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Epigenetic age acceleration in adolescence associates with BMI, inflammation and risk score for middle age cardiovascular disease

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Background:
'Accelerated ageing', assessed by adult DNA methylation predicts cardiovascular disease (CVD). Adolescent accelerated aging might predict CVD earlier. We investigated whether epigenetic age acceleration (assessed age 17-years) associated with adiposity/CVD-risk measured (ages 17, 20, 22-years), and projected CVD by middle-age.
Methods: DNA methylation measured in peripheral blood provided 2 estimates of epigenetic age acceleration; *intrinsic* (*IEAA*, (preserved across cell types) and *extrinsic* (*EEAA*, dependent on cell admixture and methylation levels within each cell type).
Adiposity was assessed by anthropometry, ultrasound and DEXA (ages 17, 20, 22 years). CVD-risk factors (lipids, HOMA-IR, blood pressure, inflammatory markers) were assessed at age 17-years. CVD development by age 47 years was calculated by Framingham algorithms. Results are presented as regression coefficients/5-year epigenetic age acceleration (*IEAA/EEAA*) for adiposity, CVD-risk factors and CVD development.
Results: In 995 participants (49.6% female, age 17.3+/-0.6 years), *EEAA* (/5-years) was associated with increased BMI of 2.4% (95%CI 1.2-3.6%) and 2.4% (0.8-3.9%) at 17 and 22 years, respectively. *EEAA* was associated with increases of 23% (3-33%) in hsCRP, 10% (4-17%) in interferon-gamma induced protein (IP-10) and 4% (2-6%) in tumour necrosis factor receptor 2 (sTNFR2), adjusted for BMI and HOMA-IR. *EEAA*(/5-years) results in a 4% increase in hard endpoints of CVD by 47 years old and a 3% increase, after adjustment for conventional risk factors.
Conclusions:
Accelerated epigenetic age in adolescence was associated with inflammation, BMI measured 5 years later, and probability of middle-age CVD. Irrespective whether this is cause or effect, assessing epigenetic age might refine disease prediction.

An estimate of biological age determined from peripheral blood DNA from approx. 1,000 adolescents, may refine prediction of cardiovascular risk above and beyond traditional risk factors.

INTRODUCTION

Obesity is a major risk factor for chronic age-related diseases, of which cardiovascular disease (CVD) (1) is a major contributor to global mortality.(2,3) Mortality attributable to obesity occurs via downstream complications, including type 2 diabetes, heart disease(1) and specific cancers(4). Individuals are not equally predisposed to these complications at the same level of obesity or obesogenic environmental exposure. Assigning an accurate risk score for development of such complications may allow individualized treatment and prevention of the complications leading to morbidity and mortality.

Epigenetic age can be calculated using DNA methylation array data from peripheral blood samples to predict chronological age.(5,6) Epigenetic age acceleration represents increased methylation age compared to chronological age, and has been shown to predict all-cause and cause-specific mortality(5,7-10). This suggests that quantifying accelerated epigenetic age might contribute to the prediction of healthy ageing and to personalization of health care.

To date, epigenetic age acceleration has been studied in older individuals.(5,7-10) Variability in DNA methylation age is heavily influenced by environmental factors, with heritability decreasing after birth as individuals are exposed to unique environments in childhood, to reach an estimated 39% by adulthood.(5) We argue that the identification of epigenetic age using DNA methylation markers would allow the early identification of individuals at increased risk of chronic disease, permitting earlier interventions to attenuate the progression of cardiovascular and metabolic disease. It is known that DNA methylation age increases logarithmically in childhood, before slowing to linear dependence in adulthood.(5) Therefore, late adolescence may be an excellent time during which to screen for epigenetic age acceleration to predict future disease risk, when DNA methylation age is stabilizing and to intervene, being a dynamic period during which changes can dramatically change health trajectories.(11) Assigning an accurate biological age to obesity related disease involves a degree of complexity. To address this, we investigated the association between epigenetic age acceleration, purporting to reflect biological age in relation to obesity and comorbidities (fat distribution, insulin resistance, components of the metabolic syndrome, and inflammatory markers) at ages 17, 20 and 22 years. We also investigated the association between epigenetic age acceleration and a Framingham risk predictor for CVD (12).

METHODS

Participants were from the Western Australian Pregnancy Cohort (Raine) Study.(13) Pregnant women (n=2900) were recruited through the public antenatal clinic at King Edward Memorial Hospital and nearby private clinics in Perth, Western Australia between May 1989 and November 1991. Total of 2868 newborns were available for follow-up. The King Edward Memorial Hospital and Princess Margaret Hospital Ethic Committees approved the study protocol. The participant and/or their primary caregiver provided written consent for their participation in the study. A total of 995 at the 17-year-old follow-up were included for analysis. All measurements were performed by research personnel trained according to the study protocols described below.

Anthropometry at 17, 20 and 22 years old

Height was measured using a Stadiometer (Holtain, Crosswell, United Kingdom) to the nearest 0.1 cm. Weight was measured using a Digital Chair Scale (Wedderburn, New South Wales, Australia) to the nearest 100 g. Body Mass Index (BMI) was calculated as weight (kg)/ height (m^2). Waist circumference was measured to the nearest centimeter.

