	0.943	0.256	0.291	0.801	
DNAmGripmax w/o Age	coefficient	0.076	0.035	0.10	0.002	0.30	−0.02	0.02	0.06	0.02	1.85E-04
	p-value	4.3E-06	0.043	0.379	0.953	0.181	0.125	0.058	0.364	0.617	
DNAmFEV1	coefficient	1.07	0.60	0.33	0.99	4.98	−0.17	0.37	0.37	−0.58	0.0062
	p-value	0.026	0.197	0.898	0.114	0.005	0.585	0.173	0.791	0.337	
DNAmVO2max	coefficient	0.06	0.03	0.26	−0.05	−0.47	−0.06	0.02	0.05	−0.03	0.113
	p-value	0.003	0.090	0.019	0.423	0.215	0.002	0.281	0.667	0.654	
DNAmGrimAge	coefficient	−0.01	−0.03	−0.08	−0.05	−0.30	-0.01	−0.03	-0.01	−0.05	1.25E-12
	p-value	0.524	5.7E-06	0.157	0.002	0.027	0.390	2.3E-05	0.794	0.007	
DNAmPhenoAge	coefficient	0.00	−0.01	−0.12	-0.02	−0.07	7.1E-05	−0.01	-0.01	−0.01	1.26E-06
	p-value	0.568	0.012	4.2E-04	0.063	0.354	0.989	0.004	0.764	0.425	
DNAmPAI1	coefficient	−3.4E-05	−4.4E-05	−7.5E-05	−1.1E-04	−3.4E-04	−1.8E-06	−2.8E-05	7.5E-05	−6.2E-05	6.36E-10
	p-value	0.021	0.002	0.358	2.5E-08	0.076	0.908	0.032	0.382	0.009	
DNAmGDF15	coefficient	−8.5E-05	−1.3E-03	-2.9E-03	−8.5E-04	−1.0E-02	−1.2E-03	−5.9E-04	−3.5E-03	−6.7E-04	6.16E-08
	p-value	0.802	0.0005	0.082	0.147	0.047	5.4E-04	0.054	0.117	0.373	

with almost doubling the mortality risk compared to the average person of the same age and sex (1.07^{10} = 1.97 risk). Similarly, increase in FitAgeAcceleration corresponds to more comorbidities (p = 9.0E-9), hypertension (p = 8.7E-5), and earlier age at menopause (p = 6.6E-9) (Figure 3, Supplementary Table 3). A lower FitAgeAcceleration was associated with disease free status (p = 1.1E-7) and lower cholesterol (p = 0.0005) (Supplementary Table 3).

Each of these associations were in the expected direction, as someone who had a low Fit-AgeAcceleration had a biological age estimate that was younger than their chronological age. Hence, people whose DNAm predicted them to be more 'physically fit' than their chronological age would suggest had better age-related outcomes. These relationships demonstrate epigenetic age acceleration can be well explained through DNAm fitness parameter biomarkers, and that FitAgeAcceleration provides a practical tool for relating fitness to the aging process.

FitAgeAcceleration is additionally informative for mortality risk beyond the information captured with AgeAccelGrim in JHS females and in InChianti males and females when comparing LRT p-values (Supplementary Table 4). FitAge Acceleration is almost always additionally informative for time-to-death compared to other epigenetic clocks, but FitAgeAcceleration is only sometimes informative beyond the epigenetic clocks for explaining number of comorbidities. Overall, our results indicate FitAgeAcceleration is informative for mortality risk and may act as a supplement (not replacement) to AgeAccelGrim.

DNAmFitAge relationship to physical activity

FitAgeAcceleration, DNAmGaitspeed, DNAmGripmax, and DNAmFEV1 have associations in the expected direction with physical activity in low to intermediate physically active individuals. Coefficients indicate the effect on physical activity for a one unit increase in each DNAm fitness biomarker after adjusting for chronological age within each sex (Table 3, Figure 4).

The relationship to DNAmFitAge is as expected; someone with a higher FitAgeAcceleration has an estimated biological age that is older than expected, which corresponds to lower physical activity or physical functioning (Table 2B). Similarly, men and women with a faster DNAmGaitspeed, stronger DNAmGripmax, and larger DNAmFEV1 are more physically active when holding age constant. In conclusion, men and women who were more active showed correspondingly 'fitter' values of FitAgeAcceleration and the DNAm fitness biomarkers. Research suggests any exercise compared to none is beneficial to health [30], and we hope DNAmFitAge may serve as a tool to motivate starting an exercise regimen at any level.

Additionally, DNAmFitAge (Stouffer *p*-value = 6.4E-13) marginally outperforms current DNAm biomarkers when comparing meta-analysis *p*-values; improvement of DNAmFitAge compared to DNAmGrimAge (*p*-value = 1.2E-12) is marginal, however the improvement compared to DNAmPhenoAge (*p*-value = 1.3E-6) and DNAmGDF-15 (*p*-value = 6.2E-8) is more pronounced. In addition, DNAmFitAge, which provides an indicator of biological age, may provide a more interpretable aging biomarker compared to DNAmGrimAge, which provides a measurement of lifespan. These comparisons demonstrate DNAmFitAge can capture the relationship to physical activity and can provide an improvement to the arsenal of current DNAm biomarkers.

DNAmFitAge relationship in body builders

Male body builders are estimated to be biologically younger and more physically fit compared to male controls of the same age. On average, DNAmFitAge is 2.74 years younger in male body builders compared to controls (p = 0.041), and DNAmVO2max is 0.4 mL/kg/sec better in male body builders (p = 0.023) (Table 4). FitAge Acceleration (p = 0.080), DNAmGaitspeed (p = 0.055), and DNAmGripmax (p = 0.075) are suggestive of having improvement in male body builders, however they were not significant at the 0.05 level. Boxplots displaying the spread of DNAmFitAge, DNAmVO2max, FitAge Acceleration, and DNAmGaitspeed between body builders and controls are presented in Figure 5. Male body builders have 5.4 more years of regular training (p = 2.6E-6) and 1.1 more training sessions per week (p = 9.4E-7) compared to male controls on average, and the DNAmFitAge and DNAmVO2max results correspond to male body builders being estimated as more physically fit, as expected. The study was underpowered for females with only 30 female body builders, however we did examine the relationship of the DNAm fitness biomarkers in females and expectedly (due to the small sample size) did not find a significant difference between female body builders and controls. Our promising results in male body builders show a physically fit lifestyle corresponds to biological aging benefits that can be captured with our new DNAm fitness biomarkers and DNAmFitAge.

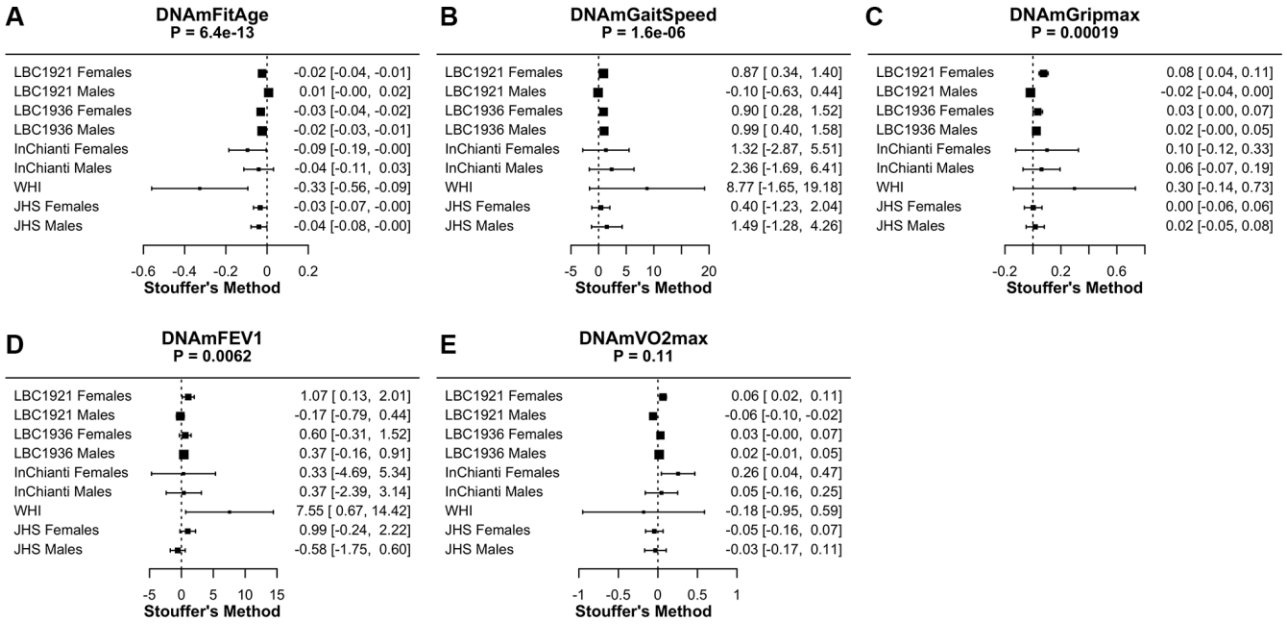

Figure 4. Meta-analysis forest plots for DNAmFitAge and DNAm fitness parameters relationship to physical activity or physical functioning in people with low to intermediate physical activity. Each panel reports the Stouffer's meta-analysis *p*-value for combining coefficients across dataset cohorts after adjusting for chronological age. (**A**) DNAmFitAge, (**B**) DNAmGaitspeed, (**C**) DNAmGripmax, (**D**) DNAmFEV1, and (**E**) DNAmVO2max. DNAmFitAge, DNAmGaitSpeed, DNAmGripmax, and DNAmFEV1 are predictive of physical activity in low to intermediate physically active individuals.

Table 4. Comparison between male controls and body builders in Polish study.

	Mean control (n = 149)	Mean body builder (n = 66)	Control - body builder	Kruskal wallis p-value
Intensity trainings per week	3	4.1	−1.1	9.43E-07
Years regular training	6.6	12	−5.4	2.61E-06
DNAmFitAge	41.1	38.4	2.74	0.041
FitAgeAcceleration	0.15	−0.56	0.72	0.08
DNAmGaitspeed	1.99	2.02	−0.03	0.055
DNAmGripmax	46.5	47.2	−0.69	0.075
DNAmVO2max	44	44.4	−0.4	0.023
DNAmFEV1	3.82	3.87	−0.05	0.199
DNAmGrimAge	44.1	41.8	2.24	0.063
DNAmPhenoAge	26.7	24.7	2.01	0.181
DNAmPAI1	19033	18238	795	0.009
DNAmGDF15	701.8	680.4	21.4	0.447

Dietary supplement use cannot explain improvement in DNAmFitAge, but multivitamin dietary supplements are associated with improvement in DNAmVO2max after controlling for athlete status and age in males. Males from the Polish Study who take multivitamins have a 0.68 mL/kg/sec fitter DNAmVO2max on average after adjusting for athlete status and age (p = 0.041, Supplementary Table 5). Multivitamins, energy, vitamin D, and Omega-3 all are disproportionately taken by the male body builders (Supplementary Table 6), however, supplement use is not sufficient to explain younger DNAmFitAge regardless of athlete status (Supplementary Table 5). These insignificant results may point to other components of athleticism that contribute to younger estimated biological ages, such as increased physical activity and decreased body fat. We note that supplement and athlete coefficients for multivitamins, proteins, and Omega-3 are statistically insignificant, but their relationships are in the expected direction for DNAmFitAge and DNAmVO2max. Our research does not establish the causative relationship of body building or supplement use on biological aging, but it does establish there are observable epigenetic benefits associated with being a male body builder.

