Research article

DNA methylation-based biological aging and cancer risk and survival: Pooled analysis of seven prospective studies

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Novelty and Impact: DNA methylation-based biological aging, "age acceleration", was found to be associated with increased mortality in previous studies. We assessed associations between age acceleration and cancer risk and survival using seven case-control studies nested within a large cohort. We found that age acceleration was associated with cancer risk and survival, independently of major health risk factors. The stronger association observed for B-cell lymphoma may reflect the relevance of the tissue used to study age acceleration.

Short title: Epigenetic aging and cancer risk and survival

Keywords: DNA methylation, biological age, aging, age acceleration, epigenetic aging, epigenetic clock, lymphoma, blood, survival, prospective study

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ABSTRACT

The association between aging and cancer is complex. Recent studies have developed measures of biological aging based on DNA methylation and called them 'age acceleration'. We aimed to assess associations of age acceleration with risk of and survival from seven common cancers. Seven case-control studies of DNA methylation and colorectal, gastric, kidney, lung, prostate and urothelial cancer, and B-cell lymphoma, nested in the Melbourne Collaborative Cohort Study were conducted. Cancer cases, vital status and cause of death were ascertained through linkage with cancer and death registries. Conditional logistic regression and Cox models were used to estimate odds ratios (OR) and hazard ratios (HR) and 95% confidence intervals (CI) for associations of five age acceleration measures derived from the Human Methylation 450K Beadchip assay with cancer risk (N=3,216 cases) and survival (N=1,726 deaths), respectively. Epigenetic aging was associated with increased cancer risk, ranging from 4% to 9% per five-year age acceleration for the 5 measures considered. Heterogeneity by study was observed, with stronger associations for risk of kidney cancer and B-cell lymphoma. An associated increased risk of death following cancer diagnosis ranged from 2% to 6% per five-year age acceleration, with no evidence of heterogeneity by cancer site. Cancer risk and mortality were increased by 15 to 30% for the fourth vs. first quartile of age acceleration. DNA methylation-based measures of biological aging are associated with increased cancer risk and shorter cancer survival, independently of major health risk factors.

INTRODUCTION

Biological aging refers to the progressive deterioration of an organism's physiological integrity and function, eventually leading to death [1]. The rate of this process is variable between individuals, and across generations [2]. Previous studies have generally reported that associations of biological age with cancer risk are weaker than those observed with other causes of death (e.g. cardiovascular disease). This has been reported for certain biomarkers of aging [3] and frailty indices [4]. Walking speed, an indicator of biological aging [5], was associated with increased cardiovascular mortality, but no association was found with cancer mortality [6]. Similar observations have been reported for grip strength and standing balance [7]. Consistent with these findings, telomere length, although strongly associated with longevity, has shown associations with cancer risk that were less clear [8, 9], and probably type-specific [10, 11].

DNA methylation varies substantially with age, with a decrease in methylation levels being observed globally [12-14]. These changes may be associated with the risk of disease, including cancer [15-18]. Recent research has defined an "epigenetic clock" based on age-associated methylation changes. Most studies have modelled chronological age using regularised regression methods to obtain a restricted set of predictive DNA methylation measures [19-23]. Two of these models have received more attention in the recent literature as they more accurately predict chronological age [19, 21]. The difference between predicted age and chronological age has been named "age acceleration" and interpreted in recent epidemiological and clinical studies as a marker of biological aging. Several studies have assessed the association between age acceleration and mortality [24-27]. Few studies have examined whether the epigenetic clock is associated with the risk of cancer. In a study based on 43 cases, the risk of lung cancer was increased by an estimated 50% per five-year age acceleration [28]. Another small study (132 cancer cases) concluded that age acceleration was associated with increased cancer risk and shorter cancer survival [29]. A recent study of 480 breast cancer cases and matched controls [30] reported a 4% increase breast cancer risk per one-year age acceleration. In another study, the magnitude of the association of age acceleration with death due to cancer was similar to estimates obtained for other causes of death [25].

We previously found that age acceleration was associated with all-cause mortality independently of major health risk factors [27]. In the present study, we aimed to assess associations of age acceleration with cancer risk and survival. We used data from seven case-

control studies nested in the Melbourne Collaborative Cohort Study (MCCS), including 3,216 incident cases (and matched controls), 1,726 of whom died during follow-up.

