Author/s:
Li, DL;Hodge, AM;Southey, MC;Giles, GG;Dugué, PA

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Research Article

Association of Epigenetic Markers of Aging With Prevalent and Incident Type 2 Diabetes

Danmeng Lily Li, MPH,[1] Allison M. Hodge, PhD,[2,3] Melissa C. Southey, PhD,[1,4] Graham G. Giles, PhD,[1,2] and Pierre-Antoine Dugué, PhD[1,2,*]

[1]Precision Medicine, School of Clinical Sciences at Monash Health, Monash University, Clayton, Victoria, Australia.
[2]Cancer Epidemiology Division, Cancer Council Victoria, Melbourne, Victoria, Australia.
[3]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Parkville, Victoria, Australia.
[4]Department of Clinical Pathology, The University of Melbourne, Parkville, Victoria, Australia.

*Address correspondence to: Pierre-Antoine Dugué, PhD. E-mail: pierre-antoine.dugue@monash.edu

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Abstract

Background: Type 2 diabetes (T2D) is characterized by elevated levels of metabolic and inflammatory markers but less is known about other molecular alterations that occur with aging. We aimed to assess the associations of DNA methylation-based measures of aging (epigenetic aging) with prevalent and incident T2D in a large sample of middle-aged and older Australians.

Methods: We used data from 5 403 participants in the Melbourne Collaborative Cohort Study (mean age = 59 years). Five blood-based epigenetic aging measures: *PCPhenoAge*, *PCGrimAge*, *DNAmFitAge*, *bAge*, and *DunedinPACE* were calculated. T2D status was assessed at baseline (1990–1994, N_{cases} = 180) and 2 waves of follow-up (1995–1998, N_{cases} = 134; 2003–2007, N_{cases} = 244). Modified Poisson regression models were used to estimate risk ratios for the associations of epigenetic age with prevalent and incident T2D.

Results: A standard deviation increase in epigenetic age was associated with 1.11-fold (*PCPhenoAge*, 95%CI: 0.98–1.26) to 1.33-fold (*bAge*, 95%CI: 1.12–1.57) higher prevalence of T2D at baseline. Prospectively, *DunedinPACE* showed the strongest association with incident T2D at follow-up 2 (risk ratio = 1.22, 95%CI: 1.07–1.38). These estimates were slightly attenuated but consistent in sensitivity analyses reclassifying participants who reported being T2D-free but had high glucose concentrations (> 7 mmol/L for fasting glucose, > 11.1 mmol/L for nonfasting glucose). No evidence of increased epigenetic age was found for participants with pre-T2D (> 5.6 mmol/L for fasting glucose, > 7.8 mmol/L for nonfasting glucose). The positive associations between epigenetic age and fasting glucose levels appeared stronger in participants with T2D.

Conclusions: In middle-aged and older Australians, epigenetic age, in particular as assessed by *bAge* and *DunedinPACE*, was positively associated with prevalent and incident T2D. Our findings may have implications for understanding the etiology and management of T2D.

Keywords: Biological aging, Epigenetic aging, Incident type 2 diabetes, Risk factors

Background

There has been a rapid increase in the occurrence of diabetes worldwide with an estimated global prevalence projected to reach over 10% by 2030 (1). Type 2 diabetes (T2D) accounts for 90% of all diabetes cases (1). Type 2 diabetes arises from the combination of insulin resistance and pancreatic beta cell impairment; it is strongly age-related, and caused by both genetic and environmental/lifestyle factors (2). People with T2D have reduced life expectancy, and are at risk of other age-related morbidities and limitations in activities of daily living (3). Although age over 40 is an important risk factor for T2D, there are an increasing number of T2D cases being diagnosed at younger ages (1,2). Biological aging is characterized by a wide range of functional and molecular alterations and captures an individual's physiological health and risk of disease (4). Molecular markers of biological aging, including epigenetic markers, have attracted interest in recent years, and these might extend our understanding of the development of T2D. In previous studies, molecular markers such as telomere length and inflammation markers (eg, cytokines and interleukin-6) were found to be associated with risk and progression of T2D (5,6).

DNA methylation (DNAm-) based biological age, or "epigenetic age," is a strong predictor of disease and mortality risk (7). Epigenetic aging measures have been assessed as both a potential risk factor for and as an adverse outcome of T2D in previous studies, which had varying designs and findings (8–14). Kawamura et al. (8) reported elevated epigenetic age (particularly *DNAmFitAge*) among participants with T2D, whereas another study found no association (9). Two cohort studies (10,11) and a nested case–control study (12) reported a positive association between epigenetic age and incident diabetes, whereas the associations were very small or null in 2 other studies (13,14). Additional to the heterogeneity of the existing evidence, early studies (9,11) only considered first-generation epigenetic clocks which were trained to

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predict chronological age; other studies (12–14) that included second- and third-generation clocks (eg, *PhenoAge*, *GrimAge*, and *DunedinPoAm*, trained to estimate biological age or rate of aging) generally had small sample size. Very few studies (10) considered the most recently developed and improved clocks, such as *DunedinPACE* (15), principal-component (PC)-based measures (16), *bAge* (17), or *DNAmFitAge* (18).

Obesity is a key risk factor for T2D and in previous work (19), we found that *DunedinPACE* was more strongly associated with body size measures than were *GrimAge* and *PhenoAge*. *DNAmFitAge* and *bAge* are recently developed measures based on *GrimAge* that incorporate additional information (about proteins and physiological fitness, respectively) and might be better measures of biological age than *GrimAge* itself (17,18). Therefore, we hypothesized that *bAge*, *DNAmFitAge*, and *DunedinPACE* might exhibit stronger associations with T2D than other clocks. We used data from a large prospective study of 5 403 middle-aged and older Australians with the aims to assess: (i) whether epigenetic age is positively associated with prevalent T2D in middle-aged and older adults; (ii) whether epigenetic age is a potential relevant marker for T2D.