Functional CpG annotation

The 627 CpG genomic locations used to construct the DNAm biomarker estimates were enriched in 5 gene

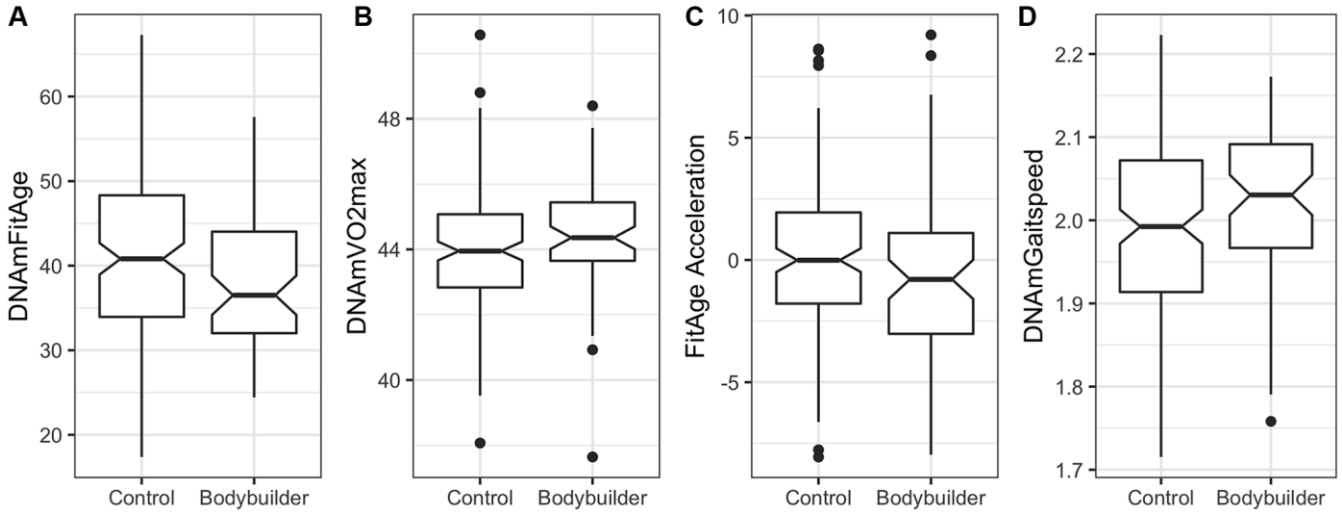

Figure 5. Boxplots showing spread of DNAm biomarkers between male controls (n = 149) and male body builders (n = 66) in the Polish study. (**A**) DNAmFitAge is younger on average in the male body builders, (**B**) DNAmVO2max is fitter on average in the male body builders, (**C**) FitAge Acceleration and (**D**) DNAmGaitspeed are suggestively improved in body builders but not significantly different at 0.05.

Table 5A. Top GREAT CpG annotation results.

	Observed regions	Fold enrichment	Binomial *p*-value	Bonferroni *p*-value
Genes				
ZNRD1	4	77.9	2.75E-07	0.0051
HLA-G	4	55.0	1.09E-06	0.020
Cellular				
MHC protein complex	9	25.1	1.86E-10	3.11E-07
Integral component of endoplasmic reticulum membrane	21	3.7	4.49E-07	0.00075
Intrinsic component of endoplasmic reticulum membrane	21	3.6	6.39E-07	0.0011
MHC class II protein complex	5	26.9	1.56E-06	0.0026
Integral component of lumenal side of Endoplasmic reticulum membrane	7	12.7	1.81E-06	0.0030
Molecular				
Peptide antigen binding	6	13.3	7.71E-06	0.032
Tapasin binding	2	421.0	1.12E-05	0.047

Table 5B. Chromatin state enrichment.

State	Description	Number of CpG loci	Odds ratio	Hypergeometric *p*-value
PromF4	Promoter; heavily acetylated - flanking tss downstream bias	25	0.45	6.5E-06
TSS1	TSS more acetylated and active	15	0.37	6.8E-06
BivProm2	Weak bivalent promoter- stronger on H3K27me3	43	1.76	0.00057
TxEx3	Exon; H3K36me3 strong	4	0.30	0.0030
DNase1	DNase I only	13	2.41	0.0041
ReprPC1	Polycomb repressed; H3K27me3 strong and H3K4me1 weak	21	1.87	0.0065
BivProm1	Weak bivalent promoter - more balanced H3K4me3/ H3K27me3	43	1.50	0.0092

sets, 11 cellular processes, and 7 molecular processes mostly related to inflammation at FDR Q-value < 0.05. Top enrichment results from GREAT analysis passing the Bonferroni *p*-value threshold of 0.05 are presented in Table 5A and complete GREAT results are presented in Supplementary Table 7. The top genes enriched include zinc ribbon domain containing 1 (ZNRD1; Bonferroni $p = 0.005$) and histocompatibility antigen (HLA-G; $p = 0.02$). Cellular processes relate to major histocompatibility complex (MHC) proteins ($p = 3.1E-7$) and molecular processes relate to peptide antigen binding ($p = 0.032$) and tapasin binding ($p = 0.047$). Tapasin is a MHC class I antigen-processing molecule present in the lumen of the endoplasmic reticulum [31]. The relationship to inflammation-based genes and processes like HLA, MHC, and tapasin support hypotheses relating physical fitness and systemic inflammation [32]. In addition, previous research found inflammation response and endoplasmic reticulum stress were down-regulated in people following a 12-week endurance exercise regime compared to the non-exercising control group [33]. Both biological findings are intriguing and may provide direction for studying modifiable methylation from fitness parameters.

Next, we examined the chromatin states of the genomic regions across the 627 CpG sites used for DNAm fitness biomarker construction and found CpG loci are significantly depleted in heavily acetylated promoters and transcription start sites (TSS) and enriched in regions with polycomb repressive complex 2 (PRC2) binding. The odds ratios (OR) are significantly less than one in the chromatin state PromF4 (heavily acetylated promoters, OR = 0.45, hypergeometric $p = 6.5E-6$) and TSS1 (acetylated TSS, OR = 0.37, $p = 6.8E-6$) (Table 5B). BivProm1 (OR = 1.50, $p = 0.009$), BivProm2 (OR = 1.76, $p = 0.0006$), and ReprPC1 (OR = 1.87, $p = 0.007$) regions are enriched in our DNAm fitness biomarkers and are known PRC2 binding sites [34]. BivProm1 and BivProm2 are weak bivalent

promoters and ReprPC1 is a polycomb repressed region. Bivalent chromatin domains control expression of *HOX* and other developmental genes in all vertebrates. PRC2 is one of the main Polycomb repressive complexes (PRC) that act as negative epigenetic regulators of transcription; it helps to initiate gene silencing via H3K27 methylation [35]. These results coincide with the increasing observation that the process of development is connected to epigenetic aging and that PRC2 targets are enriched in the age-dependent methylome in human and mammals [12, 36].

Approximately 10% of CpG sites used to construct DNAm fitness biomarkers are conserved in other epigenetic clocks with 25% of the coefficients in the same direction. Fifty-six (out of 627) CpG sites are conserved in at least one other epigenetic clock; 7 in DNAmPhenoAge, 2 in DNAmAge, 15 in DNAmAgeHannum, 23 in DNAmAgeSkinBlood (Supplementary Table 8), and 14 in DNAmGrimAge. The most conserved CpG site was cg26842024; this is used in the male DNAmGaitspeed model and is in all clocks except Hannum. CpG sites were chosen in multiple DNAm fitness models corresponding to 46 coefficients to compare to other epigenetic clocks. In total, 11 coefficients were in the same direction as other clocks. The remaining 90% of the CpG sites used for DNAm fitness biomarkers suggest new areas of the epigenome to study that may be responsive to physical activity.

DISCUSSION

DNAm biomarkers have been constructed for blood cell count [37], age [12, 13], smoking [15], and more, however, there were not yet DNAm biomarkers for fitness parameters. Our work introduces new DNAm biomarkers for the fitness parameters of maximum handgrip strength, gait speed, FEV1, and VO2max. These DNAm biomarkers represent new tools for researchers interested in studying the epigenetic components to physical fitness.

DNAm biomarkers have been improved by incorporating phenotypic information [14, 15], however, DNAm biomarkers had not yet incorporated physical fitness. DNAmFitAge provides researchers a novel indicator of biological age which combines physical fitness and epigenetic health. This biomarker integrates the established DNAm prediction of mortality risk (DNAmGrimAge) with the newly developed DNAm predictions of fitness. Higher values of DNAmGaitspeed, DNAmGripmax, DNAmFEV1, and DNAmVO2max, which reflect greater physical fitness, correspond to younger estimated biological ages in men and women. We demonstrate physically fit lifestyles have younger biological ages and fitter DNAm fitness biomarkers, which we observe in people of low to intermediate physical activity levels across five large-scale validation datasets and in male body builders who have intense, athletic exercise regimes. Furthermore, FitAgeAcceleration is strongly associated with a host of age-related conditions and predicts time-to-death and time-to-CHD across validation datasets. FitAgeAcceleration provides a novel measure of epigenetic age acceleration that is expected to be particularly sensitive to exercise interventions.

We acknowledge the following limitations. First, the DNAm fitness parameter biomarkers lead to only modest improvement to estimate fitness parameters after including age and sex as covariates in validation datasets. This reflects the relatively weak signal present in blood for fitness parameters. Because of the biomarkers' limited correlation, DNAm fitness biomarkers should *not* replace true fitness parameters. Instead, the main benefit of our biomarkers is that they show blood epigenetic changes accompany physical fitness. These biomarkers advance the molecular understanding of exercise benefits, which we hypothesize to be most pronounced in athletic populations as illustrated in our analysis of body builders. The male body builders had a mean 2.7 year reduction in DNAmFitAge compared to controls, whereas the intermediate physically active people had at most a mean 0.33 year reduction in DNAmFitAge (WHI). Second, our DNAmVO2max biomarker was only validated in one dataset with VO2max; more research is needed to evaluate how our DNAmVO2max biomarker performs across a range of independent datasets.

Overall, DNAmGaitspeed, DNAmGripmax, DNAmFEV1, DNAmVO2max, and DNAmFitAge provide epigenetic components to evaluating a person's physical fitness. Physically fit people have a younger DNAmFitAge and younger FitAgeAcceleration, and younger values are associated with more physical activity and better age-related outcomes. Our research suggests exercise and stronger fitness parameters are protective to DNAmFitAge in both sexes. We expect DNAmFitAge will be a useful biomarker for quantifying fitness benefits at an epigenetic level and can be used to evaluate exercise-based interventions.

METHODS

Study cohorts

We analyzed blood DNAm data from three datasets, Framingham Heart Study Offspring cohort (FHS, n = 1830), Baltimore Longitudinal Study on Aging (BLSA,

n = 820), and novel data (Budapest, *n* = 307) to develop DNAm biomarkers of fitness parameters. In short, the FHS cohort is a cardiovascular study which followed adults from Massachusetts starting in 1948 [38]. The BLSA cohort began in 1958 studying healthy adults and the aging process [39]. Finally, Budapest is a smaller study (*n* = 307) measuring physical fitness and DNA methylation in middle to older aged adults, some of whom are current or former athletes. More details of the Budapest study can be found in Supplementary Note 1. Dataset harmonization was performed to join multiple datasets when variables were on different scales following previously developed methods [40]. In brief, datasets were rescaled to have the same mean and standard deviation for each fitness parameter by recentering and multiplying by the ratio of standard deviations.

We conducted validation analysis in an independent group of six additional datasets: two Lothian Birth Cohorts: LBC1921 (*n* = 692) and LBC1936 (*n* = 2797), Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE, *n* = 578), InChianti (*n* = 924), Jackson Heart Study (JHS, *n* = 1746), and Women's Health Initiative (WHI, *n* = 2117). Descriptive statistics of each dataset are presented in Supplementary Table 9. Full study descriptions for validation datasets have previously been published [41–46]. We evaluate our biomarkers in a novel Polish study which collects DNA methylation and dietary supplements in body builders and controls to assess performance in an athletic population. Additional details of the Polish study can be found in Supplementary Note 1.

DNAm fitness parameter biomarker development

We developed DNAm biomarkers for four fitness parameters: gait speed, maximum handgrip strength (Gripmax), forced expiratory volume in 1 second (FEV1), and maximal oxygen uptake (VO2max). Gait speed, also known as walking speed, is measured in meters per second [47]. Maximum hand grip strength is a measurement of force taken in kg [3]. FEV1 measures lung function; it is the amount of air forced from the lungs in one second, measured in liters [8]. VO2max is a measure of cardiovascular health and aerobic endurance [4, 47]. It measures the volume of oxygen the body processes during incremental exercise in milliliters used in one minute of exercise per kilogram of body weight (mL/kg/min). VO2max has been regarded as the best indicator of an athlete's physical capacity and is the international standard of physical capacity [47].