MATERIAL AND METHODS

Study sample and blood collection

We used data collected from participants in the MCCS, a prospective study of 41,513 healthy adult volunteers (24,469 women) aged between 27 and 76 years (99.3% aged 40-69) when recruited between 1990 and 1994 [31]. DNA samples were collected from peripheral blood drawn at the time of recruitment (1990-1994) or at the wave 2 follow-up visit (2003-2007). The DNA source was dried blood spots, peripheral blood mononuclear cells or buffy coats for 70%, 28% and 2% of participants, respectively (Supplementary Methods).

Study participants provided informed consent in accordance with the Declaration of Helsinki and the study was approved by Cancer Council Victoria's Human Research Ethics Committee.

Cancer and death ascertainment

A series of case-control studies nested within the MCCS of breast, colorectal, gastric, kidney, lung, mature B-cell lymphoma, prostate and urothelial cancer were conducted [32-35]. Cancer diagnoses were identified by linkage with the Victorian Cancer Registry and the Australian Cancer Database (Australian Institute of Health and Welfare). For each nested case-control study, controls were individually matched to incident cases (diagnosed after blood sample collection) on age using incidence density sampling (i.e. they had to be free of the cancer of interest up to the age at diagnosis of the corresponding case), sex, country of birth (Australia/New-Zealand, Southern Europe, Northern Europe), blood DNA source (dried blood spots, peripheral blood mononuclear cells or buffy coat) and collection period (baseline or wave 2). Controls were also matched to cases on year of birth, except for the colorectal cancer study where controls were matched on year of baseline attendance. For the lung cancer study, controls were also matched on smoking status at the time of blood collection. Vital status to 30 November 2015 was ascertained through record linkage to the Victorian Registry of Births, Deaths and Marriages and the National Death Index. Cause of death was

known for 98.4% of deaths occurring up to 31 December 2013, and classified as "other cause" when missing.

DNA extraction and bisulfite conversion, and DNA methylation data processing

Methods relating to DNA extraction and bisulfite conversion, and DNA methylation data processing have been described previously [32-37] and are detailed in Supplementary Methods.

Methylation age and age acceleration

We used the Hannum and Horvath methods to determine methylation age and age acceleration, as these have consistently been shown to be the most accurate predictors of chronological age [24, 25, 38, 39]. Hannum methylation age was computed using a linear combination of methylation measures at 71 age-associated CpGs [24]. Horvath methylation age uses a set of methylation measures at 353 CpGs and was computed using the online calculator [21]. The CpGs used by the two predictors do not overlap and were all available in our study after quality control. Estimates of cell type composition of blood were obtained using the Houseman algorithm. [40, 41] We considered the following most recent measures of age acceleration [26, 42]: 1) epigenetic age acceleration (AA) based on the residuals from a linear regression of Horvath's estimate of epigenetic age on chronological age, referred to as AA-Horvath, 2) the corresponding measure based on Hannum (AA-Hannum), 3) intrinsic epigenetic age acceleration based on the residuals resulting from a linear regression of Horvath's estimate of epigenetic age on chronological age and measures of blood cell counts, referred to as IEAA-Horvath, 4) the corresponding measure based on Hannum (IEAA-Hannum), 5) enhanced Hannum age acceleration (EEAA), defined as AA-Hannum plus a weighted average of age-associated cell counts [26].

Statistical analysis

Correlations between age acceleration measures were assessed by calculating Spearman correlation coefficients. Missing covariate data (<1% for any individual covariate) were handled using multiple imputation with the R package *mice* [43]. The reliability of the five age acceleration measures was examined using intraclass correlation coefficients, based on 127 technical replicates, following the method described previously [37]. Relative risk estimates

were calculated per five-year increment of age acceleration [24, 25, 38]. Quartiles of age acceleration measures were based on the distribution in controls.