Measurements of adiposity

Adipose tissue thickness at 17 years was measured by ultrasound (Siemens Antares, Mountain View, CA, USA) using validated standardized criteria.(14) Visceral adipose tissue thickness was measured as the distance between the anterior wall of the aorta and the internal face of the rectus abdominis muscle perpendicular to the aorta. Subcutaneous fat thickness was measured as the thickness of the fat tissue between the skin-fat interface and the linea alba. Subcutaneous fat compression was avoided. A single specialist radiologist blinded to the clinical and laboratory characteristics of the subjects interpreted the images.(15) At 20-years-old, dual-energy x-ray absorptiometry (DEXA) was performed. A Norland XR-36 densitometer (Norland Medical Systems, Inc., Fort Atkinson, WI, USA) was used to provide estimates of whole body fat, lean and soft tissue mass (g).(16)

Measures of cardiovascular risk factors

Blood pressure was measured by oscillometric sphygmomanometer (DINAMAP vital signs monitor 8100, DINAMAP XL vital signs monitor or DINAMAP ProCare 100, GE Healthcare) after resting 5 minutes and using the appropriate cuff size. Six readings were taken while the subject was supine, every 2 minutes for 10 minutes. The average value was calculated after excluding the first reading. Venous blood samples were taken after an overnight fast. Serum insulin, glucose, triglycerides, total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), high sensitivity C-reactive protein (hsCRP), leptin and adiponectin were measured in the PathWest Laboratory at Royal Perth Hospital as described.(17) HOMA-IR (molar units) was calculated by insulin (mIU/L) x glucose (mmol/L)/22.5.(18) Plasma IL-18 was quantitated with a commercially available ELISA method (Medical Biological Laboratories, Nagoya, Japan) and plasma IL-18 binding protein (IL18BP primary isoform a) was measured using a DuoSet ELISA development system (R&D Systems, Minneapolis, MN, USA). Plasma concentrations of IP-10, sTNFR1 and sTNFR2 were quantified using cytometric Bead Array (CBA) Flex sets (BD PharMingen, San Diego, CA) on the BD FACSArray™ bioanalyser (BD Biosciences, San Jose, California, USA). Procedures followed the manufacturer's recommendations. Individual cytokine concentrations were determined using FCAP Array software (BD Biosciences).

Environmental Factors

The effect of maternal environmental factors on the biological clock of the offspring was tested. Maternal smoking habits during pregnancy were ascertained from questionnaires answered by the mother at enrolment (16-18 weeks) and 34 weeks gestation (maternal smoking (yes/no)). Maternal stress score evaluated at the same timepoints during pregnancy was assessed using a 11- item questionnaire derived from the Tennant and Andrews (1977) Life Stress Inventory collected at 16-18 and 34 weeks gestation(19). Mothers were asked whether they had experienced any of 11 major life stress events (pregnancy problems, death of a close friend or relative, separation or divorce, marital problems, problems with children, job loss (involuntary), partner's job loss (involuntary), money problems, residential move or another stressful event). At 16-18 weeks gestation mothers were asked if they had experienced any of the events since becoming pregnant and, at 34 weeks gestation, whether any of the events had been experienced in the previous four months. We calculated a total stressful life events index score throughout pregnancy from the sum of listed events, giving equal weight to each.(20)

Maternal pre-pregnancy BMI was calculated as self-reported pre-pregnancy weight (kg) divided by height $(m)^2$. Height was confirmed by standardised procedures at enrolment between 16-20 weeks' gestation, using a Harpenden Stadiometer (Holtain Ltd, Crymych, Wales, UK). Maternal weight was obtained from medical records at enrolment and 34 weeks. Gestational weight gain rate was defined as the average weekly weight gain rate

between the start of pregnancy and 34 weeks of gestation. Duration of breast feeding was obtained retrospectively from 12 month, 2 year and 3 year follow-up questionnaires.(21)

The offspring environmental factors tested for associations included dietary patterns, physical activity and adolescent smoking and alcohol consumption at age 17 years. Habitual dietary intakes were estimated at 17 years of age, by a semi-quantitative 212-item food-frequency questionnaire (FFQ) developed by the Commonwealth Scientific and Industrial Research Organization.(22) The FFQ assessed usual dietary intake over the previous year and collected information on the average frequency of consumption and usual serving sizes. Daily nutrient and food-group intakes were estimated using Australian nutrient-composition tables. The FFQ has been validated against a 3-day food diary in a sub-group of this same cohort.(23) Two dietary patterns, 'Western' and 'Healthy', were identified using factor analysis (PROC FACTOR in SAS) (maximum likelihood method) on intakes of all 38 food groups to identify major dietary patterns.(24) The two major dietary patterns explained 84% of the total variance in food group intakes. Every subject received a score for each dietary pattern measured on the z-scale. Physical activity was assessed using a self-reported questionnaire, based on exercise outside of school hours per week, where exercise was defined as activity causing breathlessness or sweating (≥4 times per week, 1-3 times per week and <once per week).(25) Smoking at 17 years of age was assessed by a confidential online questionnaire (0=never smoked, 1=smoked prior to last 12 months, 2=smoked last 12 months, 3=smoked in last 4 weeks). Alcohol consumption information was obtained from an online questionnaire that asked about the subject's intake during the past week. Alcohol consumption was defined as the average number of standard drinks consumed during the last 7 days where 1 standard drink is 10g of alcohol.

