Epigenetic biomarkers of ageing are predictive of mortality risk in a longitudinal clinical cohort of individuals diagnosed with oropharyngeal cancer

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Abstract

Background: Epigenetic clocks are biomarkers of ageing derived from DNA methylation levels at a subset of CpG sites. The difference between age predicted by these clocks and chronological age, termed “epigenetic age acceleration”, has been shown to predict age-related disease and mortality. We aimed to assess the prognostic value of epigenetic age acceleration and a DNA methylation-based mortality risk score with all-cause mortality in a prospective clinical cohort of individuals with head and neck cancer: Head and Neck 5000. We investigated two markers of intrinsic epigenetic age acceleration (IEAAHorvath and IEAAHannum), one marker of extrinsic epigenetic age acceleration (EEAA), one optimised to predict physiological dysregulation (AgeAccelPheno), one optimised to predict lifespan (AgeAccelGrim) and a DNA methylation-based predictor of mortality (ZhangScore). Cox regression models were first used to estimate adjusted hazard ratios (HR) and 95% confidence intervals (CI) for associations of epigenetic age acceleration with all-cause mortality in people with oropharyngeal cancer (n = 408; 105 deaths). The added prognostic value of epigenetic markers compared to a clinical model including age, sex, TNM stage and HPV status was then evaluated.

Results: IEAAHannum and AgeAccelGrim were associated with mortality risk after adjustment for clinical and lifestyle factors (HRs per standard deviation [SD] increase in age acceleration = 1.30 [95% CI 1.07, 1.57; p = 0.007] and 1.40 [95% CI 1.06, 1.83; p = 0.016], respectively). There was weak evidence that the addition of AgeAccelGrim to the clinical model improved 3-year mortality prediction (area under the receiver operating characteristic curve: 0.80 vs. 0.77; p value for difference = 0.069).

Conclusion: In the setting of a large, clinical cohort of individuals with head and neck cancer, our study demonstrates the potential of epigenetic markers of ageing to enhance survival prediction in people with oropharyngeal cancer, beyond established prognostic factors. Our findings have potential uses in both clinical and non-clinical contexts: to aid treatment planning and improve patient stratification.

Keywords: Epigenetic clock, Epigenetic ageing, Oropharyngeal cancer, DNA methylation, Mortality, Prediction

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Background

Oropharyngeal cancer (OPC), which includes cancers of the soft palate, base of tongue, uvula, palatine tonsils and tonsillar pillars [1], is the second most commonly diagnosed head and neck cancer (HNC) in the UK, with an age-standardised incidence rate of 2.9 per 100,000 persons [2]. Risk factors include smoking, alcohol consumption and human papillomavirus (HPV) infection. Estimated 5-year survival rates for people with OPC vary from 35 to 83% [3, 4]. As such, the ability to estimate survival probabilities at the time of diagnosis is important for clinical decision making and enrolment of low-risk individuals into treatment de-escalation trials [5].

HPV positivity, primarily HPV16, is a major determinant of OPC prognosis [6–8]. Compared to people with non-HPV-driven tumours, people with HPV-driven tumours have a 60% reduced risk of death 3-year post-diagnosis [8]. Consequently, HPV status is now included in prognostic models alongside TNM stage and comorbidity [8–11]. One such model has yielded a Harrell’s concordance statistic (C-statistic) of 0.68 (95% confidence interval [CI] 0.65, 0.71) in external validation, indicating good (but not excellent) prediction [12]. The potential for model improvement is currently being explored and the prognostic value of lifestyle factors has been evaluated [13–19]. The prognostic role of epigenetic biomarkers is less well studied.

Epigenetic biomarkers of ageing (“epigenetic clocks”), which are multivariate predictors of biological age based on DNA methylation (DNAm) levels at a subset of CpG sites across the genome, are demonstrating promise in predicting age-related disease and mortality [20–22]. Most studies evaluating the prognostic utility of these epigenetic clocks have been conducted in general (healthy) populations, however [22–24]. There is a paucity of studies focusing on clinical populations. One study used a Cox model to estimate hazard ratios (HRs) for the association between epigenetic age acceleration (EAA), that is the difference between age predicted by the epigenetic clocks and chronological age, and risk of death following cancer diagnosis ($n = 1726$ deaths) [25]. After adjusting for socio-demographic and lifestyle variables, the authors found limited evidence (OR 1.04, 95% CI 1.00–1.09) of an association with EAA based on an epigenetic clock derived from methylation at 353 CpG sites (EEAHorvath) [26]. However, mortality risk was 28% higher (OR 1.28, 95% CI 1.11–1.47) for the highest versus lowest quartile of age acceleration based on an epigenetic clock derived from methylation at 71 CpG sites (EEAHannum) [27].