We used conditional logistic regression to estimate odds ratios (ORs) for the associations between age acceleration and the risk of cancer. In Model 1, no covariates were included. In Model 2, we adjusted for smoking (never, former: quit ≤15 years prior, former: quit >15 years prior, current ≤20 cigarettes per day, current >20 cigarettes per day), body-mass index (<25, 25-30,30-35, >35 kg/m^2), height, alcohol drinking (defined by sex-specific thresholds: never, 1-39 g/d [men] and 1-19 g/d [women], 40-59 g/d [men] and 20-39 g/d [women], 60+ g/d [men] and 40+ g/d [women]), the Alternate Healthy Eating Index-2010 (AHEI) to reflect overall diet quality [44], a score of physical activity to reflect overall energy expenditure [45] socioeconomic status (deciles of relative socioeconomic disadvantage of area of residence [46]) and education (score from 1 to 8, 1: primary school only and 8: tertiary university degree or more). These models were used to analyse each cancer site separately, and all seven cancers combined. For the combined analysis, where an individual was diagnosed with several cancers, we included the first diagnosis only (respecting the incidence density sampling procedure), so that participants did not contribute twice to the pooled estimates. Heterogeneity in the associations by study was assessed by comparing models with and without an interaction term between age acceleration measure and case-control study, using a likelihood ratio test. We tested for linear trends in the ORs by attained age, age at blood draw and time since blood draw by including in the models pseudo-continuous versions of these (median value of each category) and their interaction with age acceleration measures.

We used Cox models to estimate hazard ratios (HRs) for the association between age acceleration and the risk of death following cancer diagnosis. This analysis was thus restricted to cancer cases. Time since diagnosis was used as the timescale, and person-years of follow-up were calculated from the diagnosis date until the date of death, and censored at the date of departure from Australia or end of follow-up (30 November 2015 for all-cause death and 31 December 2013 for cause-specific death), whichever came first. The proportional hazards assumption was assessed by visual inspection of Schoenfeld residuals [47]. Separate models were fitted for all-cause mortality, and for death due to cancer or another cause. Covariates included in the analysis were the same as those described above for cancer risk, plus age, sex, country of birth, sample type and plate, the latter fitted as a fixed effect. For the analysis of all cancers combined, cancer site was included as a stratification variable,

thereby allowing the underlying hazard function to vary by cancer site [48]. Where an individual was diagnosed with several cancers, only the first diagnosis was included. Heterogeneity in the HRs by cancer site and tests for trend in the HRs by age at diagnosis, time between blood draw and diagnosis and time since diagnosis were calculated using the same methodology as for the cancer risk analysis.

RESULTS

The correlations of the Horvath and Hannum age predictors with chronological age were ρ=0.73 and ρ=0.78, respectively. The median age acceleration was, in absolute terms, 3.8 years for Horvath and 3.4 years for Hannum. The correlation between AA-Horvath and AA-Hannum was ρ=0.49; the correlation between IEAA-Horvath and IEAA-Hannum was ρ=0.45 (Table 1 and Supplementary Figure 1 and 2). The reliability of age acceleration measures ranged from 0.64 to 0.77 (Table 1). Matching variables and other participant characteristics for each nested case-control study are presented in Supplementary Table 1.

For all measures, ORs for cancer risk were greater than one for most cancer types (Table 2). Some evidence of heterogeneity was observed by study for AA-Hannum and EEAA (P=0.03 and 0.001, respectively). OR estimates were greatest for kidney cancer (AA-Hannum: OR=1.46, 95%CI: 1.10-1.94, Model 2). Higher ORs were also observed for B-cell lymphomas (AA-Horvath: OR=1.16, 95%CI: 1.05-1.27, AA-Hannum: OR=1.22, 95%CI: 1.09-1.38, EEAA: OR=1.27, 95%CI: 1.14-1.41). Results were similar for Model 1 and Model 2, indicating that lifestyle and socioeconomic variables had little confounding effect on the observed associations. ORs for all cancers combined showed risk increases ranging from 4% to 9% per five-year age acceleration (Model 2: IEAA-Horvath: OR=1.04, 95%CI: 1.00-1.09; AA-Hannum: OR=1.09, 95%CI: 1.04-1.14), Table 2. For all five predictors, the risk of cancer was approximately 20% higher for the highest versus lowest quartile of age acceleration (Figure 1). Stronger associations were observed at younger ages, particularly for AA-Hannum (before age 60 years: OR=1.28, 95%CI: 1.11-1.47), but no consistent trend in the HRs was observed, as was the case for age at blood draw and time since blood draw (Table 3).