Method

Study Participants

We used data from the Melbourne Collaborative Cohort Study (MCCS), where 41 513 white European Australians (59% females) aged between 40 and 69 (99% of them) were recruited between 1990 and 1994 and were prospectively followed for over a decade (20). Between 1995 and 1998, follow-up 1 was carried out to collect self-reported lifestyle and health data via telephone or self-administered questionnaires (20). At follow-up 2 (2003–2007), 68% of the original participants attended in-person interviews (20). At baseline and follow-up 2, physical measurements and blood sample collections were performed (20).

This study used a subset of 5 403 participants from 7 prospective cancer case–control studies nested in the MCCS who had genome-wide DNAm measured from blood samples collected as dried blood spot on a Guthrie card (71.7%), peripheral blood mononuclear (26.6%), and buffy coat (1.7%) at baseline and follow-up 2 with the Illumina HumanMethylation450K BeadChip array (20). Details of DNA extraction, processing, and quality control of DNA methylation data have been described in previous papers (21–23). The incident cancer cases were identified through annual linkage to the Victorian Cancer Registry and were matched to controls on age, sex, country of birth (and smoking history for the lung cancer study) using incidence density sampling (20).

The MCCS was approved by the Human Research Ethics Committee of the Cancer Council Victoria, Melbourne, VIC, Australia, and informed consent was provided by all participants according to the Declaration of Helsinki.

Epigenetic Age

We considered 5 recently developed, improved epigenetic aging measures: *PCPhenoAge*, *PCGrimAge*, *DNAmFitAge*, *bAge*, and *DunedinPACE* (Supplementary Table 1). *GrimAge* was developed using as inputs age, sex, and DNAm markers of smoking pack-years and 7 plasma proteins (24). *PhenoAge* was developed using DNAm-based surrogate markers of a phenotypic age that include age and 9 clinical markers (25). These clocks were trained on PC to account for the multicollinearity between the CpGs, in order to reduce the technical noise and improve their reliability, resulting in *PCPhenoAge* (78464 CpGs) and *PCGrimAge* (78464 CpGs) (16). Both *DNAmFitAge* and *bAge* were developed based on *GrimAge*, where *DNAmFitAge* combined *GrimAge* with 3 DNAm markers of fitness (18), and *bAge* incorporated age, 6/8 DNAm-based surrogate markers from *GrimAge* and 28 protein EpiScores (eg, for C-reactive protein and many cytokines) (17). *DunedinPACE* (173 CpGs) measured the rate of aging and was calculated based on changes in 19 biomarkers over 20 years (15). All epigenetic aging measures were calculated using the *methscore* function in R (26); *DNAmFitAge* and *bAge* were calculated based on *PCGrimAge*.

All 5 measures were regressed on chronological age and the residuals were used as age-adjusted measures of epigenetic age in all analyses, standardized to a mean of 0 and standard deviation (*SD*) of 1.

Type 2 Diabetes and Blood Glucose Concentration

Information on diabetes status was collected via questionnaires at baseline and follow-ups. We excluded all participants who reported being diagnosed with diabetes before the age of 40 since these were more likely type 1 diabetes cases. We defined prevalent T2D as participants who reported having diabetes at baseline. Because not all participants in the MCCS attended both waves of follow-up, we defined incident T2D in 3 ways: (i) participants who reported no diabetes at baseline, but reported diabetes at follow-up 1; (ii) participants who reported no diabetes at baseline and follow-up 1 (or did not attend follow-up 1), and reported having diabetes at follow-up 2; and (iii) participants who reported no diabetes at baseline, attended both waves of follow-up, and reported having diabetes at any wave (Figure 1).

Blood glucose concentration was measured from the plasma samples at baseline using Kodak Ektachem DT60 desktop analyser (Rochester, NY), and using a glucometer (Medisense, Abbott Diabetes Care Inc., Alameda, CA) at follow-up 2. Fasting status (≥ 8 h from last food or drink except for water) was recorded at both time points. At follow-up 2, time from last food or fluid and time at measurement were recorded by trained personnel.

Confounders

At baseline, demographic and lifestyle data were collected via questionnaire during face-to-face interviews, including age (years), sex (male, female), country of birth (Australia/New Zealand/Other, Greece, Italy, UK/Malta), physical activity (score [0–16], based on weekly frequency of walking, vigorous, and less vigorous activities), smoking status (never smoker, former smoker, current smoker), smoking pack-years, educational attainment (8 levels), and alcohol consumption (grams/day in a year). Socioeconomic status (socioeconomic index for areas score, decile) was based on postcode of residence at baseline. The Alternative Healthy Eating Index 2010 (AHEI-2010) was calculated using food frequency questionnaire data. Anthropometric variables, weight (kg), height (m), and waist circumference (cm) were physically measured by trained personnel and body mass index (BMI, kg/m^2) was calculated.

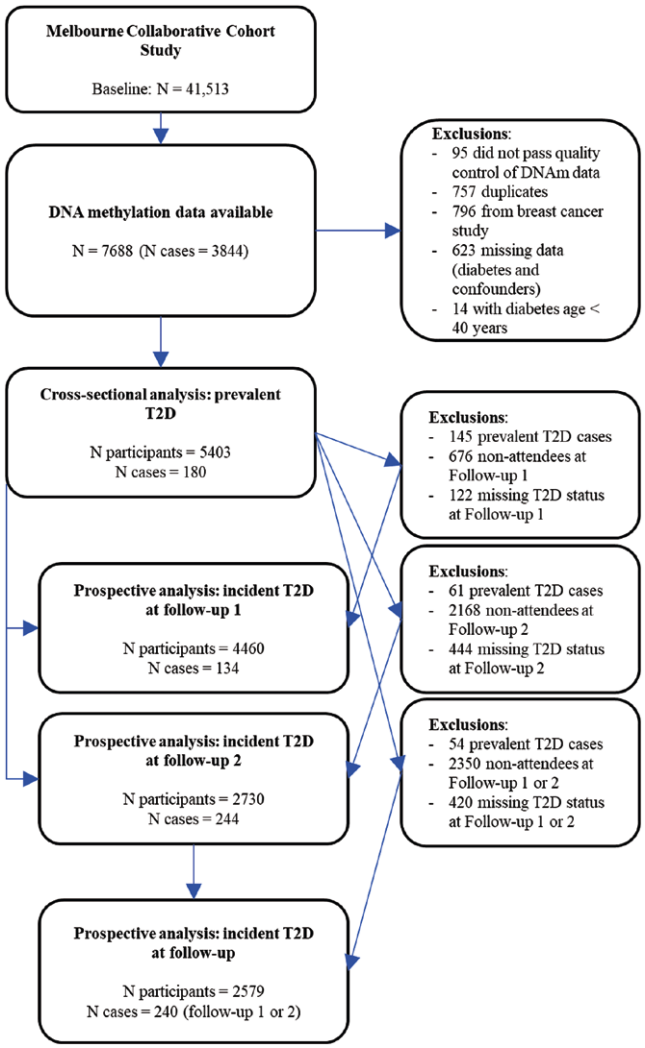

Figure 1. Study sample and analysis flowchart.