In this study, we investigated six epigenetic biomarkers in relation to survival in a cohort of individuals with OPC ($n = 408$). We examined associations between both “first generation” epigenetic clocks derived from DNAm levels at CpG sites found to be strongly associated with chronological age, and two more recently derived clocks: one optimised to predict physiological dysregulation and one optimised to predict lifespan. We also examined the association of a DNAm-based mortality risk score with survival.

In stage one of our analyses, we examined the associations of the six epigenetic biomarkers with survival using cox regression models, with and without adjustment for factors known to influence epigenetic ageing. In the second stage, we implemented flexible parametric survival models to investigate the added prognostic value of epigenetic markers compared to a standard clinical model that included age, sex, TNM stage and HPV status.

Methods

Study population

The study population included a subset of individuals with OPC enrolled in the Head and Neck 5000 (H&N5000) study, a prospective, UK-based, clinical cohort study of people with HNC ($n = 5518$) [28, 29]. H&N5000 was approved by the National Research Ethics Committee (South West Frenchay Ethics Committee, 10/H0107/57) on 5th November 2010 and approved by the Research and Development departments of participating NHS Trusts.

Individuals were selected based on pre-treatment clinical coding of OPC and the availability of baseline questionnaire and clinical data-capture information. Where possible, pathology reports of individual cases were subsequently checked to verify tumour site and subtype. Overall, 5474/5518 (99%) data-capture forms were completed, and 3361/5385 (62%) individuals returned all three baseline questionnaires.

Baseline data collection

Consent was wide-ranging, including permission to: collect, store and use biological samples; carry out genetic analyses; collect information from hospital records and through self-reported questionnaires; and obtain mortality data through electronic record linkage [28]. Baseline collection was completed pre-treatment, unless the individual’s diagnosis and treatment were the same procedure (e.g. tonsillectomy), in which case recruitment and baseline procedures were completed within a month of the diagnostic procedure. Blood samples ($n = 4676$, 85%) were sent to the study laboratory (https://www.bristol.ac.uk/population-health-sciences/research/groups/bblabs/) at ambient temperature for processing. They were centrifuged at 3500 rpm for 10 min and the buffy coat layer used for DNA extraction. Additional samples were frozen and stored at −80°C.
Assessment of HPV status
HPV serologic testing for HPV16 (E6, E7, E1, E2, E4 and L1) antibodies was conducted at the German Cancer Research Center (DKFZ) using glutathione S-transferase multiplex assays. HPV16 E6 seropositivity (a marker of HPV-transformed tumour cells [30]) was indicated if HPV16 E6 median fluorescence intensity (MFI) was > 1000 units [31, 32].

DNA methylation profiling
DNA was bisulphite-converted using the Zymo EZ DNA Methylation™ kit (Zymo, Irvine, CA, USA) and genome-wide methylation data were generated using the Infinium MethylationEPIC BeadChip (EPIC array; Illumina, USA). Raw data files were pre-processed using the R package meffil (https://github.com/perishky/meffil/) [33]. Overall, 440/448 samples passed quality control and were normalised (Fig. 1). Further details are provided in the Supplementary Material (Additional file 3).

Estimation of epigenetic age
DNAm data for a subset of CpGs on the EPIC array (n = 27,523) and an annotation file containing data on chronological age, sex and tissue type were uploaded onto the DNAm Age Calculator https://dnamage.genetics.ucla.edu/ (Additional file 3: Supplementary Methods). The following epigenetic ageing measures were obtained: intrinsic epigenetic age acceleration based on Horvath's multi-tissue predictor (IEAA) [26]; intrinsic epigenetic age acceleration based on Hannum's predictor (IEAAHannum) [27]; extrinsic epigenetic age acceleration (EEAA), an enhanced version based on Hannum's method, which up-weights the contribution of blood cell composition [21]; PhenoAge (AgeAccelPheno) [34] and GrimAge (AgeAccelGrim) [35] An overview of the age predictors is provided in Table 1. In each case, age acceleration was defined as the residual obtained from regressing predicted age, as estimated by the epigenetic clock, on chronological age.

Generation of the DNAm-based mortality predictor in H&N5000
The epigenetic predictor for mortality (ZhangScore) was generated using the equation in [36]. Two of the ten CpGs included in the DNAm score were not present in the H&N5000 epigenetic data because methylation was measured using the EPIC array rather than Illumina450K array, on which the score was developed. The score was therefore generated using the remaining 8 CpGs (See Additional file 3: Supplementary Methods).