A total of 1,726 deaths were observed during follow-up (median follow-up time=8.3 years) for individuals diagnosed with cancer (N=3,086). After adjusting for sociodemographic and lifestyle variables, there was no evidence of association for AA-Horvath or IEAA-Horvath (HR=1.02, 95%CI: 0.98-1.05, HR=1.02, 95%CI: 0.99-1.06, respectively), whereas a 4 to 6% increased risk of death per 5 years was observed for Hannum measures (AA-Hannum HR=1.05, 95%CI: 1.01-1.10, IEAA-Hannum HR=1.06, 95%CI: 1.01-1.12, and EEAA HR=1.04, 95%CI: 1.01-1.08), Table 4. Mortality was 10 to 30% higher for the highest versus lowest quartile of age acceleration (Figure 1). A total of 1,580 deaths, diagnosed up to 31 December 2013, were included in the cause-specific mortality analysis, 1,271 deaths being due to cancer and 309 due to another cause (cardiovascular deaths, N=185). The HRs did not appear to vary substantially by cause of death (P-heterogeneity ≥0.08, Table 4). Consistent results were observed for all cancers individually, with no evidence of heterogeneity by cancer site (P>0.38, Supplementary Table 2). HR estimates suggested stronger associations were also observed for cases diagnosed less than five years after blood draw but no trend was observed (Supplementary Table 3).

DISCUSSION

The measures of biological aging we considered, derived from DNA methylation in blood, [49, 50] were associated with increased cancer risk and shorter cancer survival after adjustment for major health risk factors. Although there was some evidence that the association with cancer risk varied by study for Hannum age acceleration, there was no evidence of heterogeneity for other age acceleration measures or for cancer survival. These associations were largely consistent across cancer types. Relative risks were not substantially changed after adjustment for major health risk factors and for blood cell composition (via the 'intrinsic' measures), which suggests that confounding is unlikely to fully explain the observed associations.

As in previous studies [26, 27], we observed consistently stronger associations with both cancer risk and survival for measures derived from the Hannum predictor, which was validated using blood samples. Therefore, although measures derived from the Horvath predictor may be more generalizable because they have been validated across multiple tissue types [21], our findings indicate that a predictor validated on the tissue on which it is used might be preferable. Additionally, the relatively stronger associations we observed with risk of B-cell

lymphoma suggests that the tissue in which age acceleration is measured may be important: while the risk was more strongly increased for measures that were not independent of cell composition (by 16%, 22%, and 27% for AA-Horvath, AA-Hannum and EEAA, respectively), these associations were still apparent when using intrinsic measures (risk increased by 9% and 15% for IEAA-Horvath and IEAA-Hannum, respectively). Only blood samples were available in our study, so we could not compare our measures with age acceleration in other tissues relevant to each cancer type (e.g. prostate tissue to study prostate cancer). The stronger associations observed for kidney cancer were based on a relatively small sample size, so they should be interpreted with caution.

The main strength of our study is its prospective design, particularly for the assessment of associations with cancer risk. All blood samples were obtained from participants prior to cancer diagnosis. Measures of DNA methylation in retrospective studies may reflect molecular changes due to carcinogenesis and treatment. Another strength of our study is that cases and controls were matched on several relevant variables including age, sex and country of birth, with additional adjustment for several potential confounding factors. Further, case-control pairs were placed on a same Beadchip assay, thereby minimizing potential batch effects [51]. Finally, we assessed separately associations with cancer incidence and survival, which was not done by previous studies of cancer mortality. The main limitation of our study relates to the assessment of cancer survival, as blood samples for many cases were collected well before diagnosis. Nevertheless, the assumption that age acceleration does not vary to a great extent in middle-aged and older adults is perhaps not unreasonable, as shown in a previous study [52], and because most dramatic changes to DNA methylation occur in early life through to adolescence [12, 53, 54]. Further, associations with cancer survival appeared to be strongest when restricted to cases diagnosed within five years of blood draw, so that our study might provide conservative estimates of association with overall cancer survival. Finally, we did not formally adjust our results for multiple testing because tests were largely non-independent in our study; however, the vast majority of relative risk estimates were in the direction we had hypothesised, and their magnitude was consistent with prior studies.