DNA Methylation Profiling

DNA methylation was measured in 1192 (58 technical replicates) individuals from peripheral blood samples from participants at age 17 years using the Illumina HumanMethylation450K BeadChip. Processing of the Illumina Infinium HumanMethylation450 BeadChips was carried out by the Centre for Molecular Medicine and Therapeutics (CMMT) http://www.cmmt.ubc.ca. We used Bioconductor packages *shinyMethyl* (26) and *MethylAid* (27) to perform quality control checks on the samples, and based on several diagnostic plots, three samples were found to be outliers and excluded. The rnb.execute.gender.prediction() function from the Bioconductor *RnBeads* package (28) revealed a single discrepancy between actual and predicted gender and this sample was excluded. Four participants with inconsistent results and identified as outliers (n=3) or gender misclassification (n=1) were removed. Intentional SNP DMCpGs (n=65), sex chromosome DMCpG (n=11,648) and DMCpGs with a detection *p*-value > 0.05 in any sample (n=10,777) were removed. A further 160 probes with bead counts less than 3 in more than 5% of samples were removed. DNA methylation beta-values were normalized using beta-mixture quantile dilation (BMIQ)(29). There was little evidence of a batch effect by bisulfite conversion batch and this was confirmed via permutation tests of association.

A subset of these, 995 participants (50% female, 50% male) had two parents of European descent. Ancestry was determined by self-report from the mother where the ancestry of both mother and father were reported as "Caucasian".

Epigenetic age acceleration calculation

Epigenetic age acceleration was calculated using BMIQ normalized betas using the online epigenetic age calculator(5) (https://dnamage.genetics.ucla.edu/) on the 12th of January 2017. The "Advanced Analysis for Blood Data" and "Normalize Data" options were selected. Two measures of epigenetic age acceleration, intrinsic epigenetic age acceleration (*IEAA*) and

extrinsic epigenetic age acceleration (*EEAA*) were calculated based on the Horvath(5) and Hannum(6) models.

IEAA was calculated from the epigenetic age measure defined by Horvath et al(5) using 353 cytosine phosphate guanine (CpG) sites which correlate with chronological age in sorted cell types, tissues and organs. *IEAA* is derived from the residual resulting from regression of Horvath DNA methylation age on chronological age and estimates of plasma blasts, naïve and exhausted CD8+T cells, NK, monocytes and granulocytes. *IEAA* is designed to capture cell-intrinsic properties of the ageing process that are preserved across cell types and organs.

EEAA was calculated using the predicted epigenetic age based on the 71 CpGs in Hannum et al., 2013(6), generalized by 4 epigenetic inputs. The contribution of immune blood cell types to age estimate is increased by weighting with cytotoxic T cells, exhausted (CD28-CD45RA-) cytotoxic T cells, and plasmablasts using the Klemera Doubal approach (30). The weights were determined by correlation between respective variable and chronological age. *EEAA* was defined as the residual variation resulting from a univariate model regressing the resulting age estimate on chronological age. *EEAA* is driven by both age-related changes in blood cell composition and intrinsic epigenetic changes

Statistical Methods

Associations between Cardio-metabolic Risk Factors and epigenetic age acceleration

Linear regression models were fitted, assessing associations between measures of epigenetic age acceleration (*IEAA* or *EEAA*) with adiposity measures and related cardio-metabolic risk factors adjusted for sex. *IEAA* and *EEAA* are reported per 5 years of age acceleration unless otherwise stated. The adiposity measures included anthropometric measurements (BMI, waist circumference) at 17, 20 and 22 years and more specialized measures of adiposity at a single time-points (skin folds, subcutaneous and visceral fat thickness [17 years]; DEXA measured fat, lean and soft tissue mass [20 years]). Other related classical CVD risk factors at 17 years included SBP, DBP, HOMA-IR, fasting triglycerides, cholesterol, LDL-C, HDL-C, systemic inflammatory markers (hsCRP, IP-10, sTNFR1, sTNFR2, IL-18, free IL-18 and IL-18BP) and adipokines (leptin, adiponectin). All outcome variables were transformed by natural logarithm if not normally distributed. Interactions between sex and epigenetic age acceleration were tested with sex specific results only reported if the interaction was significant $p<0.05$.

The models described thus far will be considered the main models for the study.

For these primary analyses (associations of EEAA/IEAA with either adiposity or cardiometabolic outcomes) a method controlling the false discovery rate (FDR) (31) to account for multiple testing across 54 tests was applied. The q-value threshold was selected to 0.05.

Ascertaining relationship between epigenetic age acceleration and cardiovascular risk prediction scores

Due to limitations in cohort studies for predicting long term cardiovascular endpoints, we have applied a surrogate measure validated to predict CVD events 30 years hence(12). The validated algorithm is based on data collected from the Framingham Offspring cohort when they were aged 20-59. These Framingham prediction scores were calculated in the current cohort based on measured risk factors (sex, age, systolic blood pressure, smoking, treated blood pressure, history of diabetes) and either BMI or lipid level (total cholesterol level) at 17 years old. By each method two Risk Scores (% risk) were calculated to predict either the risk of hard endpoints (coronary death, myocardial infarction, fatal or non-fatal stroke) or full CVD outcomes, which additionally includes coronary insufficiency, angina pectoris, transient ischemic attack, intermittent claudication or congestive heart failure. The four resulting Risk

Scores are designated hard endpoint (using lipid) full CVD (using lipid), hard endpoint (using BMI) and full CVD (using BMI).

The model that used epigenetic age acceleration to predict each of these Risk Scores was derived by linear regression firstly adjusted for age and sex. Then, to understand if epigenetic age acceleration predicted the Risk Score beyond traditional CVD risk factors, we added covariates consisting of classical cardiometabolic risk factors (BMI or waist circumference, HOMA-IR, SBP and triglycerides) selected in stepwise manner. If both BMI and waist circumference were independently associated with the outcome, a single measure was selected in the final model, to avoid high collinearity.