Each fitness DNAm biomarker was developed using LASSO penalized regression with 10-fold cross validation in which the fitness parameters were dependent variables and independent variables were DNAm levels at cytosine-phosphate-guanines (CpG) sites and chronological age. The LASSO-regression method uses an l_1 penalty that shrinks each coefficient towards zero. LASSO is more effective than Ridge (l_2 penalty) and elastic net (mixture of l_1 and l_2 penalty) when handling many irrelevant predictors and yields smaller number of predictors in the final model. Models were fit separately for men and women in the case of gait speed, gripmax, and FEV1 to select for sex specific CpG loci that reflect gender variation in fitness. The selected covariates and estimated coefficients were then used to form a prediction algorithm for each fitness parameter. We refer to the predicted fitness parameters generated by these algorithms as DNAmGaitspeed, DNAmGripmax, DNAmFEV1, and DNAmVO2max. Correlation of each DNAm biomarker with measured fitness values in the training data are displayed in Supplementary Figure 3.

When it came to building the biomarker for VO2max, stratifying by sex was not feasible due to the smaller sample size. This forced us to choose between using sex as a covariate or omitting sex and trusting LASSO to select X chromosome markers that best signify differences between males and females. We chose the latter, and it did. Finally, we present two models for DNAmGaitspeed and DNAmGripmax; one with chronological age and one without chronological age as potential covariates. Removing age as a potential variable for selection in LASSO was performed to remedy high collinearity discovered among these DNAm biomarkers when constructing DNAmFitAge (scatterplot matrix in Supplementary Figure 4).

DNAm fitness parameter biomarker validation

We conducted two validation analyses of DNAm biomarkers of fitness parameters using up to five independent datasets. First, we correlated DNAm biomarker values with direct measurements of the fitness parameters. In cases where direct measurement of a fitness parameter was not included in a validation dataset, substitutions were selected. Briefly, gait speed was substituted with a composite leg strength measurement and a composite physical functioning score; FEV1 was substituted by forced expiratory volume (FEV) and VO2max; VO2max was substituted by FEV. Details are reported in Supplementary Note 2.

Second, we evaluated if using our DNAm biomarkers improve estimation of fitness parameters beyond variation explained through age and sex (null models) by evaluating the significance of the DNAm biomarker as a predictor. Pearson correlations of null models are

presented in Supplementary Table 1. The reported *p*-values indicate the significance of the DNAm biomarker estimate as a predictor for the fitness parameters. The individual- dataset and fixed-effects meta-analysis *p*-values are calculated across validation datasets with the most relevant variables available in more than one dataset. Specifically, LBC1921 and LBC1936 were used for DNAmGaitSpeed and DNAmFEV1 meta-analysis *p*-value calculations. LBC1921, LBC1936, CALERIE, and WHI were used for DNAmGripmax. We did not calculate a meta-analysis *p*-value for DNAmVO2max because only one validation dataset had VO2max measurement.

DNAmFitAge: biological age estimation

DNAmFitAge development

We constructed DNAmFitAge as an indicator of biological age following the methods proposed by Klemera and Doubal [48]. In brief, the Klemera-Doubal model framework stipulates there exists an underlying trait which is unobserved (biological age) which relates to an observable trait (chronological age) and a set of additional variables. This framework posits biological age is centered on chronological age with additional noise. Weighted least squares is used to estimate the relationship of the additional variables with biological age where the weights are formed from correlations of each variable with chronological age.

DNAmFitAge is constructed separately for males and females using four DNAm variables: three of the DNAm fitness biomarkers: DNAmGripmax, DNAmGaitSpeed, and DNAmVO2max, and DNAmGrimAge, a biomarker of mortality risk [15]. We estimate biological age using the TrueTrait function from the WGCNA R package which carries out the Klemera Doubal method described above. Variable weights indicating each variable's importance for estimating biological age are presented in Table 2A. Pearson's correlation among original fitness parameters, DNAm biomarkers, and DNAmFitAge in the large training dataset (FHS + BLSA) are displayed in Supplementary Figure 2. Pearson's correlation of DNAmFitAge to chronological age in training data are presented in panels A and B of Supplementary Figure 2. Models including DNAmFEV1 as a fifth variable were explored, however no improvement in association to physical activity or age-related outcomes were observed; the parsimonious DNAmFitAge model using a subset of the DNAm fitness biomarkers was therefore chosen.

Finally, we created FitAgeAcceleration, the age-adjusted estimate of DNAmFitAge formed from taking the residuals after regressing DNAmFitAge onto chronological age. As such, FitAgeAcceleration is uncorrelated with chronological age. FitAge-Acceleration provides an estimate of epigenetic age acceleration, i.e., how much older or younger a person's estimated biological age is from expected chronological age. A positive FitAgeAcceleration means biological age is estimated to be older than chronological age. A negative FitAgeAcceleration means biological age is estimated to be younger than chronological age, which is the preferred outcome for a person.

DNAmFitAge validation

DNAmFitAge validation analysis consisted of three components: correlating DNAmFitAge to chronological age, testing FitAge Acceleration association with physical activity, and testing FitAge Acceleration association to aging-related variables in the validation datasets. First, the modeling framework posits biological age is centered on chronological age, therefore validation datasets should demonstrate good correlation and general centeredness between DNAmFitAge and chronological age. Both properties would indicate DNAmFitAge can quantify age. Second, DNAmFitAge incorporates fitness, therefore FitAgeAcceleration (age adjusted DNAmFitAge) should relate to physical activity and physical functioning. These relationships would indicate DNAmFitAge relates to fitness. Third, DNAmFitAge provides insight to the aging process through a fitness paradigm, therefore FitAgeAcceleration should relate to aging-related phenotypes.

We correlate DNAmFitAge with chronological age for males and females because (1) we cannot directly measure biological age, (2) chronological age is not used when forming DNAmFitAge estimates, and (3) the modeling framework posits biological age is centered on chronological age. In addition, because DNAmFitAge is built in males and females separately, we demonstrate what happens when the model is applied to the opposite sex (i.e., male model in females or female model in males). Median absolute deviation, mean deviation, and Pearson correlation are presented in Supplementary Table 2 and correlation is displayed in Figure 2.

We tested for associations between physical activity or physical functioning in low to intermediate physically fit individuals with FitAgeAcceleration, DNAm fitness parameter biomarkers, and other DNAm biomarkers known to relate to physical health. We restricted our analysis to people of low to intermediate fitness to determine if FitAgeAcceleration is more sensitive to small improvements in fitness compared to other current DNAm biomarkers. In addition, this separation captures low to average physically active individuals in

each dataset. In short, LBC1921, LBC1936, and JHS measure physical activity, and WHI and InChianti measure physical functioning. Higher values of any variable indicate more activity or better physical functioning. Other DNAm biomarkers which relate to physical health include DNAmPhenoAge [14], DNAmGrimAge, DNAmPAI-1, and DNAmGDF-15 [15]. See Supplementary Note 2 for a thorough description of physical activity variables and inclusion criteria. We use *p*-values across models to compare DNAm biomarker performance, however other methods could be used that may be more valuable, like likelihood ratio tests or AIC. We chose not to use other methods because of high collinearity among the DNAm biomarkers and the succinctness of *p*-values.

We tested DNAmFitAge associations to multiple aging-related variables in validation datasets. Specifically, we conducted regression analysis of physical activity, time-to-death, time-to-coronary-heart-disease (CHD), the count of age-related conditions (arthritis, cataract, cancer, CHD, CHF, emphysema, glaucoma, lipid condition, osteoporosis, and type 2 diabetes), age at menopause, cancer, hypertension, type-2 diabetes, and disease-free status. Time-to-event outcomes were analyzed using Cox regression to estimate hazard ratios (HR); continuous outcomes were analyzed using linear regression to estimate slopes; dichotomous outcomes were analyzed using logistic regression to estimate odds ratios (OR); and ordinal outcomes were analyzed using multinomial regression to estimate OR. Some of our cohorts (InChianti, LBC1921, and LBC1936) involved longitudinal measures. In these cases, linear regression models with person-level random intercepts were implemented in R using the lmer function to adjust for correlation within the same individual. Logistic regression models were estimated using generalized estimating equations with the R function gee. Multinomial models were implemented using R function multinom.

FitAgeAcceleration was also explored for explaining information in time-to-death and number of comorbidities beyond what is captured through other epigenetic clocks. DNAmFitAge is built using DNAmGrimAge, and DNAmGrimAge and other epigenetic clocks are known to be associated with age-related conditions. Therefore, FitAgeAcceleration (the age-adjusted measure) is compared to other epigenetic biomarkers using a Likelihood Ratio Test (LRT) in two nested models stratified by sex; one includes age and one other epigenetic clock, and the other includes age, the other epigenetic clock, and FitAge Acceleration. LRTs and corresponding *p*-values are presented for validation datasets in Supplementary Table 4 for DNAmGrimAge, DNAmPAI1, DNAmGDF15, DNAmAgeHannum, and DNAmAgeSkinBloodClock. We excluded LRTs for other health related outcomes (like disease free status) because those models are constructed from generalized estimating equations (GEE) which are not based on likelihoods, and likelihoods are necessary to compare LRTs.

Meta-analysis

We combine results across validation studies using fixed effect models or Stouffer's meta analysis method using the metafor R function. Fixed effect models use the inverse variance to weight estimates, and Stouffer's method uses the square root of the sample size to weight estimates. The latter is used when harmonization across cohorts was challenging; such as with physical activity variables, the number of age-related conditions, disease free status, and age at menopause. Forest plots evaluating FitAgeAcceleration hazard ratios or coefficients in models adjusted for age and sex are displayed in Figure 3 and Supplementary Table 3. We perform a test of heterogeneity for coefficients across datasets using Cochran Q test for fixed effect models; *p*-values are displayed as Het. P.

DNAmFitAge evaluation in body builders

We evaluated whether our DNAm fitness biomarkers and DNAmFitAge were significantly different in an independent study of male body builders and controls. There was a total of 66 male body builders and 149 male controls with similar age distributions (p-value > 0.05). Both groups reported the number of years they regularly trained, the average number of intensity trainings they participated in per week, and 88 total participants reported supplements or drugs they are taking. We analyzed whether the DNAm fitness biomarkers, DNAmFitAge, or FitAge Acceleration were different between male controls and body builders using a Kruskal Wallis test (Table 4).

We evaluate whether the improvement in DNAmFitAge and DNAmVO2max in male body builders can be explained by the dietary supplements taken using a linear regression model with DNAmFitAge or DNAmVO2max as the outcome with age as a covariate and indicator variables for taking the supplement and being a body builder. We adjust for age in the model because age was significantly related to taking certain supplements, therefore if age was not included, the differences observed in DNAmFitAge or DNAmVO2max may actually represent differences in chronological ages between supplement usage groups. Linear model results are presented in Supplementary Table 5. To ensure adequate power, we evaluated supplements and drugs with at least 10 people reporting

use across both body builders and controls. Only six supplements met this threshold: multivitamins (n = 19), protein (n = 17), energy (n = 17) (creatine, pre-workout, and energy gels), magnesium (n = 16), vitamin D (n = 14), and omega-3 (n = 12). We also evaluated if these supplements were disproportionately taken by male body builders compared to male controls using Fisher's Exact test (Supplementary Table 6).

Functional CpG annotation

We provide biological insight to the 627 unique CpG loci used in constructing our DNAm fitness biomarkers by exploring genomic enrichment in the entire human genome, analyzing specific enrichment in chromatin states, and comparing CpG loci and coefficients to other epigenetic clocks. We use the GREAT enrichment analysis software tool for analyzing broad genomic enrichment [49]. GREAT analyzes the genes within and nearby the genomic region covered by the CpGs. To avoid confounding the enrichment analysis by gene size, the GREAT algorithm performs a binomial test (over genomic regions) using a whole genome background. We performed the enrichment based on default settings (Proximal: 5.0 kb upstream, 1.0 kb downstream, plus Distal: up to 1,000 kb) using the hg19 assembly. We report nominal, Bonferroni, and FDR p-values for gene, biological, cellular, and molecular function in Table 5A for the top results, and complete results are presented in Supplementary Table 7.