While several studies have assessed the association of age acceleration with mortality [24-27, 38], there have been fewer analyses of cancer incidence and cancer survival. Levine et al. studied 43 lung cancer cases diagnosed during twenty years of follow-up [28]. AA-Horvath was significantly associated with risk of lung cancer (P=0.003), but the sample size was very

small. Zheng et al. studied 132 cancer cases (any site) [29] and found that these had accelerated aging compared with the rest of their cohort (HR per one-year AA-Horvath: 1.03, 95% CI: 1.00-1.06). Weak evidence of increased cancer mortality risk was reported (based on 34 deaths: HR=1.05, 95% CI: 0.99–1.12), which is consistent with the findings of our study. A recent and larger-scale European study which included 480 breast cancer cases and matched controls [30], reported an increased risk associated with IEAA-Horvath (OR per one year=1.04, 95%CI: 1.01-1.08). The association appeared to be confined to postmenopausal women, but heterogeneity by menopausal status was not tested. Finally, the study by Perna et al., which included 235 cancer deaths, found that IEAA-Horvath was associated with increased cancer mortality (HR per five years=1.22, 95% CI: 1.03–1.45), but that IEAA-Hannum was not (HR=1.03, 95% CI: 0.80–1.33) [25]. This is in contrast to our study, where we observed stronger associations for IEAA-Hannum for both cancer risk and cancer survival (including cancer-specific and other-cause deaths). While these inconsistencies in findings for different age acceleration measures are difficult to explain, and may be partly due to small sample sizes in previous studies, the balance of evidence suggests that age acceleration is weakly associated with both risk of, and survival from, at least some cancers.

The determinants and biological implications of the epigenetic clock are not well understood. On one hand, studies have shown that early-life exposures [55, 56], stress [57-59], and certain medical conditions such as HIV infection [60, 61] may accelerate the aging of the methylome. We could not control for these in our analysis, so it is not clear whether DNA methylation-based biological age is causally associated with cancer risk and survival (and overall mortality) or if it merely represents unmeasured confounding. Nevertheless, age acceleration measures may capture - in blood - the accumulation over the lifetime of exposures associated with aging and health outcomes, which could be used as an intermediate endpoint. Conversely, previous studies have suggested that strong genetic determinants of epigenetic aging exist, as illustrated by high heritability estimates of 0.43 [52] and 0.65 [62] although these studies could not account for environmental effects shared within families [39]. Observed associations with more strongly genetically determined characteristics, such as age at menopause [62] and obesity [60], or with certain genetic or neurodegenerative conditions [63-67] may also point to a genetic component of age acceleration. Few studies have attempted to assess associations with common genetic variants [68].

CONCLUSION

DNA methylation-based measures of age acceleration are associated with increased cancer risk and shorter cancer survival, after adjusting for major cancer risk factors. These findings add to the evidence regarding the use of methylation markers of biological aging as putative predictors of health outcomes and might help to better understand the relationship between aging and cancer.

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CONFLICTS OF INTEREST

None of the authors has conflicts of interest to declare.

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TABLES AND FIGURES

Table 1. Characteristics of age predictors and age acceleration measures

Age predictor	**Horvath**		**Hannum**	
Predicted age mean (SD) [chronological age, mean (SD) : 59.5 (7.5)]	60.1 (9.6)		62.2 (9.0)	
	Controls	**Cases**	**Controls**	**Cases**
All studies	**59.8**	**60.3**	**61.9**	**62.5**
Colorectal cancer	58.7	59.6	59.8	60.7
Gastric cancer	57.9	57.5	62.2	62.7
Kidney cancer	56.5	57.0	57.6	59.3
Lung cancer	59.8	59.8	61.1	61.5
B-cell lymphoma	59.4	61.0	61.2	62.8
Prostate cancer	59.3	59.5	62.5	62.4
Urothelial cell carcinoma	65.3	65.4	67.2	67.5
Correlation with chronological age				
All studies	**0.73**		**0.78**	
Colorectal cancer	0.73		0.79	
Gastric cancer	0.61		0.66	
Kidney cancer	0.74		0.80	
Lung cancer	0.66		0.75	
B-cell lymphomas	0.69		0.74	
Prostate cancer	0.73		0.80	
Urothelial cell carcinoma	0.75		0.80	
Correlation beween predictors	0.78			
Age acceleration measures	**Horvath**		**Hannum**	
Median age difference AA[a]	3.8		3.4	
Median age difference IEAA[a]	3.5		3.2	
Correlation AA-Horvath – AA-Hannum			0.49	
Correlation IEAA- Horvath – IEAA- Hannum			0.45	
Correlation AA-Horvath – IEAA-Horvath			0.93	
Correlation AA-Hannum – IEAA-Hannum			0.91	
Correlation AA-Hannum – EEAA			0.94	
Correlation IEAA-Hannum – EEAA			0.82	
ICC AA-Horvath	0.74		0.75	
ICC IEAA-Horvath	0.64		0.66	
ICC EEAA			0.77	