Statistical Analyses

Pearson correlation coefficients were calculated between chronological age and age-adjusted epigenetic age measures.

Primary analyses

Modified Poisson regression models were applied to assess the associations of baseline epigenetic age with prevalent and incident T2D (Figure 1). This approach was proposed by Zou (27) to produce a better approximation of risk ratios (RRs) which allows for easier clinical interpretation than odds ratios and compensates for the overestimation of standard errors in Poisson regression models with binary outcomes by applying a sandwich error estimator to the standard errors (28).

For each association, 3 models were considered: Model 0 was unadjusted; Model 1 adjusted for age, sex, and country of birth; Model 2 additionally adjusted for education, socioeconomic status, smoking status, smoking pack-years, alcohol consumption, physical activity, AHEI-2010, BMI, height, and waist circumference. In a previous study, we found a U-shaped pattern for the association between body size and epigenetic age (19). Alcohol consumption is known to be nonlinearly associated with various health outcomes, including T2D (29). Therefore, we applied restricted cubic spline terms (using 3 knots at 20th, 50th, and 80th percentiles) for BMI, waist circumference, and alcohol consumption. Since obesity is a major risk factor for T2D (30), both BMI and waist circumference were included to account for both overall and central obesity.

Secondary analyses

We used multivariable linear regression models to assess the association between baseline epigenetic age and glucose concentrations at baseline and follow-up 2, stratified by participants' fasting status. For the cross-sectional analyses, we used the same set of confounders as described above. For the analyses using follow-up 2 glucose concentrations, we considered 3 models: (i) adjusting for age, sex, and country of birth; (ii) additionally adjusting for time from last food or drink (except water); (iii) additionally adjusting for other lifestyle and demographic confounders described in Model 2 of the associations of T2D. To further explore whether this association was different by T2D status, we assessed the interactions between baseline epigenetic age and prevalent T2D at baseline and follow-up 2 for the associations of fasting glucose concentrations at each wave, respectively, using likelihood ratio tests (LRTs), and presented the results graphically.

Sensitivity analyses

We performed a series of sensitivity analyses to test the robustness of our effect estimates:

(i) We repeated the cross-sectional and longitudinal analyses at baseline and follow-up 2 excluding cancer cases to obtain a more representative sample of the entire MCCS cohort.

(ii) It has been estimated that approximately 1 in 2 people do not know they have diabetes (1). To explore the effect of potential misclassification, we repeated all analyses after reclassifying participants who reported having no T2D at baseline or follow-up 2 based on glucose concentration (Supplementary Figure 1), those who had fasting glucose above 7 mmol/L or nonfasting glucose above 11.1 mmol/L were reclassified as having T2D.

(iii) We hypothesized that participants who reported being T2D-free, but had abnormal glucose concentration might be either more prone to developing T2D in the future or having undiagnosed T2D. This group of participants was defined as having pre-T2D, and we assessed whether excluding pre-T2D participants, therefore resulting in a healthier control group, would enhance the association of epigenetic age with T2D. At baseline and follow-up 2, we divided participants who reported being T2D-free into 2 categories according to their glucose concentrations: no T2D (defined as glucose concentration ≤ 5.6 mmol/L for fasted participants, and ≤ 7.8 mmol/L for nonfasted participants (31)) and pre-T2D (defined as glucose concentration > 5.6 mmol/L for fasted participants, and > 7.8 mmol/L for nonfasted participants) groups. Those who reported having diabetes remained in the T2D group. Two modified Poisson models were used to analyze separately pre-T2D versus non-T2D and T2D versus non-T2D, Supplementary Figure 1. Therefore, all individual associations of epigenetic

age with pre-T2D and T2D were assessed at baseline (prevalent) and follow-up 2 (incident).

(iv) We repeated the main analyses with the age-adjusted epigenetic aging markers further adjusted for white blood cell (WBC) proportions as a comparison.

All statistical analyses were conducted with R version 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Descriptive Statistics

At baseline, the average age of the 5 403 participants was 59.2 years and 67.5% were females, Supplementary Table 2. A total of 180 (3%) participants reported having T2D at baseline, and there were 134 and 244 incident cases at follow-up 1 and 2, respectively. Correlations between epigenetic measures were moderate to strong, ranging from 0.43 for *DNAmFitAge* with *DunedinPACE*, to 0.93 for *bAge* with *PCGrimAge*, Supplementary Figure 2.

Epigenetic Age and Prevalent T2D

At baseline, older epigenetic age was associated with a higher prevalence of T2D for all markers (Model 1). *DunedinPACE* showed the strongest association with prevalent T2D with RR per *SD* (increase in *DunedinPACE*) of 1.38 (95%CI: 1.19–1.59, Table 1); other RRs ranged from 1.20 for *PCPhenoAge* and *PCGrimAge* to 1.31 for *bAge*. After adjusting for demographic and lifestyle factors, most associations were attenuated but remained strong, for *DunedinPACE*: RR = 1.26 (95%CI: 1.08–1.47); for *bAge*: RR = 1.33 (95%CI: 1.12–1.57); and for *DNAmFitAge*, from 1.23 (95%CI: 1.09–1.38) to 1.17 (95%CI: 1.04–1.33), whereas the association for *PCPhenoAge* attenuated closer to null (RR = 1.11, 95%CI: 0.98–1.26).

Epigenetic Age and Incident T2D

We observed weak to null associations between epigenetic age and incident T2D, except for DunedinPACE, of which the associations were consistently strong.