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**Fig. 1 Flow of participants included in the analysis. OPC, oropharyngeal cancer; QC, quality control**
Study follow-up and survival

Regular vital status updates were received from the NHS Central Register and NHS Digital, notifying on subsequent cancer registrations/deaths among cohort members. Recruitment finished December 2014 and follow-up information on survival status was obtained on 1 September 2018. The median duration of follow-up was 4.3 years (inter-quartile range [IQR] 3.3–5.2).

Covariates

Information on age at diagnosis, sex, weight, height, marital status, highest educational attainment (school education, college or degree-level), annual household income, smoking status (defined as “current”, “former” or “never” user of tobacco) and alcohol intake (units per week) were obtained from baseline questionnaires, which are available on the study website (http://www.headandneck5000.org.uk/). We were unable to include lifetime exposure to tobacco (i.e. pack-years) in the current analysis because categories of drinking form the basis of clinical advice, i.e. they are more clinically relevant, and many governments and public health bodies have sought to promote public guidelines for “low risk” or “sensible” drinking based on cut-offs of intake. In addition, these alcohol exposure variables are consistent with previous publications [17, 38].

Body mass index (BMI) was calculated as: weight (kg)/(height (m))^2. Comorbidity was defined as “none”, “mild”, “moderate” or “severe” based on the extent of functional deterioration, as measured by the ACE-27. Ethnicity was not included because only two individuals reported being non-white.

Sex, diagnosis, stage and comorbidity were recorded on the data-capture form. Diagnosis was coding using the International Classification of Diseases (ICD) version 10 [39]. Clinical staging of the tumour from T (characteristics of the tumour site), N (degree of lymph node involvement) and M (the absence or presence of metastases) were based on the American Head and Neck Society TNM staging of head and neck cancer [40]. Comorbidity was determined using the Adult Comorbidity Evaluation-27 [ACE-27] [41].

Statistical analysis

Stata 15.0 (StataCorp. 2017) was used for all analyses. Firstly, we examined whether EAA measures were associated with survival, after controlling for established HNC

| Table 1 | Overview of various measures of epigenetic age acceleration and mortality risk used in this analysis |
|---------|---------------------------------------------------------|
| **Epigenetic marker** | **Abbreviation** | **CpGs** | **Description** | **References** |
| Intrinsic Epigenetic age acceleration based on Horvath | IEAA | 353 | The residual resulting from regressing DNAm age on chronological age and estimates of major blood immune cells | [26] |
| Intrinsic epigenetic age acceleration based on Hannum | IEAAHannum | 71 | The residual resulting from a univariate model regressing a weighted age estimate (which increases the contribution of 3 cell types known to change with age) on chronological age | [27] |
| Extrinsic age acceleration based on Hannum | EEAA | 71 | The residual resulting from a univariate model regressing PhenoAgeAccel on chronological age, where PhenoAgeAccel is an ageing measure based on a linear combination of chronological age and nine clinical biomarkers | [21] |
| Age acceleration based on PhenoAge | AgeAccelPheno | 513 | The residual resulting from a linear model when regressing PhenoAgeAccel on chronological age, where PhenoAge is an ageing measure based on a linear combination of chronological age and nine clinical biomarkers | [34] |
| Age acceleration based on GrimAge | AgeAccelGrim | 1030 | The residual resulting from a linear model when regressing GrimAge on chronological age, where GrimAge is an ageing measure based on a linear combination of chronological age, sex and DNAm-based surrogate biomarkers for smoking pack-years (DNAm-packyears) and seven plasma protein levels | [35] |
| Mortality risk score based on Zhang | ZhangScore | 8 | A linear combination of LASSO regression coefficient weighted methylation values of the ten CpGs | [36] |

* Naïve CD8+ T cells, exhausted CD8+ T cells, plasmablasts, CD4+ T cells, natural killer cells, monocytes and granulocytes. ** naïve (CD45RA+ CCR7+) cytotoxic T cells, exhausted (CD28-CD45RA-) cytotoxic T cells and plasmablasts
prognostic factors; secondly, we investigated whether these measures provide any additional prognostic information, over and above factors that are considered in routine clinical practice.

**Step 1: examining associations of EAA measures with survival**

Descriptive analyses were performed to explore the distribution of, and correlations between EAA measures. Baseline descriptive data were stratified by survival at 3 years. The univariate association of covariates on all-cause mortality risk was assessed using Kaplan–Meier curves and log-rank tests.