[a] Median age difference = absolute value of the measure; age and age acceleration measured in years

Table 2. Age acceleration measures and cancer risk; seven case-control studies nested within the Melbourne Collaborative Cohort Study, N=3,216 cases and matched controls

Outcome	N cases	Age acceleration measure	Model 1: minimally adjusted[a] OR (95 % CI)	P	Model 2: Model 1 + lifestyle factors[b] OR (95 % CI)	P	
Colorectal cancer	835	AA-Horvath	1.08 (0.99-1.17)	0.07	1.07 (0.99-1.17)	0.09	
		AA-Hannum	1.09 (0.99-1.20)	0.09	1.10 (0.99-1.21)	0.06	
		IEAA-Horvath	1.07 (0.98-1.16)	0.15	1.05 (0.96-1.15)	0.25	
		IEAA-Hannum	1.05 (0.95-1.17)	0.33	1.05 (0.94-1.17)	0.37	
		EEAA	1.07 (0.99-1.16)	0.10	1.08 (0.99-1.17)	0.07	
Gastric cancer	170	AA-Horvath	0.95 (0.80-1.12)	0.54	0.96 (0.81-1.15)	0.68	
		AA-Hannum	1.08 (0.88-1.31)	0.46	1.09 (0.88-1.35)	0.41	
		IEAA-Horvath	1.00 (0.84-1.20)	0.99	1.02 (0.85-1.23)	0.82	
		IEAA-Hannum	1.12 (0.90-1.41)	0.32	1.15 (0.90-1.46)	0.26	
		EEAA	1.05 (0.91-1.20)	0.52	1.05 (0.91-1.21)	0.52	
Kidney cancer	143	AA-Horvath	1.08 (0.89-1.32)	0.43	1.10 (0.89-1.37)	0.37	
		AA-Hannum	1.40 (1.10-1.79)	0.01	1.46 (1.10-1.94)	0.01	
		IEAA-Horvath	1.07 (0.87-1.31)	0.52	1.12 (0.90-1.40)	0.32	
		IEAA-Hannum	1.47 (1.11-1.96)	0.01	1.63 (1.16-2.28)	0.005	
		EEAA	1.32 (1.08-1.61)	0.01	1.35 (1.08-1.70)	0.01	
Lung cancer	332	AA-Horvath	0.99 (0.88-1.12)	0.90	0.99 (0.88-1.12)	0.89	
		AA-Hannum	1.07 (0.92-1.24)	0.37	1.07 (0.92-1.25)	0.39	
		IEAA-Horvath	0.99 (0.88-1.11)	0.83	0.99 (0.88-1.12)	0.86	
		IEAA-Hannum	1.04 (0.89-1.22)	0.59	1.05 (0.89-1.23)	0.56	
		EEAA	1.08 (0.96-1.21)	0.21	1.08 (0.95-1.22)	0.23	
B-cell lymphomas	439	AA-Horvath	1.16 (1.05-1.28)	0.002	1.16 (1.05-1.27)	0.003	
		AA-Hannum	1.23 (1.09-1.38)	<0.001	1.22 (1.09-1.38)	<0.001	
		IEAA-Horvath	1.09 (0.99-1.21)	0.09	1.09 (0.98-1.20)	0.11	
		IEAA-Hannum	1.15 (1.01-1.30)	0.03	1.15 (1.01-1.30)	0.04	
		EEAA	1.27 (1.14-1.40)	<0.001	1.27 (1.14-1.41)	<0.001	
Prostate cancer	869	AA-Horvath	1.03 (0.95-1.11)	0.46	1.03 (0.95-1.11)	0.49	
		AA-Hannum	0.96 (0.87-1.06)	0.45	0.96 (0.87-1.07)	0.48	
		IEAA-Horvath	1.04 (0.96-1.13)	0.38	1.04 (0.96-1.13)	0.37	
		IEAA-Hannum	0.97 (0.87-1.08)	0.55	0.98 (0.88-1.09)	0.68	
		EEAA	0.96 (0.88-1.04)	0.30	0.96 (0.89-1.05)	0.37	
Urothelial cell carcinoma	428	AA-Horvath	1.02 (0.91-1.13)	0.78	1.03 (0.92-1.15)	0.63	
		AA-Hannum	1.07 (0.94-1.21)	0.33	1.06 (0.93-1.22)	0.36	
		IEAA-Horvath	1.03 (0.92-1.15)	0.60	1.03 (0.92-1.16)	0.57	
		IEAA-Hannum	1.07 (0.93-1.22)	0.34	1.06 (0.92-1.22)	0.45	
		EEAA	1.08 (0.98-1.20)	0.13	1.07 (0.96-1.20)	0.20	***P-het***[d]
Pooled	**3,086**[c]	**AA-Horvath**	**1.05 (1.01-1.10)**	**0.01**	**1.05 (1.01-1.10)**	**0.01**	***0.28***
		AA-Hannum	**1.09 (1.03-1.14)**	**<0.001**	**1.09 (1.04-1.14)**	**<0.001**	***0.03***
		IEAA-Horvath	**1.05 (1.00-1.09)**	**0.04**	**1.04 (1.00-1.09)**	**0.05**	***0.94***
		IEAA-Hannum	**1.07 (1.01-1.13)**	**0.01**	**1.07 (1.01-1.12)**	**0.02**	***0.13***
		EEAA	**1.08 (1.04-1.12)**	**<0.001**	**1.08 (1.04-1.13)**	**<0.001**	***0.001***