Potential confounder or mediators

There are a multitude of potential confounder and mediators for the relationships tested in this study, including exposures which span the periconceptional, pregnancy and postnatal periods. We have ascertained many of these including socioeconomic status (family income measured at recruitment), measures from early life (maternal stress and smoking during pregnancy, pre-pregnancy BMI, pregnancy weight gain rate, and duration of breast feeding) and environmental factors at 17 years old (including dietary patterns, physical activity and adolescent smoking and weekly alcohol consumption at age 17 years). To select for potential confounders, we considered variables with a significant associations with environmental factors judged by a nominal $p<0.05$, which were taken forward in stepwise fashion to assess their influence on the association between biological age and BMI.

Due to variable missingness of covariates and the likelihood that unaccounted confounding still exists, these results were reported selectively for the major outcome of BMI and were not considered the main models for this analysis.

All analyses were undertaken on R version 3.3.2 and IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY. Significance criteria were set at $\alpha=0.05$. (31)

RESULTS

Characteristics of the population (Table 1)

Of the 1192 participants who had Infinium 450K array data, 995 participants (49.6% female,) had two parents of European descent. Of these participants, at the 17-year-old follow up, 870 (50.3% female) had fasting blood samples and 744 (49.8% female) physical measurements taken. (Supplemental Figure 1(32)) A subset of the 995 participants had clinical data at the 20 (n=757;50.6% females) and 22 (n=618; 50.3% female) year-old follow-ups. In males, BMI was 22.6 (95% CI=22.2 to 23.0), 24.2 (95% CI=23.7 to 24.8) and 25.1 (95% CI=24.6 to 25.5) kg/m^2 at 17, 20 and 22-year-old follow-ups respectively. In females, BMI was 23.1 (95% CI=22.7 to 23.5), 24.2 (95% CI=23.7 to 24.8) and 25.0 (95% CI=24.3 to 25.6) kg/m^2 at 17, 20 and 22-year-old follow-ups respectively. The percentage risk for developing any CVD in 30 years' time was 2.1% (95% CI 2.0-2.2) in males and 1.4% (95% CI=1.3-1.4) in females.

Chronological age ranged from 16.0-19.8 years with [mean (SD)] 17.3 (1.9) years with no difference between males and females. Across both sexes, *IEAA* had a mean (SD) of 0.08 (3.7) years and *EEAA* 0.13 (5.3) years. Males had greater *intrinsic and extrinsic EAA* compared to females.

Interaction terms between sex and each of the measures of DNA methylation biological age were tested and none were significant. Hence for the subsequent analyses, results are reported with males and females combined.

Adiposity Associations with epigenetic age acceleration

The relationships between 17-year-old *IEAA* and *EEAA* and measures of adiposity at three time-points (17, 20 and 22 years old) were investigated. (Supplemental Figure 2 (33), Table

2) *IEAA* was not associated with any measures of adiposity. However, *EEAA* was associated with BMI, waist circumference and total lean mass. Log transformed BMI was associated with increased *EEAA* (per 5-years) at 17 years (β=0.024, p=1.1 x 10-^{4}), 20 years (β=0.023, p=0.001) and 22 years (β=0.023, p=0.002). This is equivalent to *EEAA* (per 5-years) being associated with 2.4% (95% CI 1.2 to 3.6%), 2.3% (95% CI 1.0 to 3.6%), and 2.4% (95% 0.8 to 3.9%) increases in BMI at 17, 20 and 22 years respectively. It was also associated with 1.8% (95% CI 0.7 to 2.9%), and 1.7% (95% 0.4 to 3.0%) increases in waist circumference at 20 and 22 years respectively.

Beyond anthropometry, two further methods of measurement of fat distribution were undertaken in this cohort at two timepoints; namely ultrasound measurements of fat thickness at 17 years of age (visceral and subcutaneous) (n=548), and DEXA at 20 years of age (lean, soft tissue and fat mass) (n=565). Of these measurements, only the association of *EEAA* with (log) lean mass measured by DEXA approached significance after correction for multiple testing (β=0.014, 95% CI 0.010 to 0.15, p=0.005). This is equivalent to *EEAA* (per 5-years) associating with a 1.4% (95% CI 0.4 to 2.4%) increase in lean mass.

There were no associations between *EEAA* or *IEAA* and total fat mass or visceral fat thickness (all p>0.25).

Associations between associated cardiometabolic factors and epigenetic age acceleration at 17 years

EEAA (per 5-years) was associated with log HOMA-IR (molar units) at 17 years (β=0.061, 95% CI 0.017 to 0.104, p=0.007) (Table 3). Therefore, *intrinsic* and e*xtrinsic* age acceleration (per 5-years) were associated with 6.1% (95% CI 1.7 to 11.0%) and 5.4% (95% CI 0.0 to 12.1%) increases, respectively, in HOMA-IR at 17 years of age. Associations were not detected with lipids or blood pressure .

Association of adipokines and inflammatory markers with epigenetic age acceleration

Log serum leptin was associated with *IEAA* (β=0.12, p=0.005) at 17 years-old, but not with *EEAA* (Table 3). Serum adiponectin was not associated with *extrinsic* or *intrinsic EAA* at 17 years.

EEAA was associated with log hsCRP (β=0.28 p=4.8 x10-^{10}) and four circulating cytokines, namely IP10 (β=0.10, p=0.001), sTNFR2 (β=0.05, p=4.6 x10-^{5}), IL-18 (β=0.04, p=0.011) and IL-18BP (β=0.029, p=0.002). As BMI and insulin resistance are both highly pro-inflammatory states, we further adjusted these models for BMI and HOMA-IR. This modestly reduced the coefficient to hsCRP (β=0.21, p=1.0 x10-^{6}). These adjustments minimally altered the coefficients to IP-10 (β=0.12, p=9.5 x10-^{5}), sTNFR2 (β=0.04, p=3.8 x10-^{4}), IL-18 (β=0.03, p=0.055), IL-18BP (β=0.025, p=0.008) respectively. These adjusted coefficients can be interpreted as increasing hsCRP, IP10 and sTNFR2 by 23% (95% CI 13 to 33%), 10% (95% CI 4 to 17%) and 4% (2 to 6%) respectively.