Multivariable Cox proportional hazards models were used to examine associations of EAA measures and the mortality predictor with overall survival, defined as the time in years from study enrolment to date of death from any cause or date of censorship (i.e. the last date of follow-up). Measures were standardised using z-scores to allow comparison of effect estimates. Hazard ratios (HRs) and 95% CIs for all-cause mortality were calculated for each standard deviation (SD) increase in EAA.

For each epigenetic ageing marker, four separate Cox models were run: (1) a minimally adjusted model that controlled for sex; (2) a model that additionally controlled for clinical factors (TNM stage, HPV status, comorbidity and BMI); (3) a model that additionally controlled for socio-demographic and economic factors (education, annual household income, marital status) and (4) a fully adjusted model that additionally controlled for lifestyle behaviours (self-reported smoking and alcohol consumption). Models were selected a priori based on the existing literature linking these covariates with survival [15, 42–46]. As a sensitivity analysis, we used the continuous measure of alcohol intake (units/week) rather than categories of intake in model 4.

For the DNAm-based mortality predictor (Zhang-Score), the same models were run, with the exception that the minimally adjusted model also included age at time of diagnosis, since chronological age was not factored in when generating this score.

The proportional hazards assumption was checked using statistical tests and graphical diagnostics based on the Schoenfeld residuals. Missing covariate values were imputed using the ICE package for multiple chained equations in Stata [47] (Additional file 3: Supplementary Methods). As a further sensitivity analysis, we created a complete case dataset and analysed as above [48]. We chose not to include chronological age as a covariate in the (EAA) primary survival models because, by definition, age acceleration residuals from a DNAm age predictor should be zero (i.e. not correlated with chronological age). However, since chronological age is positively correlated with mortality, we re-ran the cox models adjusting for chronological age (imputed and complete case).

**Step 2: assessing the prognostic value of EAA measures**

Evidence of an association with survival is not enough to include novel biomarkers in prediction models; to aid clinicians they must provide added prognostic value to existing models [49]. We explored whether the addition of EAA measures to existing models based on established mortality risk factors (i.e. those currently considered in clinical decision making), improved model performance.

Flexible parametric survival models were fitted using the methods of Royston and Parmar [50, 51] (Additional file 3: Supplementary Methods). Models were fitted using maximum likelihood estimation via the “stpm2” command. Nonlinear relationships with continuous predictors were considered using the multivariable fractional polynomial (MFP) algorithm [52] and implemented in Stata using the “mfp” command.

The following models were fit: (1) a “clinical model”, which comprised age, sex, TNM stage, HPV status and comorbidity; (2) clinical + IEAA; (3) clinical + EEA; (4) clinical + IEAAHannum; (5) clinical + AgeAccelGrim; (6) clinical + AgeAccelPheno; (7) clinical + ZhangScore.

Models were fit in a sub-sample of participants with data available for the clinical covariates included in the model (age, sex, tumour stage, comorbidity and HPV status).

The performance measures examined were the Akaike Information Criterion (AIC) and the C-statistic, an extension of the area under the receiver operating curve (AUC) to survival analysis [53, 54]. ROC curves and AUC functions were also calculated to characterise how well the models distinguished between people who were and were not alive at 3 years. Internal validation was performed using 500 bootstrap samples to adjust performance for optimism and calculate a shrinkage factor to be applied to model regression coefficients. Where there was evidence of model improvement with addition of the epigenetic markers, assessed based on the C-statistic, we also examined the complementary role of these markers in the prediction of mortality through inclusion in the same model.

**Results**

In total, 408 out of 1896 participants with pathologically confirmed OPC had epigenetic data available (Fig. 1). There were 105 deaths during follow-up (median = 5.3 years, IQR 4.9–6.0). The proportion of missing data is presented in Additional file 1: Table S1.

**Baseline descriptives**

Participants who were alive at 3 years had a mean age of 57.4 years at diagnosis (SD = 8.9) compared to 62.9 years...
The results of the sensitivity analysis, where we included chronological age as a covariate in the epigenetic age models, are presented in Additional file 1: Table S3 (imputed) and Table S4 (complete case). On adjusting for age, the associations of AgeAccelGrim and IEAAHannum with survival remained in the imputed analysis (fully adjusted HRs 1.50 [1.14, 1.97; \( p = 0.004 \)] and 1.22 [1.00, 1.49; \( p = 0.052 \)], respectively). The strength of the evidence associating these measures with survival was reduced in the complete case analysis, although effect estimates were similar (fully adjusted HRs 1.42 [0.94, 2.14; \( p = 0.095 \)] and 1.23 [0.88, 1.72; \( p = 0.234 \)], respectively)."
Table 2 Baseline characteristics of the study sample stratified by 3-year mortality status (\(n=408\))