To annotate the CpGs used to construct the DNAm fitness biomarkers based on chromatin state, we assigned a state for the CpGs based on the detailed universal ChromHMM chromatin state annotation of the human genome in which chromatin structure and their associated characteristics are annotated [34]. This annotation generated 100 distinct states using 1,032 experiments into 16 major categories such as active and weak enhancers (EnhA, EnhW), bivalent states associated with promoters (BivProm), flanking promoter states (PromF), polycomb repressed states associated with H3K27me3 (ReprPC), and states associated with exons and transcription. We used one-sided hypergeometric tests to study both the enrichment (OR >1) and depletion (OR <1) patterns of CpGs across the chromatin states as detailed in [50]. Genomic CpG regions on the 450K array with chromatin state information were used as background (n = 483,090). The genomic regions of DNAm fitness biomarker CpG sites with chromatin state information were used as foreground (n = 626), which only excluded 1 CpG. This yielded one-sided hypergeometric p-values not confounded by the number of CpGs within a gene. We report the chromatin state, number of CpG loci enriched in each state, Odds Ratios, and hypergeometric p-values in Table 5B, and complete results are presented in Supplementary Table 10. Because the underlying chromatin states follow a multinomial distribution, we do not adjust our p-values for multiple comparisons.

Finally, we compared the CpG loci and model coefficients used in construction of the DNAm fitness biomarkers to the CpG loci used in DNAmPhenoAge, DNAmAge (Horvath 2013), DNAmAgeHannum, and DNAmAgeSkinBlood. For CpG loci conserved across other epigenetic clocks, we report the DNAm fitness biomarker coefficients, other clock coefficients, overlap with other clocks, and whether the coefficient direction is the same in Supplementary Table 8. We also compare the overlap of CpG loci used in our models and DNAmGrimAge but omit the comparison of coefficient direction to prevent disclosure of intellectual property. For a full list of coefficients and CpG loci used in DNAm fitness biomarker construction, see our GitHub repository at https://github.com/kristenmcgreevy/DNAmFitAge.

Abbreviations

DNAm: DNA methylation; FEV: forced expiratory volume; FEV1: forced expiratory volume in one second; VO2max: maximal oxygen uptake; Gripmax: maximum handgrip force; FHS: Framingham Heart Study Offspring Cohort; BLSA: Baltimore Longitudinal Study on Aging; LBC1921: Lothian Birth Cohort 1921; LBC1936: Lothian Birth Cohort 1936; CALERIE: Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy; JHS: Jackson Heart Study; WHI: Women's Health Initiative; CpG: cytosine-phosphate-guanine; CHD: time-to-coronary-heart-disease; OR: odds ratios; GREAT: Genomic Regions Enrichment of Annotations Tool; FDR: false discovery rate.

AUTHOR CONTRIBUTIONS

Conceptualization: KMM, ZR, SH, FT; methodology: KMM, SH, ATL, AB; statistical analysis: KMM, ATL, FT; writing and editing: KMM, DWB, SH, ZR, EP, FT, REM, LMR, and others; data contribution: ZR, SH, EP, WB, DWB, LF, REM, MK, DLC, AO, AS, MS, LMR, APR. All authors helped with manuscript preparation and interpreted results.

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CONFLICTS OF INTEREST

S.H. is a founder of the non-profit Epigenetic Clock Development Foundation which plans to license several patents from his employer UC Regents. These patents list SH as inventor. R.E.M has received a speaker fee from Illumina and is an advisor to the Epigenetic Clock Development Foundation. The other authors declare no conflicts of interest.

ETHICAL STATEMENT AND CONSENT

The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Aging (Albany NY, USA). Consent to conduct this research has been approved by University of California Los Angeles IRB board under IRB#15-001479 for Epigenetic Analyses of Aging, IRB#16-002028 for building DNA methylation based biomarkers, and IRB#18-000315 for Validation of Epigenetic Clocks.

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SUPPLEMENTARY MATERIALS

Supplementary Note 1: Datasets

The Budapest dataset was used as the training dataset for the DNAmVO2max biomarker. For the other biomarkers, this dataset was used for validation. The additional validation datasets involved six cohorts: the Lothian Birth Cohorts (1921 and 1936), Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE), the Women's Health Initiative (WHI), Jackson Heart Study (JHS), and Invecchiare in Chianti, aging in the Chianti area (InChianti). The Polish Study is used to evaluate biomarkers across body builders and controls. Below we describe each study cohort/datasets in more detail.

Budapest

Budapest is a small, novel study (n = 307) measuring physical fitness and DNA methylation in middle to older aged adults, some of whom are current or former athletes. A total of n = 205 participants previously participated in the World Rowing Masters Regatta in Velence, Hungary. The study was approved by the National Public Health Center in accordance with the Helsinki Declaration and the regulations applicable in Hungary (25167-6/2019/EÜIG). This research study was undertaken by the Research Institute of Sport Science, Hungarian University of Sport Science, Budapest. Subjects completed a questionnaire regarding their health, educational status, and life-style- including exercise habits. Maximum hand gripping force was assessed using the CAMRY EH101 dynamometer. Relative maximal oxygen uptake (VO2max) was measured using the Chester step test on a treadmill. The strength of the legs (Jumpmax) was assessed by a person's maximal vertical jump, measured using a linear encoder.

Budapest DNAm methylation quantification

Epigenome wide DNA methylation was measured with the Infinium MethylationEPIC BeadChip (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. DNA methylation was derived from whole blood samples and 500 ng of genomic DNA was bisulfite converted using the EZ-96 DNA Methylation MagPrep Kit (Zymo Research, Irvine, CA, USA) with the KingFisher Flex robot (Thermo Fisher Scientific, Breda, Netherlands). The samples were plated in randomized order. The bisulfite conversion was performed according to the manufacturer's protocol with the following modifications: For binding of the DNA 15 µl MagBinding Beads was used. The conversion reagent incubation was done according to the following cycle protocol: 16 cycles of 95°C for 30 seconds followed by 50°C for 1 hour. After the cycle protocol the DNA was incubated for ten minutes at 4°C. Next, DNA samples were hybridized on the Infinium MethylationEPIC BeadChip (Illumina Inc., San Diego, CA, USA) according to the manufacturers protocol with the modification that 8 µl bisulfite treated DNA was used as start material.

Quality control of DNA methylation was performed using minfi, Meffil, and ewastools packages with R version 4.0.0. Samples which failed technical controls, including extension, hybridization and bisulfite conversion, according to the criteria set by Illumina, were excluded. Samples with a call rate <96% or at least with 4% of undetected probes were also excluded. Probes with a detection p-value >0.01 in at least 10% of the samples were set as undetected. Probes with a bead number <3 in at least 10% of the samples were excluded. Methylation beta values were generated using the Bioconductor minfi package in R with Noob normalization background correction.

Lothian birth cohorts

The Lothian Birth Cohorts (LBC) consists of two longitudinal studies evaluating cognition and brain aging of older adults who were born in either 1921 (LBC1921) or 1936 (LBC1936) and lived in Edinburgh or the surrounding Lothian regions of Scotland. LBC1921 was started in 1999 and LBC1936 began in 2004. LBC1936 was established to study cognitive aging in surviving members of the 1947 Scottish Mental Survey. Ethical approval was obtained from the Multi-Centre Ethics Committee for Scotland and Lothian Research Ethics Committee. National Records of Scotland provided regular updates on mortality data for the LBC participants via data linkage with the National Health Service Central Register.

LBC1921

Participants were born in 1921 and most completed a cognitive ability test around age of 11 years in the Scottish Mental Survey 1932 (SMS1932). The SMS1932 was administered nationwide to almost all 1921-born children who attended school in Scotland in June 1932. The cognitive test was the Moray House Test No. 12. The LBC1921 study attempted to follow up individuals who might have completed the SMS1932 and resided in the Lothian region (Edinburgh and its

surrounding areas) of Scotland; 550 people ($N = 234$, 43% men) were successfully traced and participated in the study from the age of 79 years. To date, there have been four additional follow-up waves at average ages of 83, 87, 90, and 92 years. The cohort has been studied during the later-life waves, including blood biomarkers, cognitive testing, and psycho-social, lifestyle, and health measures.

LBC1936

The methylation mortality survival analysis was investigated in LBC1936. All participants were born in 1936 and most had taken part in the Scottish Mental Survey 1947. These participants attended Scottish schools in June 1947. The cognitive test administered was the same Moray House Test No. 12. A total of 1,091 participants ($n = 548$, 50% men) who were living in the Edinburgh and Lothian area of Scotland were re-contacted in later life. Data has since been collected in waves at five time points.

LBC DNAm methylation quantification

Whole blood DNA methylation was measured using the Illumina HumanMethylation450BeadChips from 514 whole blood samples in LBC1921 and from 1,004 samples in LBC1936. Samples were extracted at MRC Technology, Western General Hospital, Edinburgh (LBC1921) and the Wellcome Trust Clinical Research Facility (WTCRF), Western General Hospital, Edinburgh (LBC1936), using standard methods. Methylation typing of 485,512 probes was performed at the WTCRF. Raw intensity data were background-corrected and methylation beta-values generated using the R minfi package. Quality control analysis was performed to remove probes with a low (<95%) detection rate at $P < 0.01$. Manual inspection of the array control probe signals was used to identify and remove low quality samples (for example, samples with inadequate hybridization, bisulfite conversion, nucleotide extension, or staining signal). The Illumina-recommended threshold was used to eliminate samples with a low call rate (samples with <450,000 probes detected at $P < 0.01$). Since the LBC samples had previously been genotyped using the Illumina 610-Quadv1 genotyping platform, genotypes derived from the 65 SNP control probes on the methylation array using the wateRmelon package were compared to those obtained from the genotyping array to ensure sample integrity. Samples with a low match of genotypes with SNP control probes, which could indicate sample contamination or mix-up, were excluded ($n = 9$). Moreover, eight subjects whose predicted sex, based on XY probes, did not match reported sex were also excluded.

CALERIE

Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) was a Phase 2 clinical trial started in 2007 studying young to middle-aged healthy adults [13]. CALERIE is the first clinical trial to focus on the effects of sustained CR in humans. It was completed in May 2013 as a two-year three-site randomized controlled trial in young and middle-aged non-obese healthy men and women ($N = 220$). Participants were randomized in a 2:1 fashion to 25% caloric restriction (CR) or ad libitum control group (diet is available at all times). All participants needed to have a baseline body mass index (BMI) of 22-27.9 kg/m^2 (lean to slightly overweight). Each participant has 1) behavioral counselor (Masters of doctoral in psychology) AND 2) registered dietician who follow with them for the whole 2 years. 25% reduction and caloric goals are calculated based on each person's initial food intake at baseline. They must meet with the dietician 2–3 times a week and record food intake. Two consecutive 14-day doubly labeled water studies are conducted with each participant at baseline with the average used to determine AL TEE (total energy expenditure); from this, the 25% CR prescription for that participant is derived. An average of 12% caloric reduction was achieved in the CR group throughout the study.

CALERIE DNAm methylation quantification

DNA methylation was measured from Illumina EPIC 850k Arrays (Illumina Inc., San Diego, CA) as per the manufacturer's protocol. DNA methylation was derived from whole blood samples. CALERIE methylation assays were run by the Molecular Genomics Shared Resource at Duke Molecular Physiology Institute, Duke University (USA). Quality control of sample handling included comparison of clinically reported sex versus sex of the same samples determined by analysis of methylation levels of CpG sites on the X chromosome. Methylation beta values were generated using the Bioconductor minfi package with Noob background correction.

CALERIE data are available at https://calerie.duke.edu/samples-data-access-and-analysis.

Women's health initiative

The WHI is a national study that enrolled postmenopausal women aged 50–79 years into the clinical trials (CT) or observational study (OS) cohorts between 1993 and 1998 [4, 5]. We included 4,079 WHI participants with available phenotype and DNA methylation array data: 2,107 women from "Broad

Agency Award 23" (WHI BA23). WHI BA23 focuses on identifying miRNA and genomic biomarkers of coronary heart disease (CHD), integrating the biomarkers into diagnostic and prognostic predictors of CHD and other related phenotypes.

The total number of age-related conditions was based on Alzheimer's disease, amyotrophic lateral sclerosis, arthritis, cancer, cataract, CVD, glaucoma, emphysema, hypertension, and osteoporosis.