Prediction of Cardiovascular risk using epigenetic acceleration age prediction scores

Having identified associations between epigenetic age acceleration and cardiometabolic risk factors, we investigated whether epigenetic age acceleration was associated with CVD Risk Scores. (Figure). *EEAA* was associated with (log) CVD Risk Scores for hard endpoints (using lipids) (β=0.052, 95% CI 0.016 to 0.089, p=0.005), full CVD outcomes (using lipids) (β=0.046, 95% CI 0.015 to 0.077, p=0.004), hard endpoints (using BMI) (β=0.076, 95% CI 0.033 to 0.119, p=0.001) and full CVD outcomes (using BMI) (β=0.067, 95% CI 0.029 to 0.105, p=0.001). All effects were attenuated but remained significant after adding classical cardiovascular risk factors. *EEAA* remained independently associated with (log) CVD Risk Scores using lipids for calculation with respect to hard endpoints (β=0.038, 95% CI 0.008 to 0.069, p=0.014) and full CVD outcomes (β=0.033, 95% CI 0.008 to 0.058, p=0.011) after adjustment for additional cardiovascular risk factors. *EEAA* also remained independently

associated with (log) CVD Risk Scores using BMI for calculation with respect to hard endpoints (β=0.039, 95% CI 0.003 to 0.075, p=0.035) and full CVD outcomes (β=0.034, 95% CI 0.002 to 0.065, p=0.035) after adjustment for additional cardiovascular risk factors.

The results equate to an interpretation that every 5-years of *EEAA* results in a 4% increase in hard endpoints of CVD by 47 years old and a 3% increase, after adjustment for conventional risk factors. There was no association between *IEAA* with CVD prediction scores.

Associations between biological age and environmental influences early in life and at 16 years

IEAA was not associated with socioeconomic status (family income) or any of the environmental influences tested (Supplemental table 1 (34)). *EEAA* (per 5-year) was positively associated with the lowest (β=0.199, p=0.005) compared to the highest income bracket, prepregnancy BMI of the mother (β=0.015, p=0.048), duration of breastfeeding (months) (β=-0.01 p=0.047) and adolescent smoking in last 4 weeks compared to never smoked (β=0.289, p=0.003).

Adjusting for potential confounders

The significant associations between accelerated biological age and environmental influences were considered for inclusion as covariates in the model. Significant associations ($p<0.05$) were observed between *EEAA* and potential confounders (prepregnancy maternal BMI, breastfeeding duration, socioeconomic status (measured by family income) and smoking history of adolescent at 17 years). Hence these covariates were considered in stepwise fashion in further models predicting BMI at 17, 20 and 22 years old, in subsets with complete data for these variables.

In Supplementary Table 2 (35), modest amelioration of the coefficient for *EEAA* was noted after consideration of these covariates. The coefficients varied from between [0.019 to 0.023] without adjustment and between [0.013 to 0.016] after adjustment for the potential confounders. After stepwise selection, only prepregnancy maternal BMI consistently remained significant in these models.

DISCUSSION

This study has shown that during the period of transition from adolescence to young adulthood, measures of epigenetic age acceleration, determined by DNA methylation from whole blood DNA, were associated with anthropometric and inflammation measurements. *IEAA*, theoretically preserved across cell types and organs after accounting for cell subtypes(36), was associated with increased leptin; while *EEAA*, theoretically dependent on both cell admixture and methylation levels within each cell type(36), was positively associated with BMI, waist circumference, HOMA-IR, hsCRP, IP-10, sTNFR2, IL-18 and IL-18BP. There was no evidence for associations between either epigenetic age measure and fat thickness or mass, lipids, blood pressure or adiponectin.

(36)Obesity induces a highly inflammatory systemic milieu with perturbations of white blood cell counts in adults(37) and children (38). This is consistent with our findings that markers of adiposity (waist circumference and BMI) were positively associated with *EEAA*, whose key difference from *IEAA* being dependence on cell type proportions. Similarly, systemic inflammatory markers (hsCRP, IP-10, sTNFR2, IL-18 and IL-18BP) were associated with *EEAA*. As expected, obesity and some related inflammatory markers have partial dependency on counts of white cell subtypes. (36)

Not all inflammatory markers tested were associated with epigenetic age acceleration. Those that were strongly associated with extrinsic (*EEAA*) could be characterized as marking inflammation that is non-specific and systemic. Elevated C-reactive protein (CRP) is well documented in children and adolescents with the metabolic syndrome(39) and within adults

is associated with CVD (40,41). Tumour necrosis factor (TNF-α) is produced primarily by mononuclear phagocytes(42). Elevated plasma levels of TNF-receptor 1 and 2 have been associated with cardiovascular events(43) and childhood obesity(44). In this study, only TNFR2 was associated with *EEAA*. Interferon-gamma induced protein of 10 kDa (IP-10) is a monocyte-derived pro-inflammatory chemokine that promotes the recruitment of lymphocytes and monocytes to sites of inflammation. IP10 is expressed in human atherosclerotic plaques(45) and plasma levels are elevated in patients with diabetes(46) and the metabolic syndrome(47). Interleukin-18 (IL-18) and its binding protein (IL-18BP) were also associated with *EEAA*. IL-18 regulates both the innate and acquired immune responses(48) with high plasma levels being associated with central obesity, the metabolic syndrome(49) and CVD(50). Soluble receptors for IL-18 exist but have low affinity for IL-18. By contrast, a secreted inhibitor, IL-18BP binds with a high affinity effectively preventing signal transduction.(51) Therefore, IL-18BP may be a useful functional marker for IL-18 activity. In a mouse model, *in vivo* electrotransfer of an expression-plasmid DNA encoding for murine IL-18BP induced a switch from unstable to stable aortic plaque phenotype.(52) From this study, we are unable to distinguish the direction of cause and effect. However, our findings are consistent with age-related changes in immune functioning being associated with increased susceptibility to a wide range of diseases in later life.