| Characteristic                  | Overall \((n=408)\) | Dead at 3 years \((n=77)\) | Alive at 3 years \((n=331)\) | \(p\) value |
|---------------------------------|---------------------|-----------------------------|-------------------------------|-------------|
|                                | \(N\)   & \(\%\) | \(N\)   & \(\%\) | \(N\)   & \(\%\) |              |
| Sex                             |         |                  |                               |             |
| Male                            | 317     & 77.70       | 60    & 77.90       | 257   & 77.60       |             |
| Female                          | 91      & 22.30       | 17    & 22.10        | 74    & 22.40        | 0.958       |
| TNM stage group                 |         |                  |                               |             |
| I                               | 17      & 4.20        | 1     & 1.30         | 16    & 4.80         |             |
| II                              | 39      & 9.60        | 4     & 5.20         | 35    & 10.60        |             |
| III                             | 58      & 14.20       | 14    & 18.20       | 44    & 13.30        |             |
| IV                              | 294     & 72.10       | 58    & 75.30       | 236   & 71.30        | 0.175       |
| HPV status                      |         |                  |                               |             |
| Negative                        | 122     & 29.90       | 45    & 58.40       | 77    & 23.30        |             |
| Positive                        | 286     & 70.10       | 32    & 41.60       | 254   & 76.70        | <0.001      |
| Comorbidity status*             |         |                  |                               |             |
| None                            | 211     & 52.10       | 26    & 34.20       | 185   & 56.20        |             |
| Mild                            | 119     & 29.40       | 27    & 35.50       | 92    & 28.00        |             |
| Moderate/severe                 | 75      & 18.50       | 23    & 30.30       | 52    & 15.80        | 0.001       |
| Smoking                         |         |                  |                               |             |
| Never                           | 110     & 28.10       | 8     & 11.00       | 102   & 32.00        |             |
| Former                          | 205     & 52.30       | 40    & 54.80       | 165   & 51.70        |             |
| Current                         | 77      & 19.60       | 25    & 34.20       | 52    & 16.30        | <0.001      |
| Alcohol                         |         |                  |                               |             |
| Non-drinker                     | 104     & 26.00       | 14    & 18.90       | 90    & 27.60        |             |
| Moderate                        | 90      & 22.50       | 11    & 14.90       | 79    & 24.20        |             |
| Hazardous/harmful               | 206     & 51.50       | 49    & 66.20       | 157   & 48.20        | 0.019       |
| Education                       |         |                  |                               |             |
| School education                | 170     & 43.70       | 37    & 50.00       | 133   & 42.20        |             |
| College                         | 158     & 40.60       | 28    & 37.80       | 130   & 41.30        |             |
| Degree                          | 61      & 15.70       | 9     & 12.20       | 52    & 16.50        | 0.422       |
| Annual household income         |         |                  |                               |             |
| £18,000–£34,999                 | 138     & 38.70       | 36    & 56.30       | 102   & 34.80        |             |
| £35,000–£74,999                 | 103     & 28.90       | 13    & 20.30       | 90    & 30.70        |             |
| < £18,000                      | 116     & 32.50       | 15    & 23.40       | 101   & 34.50        | 0.006       |
| Marital status                  |         |                  |                               |             |
| Single (never married)          | 47      & 11.70       | 11    & 14.70       | 36    & 11.00        |             |
| Currently in relationship       | 280     & 69.70       | 38    & 50.70       | 242   & 74.00        |             |
| No longer with spouse           | 75      & 18.70       | 26    & 34.00       | 49    & 15.00        | <0.001      |