There were 4 460 participants who attended follow-up 1 (prevalent cases from baseline excluded). In Model 1, *bAge*, *DunedinPACE*, and *DNAmFitAge* were associated with higher risks of incident T2D (RRs of 1.18 [*DNAmFitAge*]–1.29 [*DunedinPACE*], Table 2). After adjusting for all confounders, only *bAge* retained some association with incident T2D (per *SD*: RR = 1.21, 95%CI: 1.00–1.46), RRs for *DNAmFitAge* and *DunedinPACE* reduced to 1.09 and 1.06, respectively. At follow-up 2, with 2 730 attendees (prevalent cases from baseline excluded), only *DunedinPACE* was associated with T2D in Model 2: RR = 1.22, 95%CI: 1.07–1.38. The associations for *PCPhenoAge* or *PCGrimAge* with incident T2D were close to null at both waves.

Of 2 579 participants who attended both waves of follow-up, 240 in total developed T2D between baseline and follow-up 2. The associations were virtually the same as in the analysis including only incident cases at follow-up 2 (eg, for *DunedinPACE*, Model 2: per *SD*: RR = 1.21, 95%CI: 1.06–1.39).

Epigenetic Age and Glucose Concentration

After stratifying the study sample by fasting status at blood draw, no association was found for baseline epigenetic age and nonfasting glucose at either baseline or follow-up 2 (Supplementary Table 3). At baseline, epigenetic age was positively associated with fasting glucose concentration: Model 0, per *SD*: 0.07 mmol/L ($p = .05$) for *PCPhenoAge* to 0.13 mmol/L ($p = 2 \times 10^{-6}$) for *bAge*. Adjusting for all confounders reduced these associations to almost null for *PCGrimAge* ($\beta = 0.03$) whereas these remained positive for *PCPhenoAge* ($\beta = 0.04$), *DNAmFitAge* ($\beta = 0.04$), *bAge* ($\beta = 0.06$), and *DunedinPACE* ($\beta = 0.05$).

Similar findings were obtained for fasting glucose at follow-up 2. In Model 1, a *SD* increase in baseline epigenetic age was associated with 0.04 mmol/L (*PCGrimAge*) to 0.08 mmol/L (*DunedinPACE*) higher fasting glucose concentration. Time at glucose test and time from last food/drink had little effect on these associations (eg, Model 2, *DunedinPACE*: $\beta = 0.09$, $p = 2 \times 10^{-7}$). However, after accounting for all confounders, only *DunedinPACE* remained associated with higher glucose concentration ($\beta = 0.04$, $p = .03$).

Evidence of interactions was found between epigenetic age, particularly for *DunedinPACE*, and T2D in their associations with fasting glucose (LRTs: $p < .05$, Figure 2). The associations for fasting glucose with epigenetic age were much stronger among participants with T2D at both baseline and follow-up 2, particularly for *DunedinPACE*.

Sensitivity Analyses

After excluding pre-T2D participants or cancer cases, or reclassifying participants who did not report having diabetes at baseline or follow-up 2 according to glucose concentration, both cross-sectional and longitudinal associations of epigenetic age with prevalent and incident T2D slightly attenuated, but were generally consistent with the results from main analyses (Tables 1 and 2, Figure 3). For example, after reclassifying participants who reported T2D-free but had high glucose concentration as T2D cases, the associations between *DunedinPACE* and incident T2D at follow-up 2 attenuated from RR = 1.22 to RR = 1.17, 95%CI: 1.10–1.55.

When pre-T2D participants were evaluated separately, epigenetic age showed no association with pre-T2D at baseline or follow-up 2 (eg, *DunedinPACE*: baseline: RR = 1.00, 95%CI: 0.97–1.04; follow-up 2: RR = 0.96, 95%CI: 0.90–1.02, Supplementary Table 4).

The associations with prevalent and incident T2D were stronger for WBC-adjusted epigenetic aging markers, Supplementary Table 5. For example, in Model 2, the associations with prevalent T2D increased to 1.22 (95%CI: 1.07–1.39) for *DNAmFitAge*, 1.45 (95%CI: 1.23–1.72) for *bAge*, and 1.27 (95%CI: 1.09–1.48) for *DunedinPACE*; the prospective associations with incident T2D at follow-up 2 were 1.26 (95%CI: 1.07–1.49) for *bAge*, and 1.31 (95%CI: 1.16–1.49) for *DunedinPACE*.

Discussion

This study examined the associations of 5 epigenetic markers of aging with prevalent and incident T2D. Epigenetic age at baseline was positively associated with prevalent T2D, with the strongest associations observed for *bAge* and *DunedinPACE*. Associations with incident T2D were also positive but weaker for *DNAmFitAge*, *bAge*, and *DunedinPACE*, and null for *PCGrimAge* and *PCPhenoAge*. All results were robust to sensitivity analyses: excluding participants who were selected for their incident cancer, reclassifying participants who

Table 1. Risk Ratios for the Association Between Epigenetic Age and Prevalent T2D at Baseline