In our study, leptin was associated with *IEAA*, but not with *EEAA*. *IEAA* is theoretically preserved across cell types and organs. Leptin is an adipokine, while produced by adipose tissue has a direct etiological role on multiple tissues such as pancreas, liver and muscle in control of insulin sensitivity (53) and resistance (54).

In a much older population of postmenopausal women, Quach et al (55) also observed that *EEAA* was associated with CRP, and BMI, but in contrast to our study also observed associations with lipids and SBP. These differences may be due to our younger population who may have had less exposure to potentially confounding environmental factors. However, Quach et al in a second younger cohort used for replication (Chianti cohort -aged 30 and over) did not find associations with lipid levels and SBP. (55) These latter findings are more in line with ours. The Chianti cohort is of an age between our cohort and the original Quach discovery cohort. Overall, the coefficients observed in our 17-year-olds were of a smaller magnitude than observed in the older cohorts. This implies that associations with lipids and SBP occur less consistently and probably later in life, and hence *EEAA* may not be good predictors of these.

Epigenetic age acceleration predicts future obesity and risk of CVD

Biological age acceleration was associated with BMI and waist circumference up to 5 years hence, and with downstream complications of obesity (insulin resistance). Quach et al showed that an increase in BMI was associated with an increase in *EEAA* in later life.(55) It is difficult to untangle whether this association may be due to the known tracking of BMI from childhood into adulthood(56), however, measurement of *EEAA* in late childhood or early adulthood appears to predict future obesity.

The use of CVD risk factor calculators (12,57) have been employed in the research and clinical setting for secondary and primary prevention. To date their widespread use has been mostly in older adults where validation is quicker due to proximity to disease endpoints and death. Increasingly they have been applied in younger participants, as young as 18 years of age. We applied a Framingham calculation(12) that has been validated on younger participants of similar age to the current study. We showed that *EEAA* independent of a traditional panel of cardiovascular risk factors is associated with all Framingham risk scores. This suggests that epigenetic biological age may be useful in refining the prediction of risk of developing downstream CVD from obesity.

This study is limited by its observational nature, whereby causation cannot be attributed, but only inferred. The lack of intervention does make it impossible to determine if lifestyle or pharmaceutical interventions could modify DNA methylation age, and secondly if modifications are a consequence or causative of changes in CVD risk. We have attempted to partly address these issues by using prospectively collected (avoiding recall bias) and longitudinal data (permitting direct observation 5 years in the future) and use of CVD risk scores validated for similarly aged subjects. We acknowledge that these CVD risk scores are still an estimation and that we will not be able to verify how well these scores actually perform in this cohort for 2-3 decades. The longitudinal data allowed us to demonstrate that the associations with adiposity persist up to 5 years subsequently. Together with this, we demonstrated that epigenetic age acceleration refined prediction of Framingham risk scores. Therefore, epigenetic age acceleration appears to be a promising tool for prediction of CVD. Nevertheless, confirmation of causation will require future functional and interventional studies. The study was also limited by missing data for adolescent smoking and alcohol consumption preventing any investigation of how it affected the association between *EEAA* and obesity. A further limitation is that DNA methylation was measured from whole blood samples. This introduces complexity in interpretation of the contribution of cellular heterogeneity in these tissues. Nevertheless, measurement in whole blood may have advantages for large scale screening, being more practical as any screening requiring cell sorting would be cost prohibitive and impractical due to lack of widespread laboratory expertise.

This study shows that epigenetic age acceleration may further refine ability to identify those likely to develop hard endpoints of disease as a consequence of obesity but confirmation will require long term follow up of cohorts and replication in other studies. Screening specifically at the time of transition from adolescence to adulthood, might allow years for prevention of end points such as acute myocardial infarction, stroke, and vascular disease. Further work is required to understand how acceleration in DNA methylation age is partitioned between environment and fixed genetics, and mediated by inflammation, to enable understanding of how modifiable this measurement is over time.

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Figure: Linear regression models to determine association between extrinsic epigenetic age acceleration (*EEAA*) and 30 year Framingham cardiovascular risk scores(12). The coefficient and 95% confidence interval pertaining to *EEAA* are shown. The Cardiovascular Risk Scores (%) predict the occurrence within 30 years of hard endpoints (coronary death, myocardial infarction, fatal or non-fatal stroke) and full CVD outcomes (additionally coronary insufficiency, angina pectoris, transient ischemic attack, intermittent claudication or congestive heart failure). Model a is a minimally adjusted model (includes covariates of age and sex only). Model b includes adjustment for classical cardiometabolic risk factors at 17-years-old, where BMI or waist circumference, HOMA-IR, SBP and triglycerides were considered for inclusion in model using stepwise selection. c Included in final model were age, sex, systolic blood pressure, triglyceride levels, HOMA, waist circumference at 17 years old, duration of exclusive breastfeeding, prepregnancy BMI, pregnancy income bracket. d Included in final model were age, sex, systolic blood pressure, triglyceride levels, HOMA, BMI at 17 years old, duration of exclusive breastfeeding, prepregnancy BMI, pregnancy income bracket.