| Characteristic                  | Overall \((n=408)\) | Dead at 3 years \((n=77)\) | Alive at 3 years \((n=331)\) | \(p\) value |
|---------------------------------|---------------------|-----------------------------|-------------------------------|-------------|
|                                | \(N\)   & \(\%\) | \(N\)   & \(\%\) | \(N\)   & \(\%\) |              |
| Age at baseline                 | 403     & 58.4 (9.6)  | 77    & 62.86 (11.25) | 326   & 57.39 (8.91)  | <0.001      |
| Body mass index                 | 272     & 26.4 (4.9)   | 46    & 24.33 (4.76) | 226   & 26.68 (4.87) | 0.001       |
| EEAA                            | −0.03 (5.78)      | 77    & 1.68 (6.52)   | 331   & −0.42 (5.53)  | 0.004       |
| IEAA                            | −0.07 (4.37)      | 77    & 0.36 (4.34)   | 331   & −0.17 (4.38)  | 0.333       |
| IEAA Hannum                     | −0.01 (3.94)      | 77    & 1.10 (4.52)   | 331   & −0.27 (3.76)  | 0.006       |
| AgeAccelGrim                    | −0.10 (5.61)      | 77    & 3.16 (5.37)   | 331   & −0.86 (5.40)  | <0.001      |
| AgeAccelPheno                   | −0.12 (6.57)      | 77    & 2.00 (7.04)   | 331   & −0.62 (6.36)  | 0.002       |
| ZhangScore                      | −2.20 (0.28)      | 77    & −2.15 (0.29)  | 331   & −2.21 (0.27)  | 0.096       |

EEAA, extrinsic epigenetic age acceleration; IEAA, intrinsic epigenetic age acceleration; TNM, Tumour, Node, Metastasis. \(p\) value for difference based on the chi-squared test (categorical) and one-way ANOVA (continuous). * Based on the Adult Comorbidity Evaluation-27 (ACE-27). **For the epigenetic clock measures (IEAA, IEAA Hannum, EEAA, AgeAccelPheno and AgeAccelGrim), mean values represent the difference in chronological age and age predicted by the clock, e.g. a mean value of 1.68 indicates that, on average, people who had died at 3 years were predicted to be 1.68 years older than their chronological age at baseline based on
model, each SD unit increase in AgeAccelGrim was associated with a 1.5-fold increased risk of death at 3 years (optimism-adjusted HR: 1.54, 95% CI 1.2, 1.92; $p \leq 0.001$).

Smoking has been shown to be independently predictive of mortality in H&N5000 [17]. The reduced effect estimate observed between AgeAccelGrim and mortality with adjustment for smoking status suggests that the enhanced prognostic ability gained from adding AgeAccelGrim to the clinical model could be due to the inclusion of a smoking predictor [35]. We conducted an additional sensitivity analysis (Additional file 2: Fig. S1) whereby we compared the prognostic ability of the following models:

1. clinical + AgeAccelGrim;
2. clinical + self-reported smoking;
3. clinical + DNAmpackyears, the DNAm-based surrogate biomarker for pack-years of smoking used to derive GrimAge ($n = 384$ participants with smoking data available; no. deaths = 72).

At 3 years, there was a suggestion that the clinical + AgeAccelGrim model had better discrimination (AUC value of 0.80 [95% 95% CI 0.74, 0.85]) than the clinical models including both

Table 2 (continued)

their epigenome. A mean age value of $-0.42$ indicates that people who were still alive at 3 years were predicted to be, on average, 0.42 years younger than their chronological age. The mortality risk score (ZhangScore), values represent methylation values (rather than years).

**Fig. 2** Pairwise correlations between measures of epigenetic age acceleration and the mortality risk score. EEAA, extrinsic epigenetic age acceleration; IEAA, intrinsic epigenetic age acceleration.

**Fig. 3** Association of epigenetic age acceleration measures with mortality risk ($n = 408$). EEAA, extrinsic epigenetic age acceleration; IEAA, intrinsic epigenetic age acceleration. Minimally adjusted model included sex (and age for ZhangScores); fully adjusted model included tumour stage, HPV status, comorbidity, BMI, education, income, marital status, smoking status and alcohol consumption.
Table 3 Measures of model performance for survival prediction

| Model                        | AIC     | C-statistic (95% CI) |
|------------------------------|---------|----------------------|
| Clinical                     | 486.93  | 0.75 (0.70, 0.80)    |
| Clinical + EEAA              | 483.36  | 0.76 (0.71, 0.81)    |
| Clinical + IEAA              | 488.14  | 0.76 (0.71, 0.81)    |
| Clinical + IEAAHannum        | 480.10  | 0.77 (0.72, 0.82)    |
| Clinical + AgeAccelGrim      | 473.14  | 0.78 (0.73, 0.83)    |
| Clinical + AgeAccelPheno     | 485.52  | 0.76 (0.71, 0.81)    |
| Clinical + ZhangScore        | 488.72  | 0.75 (0.70, 0.80)    |

AgeAccelGrim, age acceleration based on DNAmGrimAge; AgeAccelPheno, age acceleration based on PhenoAge; AIC, Akaike information criterion; C-statistic, Harrell’s concordance statistic; EEAA, extrinsic epigenetic age acceleration; IEAA, intrinsic epigenetic age acceleration; ZhangScore, DNA methylation score based on CpG sites found to be associated with mortality risk; 95% CI, 95% confidence interval.