Models		T2D status		*PCPhenoAge*			*PCGrimAge*			*DNAmFitAge*			*bAge*			*DunedinPACE*		
			N	RR	95% CI	*p*	RR	95% CI	*p*	RR	95% CI	*p*	RR	95% CI	*p*	RR	95% CI	*p*
Main analysis (*N* = 5 403)	Model 0*	No T2D	5 223	Ref.														
		T2D	180	1.24	1.09, 1.42	**.001**	1.28	1.11, 1.46	**4×10^{-4}**	1.34	1.21, 1.50	**1×10^{-7}**	1.41	1.24, 1.60	**8×10^{-8}**	1.46	1.28, 1.66	**2×10^{-8}**
	Model 1†	No T2D	5 223	Ref.														
		T2D	180	1.20	1.06, 1.37	**.005**	1.20	1.04, 1.38	**.013**	1.23	1.09, 1.38	**8×10^{-4}**	1.31	1.14, 1.50	**1×10^{-4}**	1.38	1.19, 1.59	**2×10^{-5}**
	Model 2‡	No T2D	5 223	Ref.														
		T2D	180	1.11	0.98, 1.26	.11	1.21	1.03, 1.43	**.024**	1.17	1.04, 1.33	**.011**	1.33	1.12, 1.57	**.001**	1.26	1.08, 1.47	**.004**
Excluding incident cancer cases (*N* = 2 654)	Model 1	No T2D	2 567	Ref.														
		T2D	87	1.18	0.99, 1.41	.07	1.17	0.96, 1.43	.13	1.24	1.05, 1.46	**.010**	1.34	1.11, 1.63	**.002**	1.39	1.17, 1.67	**3×10^{-4}**
	Model 2	No T2D	2 567	Ref.														
		T2D	87	1.11	0.93, 1.32	.24	1.20	0.97, 1.49	.09	1.21	1.03, 1.43	**.022**	1.40	1.13, 1.74	**.002**	1.31	1.08, 1.60	**.007**
T2D reclassified by glucose levels (*N* = 5 396)	Model 1	No T2D	5 103	Ref.														
		T2D	293	1.11	1.00, 1.22	**.045**	1.14	1.02, 1.27	**.02**	1.18	1.07, 1.30	**7×10^{-4}**	1.26	1.14, 1.40	**1×10^{-5}**	1.29	1.15, 1.44	**1×10^{-5}**
	Model 2	No T2D	5 103	Ref.														
		T2D	293	1.04	0.94, 1.14	.48	1.10	0.97, 1.25	.13	1.11	1.01, 1.23	**.028**	1.23	1.08, 1.39	**.001**	1.16	1.03, 1.31	**.016**

Notes:
*Model 0 was the unadjusted model.
†Model 1 adjusted for age, sex, and country of birth.
‡Model 2 additionally adjusted for education level, SES, AHEI-2010, smoking status, smoking pack-years, physical activity, alcohol consumption (spline), BMI (spline), waist circumference (spline), and height.
§All epigenetic aging measures were age-adjusted and standardized to a mean of 0 and standard deviation of 1.
Abberviations: T2D: type 2 diabetes
RR: risk ratio
CI: confidence interval

Table 2. Risk Ratios for the Associations of Baseline Epigenetic Age With Incident T2D at Follow-ups

	Models		T2D status	*N*	*PCPhenoAge*			*PCGrimAge*			*DNAmFitAge*			*bAge*			*DunedinPACE*		
					RR	95% CI	*p*	RR	95% CI	*p*	RR	95% CI	*p*	RR	95% CI	*p*	RR	95% CI	*p*
Follow-up 1	Main analysis (*N* = 4 460)	Model 0*	No T2D	4 326	Ref.														
			T2D	134	1.13	0.96, 1.33	0.13	1.10	0.94, 1.30	.24	1.26	1.08, 1.47	**.003**	1.30	1.13, 1.50	**3×10^{-4}**	1.32	1.11, 1.57	**.002**
		Model 1†	No T2D	4 326	Ref.														
			T2D	134	1.12	0.95, 1.31	0.17	1.10	0.93, 1.30	.27	1.18	1.00, 1.39	**.05**	1.28	1.10, 1.50	**.002**	1.29	1.07, 1.55	**.008**
		Model 2‡	No T2D	4 326	Ref.														
			T2D	134	1.04	0.90, 1.21	0.61	1.04	0.86, 1.26	.68	1.09	0.92, 1.28	.31	1.21	1.00, 1.46	**.05**	1.06	0.87, 1.28	.57
Follow-up 2	Main analysis (*N* = 2 730)	Model 0	No T2D	2 486	Ref.														
			T2D	244	1.04	0.90, 1.19	0.61	1.05	0.91, 1.21	.49	1.20	1.06, 1.36	**.005**	1.18	1.03, 1.35	**.02**	1.38	1.22, 1.56	**2×10^{-7}**
		Model 1	No T2D	2 486	Ref.														
			T2D	244	1.02	0.89, 1.17	0.74	1.04	0.90, 1.20	.63	1.15	1.01, 1.31	**.04**	1.16	1.01, 1.33	**.04**	1.36	1.20, 1.54	**1×10^{-6}**
		Model 2	No T2D	2 486	Ref.														
			T2D	244	0.97	0.85, 1.10	0.62	0.98	0.84, 1.14	.81	1.09	0.96, 1.24	.19	1.09	0.92, 1.28	.32	1.22	1.07, 1.38	**.003**
	Excluding incident cancer cases (*N* = 1 471)	Model 1	No T2D	1 348	Ref.														
			T2D	123	1.08	0.89, 1.30	0.43	1.04	0.84, 1.29	.70	1.12	0.92, 1.36	.25	1.19	0.98, 1.46	.08	1.48	1.24, 1.76	**1×10^{-5}**
		Model 2	No T2D	1 348	Ref.														
			T2D	123	1.01	0.84, 1.20	0.95	0.98	0.79, 1.22	.86	1.06	0.89, 1.27	.52	1.13	0.91, 1.40	.27	1.31	1.10, 1.55	**.003**
	T2D reclassified by glucose levels (*N* = 2 619)	Model 1	No T2D	2 318	Ref.														
			T2D	301	1.03	0.92, 1.16	0.63	1.08	0.95, 1.22	.22	1.13	1.01, 1.27	**.04**	1.19	1.05, 1.34	**.01**	1.32	1.18, 1.47	**8×10^{-7}**
		Model 2	No T2D	2 318	Ref.														
			T2D	301	0.98	0.87, 1.10	0.71	1.00	0.88, 1.14	.97	1.06	0.94, 1.19	.36	1.09	0.95, 1.25	.23	1.17	1.04, 1.31	**.01**
All incident cases who attended both waves (*N* = 2 579)		Model 0	No T2D	2 339	Ref.														
			T2D	240	1.05	0.92, 1.21	0.46	1.05	0.91, 1.21	.49	1.21	1.06, 1.37	**.005**	1.19	1.03, 1.36	**.02**	1.38	1.22, 1.58	**8×10^{-7}**
		Model 1	No T2D	2 339	Ref.														
			T2D	240	1.04	0.90, 1.19	0.61	1.02	0.89, 1.18	.75	1.16	1.01, 1.32	**.03**	1.16	1.00, 1.34	**.05**	1.36	1.19, 1.56	**6×10^{-6}**
		Model 2	No T2D	2 339	Ref.														
			T2D	240	0.98	0.86, 1.11	0.74	0.98	0.84, 1.15	.84	1.09	0.95, 1.25	.21	1.10	0.94, 1.29	.25	1.21	1.06, 1.39	**.004**