Table 1: Characteristics of subjects included within the study. For categorical variables the number of cases and percentage are shown (n(%)). For continuous variables, the mean and 95% confidence interval are shown. Where appropriate, the geometrical mean and 95% confidence interval around the geometrical mean is shown and indicated by #. * indicates a difference detected between males and females with p-value <0.05.

	Males	**Females**
	N=995	
n	501	494
General/Demographic Features		
Low annual family income during pregnancy (<$24 000)	163 (34%)	170 (34%)
Chronological and Biological Age		
Chronological Age at 17 year follow up	17.3 (17.2-17.3)	17.2 (17.2-17.3)
IEAA (years)	0.41 (0.09 to 0.74)	-0.26 (-0.58 to 0.06)*
EEAA (years)	0.90 (0.47 to 1.34)	-0.66 (-1.14 to -0.17)*
Environmental Exposures		
Early life factors		
Prepregnancy BMI (kg/m2)	22.4 (22.0 to 22.8)	22.6 (22.2 to 22.8)
Total Weight Gain Rate (g/week)	497 (480 to 514)	500 (481 to 518)
Maternal Stress#	1.53 (1.45 to 1.62)	1.56 (1.48 to 1.65)
Maternal Smoking at 18 weeks	110 (22%)	123 (25%)
Maternal Smoking at 34 weeks	57 (16%)	77 (16%)*
Age breast feeding stopped (years)#	6.1 (5.6 to 6.7)	5.7 (5.2 to 6.3)
17-year-old follow up	N=870	
	432	438
Cardiometabolic Parameters		
Weight (kg)	72.5 (71.1 to 74.0)	63.8 (62.6 to 65.0)*
Height (cm)	179.1 (178.4 to 179.9)	166.3 (165.6 to 167.0)*
BMI (kg/m2)#	22.6 (22.2 to 23.0)	23.1 (22.7 to 23.5)
Waist circumference (cm)#	80.7 (79.6 to 81.8)	77.5 (76.3 to 78.7)*
Glucose (mmol/L)#	4.8 (4.78 to 4.9)	4.6 (4.6 to 4.7)*
Insulin (mIU/L) #	7.0 (6.6 to 7.5)	8.0 (7.4 to 8.6)*
HOMA-IR (molar units)#	1.6 (1.5 to 1.7)	1.6 (1.5 to 1.7)
Triglycerides (mmol/L)#	0.98 (0.94 to 1.02)	0.96 (0.92 to 0.99)
HDL-C (mmol/L)	1.20 (1.18 to 1.23)	1.38 (1.35 to 1.41)*
LDL-C (mmol/L)	2.23 (2.17 to 2.29)	2.46 (2.40 to 2.53)*
Systolic Blood Pressure (mmHg)	118 (117-119)	109 (108-110)*
Diastolic Blood Pressure (mmHg)	58 (57-58)	59 (59-60)*
Inflammatory Markers		
hsCRP (mg/L)#	0.56 (0.49 to 0.63)	1.0 (0.86 to 1.11)*
Leptin (µg/L) #	4.0 (3.6 to 4.4)	26.6 (24.8 to 28.6)*

Adiponectin (mg/L)#	7.2 (6.9 to 7.6)	9.9 (9.4 to 10.3)*
IL-18 (pg/ml)#	302 (291 to 314)	291 (280 to 303)*
IL18BP (ng/ml)#	14.2 (13.9 to 14.6)	12.6 (12.3 to 14.6)*
IP10 (pg/ml)#	110 (101 to 119)	99 (91 to 108)*
sTNFR1 (pg/ml)#	363 (350 to 376)	310 (298 to 324)*
sTNFR2 (pg/ml)#	3242 (3148 to 3339)	3153 (3054 to 3255)*
Environmental factors at 17-years-old		
Dietary Patterns (z-score)^		
Healthy	-0.12 (-0.23 to -0.01)	0.09 (-0.01 to 0.19)*
Western	0.32 (0.21 to 0.43)	-0.37 (-0.44 to -0.29)*
Physical Activity∫		
≤1/week	91 (28%)	191 (51%)
2-3 times per week	126 (38%)	108 (29%)
≥4/week	114 (34%)	75 (20%)*
Smoking (yes)¥	73 (64%)	87 (62%)
Alcohol consumption (number of standard drinks during the last 7 days)^^	12.3 (10.6 to 13.9)	7.2 (6.4 to 8.1)
CVD risk scores		
All CVD (using lipids) (%)	2.1 (2.0 to 2.2)	1.4 (1.3 to 1.4)*
Hard Endpoints (using lipids) (%)	0.9 (0.9 to 1.0)	0.5 (0.5 to 0.5)*
All CVD (using BMI) (%)	2.7 (2.5 to 2.8)	1.5 (1.4 to 1.6)*
Hard Endpoints (using BMI) (%)	1.2 (1.1 to 1.3)	0.5 (0.5 to 0.6)*
20-year-old follow up	N=757	
	374	383
BMI (kg/m2)#	24.2 (23.7 to 24.8)	24.2 (23.7 to 24.8)
Waist circumference (cm)#	82.7 (81.5 to 83.9)	77.2 (75.3 to 78.4)*
Fat Mass (kg)‖	15.88 (14.97 to 16.84)	25.63 (24.57 to 26.74)*
Lean Mass (kg)	57.35 (56.54 to 58.17)	36.76 (36.25 to 37.28)*
22 year old follow up	N=618	
	307	311
BMI (kg/m2)#	25.1 (24.6 to 25.5)	25.0 (24.3 to 25.6)
Waist circumference (cm)#	86.0 (84.7 to 87.3)	80.2 (78.6 to 81.8)*

Geometric mean and 95% confidence interval shown.
*Difference detected between males and females with p<0.05
^ n=548
∫ n=705
¥ n=255, percentage of those who answered the question is shown.
^^n=677
‖ n=693

Table 2: Associations between epigenetic age acceleration (*IEAA* and *EEAA*) with measures of adiposity. Anthropometry includes BMI and waist at 17, 20 and 22-year-old follow ups. Measures of adiposity include 17-year-old skin folds, ultrasound fat tissue thickness at 17 years, and 20-year-old DEXA body composition measurements.