Discussion

In this study of 408 OPC cases with a median of 5 years of follow-up, we demonstrate that epigenetic markers derived from blood are associated with increased risk of all-cause mortality and these associations are independent of established mortality risk factors. In particular, AgeAccelGrim, an “extrinsic” age acceleration measure which captures exogenous lifestyle factors and extracellular changes related to ageing, had the strongest effect estimate, with each SD increase in EAA resulting in a 40% increase in risk of death in the fully adjusted model (HR 1.40; 95% CI 1.06, 1.83; \( p = 0.016 \)). IEAAHannum, an “intrinsic” measure of EAA, was also associated with mortality risk, but to a lesser extent. The addition of AgeAccelGrim to the clinical model showed marginal improvement in mortality risk prediction at 3 years (Clinical AUC: 0.77, Clinical + AgeAccelGrim AUC: 0.80; \( p = 0.069 \)). Our findings support the literature which suggests that age acceleration as measured by GrimAge is a better predictor of mortality risk in healthy populations compared to first-generation DNAm-based predictors (i.e. Horvath and Hannum’s clocks) [35].

It is unclear why some epigenetic ageing measures can predict mortality risk better than others in this population. The DNAm clocks used to derive these measures reflect different aspects of cellular processes and exogenous factors (i.e. lifestyle factors). Smoking has been shown to be independently predictive of mortality among HNC cases [17], therefore it is possible that the relatively strong association of AgeAccelGrim with mortality risk may be explained by the inclusion of the surrogate measure for smoking in the GrimAge biomarker. When we compared the prognostic performance of the clinical + AgeAccelGrim model with clinical models including both self-reported smoking and the DNAm surrogate biomarker for pack-years of smoking, clinical + AgeAccelGrim had better discrimination. While the difference in model performance was modest, nonetheless suggests that the methylation-based measure of smoking provides a better indicator with less misclassification than self-report. Moreover, the prognostic utility of AgeAccelGrim does not appear to be solely driven by the inclusion of the DNAm-based biomarker for smoking. GrimAge is also trained on a set of proteins known to be associated with mortality [35]. One of these, Plasminogen activator inhibitor 1 (PAI-1), is overexpressed in a variety of tumours and is a strong predictor of poor clinical outcomes [57–59]. Another, growth differentiation factor 15 (GDF15) is involved in the pathogenesis of oral squamous cell carcinoma (OSCC) [60–62]. Further studies are needed to examine whether these factors may be contributing to the prognostic utility of GrimAge.

Hannum and Horvath’s clocks were built using similar regression techniques and show moderate correlation, yet, in our analysis, only IEAAHannum was associated with survival. This finding is consistent with previous work [25]. It is possible that, because the Hannum predictor was developed and validated in blood samples—the tissue type used in our analysis—it may be better able to capture cell-intrinsic processes in blood compared with a predictor that was developed across multiple tissue type, i.e. Horvath’s predictor.

Our investigation has several strengths including the relatively long follow-up period, the fact that individuals were sampled at the time of diagnosis and that DNAm was assayed in the same laboratory. We were also able to...
account for a range of factors which are known to influence both DNAm and HNC risk [63, 64] and missing covariate data were imputed to minimise possible biases [65, 66].