WHI DNA methylation quantification

Bisulfite conversion using the Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) as well as subsequent hybridization of the HumanMethylation450k Bead Chip (Illumina, San Diego, CA, USA), and scanning (iScan, Illumina) were performed according to the manufacturers protocols by applying standard settings. DNA methylation levels (β values) were determined by calculating the ratio of intensities between methylated (signal A) and un-methylated (signal B) sites. Specifically, the β value was calculated from the intensity of the methylated (M corresponding to signal A) and un-methylated (U corresponding to signal B) sites, as the ratio of fluorescent signals $\beta = Max(M,0)/[Max(M,0) + Max(U,0) + 100]$. Thus, β values range from 0 (completely un-methylated) to 1 (completely methylated).

Jackson heart study

The JHS is a large, population-based observational study evaluating the etiology of cardiovascular, renal, and respiratory diseases among African Americans residing in the three counties (Hinds, Madison, and Rankin) that make up the Jackson, Mississippi metropolitan area. The age at enrollment for the unrelated cohort was 35–84 years; the family cohort included related individuals >21 years old. Participants provided extensive medical and social history, had an array of physical and biochemical measurements and diagnostic procedures, and provided genomic DNA during a baseline examination (2000–2004) and two follow-up examinations (2005–2008 and 2009–2012). Annual follow-up interviews and cohort surveillance are ongoing. In our analysis, we used the visits at baseline from 1747 individuals as part of project JHS ancillary study ASN0104, available with both phenotype and DNA methylation array data. Total numbers of age-related conditions were based on hypertension, type 2 diabetes, kidney dysfunction based on ever dialysis, and CVD. Disease free status was classified if the number of age-related conditions was 0 and they did not take medication for blood pressure or diabetes.

JHS DNA methylation quantification

Peripheral blood samples were collected at the baseline. DNA was extracted using the Gentra Puregene blood kit (Gentra System, MN, Minnesota, USA). Methylation beta values were generated using the Bioconductor minfi package with Noob background correction.

Invecchiare in Chianti, aging in the Chianti area (InChianti)

The InChianti (Invecchiare in Chianti, aging in the Chianti area) cohort is a representative population-based study of older persons enrolling individuals aged 20 years and older from two areas in the Chianti region of Tuscany, Italy. One major goal of the study is to translate epidemiological research into geriatric clinical tools, ultimately advancing clinical applications in older persons. Of the cohort, 924 observations from 484 individuals with both phenotype information and DNA methylation data were including in our studies. The observations were collected from baseline in 1998 and the third follow-up visit in 2007. All participants provided written informed consent to participate in this study. The study complied with the Declaration of Helsinki. The Italian National Institute of Research and Care on Aging Institutional Review Board approved the study protocol. We computed the total number of age-related conditions based on cancer, hypertension, myocardial infarction, Parkinson's disease, stroke and type 2 diabetes.

InChianti DNA methylation quantification

Genomic DNA was extracted from buffy coat samples using an AutoGen Flex and quantified on a Nanodrop1000 spectrophotometer prior to bisulfite conversion. Blood DNA methylation was taken twice over the span of nine years in a total of 966 people. Genomic DNA was bisulfite converted using Zymo EZ-96 DNA Methylation Kit (Zymo Research Corp., Irvine, CA USA) as per the manufacturer's protocol. CpG methylation status of 485,577 CpG sites was determined using the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA, USA) as per the manufacturer's protocol and as previously described [11]. Initial data analysis was performed using GenomeStudio 2011.1 (Model M Version 1.9.0, Illumina Inc.). Threshold call rate for inclusion of samples was 95%. Quality control of sample handling included comparison of clinically reported sex versus sex of the same samples determined by analysis of methylation levels of CpG sites on the X chromosome. Methylation beta values were generated using the Bioconductor minfi package with Noob background correction.

Polish study

The Polish Study is a small, novel study (n = 416) measuring blood DNA methylation and lifestyle behaviors in Polish body builders and similar aged healthy controls ranging from 17 to 56 years of age. It is part of a larger cohort representing the general population of Poland, for which blood samples, buccal swabs or semen samples were collected as part of the local project EPIGENOME (DOB-BIO10/06/2019). Participants of the Polish Study recorded the total number of years they regularly trained, average number of intensity trainings per week, sports training they participate in, and dietary supplements or drugs they take. There were a total of 66 male body builders and 30 female body builders. Because of the small sample size in females, we restricted the analysis to males only, which decreases the sample size to 215 individuals total, 149 controls and 66 body builders. 88 males in the study reported dietary supplements or drugs, and a total of 147 unique substances were reported. The use of each analyzed supplement was coded based on presence of multiple phrases in the open question of the questionnaire about drug/supplements intake. Specifically, multivitamins include reported use of vitamins, multivitamins, and vitamins + minerals. Proteins included reported use of protein supplement, branched chain amino acids (bcaa), amino acids, and training supplements. Energy supplements included creatine, energy gels, and pre-workout. Magnesium included mg and magnesium. Vitamin D consisted of vitamins D and D3. Omega-3 consisted of Omega-3 and cod liver oil.

The study was approved by the Bioethics Committee of the Jagiellonian University in Kraków (decision no. 1072.6120.132.2018) and all participants provided written informed consent. This work was financed by the National Centre for Research and Development (NCBR) in Poland within the framework of call 10/2019 related to scientific research and studies for national defense and security [project no. DOB-BIO10/06/2019].

Polish study DNAm methylation quantification

Epigenome wide DNA methylation was measured with the Infinium MethylationEPIC BeadChip (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. DNA from whole blood was extracted using Maxwell® RSC Blood DNA Kit. The quality and quantity of DNA isolates were assessed using NanoDrop 8000 UV-Vis Spectrophotometer and Qubit 4 Fluorometer. Then, the DNA concentration was normalized to 50 ng/µl and subjected to microarray analysis. Quality control and preprocessing were done using minfi and ENmix packages with R version 4.2.1. Methylation beta values were generated using the Bioconductor minfi package with Noob normalization background correction.

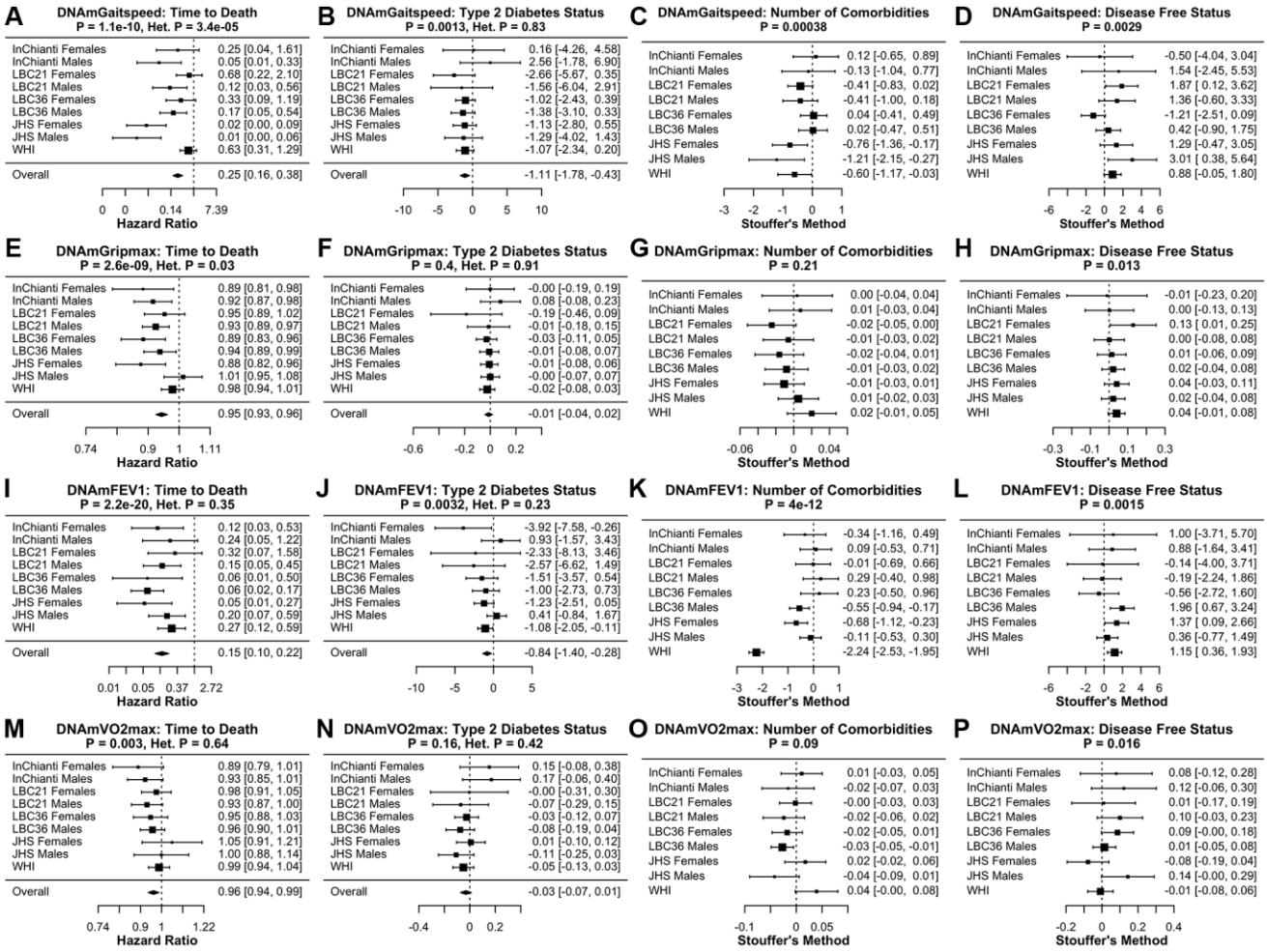
Supplementary Note 2: Other variables

Not all validation datasets have measurements of VO2max, FEV1, handgrip strength, or gait speed. In this case, we correlate similar fitness parameters; VO2max as a substitute for FEV1 and composite leg strength or composite physical functioning score as substitutes for gait speed. Composite leg strength is a measure of absolute peak leg flexion and extension torque, measured in Newton-meters. Composite physical functioning score combines walking and chair activities and ranges from 0 to 12 with 12 being best physical functioning. We expect VO2max, composite leg strength, and composite physical functioning to have positive correlation with their respective DNAm fitness parameter biomarker. VO2max and composite leg strength are used in CALERIE, and composite physical functioning score is used in InChianti and WHI.