Notes:
*Model 0 were the univariate models.
†Model 1 adjusted for age, sex, and country of birth.
‡Model 2 additionally adjusted for education level, SES, AHEI-2010, smoking status, smoking pack-years, alcohol consumption (spline), physical activity, BMI (spline), waist circumference (spline), and height.
§All epigenetic aging measures were age-adjusted and standardized to a mean of 0 and standard deviation of 1.
T2D: type 2 diabetes
RR: risk ratio
CI: confidence interval

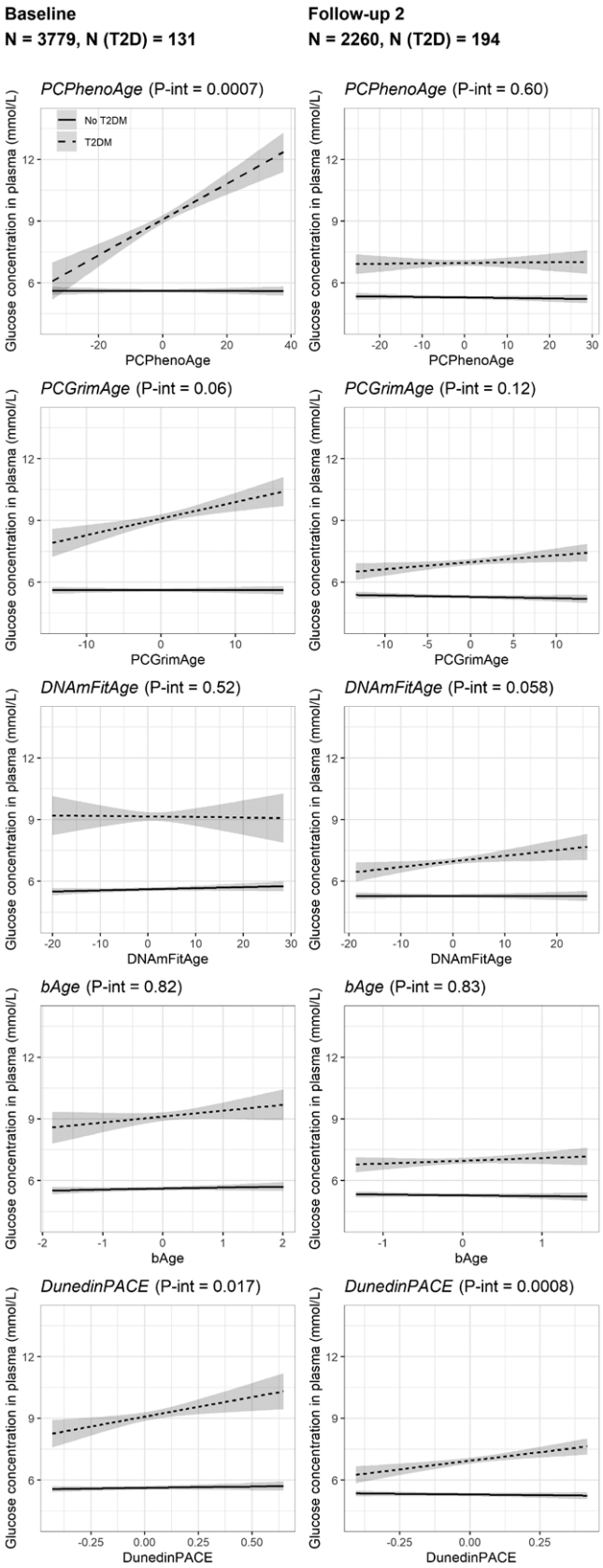

Figure 2. Interactions between epigenetic age and prevalent T2D in their association with fasting glucose concentration. Lines represent the predicted fasting glucose in relation to epigenetic age in participants with and without T2D at baseline and follow-up 2. *Baseline Model 2 included an interaction term between epigenetic age and prevalent T2D (at baseline), and adjusted for age, sex, country of birth, education level, SES, AHEI-2010, smoking status, smoking pack-years, alcohol consumption (spline), physical activity, BMI (spline), waist circumference (spline), and height. **Follow-up 2 Model 3 included an interaction term between epigenetic age and prevalent T2D (at follow-up 2), and adjusted for age, sex, country of birth, time at glucose test, time from last fluid or food, education level, SES, AHEI-2010, smoking status, alcohol consumption (spline), physical activity, BMI (spline), waist circumference (spline), and height. ***P-int refers to the *p* value for interaction tested using likelihood ratio tests comparing models with and without interaction terms between epigenetic aging and T2D. ****All epigenetic aging measures were age-adjusted and standardized to a mean of 0 and standard deviation of 1.

reported being T2D-free but had abnormal glucose concentrations, and separating the pre-T2D group from the non-T2D group (Supplementary Table 6). *DNAmFitAge*, *bAge*, and *DunedinPACE* were positively associated with higher fasting glucose levels in the cross-sectional analysis, whereas only *DunedinPACE* showed a strong association with fasting glucose longitudinally. For both prevalent and incident T2D, the associations were slightly stronger for epigenetic aging markers adjusted for WBC proportion.

The associations between epigenetic age and fasting glucose were consistent with the epigenetic age/T2D associations and were much stronger in participants with T2D than in their T2D-free counterparts. The near-null associations with glucose levels in non-T2D participants aligned with the observation that pre-T2D participants did not have higher epigenetic age, which might be explained by the fairly narrow range of glucose levels (typically 3.3–7.8 mmol/L) in non-T2D participants, compared with T2D cases (3.9 –27.8 mmol/L) (32).