[a] Model 1 included no covariates. Cases and controls were matched on age, sex, ethnicity, sample type (and smoking for the lung cancer study), and placed consecutively on a same chip of the assay.
[b] Model 2, we added to Model 1 anthropometric measures, lifestyle factors and socioeconomic variables (BMI, smoking, alcohol intake, diet quality, physical activity, socioeconomic status and education)
[c] Only the first cancer diagnosis was considered for the pooled analysis
[d] P-het: p-value for heterogeneity between studies
OR: odds ratio, per 5-year age acceleration increase, CI: confidence interval

Table 3. Age acceleration (per 5-year increment) and cancer risk, by attained age, age at blood draw, and follow-up time, Melbourne Collaborative Cohort Study (N cases=3,086)

		N cases	AA-Horvath OR[a] (95 % CI)	AA-Hannum OR[a] (95 % CI)	IEAA-Horvath OR[a] (95 % CI)	IEAA-Hannum OR[a] (95 % CI)	EEAA OR[a] (95 % CI)
Attained age	<60 years	429	1.09 (0.98-1.21)	1.28 (1.11-1.47)	1.06 (0.95-1.19)	1.22 (1.05-1.43)	1.23 (1.10-1.38)
	60-70	1,143	1.11 (1.04-1.19)	1.10 (1.01-1.19)	1.09 (1.02-1.17)	1.04 (0.95-1.14)	1.08 (1.01-1.16)
	70-80	1,256	0.99 (0.93-1.05)	1.02 (0.95-1.10)	1.00 (0.93-1.06)	1.04 (0.96-1.12)	1.03 (0.97-1.09)
	>80	258	1.09 (0.95-1.25)	1.14 (0.98-1.33)	1.03 (0.89-1.19)	1.08 (0.92-1.27)	1.16 (1.03-1.32)
		P trend	*0.13*	*0.04*	*0.20*	*0.19*	*0.08*
Age at blood draw	<55 years	797	1.09 (1.01-1.18)	1.20 (1.08-1.33)	1.06 (0.97-1.15)	1.15 (1.02-1.28)	1.16 (1.07-1.26)
	55-60	570	1.01 (0.91-1.11)	1.01 (0.90-1.13)	1.00 (0.90-1.11)	1.00 (0.89-1.12)	1.01 (0.92-1.11)
	60-65	743	1.09 (1.01-1.18)	1.08 (0.99-1.19)	1.08 (1.00-1.18)	1.06 (0.96-1.18)	1.09 (1.00-1.17)
	>65	976	1.02 (0.96-1.09)	1.06 (0.98-1.15)	1.02 (0.95-1.09)	1.05 (0.96-1.15)	1.07 (1.00-1.14)
		P trend	*0.37*	*0.11*	*0.67*	*0.35*	*0.18*
Time since blood draw	<5 years	721	1.11 (1.03-1.20)	1.20 (1.08-1.32)	1.11 (1.02-1.20)	1.18 (1.05-1.31)	1.16 (1.07-1.26)
	5-10	911	1.00 (0.93-1.08)	0.98 (0.89-1.07)	0.99 (0.92-1.08)	0.95 (0.86-1.05)	0.98 (0.91-1.06)
	10-15	953	1.08 (1.00-1.15)	1.14 (1.05-1.24)	1.06 (0.98-1.14)	1.13 (1.03-1.24)	1.13 (1.06-1.21)
	>15	501	1.01 (0.91-1.12)	1.03 (0.91-1.15)	0.97 (0.87-1.09)	0.98 (0.86-1.11)	1.05 (0.95-1.15)
		P trend	*0.36*	*0.34*	*0.16*	*0.30*	*0.50*