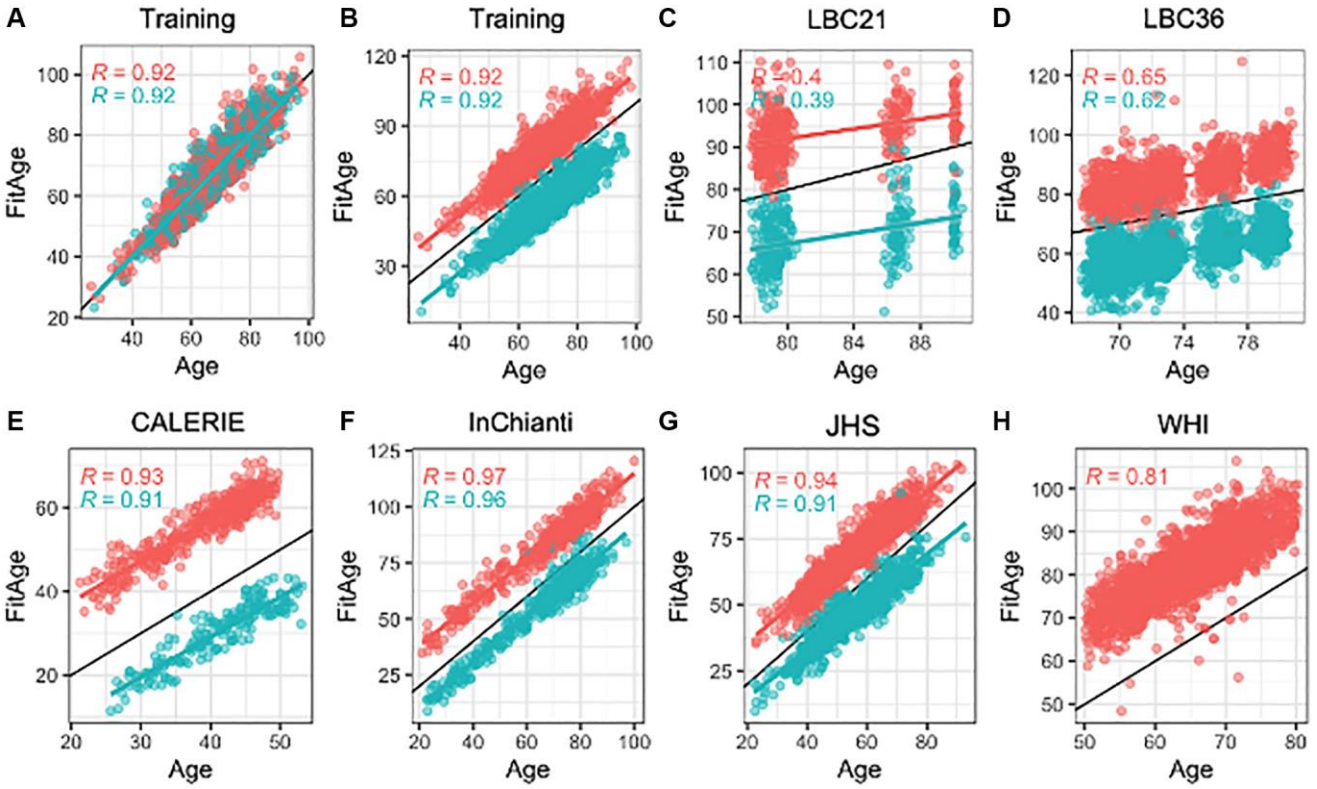
LBC21 measures self-reported days per month spent exercising; participants with at most 12 days of reported exercise per month were included. LBC36 measures level of physical activity using an electronic activity monitor and then categorizes people into one of six categories: sedentary, light, low-light activity, high-light, moderate to vigorous, and vigorous activity. LBC36 participants with sedentary to low-light activity were included for analysis. JHS categorizes participants into poor, intermediate, or ideal physical activity health; participants with poor or intermediate categorization were included. InChianti measures physical functioning as a composite score from 0 to 12 with 12 being a perfect score; participants with scores at or below 11 were included. WHI measures physical functioning as a composite score from 0 to 100 with 100 being a perfect score. WHI participants with scores at or below 85 were included; outliers were also excluded using scores beyond 1.5 times the interquartile range.

DNAmPhenoAge is an estimate of epigenetic age constructed using DNAm composite clinical measures of phenotypic age [27]. DNAmPAI-1 is a surrogate marker of plasma protein plasminogen activator inhibitor level 1, and DNAmGDF-15 is a surrogate marker for growth differentiation factor 15 [28].

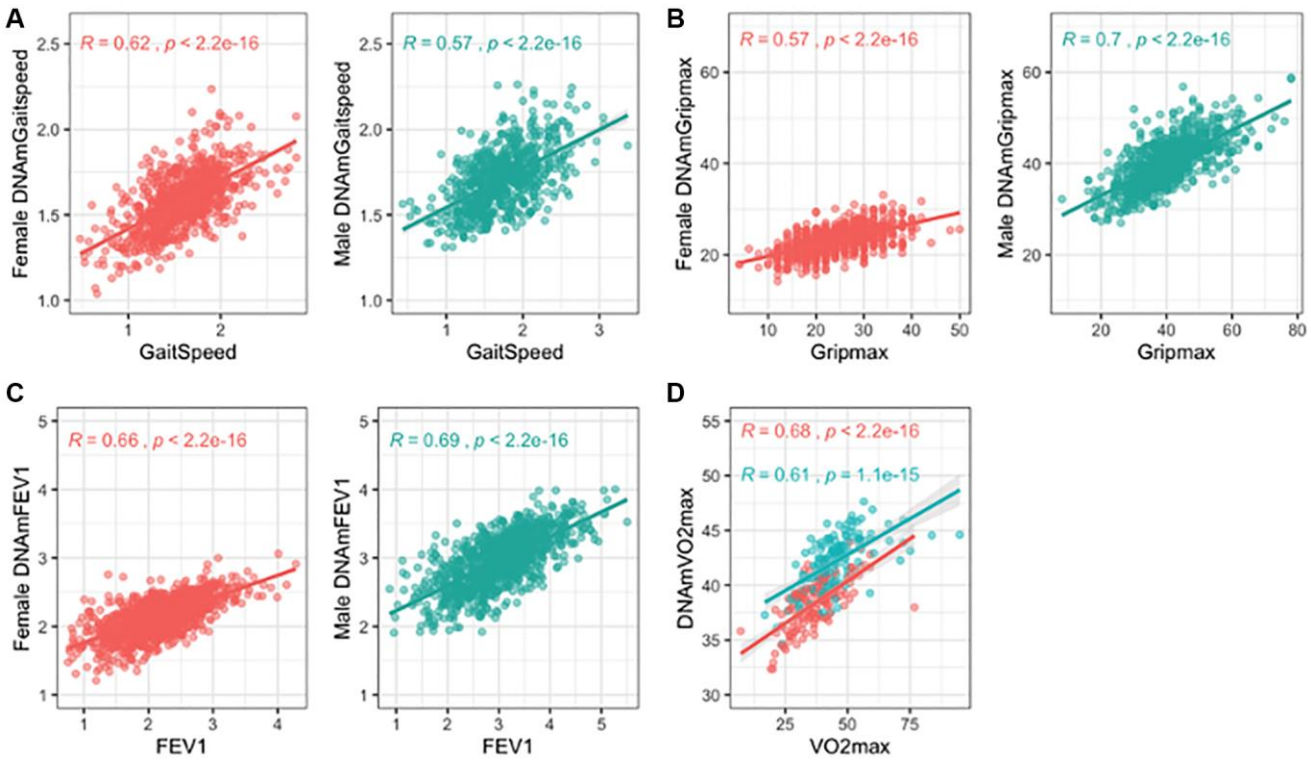
Supplementary Figures



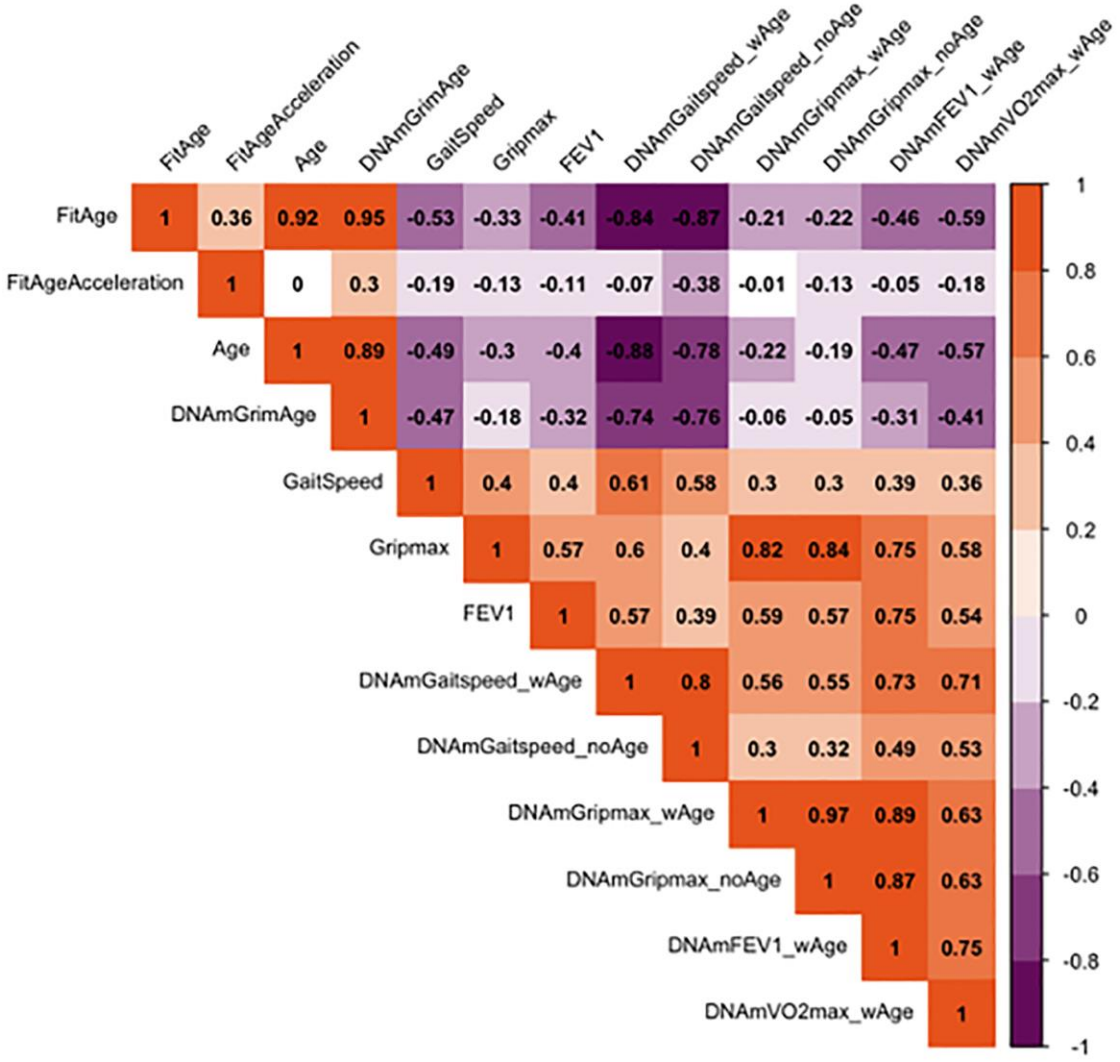
Supplementary Figure 1. Meta-analysis forest plots for DNAm fitness parameter biomarkers with age-related conditions. Each row reports a meta analysis forest plot for combining hazard ratios or regression coefficients across dataset cohorts for one DNAm biomarker estimate. (**A**–**D**) DNAmGaitSpeed without age, (**E**–**H**) DNAmGripmax without age, (**I**–**L**) DNAmFEV1, and (**M**–**P**) DNAmVO2max. Time-to-death, type 2 diabetes, comorbidity count, and disease-free status are presented. Meta-analysis *p*-values are displayed in the header of each panel, and test of heterogeneity Cochran *Q* test *p*-value (Het. P) are displayed for fixed effect models. Fixed effects models were used for time-to-death and type 2 diabetes whereas Stouffer's method was used for comorbidity count and disease-free status. All DNAm fitness biomarkers are predictive of mortality, and DNAmGaitspeed and DNAmFEV1 are predictive of number of comorbidities.



Supplementary Figure 2. Scatterplots of DNAmFitAge versus age in training and test datasets separated by sex. Pink indicates females, and blue indicates males. Each panel corresponds to the performance of DNAmFitAge across datasets displayed with Pearson correlation to chronological age and corresponding *p*-values. (**A**) DNAmFitAge performance in training dataset built in each sex separately. (**B**–**H**) DNAmFitAge models applied to the opposite sex it was built in (ie DNAmFitAge built for females tested in males and DNAmFitAge built for males tested in females). Females are estimated to be older than they are, and males are estimated to be younger than they are in training and test datasets.



Supplementary Figure 3. Scatterplots of DNAm fitness biomarker models versus true fitness values in training data. Pink indicates females, and blue indicates males. Each panel corresponds to the training performance of one DNAm biomarker model built with chronological age displayed with Pearson correlation and *p*-values. (**A**) DNAmGaitspeed, (**B**) DNAmGripmax, (**C**) DNAmFEV1, and (**D**) DNAmVO2max. (**A**–**C**) (DNAmGaitspeed, DNAmGrip, and DNAmFEV1) were built in each sex separately while (**D**) (DNAmVO2max) was built in both sexes jointly.



Supplementary Figure 4. Correlation matrix in largest training data (FHS and BLSA) among fitness parameters, DNAm fitness parameter biomarkers, and DNAmFitAge. Pearson r correlation between two variables are displayed. Correlation among VO2max is not displayed because this variable was not present in the large training data. Variables labeled "_wAge" indicate models built with chronological age as a predictor, and variables labeled "_noAge" indicate models built using only CpG loci (ie without chronological age as a predictor).