		IEAA (5 years)[b]		**EEAA (5 years)**	
		B (95% CI)	**p**	**B (95%CI)**	**p**
Anthropometry					
BMI (kg/m2)[a]	17 year old	0.016	0.069	**0.024**	**1.1 x 10^{-4}***
		(-0.001 to 0.033)		**(0.012 to 0.036)**	
	20 year old	0.010	0.27	**0.023**	**0.001***
		(-0.008 to 0.029)		**(0.010 to 0.036)**	
	22 year old	0.020	0.062	**0.023**	**0.002***
		(-0.001 to 0.041)		**(0.009 to 0.038)**	
Waist (cm)[a]	17 year old	0.012	0.083	0.008	0.110
		(-0.001 to 0.026)		(0.000 to 0.019)	
	20 year old	0.009	0.22	**0.018**	**0.002***
		(-0.005 tp 0.024)		**(0.005 to 0.026)**	
	22 year old	0.014	0.10	**0.017**	**0.008***
		(-0.002 to 0.031)		(0.004 to 0.027)	
Ultrasound measured thickness of fat layers	17 year old				
Subcutaneous Fat (cm)[a]		0.084	0.015	0.033	0.172
		(0.017 to 0.153)		(-0.013 to 0.084)	

Visceral Fat (cm) [a]		0.008	0.73	0.004	0.788
		(-0.034 to 0.051)		(-0.024 to 0.037)	
DEXA	20 year old				
Lean Mass (kg) [a]		0.000	0.98	**0.014**	**0.005***
		(-0.013 to 0.014)		**(0.004 to 0.023)**	
Soft Tissue mass (kg) [a]		0.009	0.38	0.070	0.037
Fat Mass (kg) [a]		0.03	0.18	0.02	0.44
		(-0.01 to 0.08)		(-0.02 to 0.05)	

* Values which remain significant after FDR correction for multiple testing are shown in bold font and indicated with *. FDR was applied across 54 tests encompassing results presented in tables 2 and 3.
[a] Log transformed

Table 3: Associations between epigenetic age acceleration (*IEAA* and *EEAA*) with measured cardiometabolic risk factors and inflammatory markers at 17 years old.

	IEAA (5 years)		**EEAA (5 years)**	
	B (95%CI)	**p**	**B (95%CI)**	**p**
Other cardiometabolic risk factors				
HOMA (molar Units) [a]	0.052	0.097	**0.061**	**0.007***
	(-0.009 to 0.114)		**(0.017 to 0.104)**	
SBP (mm Hg)	0.27	0.60	0.5	0.11
	(-0.60 to 1.22)		(-0.1 to 1.2)	
DBP (mm Hg)	-0.08	0.80	-0.06	0.78
	(-0.65 to 0.56)		(-0.42 to 0.42)	
Triglycerides (mmol/L) [a]	-0.007	0.71	0.012	0.40
	(-0.046 to 0.031)		(-0.016 to 0.039)	
Cholesterol (mmol/L)	-0.014	0.70	0.029	0.24
	-0.083 to 0.055		-0.020 to 0.078	
LDL-C (mmol/L)	-0.009	0.78	0.043	0.049
	(-0.069 to 0.052)		(0.000 to 0.086)	
HDL-C (mmol/L)	-0.001	0.96	-0.022	0.014
	(-0.025 to 0.024)		-0.040 to -0.005)	
Inflammatory Markers				
Adipokines				
Leptin [a]	**0.120**	**0.005***	0.042	0.165
	(0.037 to 0.203)		(-0.017 to 0.101)	
Adiponectin [a]	0.024	0.277	-0.037	0.020
	(-0.022 to 0.066)		(-0.068 to 0.006)	
Systemic inflammatory markers				
hsCRP [a]	-0.012	0.84	**0.276**	**4.8 x10^{-10} ***
	(-0.128 to 0.104)		**(0.178 to 0.340)**	
Circulating cytokines				
IP-10 [a]	-0.026	0.45	**0.100**	**0.001***
	(-0.106 to 0.054)		**(0.0143 to 0.156)**	
sTNFR1 [a]	-0.011	0.57	0.002	0.88
	(-0.048 to 0.027)		(-0.025 to 0.029)	
sTNFR2	-0.010	0.52	**0.046**	**4.6 x10^{-5} ***
	(-0.039 to 0.020)		**(0.021 to 0.063)**	
IL-18	-0.023	0.24	**0.037**	**0.011***
	(-0.060 to 0.015)		**(0.006 to 0.059)**	
Free IL-18	-0.024	0.23	0.000	0.97
	(-0.063 to 0.015)		(-0.028 to 0.028)	
IL-18 BP	0.000	1.00	**0.029**	**0.002***
	(-0.024 to 0.024)		**(0.011 to 0.045)**	

* Values which remain significant after FDR correction for multiple testing are shown in bold font and indicated with *. FDR was applied across 54 tests encompassing results presented in tables 2 and 3. [a] Log transformed