Our results aligned with our hypotheses: *DunedinPACE* and *bAge* demonstrated the strongest associations with prevalent and incident T2D, and *DNAmFitAge* showed stronger associations with T2D than *PCGrimAge*. We applied thorough adjustment for lifestyle factors, particularly adiposity and smoking (eg, including both BMI and waist circumference modeled using spline terms, and including both smoking status and smoking pack-years). The associations for *DunedinPACE* showed more substantial attenuation after adjustment for lifestyle factors, presumably because it incorporates more obesity- and diabetes-related surrogate markers than other measures (15,17,18,24,25). Similar attenuation was observed for *DNAmFitAge*, which encompasses physiological fitness-related aspects of aging (18). Although *PCPhenoAge* incorporates blood glucose as one of its 10 components (25), it showed no association with blood glucose concentration in our study. This is in line with the original *PhenoAge* study (25), which reported only weak correlation with blood glucose ($r = 0.10$).

Our study adds robust evidence to the inconsistent results of previous studies investigating the effect of epigenetic age on prevalent or incident T2D. A nested case–control study including 264 participants ($N_{cases} = 132$) found that those who did not develop diabetes over 10 years follow-up had younger epigenetic age at baseline than those who developed diabetes (12). Using 1 067 participants (126 prevalent T2D cases) from the Berlin Aging Study II, Vetter et al. found no cross-sectional association between epigenetic age and T2D (14). Hillary et al. used a larger sample from Generation Scotland to explore the cross-sectional and longitudinal associations between epigenetic age and T2D (10). They found that epigenetic age was positively associated with prevalent T2D (mean age ~ 50 years in both discovery and replication cohorts). With 149

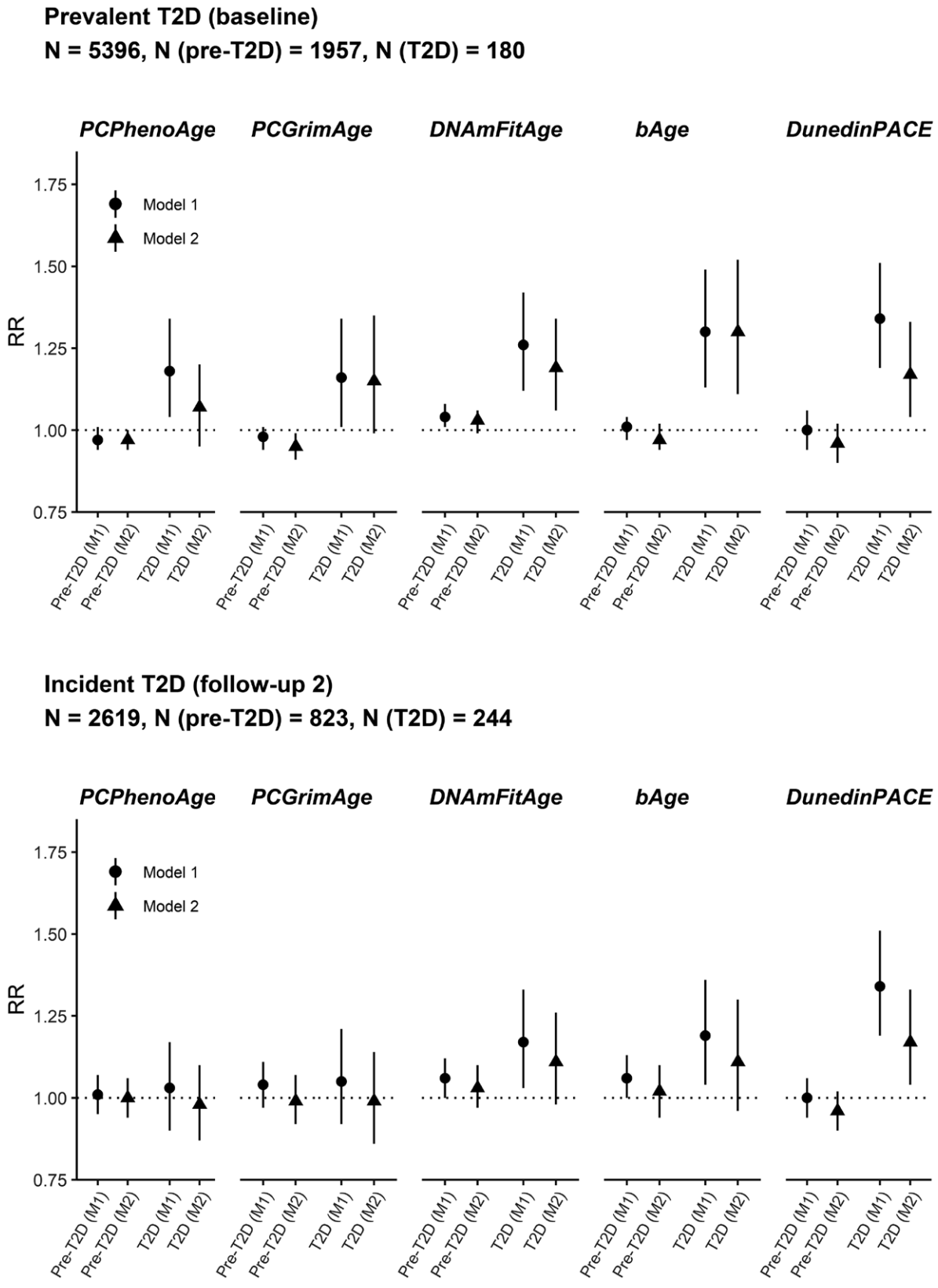

Figure 3. Risk ratios for the associations of epigenetic age with pre-T2D and T2D (in comparison with no T2D). [a]Model 1 adjusted for age, sex, and country of birth. [b]Model 2 additionally adjusted for education level, SES, AHEI-2010, smoking status, smoking pack-years, alcohol consumption (spline), physical activity, BMI (spline), waist circumference (spline), and height. *All epigenetic aging measures were age-adjusted and standardized to a mean of 0 and standard deviation of 1.

incident T2D cases over approximately 13-year follow-up (N = 9 219), the same study reported a strong positive association between epigenetic age and T2D risk, particularly for *GrimAge* and *DunedinPoAm* (10). Conversely, in a cohort of younger participants (mean age ~ 40 years, N_{cases} = 115) from the Coronary Artery Risk Development in Young Adults Study, *GrimAge* was only weakly associated with incident T2D (13). A Swedish study based on 536 participants (122 cases) found that *DunedinPACE* showed higher values close to diabetes onset, but no prospective associations with diabetes risk was observed for the 8 epigenetic aging markers they analyzed (including *PCGrimAge*, *PCPhenAge*, and *DunedinPACE*) (33).