Supplementary Tables

Supplementary Table 1. Validation dataset null model pearson correlation and DNAm biomarker significance.

DNAm Biomarker	Sex	Age in Model	LBC1921		LBC1936		CALERIE		InChianti		WHI		Meta Analysis *p*-value
			Null R	DNAm *p*-value	Null R	DNAm *p*-value	Null R	DNAm *p*-value	Null R	DNAm *p*-value	Null R	DNAm *p*-value	
Gaitspeed	Females	Y	0.412	0.437	0.382	0.118	0.100*	0.519	0.478+	0.011	0.107+	1.7E-05	0.082
		N		0.069		0.230		0.736		0.427		0.0002	0.051
	Males	Y	0.418	0.092	0.413	0.118	0.264*	0.519	0.334+	0.011			0.0048
		N		0.011		0.230		0.736		0.427			0.0037
Gripmax	Females	Y	0.422	0.556	0.114	7.7E-05	0.170	0.258			0.128+	0.012	0.056
		N		0.0014		0.028		0.312				0.430	0.056
	Males	Y	0.363	0.026	0.203	0.034	0.115	0.095					0.702
		N		0.0037		0.005		0.939					0.0015
FEV1	Females	Y	0.163^	0.0017	0.214^	0.0061	0.193	4.3E-05					1.99E-04
	Males	Y	0.196^	0.0001	0.206^	9.2E-09	0.483	0.655					2.01E-09
VO2max	Overall	Y	0.623^	0.0065	0.606^	0.809	0.706	0.00013					-

*Composite Leg Strength, +Physical Functioning, ^FEV

Supplementary Table 2. DNAmFitAge to chronological age performance in validation datasets.

		Females	Males	Male model in females	Female model in males
Training Data	Median Absolute Deviation	2.7	3.0	11.9	13.5
	Mean Deviation	0.0	0.0	−12.2	13.1
	R	0.923	0.925	0.925	0.922
LBC1921	Median Absolute Deviation	3.7	4.8	11.0	14.5
	Mean Deviation	0.8	1.1	−11.1	13.8
	R	0.409	0.386	0.404	0.391
LBC1936	Median Absolute Deviation	3.2	3.4	11.6	13.3
	Mean Deviation	0.0	0.2	−11.9	12.9
	R	0.635	0.635	0.647	0.624
CALERIE	Median Absolute Deviation	4.9	2.3	17.1	11.0
	Mean Deviation	−5.0	−2.0	−17.1	11.0
	R	0.926	0.915	0.928	0.912
InChianti	Median Absolute Deviation	3.9	3.9	16.0	9.6
	Mean Deviation	−3.8	−4.3	−16.1	9.1
	R	0.969	0.964	0.969	0.963
JHS	Median Absolute Deviation	2.9	3.4	13.6	9.2
	Mean Deviation	−1.6	−2.8	−13.9	8.6
	R	0.937	0.917	0.940	0.914
WHI	Median Absolute Deviation	3.8		16.8	
	Mean Deviation	−3.4		−16.8	
	R	0.808		0.812	

Supplementary Table 3. FitAgeAcceleration association to phenotypic outcomes.

Meta analysis *p*-values		Time to death*	Time to coronary heart disease*	Type 2 diabetes	Number of comorbidities	Disease free status	Total cholesterol	Age at menopause	Any cancer	hypertension
		***p* = 7.2 E-51**	***p* = 2.6 E-8**	***p* = 2.7 E-9**	***p* = 9.0 E-9**	***p* = 1.1 E-7**	***p* = 0.00048**	***p* = 6.6 E-9**	***p* = 0.157**	***p* = 8.7 E-5**
LBC1921 Females	Coefficients	1.03		0.042	0.007	0.009	-0.039			
	p-values	0.013		0.230	0.171	0.922	0.0017			
	No. of events	375		16	96	266	-			
LBC1921 Males	Coefficients	1.06		0.033	0.007	−0.027	-0.027			
	p-values	1.62E-06		0.392	0.273	0.161	0.029			
	No. of events	277		21	102	157	-			
LBC1936 Females	Coefficients	1.08		0.046	0.008	−0.006	-0.010	−0.081		
	p-values	3.76E-08		0.0015	0.153	0.672	0.112	0.043		
	No. of events	334		109	1171	179	-	-		
LBC1936 Males	Coefficients	1.09		0.048	0.017	−0.038	-0.011			
	p-values	9.24E-12		0.0032	0.0031	0.0048	0.045			
	No. of events	499		183	1219	211	-			
InChianti Females	Coefficients	1.06		0.018	−0.002	−0.006	-0.046	−2.96	−0.059	0.0037
	p-values	0.011		0.635	0.842	0.868	0.223	0.00034	0.176	0.881
	No. of events	167		33	235	104	-	-	37	140
InChianti Males	Coefficients	1.07		−0.070	2.11E-05	−0.045	−0.004		0.045	0.026
	p-values	1.01E-06		0.135	0.998	0.215	0.905		0.292	0.219
	No. of events	162		33	221	87	-		31	143
WHI	Coefficients	1.05	1.04	0.050	0.021	−0.031	−0.008	−0.060	0.025	0.024
	p-values	8.06E-09	1.20E-05	0.00052	0.0011	0.0041	0.570	3.82E-05	0.063	0.014
	No. of events	771	2117	392	1539	793	-	-	388	918
JHS Females	Coefficients	1.15	1.13	0.054	0.029	−0.062	−0.116	0.747**		0.057
	p-values	1.96E-15	0.00025	0.0016	4.12E-07	0.00024	0.696	0.152		0.00071
	No. of events	156	1001	267	721	352	-	-		664
JHS Males	Coefficients	1.06	1.06	0.012	0.012	−0.034	−1.148			0.021
	p-values	9.25E-07	0.041	0.495	0.066	0.082	0.0006			0.232
	No. of events	125	579	135	406	226	-			363

*Hazard Ratios; **Not age at menopause, menopause status; number of events for comorbidities is people with at least 1 comorbidity.

Supplementary Table 4. Comparing DNAmFitAge importance with other DNAm biomarkers for time-to-death and number of comorbidities after controlling for age and sex.

		LBC1921		LBC1936		InChianti		WHI		JHS	
Time-to-Death Model Comparison		**LRT**	**LRT *p*-value**	**LRT**	**LRT *p*-value**	**LRT**	**LRT *p*-value**	**LRT**	**LRT *p*-value**	**LRT**	**LRT *p*-value**
DNAmGrimAge + DNAmFitAge to DNAmGrimAge	Females	0.5	0.479	2.9	0.091	7.2	0.007	1.1	0.286	4.6	0.032
	Males	3.7	0.054	2.6	0.110	7.7	0.005			0.2	0.628
DNAmPhenoAge + DNAmFitAge to DNAmPhenoAge	Females	9.1	0.003	36.0	1.98E-09	1.2	0.269	17.0	3.70E-05	30.4	3.53E-08
	Males	11.3	7.64E-04	91.0	< 1.0E-16	26.4	2.76E-07			4.3	0.039
DNAmPAI1 + DNAmFitAge to DNAmPAI1	Females	9.8	0.002	51.1	8.67E-13	7.3	0.007	17.0	3.76E-05	38.5	5.36E-10
	Males	30.4	3.49E-08	83.6	< 1.0E-16	22.9	1.67E-06			7.1	0.008
DNAmGDF15 + DNAmFitAge to DNAmGDF15	Females	5.2	0.023	44.8	2.16E-11	6.1	0.014	23.1	1.54E-06	46.6	8.88E-12
	Males	25.0	5.78E-07	70.7	< 1.0E-16	14.1	1.73E-04			4.4	3.66E-02
DNAmAgeHannum + DNAmFitAge to DNAmAgeHannum	Females	13.2	2.79E-04	60.8	6.11E-15	2.0	0.157	31.5	1.95E-08	41.6	1.11E-10
	Males	15.0	1.09E-04	104.0	< 1.0E-16	22.0	2.70E-06			7.0	0.008
DNAmAgeSkinBlood Clock + DNAmFitAge to DNAmAgeSkinBlood Clock	Females	16.4	5.05E-05	92.3	< 1.0E-16	3.6	0.058	34.7	3.79E-09	57.2	3.89E-14
	Males	22.4	2.17E-06	133.8	< 1.0E-16	21.8	3.01E-06			10.9	9.46E-04
Number of Comorbidities Model Comparison											
DNAmGrimAge + DNAmFitAge to DNAmGrimAge	Females	2.1	0.148	1.2	0.269	0.4	0.513	3.4	0.065	0.0	0.910
	Males	2.5	0.117	3.1	0.080	0.05	0.828			1.2	0.267
DNAmPhenoAge + DNAmFitAge to DNAmPhenoAge	Females	0.05	0.828	2.6	0.110	3.4	0.067	2.9	0.091	8.6	0.003
	Males	5.2	0.023	38.7	4.98E-10	0.01	0.927			0.7	0.412
DNAmPAI1 + DNAmFitAge to DNAmPAI1	Females	0.7	0.401	1.3	0.255	0.9	0.344	1.4	0.230	2.7	0.101
	Males	1.4	0.233	26.5	2.70E-07	0.1	0.800			0.1	0.817
DNAmGDF15 + DNAmFitAge to DNAmGDF15	Females	0.5	0.476	5.3	0.021	0.01	0.944	6.7	0.010	22.1	2.61E-06
	Males	2.6	0.105	31.6	1.86E-08	0.6	0.453			4.2	0.041
DNAmAgeHannum + DNAmFitAge to DNAmAgeHannum	Females	0.03	0.871	2.4	0.123	0.7	0.411	6.5	0.011	13.1	2.88E-04
	Males	2.6	0.108	39.2	3.78E-10	0.2	0.624			1.1	0.305
DNAmAgeSkinBlood Clock + DNAmFitAge to DNAmAgeSkinBlood Clock	Females	0.3	0.596	5.6	0.018	0.2	0.682	8.3	0.004	16.5	4.83E-05
	Males	1.3	0.256	55.0	1.21E-13	0.2	0.676			2.9	0.089

Supplementary Table 5. Linear models evaluating dietary supplement usage to DNAmFitAge and DNAmVO2max in males after adjusting for age.

Supplement in model		Outcome: DNAmFitAge		Outcome: DNAmVO2max	
		Supplement	BodyBuilder	Supplement	BodyBuilder
Multivitamins	coefficient	–0.32	–0.62	0.68	0.07
	p-value	0.690	0.208	0.041	0.746
Proteins	coefficient	–0.05	–0.65	0.45	0.10
	p-value	0.961	0.184	0.241	0.607
Energy	coefficient	0.16	–0.66	0.24	0.13
	p-value	0.852	0.175	0.518	0.513
Magnesium	coefficient	–1.03	–0.60	-0.12	0.15
	p-value	0.213	0.219	0.727	0.472
Vitamin D	coefficient	–0.56	–0.62	-0.32	0.16
	p-value	0.570	0.207	0.439	0.431
Omega-3	coefficient	–1.23	–0.46	0.33	0.08
	p-value	0.157	0.366	0.355	0.687

Supplementary Table 6. Dietary supplement use by male athlete status.

		Control	Body builder	Fisher's exact *p*-value
Multivitamins	No	141	55	0.016
	Yes	8	11	
Proteins	No	140	58	0.169
	Yes	9	8	
Energy	No	145	53	6.81E-05
	Yes	4	13	
Magnesium	No	140	59	0.265
	Yes	9	7	
Vitamin D	No	143	58	0.036
	Yes	6	8	
Omega-3	No	144	59	0.050

Supplementary Table 7. Complete GREAT analysis CpG annotation.