[a] Model 2, adjusted for BMI, smoking, alcohol intake, diet quality, physical activity, socioeconomic status, education. Cases and controls were matched on age, sex, ethnicity, sample type (and smoking for the lung cancer study), and placed consecutively on a same chip of the assay.
HR: hazard ratio, CI: confidence interval

Table 4. Age acceleration (per 5-year increment) and cancer mortality, overall and by cause of death, Melbourne Collaborative Cohort Study (N cases=3,086; N deaths=1,726 for overall analysis, and N=1,580 for cause-specific analysis)

Mortality outcome	N cases / N deaths	Age acceleration measure	Model 1: minimally adjusted[a]		Model 2: Model 1 + lifestyle factors[b]	
			HR (95 % CI)	p	HR (95 % CI)	p
All-cause	3,086 / 1,726	AA-Horvath	1.02 (0.98-1.05)	0.38	1.02 (0.98-1.05)	0.33
		AA-Hannum	1.05 (1.01-1.10)	0.01	1.05 (1.01-1.10)	0.02
		IEAA-Horvath	1.02 (0.99-1.06)	0.22	1.02 (0.99-1.06)	0.22
		IEAA-Hannum	1.07 (1.02-1.13)	0.003	1.06 (1.01-1.12)	0.01
		EEAA	1.05 (1.01-1.08)	0.01	1.04 (1.01-1.08)	0.02
Cancer	3,086 / 1,271	AA-Horvath	1.02 (0.98-1.06)	0.38	1.02 (0.98-1.06)	0.36
		AA-Hannum	1.05 (1.00-1.10)	0.05	1.05 (1.00-1.10)	0.06
		IEAA-Horvath	1.03 (0.99-1.08)	0.15	1.03 (0.99-1.08)	0.16
		IEAA-Hannum	1.08 (1.03-1.14)	0.004	1.08 (1.02-1.14)	0.01
		EEAA	1.04 (1.00-1.09)	0.04	1.04 (1.00-1.08)	0.05
Other cause	3,086 / 309	AA-Horvath	1.01 (0.93-1.10)	0.82	1.01 (0.93-1.10)	0.87
		AA-Hannum	1.15 (1.05-1.27)	0.004	1.14 (1.03-1.26)	0.01
		IEAA-Horvath	1.00 (0.91-1.09)	0.97	1.00 (0.91-1.09)	0.93
		IEAA-Hannum	1.14 (1.02-1.27)	0.02	1.12 (1.00-1.25)	0.05
		EEAA	1.12 (1.03-1.22)	0.005	1.11 (1.02-1.20)	0.02

[a] Model 1 (minimally adjusted) was adjusted for sex, ethnicity, sample type and batch effects

[b] Model 2, we added to Model 1 anthropometric measures, lifestyle factors and socioeconomic variables (BMI, smoking, alcohol intake, diet quality, physical activity, socioeconomic status and education)

In Model 2, p-values for heterogeneity by cause of death (data duplication method) were: AA-Horvath: P=0.77, AA-Hannum: P=0.08, IEAA-Horvath: P=0.92, IEAA-Hannum: P=0.30, EEAA: P=0.40

HR: hazard ratio, CI: confidence interval

Figure 1: Quartiles of age acceleration measures and cancer risk and all-cause mortality following cancer diagnosis

Legend:

A) Association of age acceleration with cancer risk (Model 2)

B) Association of age acceleration with mortality after cancer (Model 2)

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