A few cross-sectional studies assessed the association using epigenetic age as the endpoint and found that participants with T2D tend to have elevated epigenetic age. Using the MCCS data, Dugué et al. found that having T2D was associated with elevated *HorvathAge* and *HannumAge* (9). In Kawamura et al., which included 144 participants, a weak association was found for *DNAmFitAge*, but not for *HannumAge*, *HorvathAge*, *GrimAge*, or *PhenoAge* (8). In our study, despite the strong prospective association observed for *DunedinPACE*, prospective associations with other epigenetic aging measures with T2D were quite weak. We also considered the possibility that T2D could cause increased epigenetic age, but had limited DNAm data were available at follow-up 2 (~1 000 participants) to address this hypothesis with sufficient power. The fact that baseline epigenetic age was not associated with prevalent or incident pre-T2D in our data nevertheless suggests that our results were not due to reverse causation. The stronger associations observed for *DunedinPACE* are likely due to the fact that it incorporates changes in obesity- or diabetes-related clinical indicators such as BMI, waist-hip ratio, and glycated hemoglobin A1C (15).

A plausible link exists between T2D and methylation-based biological aging since hallmarks of aging, such as insulin resistance, low-grade inflammation, cellular dysfunction, and metabolic dysfunction (34), contribute significantly to hyperglycemia and the development of T2D (2,35). Epigenetic aging markers capture and summarize some of these features, and therefore, have the potential to contribute to the prediction of T2D. Besides, epigenetic age is modifiable by various lifestyle factors, such as diet and weight management (19), which also play important roles in glycemic control. Our results, therefore, support the importance of maintaining a healthy lifestyle to prevent T2D. Although we could not assess it in our study, it is plausible that the association of epigenetic age with T2D is bidirectional, since developing T2D might in return, accelerate the aging process. Another important finding of our study that corroborates this possibility is the fact that in participants with T2D, those with higher fasting glucose levels tended to have higher epigenetic age. A small longitudinal twin study (N = 314) found that glycemic traits (including fasting glucose, hemoglobin A1c, and triglyceride glucose index) preceded the increase in epigenetic age using cross-lagged models (36). This however does not necessarily indicate that T2D increases epigenetic age since these traits precede T2D onset. Metformin, one of the medications used to treat T2D and lower blood glucose, is considered to have potential for slowing the aging process (37). Due to limited data collected on T2D-related treatment, complications, metabolic biomarkers, and relatively small number of T2D cases, we were unable to thoroughly explore the potential effects of T2D treatment and progression of T2D on epigenetic age. Our results on the associations between epigenetic age and blood glucose might therefore have been confounded by factors such as treatment initiation and success and the health and socioeconomic profiles of participants with uncontrolled T2D. This aspect warrants further exploration in datasets with detailed medical history information. While our study highlights that epigenetic age is a relevant marker for T2D, additional research is required to investigate the temporality of these associations and whether proper T2D management and medication would reduce epigenetic age. The MCCS participants at baseline were overall healthier than the general population (38). We used participants from 7 nested cancer case–control studies of which only 54% of the incident cancer cases (most of them diagnosed after follow-up 2) attended follow-up 2, while this percentage was 66% in the control group. These cancer cases were nevertheless all cancer-free at blood draw and excluding them left the results essentially unchanged for both cross-sectional and longitudinal analyses, likely due to the overall weak association of cancer risk with epigenetic age (39) and diabetes (40). Previous analyses of the MCCS showed that the associations of blood DNA methylation with several health-related factors were very similar in cases and controls (22,41,42). Although the associations for *DNAmFitAge* and *bAge* with T2D appeared slightly weaker in analyses of incident versus prevalent T2D cases, it should be noted that due to attrition in the follow-ups, the RRs at different time points are not strictly comparable. Therefore, our results do not fully describe the trajectory of the effect of epigenetic age on T2D occurrence. In addition, T2D status was self-reported, but when we reclassified those with high glucose levels as being T2D cases, this had negligible influence on the results. The methylation data derived from dried blood spots were found to have somewhat lower reliability than other blood sample types (21), which might have weakened the observed association as this measurement error would likely be nondifferential. Methylation data obtained from dried blood spots are nevertheless considered to be adequate for use in large-scale epigenetic studies (43,44). There is ongoing debate on whether analyses of epigenetic aging measures should adjust for WBC proportion (45–48). A somewhat stronger association of epigenetic aging with breast cancer risk after adjustment for WBC proportions was also observed in Kresovich et al. (49), but minimal differences were observed in a similar study carried out in the MCCS (50). Fiorito et al. pooled data from 18 cohorts and found similar associations for age-adjusted and age- and WBC-adjusted epigenetic age with lifestyle and socioeconomic factors (51). A Mendelian randomization study reported associations of lymphocyte and neutrophil cell counts with epigenetic age (52).

In conclusion, epigenetic age was elevated in middle-aged and older Australians with prevalent T2D and also associated with future risk of T2D. Associations appeared greater for *DunedinPACE* and *bAge* compared with *PCGrimAge* and *PCPhenoAge*. This highlights that epigenetic markers of aging, which incorporate various clinical markers of aging, may contribute to understanding the etiology and risk prediction of T2D. Further studies are warranted on the effect of T2D management on epigenetic age and the temporality of the T2D/epigenetic age association.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None.

Data Availability

Due to ethical constraints related to the consent of participants, we cannot share the full deidentified dataset. For consented participants, the data are publicly available under controlled access at dbGaP (#phs003213.v1.p1, for which more details can be found at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs003213.v1.p1).

Author Contributions

Danmeng Lily Li: Conceptualization, Methodology, Data analysis, Manuscript drafting; Allison M. Hodge: Conceptualization, Methodology, Supervision, Manuscript review; Melissa C. Southey: Funding, Resources, Data curation, Manuscript review; Graham G. Giles: Conceptualization, Funding, Resources, Manuscript review; Pierre-Antoine Dugué: Conceptualization, Methodology, Data analysis, Supervision, Manuscript drafting.

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