Genes	Observed Regions	Fold Enrichment	Binomial *p*-value	Bonferroni *p*-value	FDR Q-value
ZNRD1	4	77.9	2.8E-07	0.0051	0.0051
HLA-G	4	55.0	1.1E-06	0.020	0.010
KCNS1	3	93.3	5.4E-06	0.100	0.033
HOXA2	2	518.0	7.4E-06	0.138	0.034
TAP2	2	421.0	1.1E-05	0.208	0.042
Cellular					
MHC protein complex	9	25.1	1.9E-10	3.1E-07	3.1E-07
integral component of endoplasmic reticulum membrane	21	3.7	4.5E-07	7.5E-04	3.7E-04
intrinsic component of endoplasmic reticulum membrane	21	3.6	6.4E-07	0.0011	3.5E-04
MHC class II protein complex	5	26.9	1.6E-06	0.0026	6.5E-04
integral component of lumenal side of endoplasmic reticulum membrane	7	12.7	1.8E-06	0.0030	6.0E-04
MHC class I protein complex	4	23.1	3.2E-05	0.054	0.0089
TAP complex	2	195.9	5.2E-05	0.086	0.012
DNA-directed RNA polymerase I complex	5	12.2	6.7E-05	0.112	0.014
MHC class I peptide loading complex	2	169.4	6.9E-05	0.115	0.013
A band	10	4.2	1.7E-04	0.286	0.029
lysosomal membrane	30	2.0	2.9E-04	0.479	0.044
Molecular					
peptide antigen binding	6	13.3	7.7E-06	0.032	0.032
tapasin binding	2	421.0	1.1E-05	0.047	0.023
MHC class II receptor activity	4	28.5	1.4E-05	0.060	0.020
antigen binding	11	4.8	2.8E-05	0.116	0.029
peptide antigen-transporting ATPase activity	2	249.6	3.2E-05	0.133	0.027
DNA-directed 5′–3′ RNA polymerase activity	8	6.7	3.5E-05	0.148	0.025
5′–3′ RNA polymerase activity	8	6.5	4.4E-05	0.185	0.026

Supplementary Table 8. DNAm fitness CpG loci overlap with other epigenetic clocks.

	CpG	DNAm Fitness Coefficient	Clocks Present in	Same Coefficient Direction	PhenoAge Coefficient	DNAmAge Coefficient	Hannum Coefficient	Skin and Blood Coefficient	DNAm Fitness Model
					(*n* = 7)	(*n* = 2)	(*n* = 15)	(*n* = 23)	
1	cg26842024	−0.16	3	0	0.06	0.36		0.48	DNAmGaitspeed Males no Age
2	cg00748589	−0.37	2	0			8.21	0.17	DNAmGaitspeed Males no Age
3	cg02867102	0.32	2	0			−12.50	-0.12	DNAmGaitspeed Males no Age
4	cg03607117	−0.63	2	0			10.70	0.17	DNAmGaitspeed Females no Age
		−6.47		0			10.70	0.17	DNAmGripmax Females no Age
5	cg04424621	0.01	2	1	-14.45			0.04	DNAmGaitspeed Males no Age
6	cg04875128	−4.72	2	1			−4.37	0.0004	DNAmGripmax Males no Age
7	cg06639320	−8.60	2	0			8.95	0.02	DNAmGripmax Males no Age
8	cg07082267	0.22	2	1			2.87	-0.47	DNAmGaitspeed Males no Age
9	cg10917602	0.02	2	0	-2.82			-0.02	DNAmGaitspeed Females w/ Age
10	cg16419235	−0.23	2	1			−1.60	0.11	DNAmGaitspeed Females no Age
		−3.81		1			−1.60	0.11	DNAmGripmax Females no Age
11	cg16867657	−0.21	2	0			10.80	0.90	DNAmGaitspeed Males no Age
		−1.26		0			10.80	0.90	DNAmGaitspeed Females no Age
		−8.32		0			10.80	0.90	DNAmGripmax Males no Age
		−12.72		0			10.80	0.90	DNAmGripmax Females no Age
12	cg20822990	1.53	2	0			−15.70	−0.01	DNAmGripmax Males no Age
13	cg22454769	−0.17	2	0			4.85	0.05	DNAmGaitspeed Males no Age
14	cg23500537	−0.22	2	0			5.67	0.23	DNAmGripmax Males no Age
15	cg25410668	−1.90	2	0			3.87	0.28	DNAmGripmax Males no Age
16	cg26581729	4.08	2	1	-4.00			0.02	DNAmGripmax Males no Age
17	cg00481951	−0.52	1	1			−2.72		DNAmGaitspeed Males no Age
18	cg05228408	0.05	1	0	-4.49				DNAmGaitspeed Females w/ Age
19	cg07502389	−0.36	1	0				0.32	DNAmGaitspeed Females no Age
20	cg08622677	−4.43	1	0				0.23	DNAmGripmax Females no Age
21	cg12753631	0.11	1	0				−0.34	DNAmGaitspeed Males no Age
22	cg12864235	0.06	1	1	0.66				DNAmFEV1 Females w/ Age
23	cg13649056	−4.04	1	0				0.04	DNAmGripmax Females no Age
		−0.43		0				0.04	DNAmGaitspeed Females no Age
24	cg17110586	−0.32	1	1				−0.07	DNAmGaitspeed Females no Age
		−7.53		1				−0.07	DNAmGripmax Females no Age
		−12.20		0	4.54				DNAmGripmax Males no Age
25	cg18691434	−0.13	1	0	4.54				DNAmGaitspeed Males no Age
		−3.40		0	4.54				DNAmGripmax Males w/ Age
26	cg18933331	0.06	1	0				−0.23	DNAmGaitspeed Males no Age
27	cg19702785	−0.23	1	0				0.13	DNAmGaitspeed Males no Age
28	cg22285878	−4.14	1	1			−20.70		DNAmGripmax Females no Age
29	cg25101936	0.004	1	0		−0.06			DNAmGaitspeed Females w/ Age
30	ch.13.39564907R	0.30	1	0			−20.60		DNAmGaitspeed Males no Age

Supplementary Table 9. Descriptive statistics for each dataset.

Total Observations	BLSA	Budapest	LBC1921	LBC1936	CALERIE	InChianti	JHS	WHI	Polish
	820	307	692	2797	578	924	1746	2117	215
Age mean (sd)	69.2 (13.6)	60.3 (11.7)	82.3 (4.3)	73.6 (3.7)	39.4 (7.2)	67.0 (16.6)	56.2 (12.3)	65.4 (7.1)	35.5 (8.8)
< 40	24 (3%)	8 (3%)	0 (0%)	0 (0%)	265 (46%)	100 (11%)	173 (10%)	0 (0%)	144 (67%)
40–59	178 (22%)	133 (43%)	0 (0%)	0 (0%)	313 (54%)	128 (14%)	856 (49%)	525 (25%)	71 (33%)
60–79	400 (49%)	151 (50%)	410 (59%)	2719 (97%)	0 (0%)	502 (54%)	691 (40%)	1589 (75%)	0 (0%)
80+	218 (27%)	15 (5%)	282 (41%)	78 (3%)	0 (0%)	194 (21%)	26 (2%)	3 (0%)	0 (0%)
Sex									
Males	417 (51%)	148 (48%)	291 (42%)	1141 (52%)	178 (31%)	426 (46%)	649 (37%)	0 (0%)	215 (100%)
Females	403 (50%)	159 (52%)	401 (58%)	1356 (49%)	400 (69%)	498 (54%)	1097 (63%)	2117 (100%)	0 (0%)
Race									
White	572 (70%)	297 (97%)	692 (100%)	2797 (100%)	442 (77%)	924 (100%)	0 (0%)	1007 (48%)	215 (100%)
Black	216 (26%)	0 (0%)	0 (0%)	0 (0%)	71 (12%)	0 (0%)	1746 (100%)	677 (32%)	0 (0%)
Asian	24 (3%)	10 (3%)	0 (0%)	0 (0%)	unknown	0 (0%)	0 (0%)	unknown	0 (0%)
Other	8 (1%)	0 (0%)	0 (0%)	0 (0%)	65 (11%)	0 (0%)	0 (0%)	433 (21%)	0 (0%)

Supplementary Table 10. Complete chromatin state analysis.

	State	Number of CpG loci	Odds Ratio	Hypergeometric *p*-value		State	Number of CpG loci	Odds Ratio	Hypergeometric *p*-value
1	PromF4	25	0.45	6.5E-06	51	Acet1	5	1.43	0.277
2	TSS1	15	0.37	6.8E-06	52	EnhA1	8	1.30	0.280
3	BivProm2	43	1.76	0.00057	53	EnhA16	4	1.49	0.286
4	TxEx3	4	0.30	0.0030	54	HET9	0	0.00	0.287
5	DNase1	13	2.41	0.0041	55	EnhA12	2	0.56	0.312
6	ReprPC1	21	1.87	0.0065	56	GapArtf2	1	2.64	0.316
7	BivProm1	43	1.50	0.0092	57	znf2	2	1.74	0.320
8	ReprPC5	18	1.87	0.011	58	HET3	1	2.47	0.334
9	EnhA3	9	2.53	0.011	59	Tx2	1	0.44	0.336
10	TxWk1	0	0.00	0.014	60	EnhWk2	8	1.22	0.338
11	PromF6	11	2.13	0.018	61	HET8	1	0.44	0.338
12	EnhA8	11	2.09	0.020	62	TxEx1	2	0.59	0.342
13	ReprPC7	21	1.66	0.021	63	Acet4	5	1.29	0.346
14	PromF2	17	1.70	0.028	64	EnhA14	3	0.67	0.350
15	BivProm4	12	1.82	0.039	65	Quies4	3	1.43	0.352
16	EnhA18	11	1.87	0.039	66	Tx1	3	1.40	0.364
17	Acet2	0	0.00	0.042	67	Acet3	3	0.69	0.367
18	TxEnh5	6	0.49	0.044	68	PromF5	25	0.91	0.368
19	TxEnh2	0	0.00	0.047	69	ReprPC3	3	0.69	0.374
20	TxEnh8	8	2.02	0.049	70	PromF3	23	0.91	0.380
21	BivProm3	20	1.51	0.053	71	TxWk2	10	0.86	0.382
22	TxEx2	4	0.44	0.054	72	EnhA9	5	1.23	0.386
23	EnhWk8	2	0.32	0.055	73	ReprPC9	3	1.30	0.408

24	EnhWk6	5	2.42	0.060	74	EnhWk1	1	0.50	0.408
25	EnhWk4	0	0.00	0.083	75	EnhA11	1	0.50	0.408
26	Tx7	0	0.00	0.086	76	ReprPC4	4	0.78	0.418
27	Quies3	4	0.49	0.089	77	Acet6	2	0.66	0.420
28	ReprPC6	5	0.53	0.096	78	Acet7	4	0.78	0.420
29	Quies2	0	0.00	0.102	79	TSS2	23	0.93	0.422
30	TxEnh1	0	0.00	0.106	80	Tx4	1	0.52	0.425
31	EnhA4	6	1.84	0.114	81	TxEnh6	2	0.67	0.427
32	EnhA2	2	0.39	0.119	82	EnhA17	6	1.11	0.456
33	EnhA15	5	1.86	0.136	83	EnhA10	3	0.78	0.464
34	TxEx4	6	1.74	0.136	84	PromF7	2	0.71	0.465
35	TxEnh7	8	1.59	0.139	85	TxEnh4	8	1.06	0.482
36	Tx3	6	1.72	0.142	86	HET7	4	0.85	0.489
37	EnhA6	1	0.30	0.150	87	EnhA19	6	1.03	0.529
38	HET5	0	0.00	0.185	88	Tx5	5	1.03	0.534
39	HET6	7	1.50	0.191	89	Acet8	3	1.07	0.534
40	Tx8	1	0.34	0.204	90	HET2	10	0.96	0.538
41	Quies1	0	0.00	0.208	91	GapArtf1	1	0.68	0.566
42	HET1	5	1.59	0.210	92	ReprPC2	5	0.96	0.579
43	EnhWk3	2	0.48	0.215	93	TxEnh3	3	0.93	0.595
44	Tx6	0	0.00	0.222	94	EnhA13	1	0.73	0.601
45	EnhA7	8	1.38	0.233	95	Quies5	1	1.03	0.623
46	Acet5	1	0.36	0.235	96	EnhA20	0	0.00	0.630
47	PromF1	12	1.26	0.251	97	znf1	1	0.79	0.638
48	EnhA5	4	1.56	0.257	98	ReprPC8	1	0.85	0.672
49	EnhWk5	3	1.68	0.266	99	EnhWk7	1	0.94	0.714
50	HET4	1	3.16	0.272	100	GapArtf3	0	0.00	0.720