Our study has several limitations. First, the sample size for our analysis was relatively small and we were unable to identify independent prospective datasets to validate our findings. This limits the translation impact of our work. To mitigate this, we obtained estimates of a uniform shrinkage factor and multiplied this by the original β-coefficients from the fitted model to obtain optimism-adjusted coefficients. Second, various unmeasured confounders may influence the outcome of these age predictors, including genetic and environmental factors. While we found that the associations of GrimAge and IEAAHannum persisted after controlling for smoking and alcohol intake in our primary analyses, residual confounding is likely to be present. This is especially likely since we used categories of exposure which were derived via participants’ self-report, which is prone to recall bias and/or misreporting. We conducted sensitivity analyses to evaluate residual confounding by alcohol based on a continuous variable of units/week and found that the effect estimates for AgeAccelGrim were comparable to those of our primary analysis (HR 1.40 [1.05, 1.85]; p = 0.020 in the model that included units of alcohol consumed vs 1.40 [1.06, 1.83]; p = 0.016 in the model that included categories of alcohol consumption). The association of IEAAHannum with mortality remained when alcohol units were used but the HR was lower (HR 1.22 [0.99, 1.50]; p = 0.066 vs 1.30 [1.07, 1.57]; p = 0.007 for the model that included alcohol categories). While we were unable to derive a continuous measure for lifetime smoking, we utilised a DNAm-derived measure of pack-years of smoking in our sensitivity analysis for Grim-Age. We found that the addition of AgeAccelGrim to a clinical model that included age, tumour stage and HPV status had slightly better discrimination (AUC = 0.80) compared to a clinical model that additionally included the DNAm surrogate marker for smoking (AUC = 0.78). Genome-wide DNA methylation (DNAm) profiling has allowed for the development of molecular predictors for a multitude of traits and diseases, including smoking and alcohol intake [60]. Future studies could implement the use of other methylation scores to index these variables [63, 64]. Third, there is a disparity in coverage between Illumina 450 K and EPIC platforms meaning that 17 of the 353 CpGs (4.8%), and 6 of the 71 CpGs (8.5%) necessary to calculate epigenetic age via the Horvath and Hannum methods, respectively, were missing [67]. Similarly, two of the CpGs included in the DNAm risk score for mortality were missing from the DNA methylation dataset for the same reason. Previous work suggests that the lack of the clock-CpGs on the EPIC array does not undermine the utility of the epigenetic age predictors [68]. Fourth, we did not account for multiple testing, although evidence of correlation between some of the epigenetic measures suggests that correction may not have been appropriate. Finally, it was not possible to examine cancer-specific mortality.

Table 4 Estimated coefficients (uncorrected and corrected) for the clinical + AgeAccelGrim model

| Variable          | Original model | Final model after adjustment for overfitting |
|-------------------|----------------|---------------------------------------------|
|                   | β              | 95% CI | β              | 95% CI | β              | 95% CI |
| Age               | 0.05           | 0.02   | 0.07           |       | 0.04           | 0.02   | 0.06   |
| Sex               |                |        |                |       |                |        |        |
| Female            | 0.42           | −0.14  | 0.99           |       | 0.35           | −0.12  | 0.82   |
| Tumour stage      |                |        |                |       |                |        |        |
| II                | 0.64           | −1.56  | 2.85           |       | 0.53           | −1.29  | 2.36   |
| III               | 1.65           | −0.38  | 3.69           |       | 1.37           | −0.32  | 3.06   |
| IV                | 1.85           | −0.14  | 3.84           |       | 1.54           | −0.12  | 3.19   |
| HPV status        |                |        |                |       |                |        |        |
| Positive          | −0.95          | −1.47  | −0.44          |       | −0.79          | −1.22  | −0.36  |
| Comorbidity*      |                |        |                |       |                |        |        |
| Mild              | 0.33           | −0.23  | 0.90           |       | 0.28           | −0.19  | 0.75   |
| Moderate/severe   | 0.24           | −0.38  | 0.85           |       | 0.20           | −0.31  | 0.70   |
| AgeAccelGrim      | 0.52           | 0.26   | 0.78           |       | 0.43           | 0.22   | 0.65   |

Regression coefficients (β) and 95% confidence intervals (CI) for 3-year overall survival

Hazard ratios can be obtained by exponentiating model estimates.
Conclusion

DNAm-based estimators of ageing could provide prognostic utility in people with OPC, above established prognostic factors, though the mechanisms of association are currently unclear. That an accurate, easy-to-measure biomarker could serve as a better predictor of mortality risk is important as it could aid treatment planning and improve patient stratification in study design. These findings should be investigated in further, independent samples.

Abbreviations

AIC: Akaike information criterion; AUC: Area under the receiver operating characteristic curve; CI: Confidence interval; Cpg: Cytosine–phosphorus–guanine site; EAA: Epigenetic age acceleration; HNC: Head and neck cancer; HPV: Human papillomavirus; HR: Hazard ratio; IQR: Inter-quartile range; MAR: Missing at random; OPC: Oropharyngeal cancer; SD: Standard deviation.

Supplementary Information

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Authors’ contributions

RAB, RCR, MM, RMW and AN contributed to the study design. RAB, MM, SMI and RCR contributed to the data analysis. RAB, SMI, MM, AN, RMW, MS, KI, RL, REM, DLW, TW, MP, CR, GDS and RCR all contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used in this analysis are available from the Head and Neck 5000 study upon submission of a research proposal. If you would like to access this resource, please contact the Head and Neck 5000 Executive on head-andneck5000@uhbristol.nhs.uk. The study website http://www.headandneck5000.org.uk/ describes the resource and the types of data available.

Declarations

Competing interests

The authors declare that they have no competing interests.

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