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Anna Kankaanpää

Genetic and Environmental Influences on Biological Ageing across Different Ages

Epigenetic Clocks as Markers of Biological Ageing

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF SPORT AND
HEALTH SCIENCES

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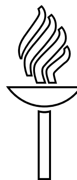

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ABSTRACT

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Ageing is a complex phenotype regulated by both genetic influences and environmental factors, such as modifiable lifestyle-related factors, over the life course. DNA methylation (DNAm)-based estimators, including 'epigenetic clocks', are promising candidates for biomarkers of ageing. The purpose of this study was to investigate genetic and environmental influences on biological ageing using DNAm-based estimators at different ages. This thesis includes four studies based on data retrieved from the Finnish Twin Cohort (FTC). The FTC comprises three large cohort studies. The older FTC study included twins born before 1958, Finntwin16 focused on twins born from 1975–1979 and Finntwin12 examined twins born from 1983–1987. For the subsamples (N: ~2,600), DNAm levels were determined based on blood samples, and DNAm-based estimators were used to assess biological ageing in adulthood. Based on the results, biological ageing is highly heritable. Unhealthy lifestyles and overweight in adolescence were associated with accelerated biological ageing in young adulthood, but genetic factors largely explained the observed associations. Men were biologically older than women, and this sex difference increased with age. The difference was partially explained by more frequent smoking and a higher body mass index in men than in women. Leisure-time physical activity was associated with biological ageing, but this association seems to be susceptible to bias from multiple sources. The findings of this study suggest that DNAm-based estimators of biological ageing are valuable tools for epidemiological studies of life course, as they seem to capture the effects of lifestyle-related factors that accumulate over the life course. The confounding role of genetic factors and exposure to smoking should be carefully considered when using these measures.

Keywords: biological ageing, epigenetic clock, twin study, lifestyle

TIIVISTELMÄ (ABSTRACT IN FINNISH)

Kankaanpää, Anna
Perintö- ja ympäristötekijöiden vaikutukset biologiseen vanhenemiseen – Epigeneettiset kellot biologisen vanhenemisen mittareina
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Vanhenemista säätelevät läpi elämän sekä perintö- että ympäristötekijät, kuten muokattavissa olevat elintapoihin liittyvät tekijät. DNA:n metylaatioon perustuvat estimaattorit, mukaan lukien "epigeneettiset kellot", ovat lupaavia biologisen vanhenemisen mittareita. Tämän tutkimuksen tarkoituksena oli selvittää perintö- ja ympäristötekijöiden vaikutuksia biologiseen vanhenemiseen eri ikäryhmissä DNA:n metylaatioon perustuvia mittareita hyödyntäen.

Tämä väitöskirja sisältää neljä tutkimusta, jotka perustuvat suomalaisen kaksoskohortin aineistoon. Kaksoskohortti koostuu kolmesta suuresta kohorttitutkimuksesta: vanhempi kaksoskohortti (ennen vuotta 1958 syntyneet kaksoset), Nuorten kaksosten terveystutkimus (FinnTwin16, vuosina 1975–79 syntyneet kaksoset) ja Kaksosten kehitys ja terveys -tutkimus (FinnTwin12, vuosina 1983–1987 syntyneet kaksoset). DNA:n metylaatiotasot määritettiin osajoukolle verinäytteistä ja biologisen vanhenemisen arvioimiseen aikuisiässä käytettiin useita DNA:n metylaatioon perustuvia mittareita (N: ~2 600). Tulosten perusteella biologinen vanheneminen on voimakkaasti periytyvää. Epäterveelliset elämäntavat ja ylipaino nuoruudessa liittyivät kiihtyneeseen biologiseen vanhenemiseen varhaisessa aikuisuudessa, mutta perintötekijät selittivät suurelta osin havaitut yhteydet. Miehet olivat biologisesti vanhempia kuin naiset, ja sukupuoliero kasvoi iän myötä. Sukupuoliero selittyi osittain miesten yleisemmällä tupakoinnilla ja korkeammalla painoindeksillä. Vapaa-ajan fyysinen aktiivisuus liittyi biologiseen vanhenemiseen, mutta yhteys näyttää olevan altis useasta lähteestä johtuvalle harhalle.

Tutkimuksen tulokset viittaavat siihen, että DNA:n metylaatioon perustuvat biologisen vanhenemisen mittarit ovat hyödyllisiä elämänkulun epidemiologisissa tutkimuksissa, koska niissä näkyy elämän aikana kertyvien elintapatekijöiden vaikutukset. Perintötekijöiden ja tupakoinnin sekoittavat vaikutukset tulisi ottaa tarkasti huomioon DNA:n metylaatioon perustuvia mittareita käytettäessä.

Avainsanat: biologinen vanheneminen, epigeneettinen kello, kaksostutkimus, elintavat

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Jyväskylä 3.3.2024
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ORIGINAL PUBLICATIONS AND AUTHOR CONTRIBUTION

This thesis is based on the following original research articles, which are referred to in the text by their Roman numerals:

I Kankaanpää, A., Tolvanen, A., Heikkinen, A., Kaprio, J., Ollikainen, M., & Sillanpää, E. (2022). The role of adolescent lifestyle habits in biological aging: A prospective twin study. *eLife*, 11, e80729. https://doi.org/10.7554/eLife.80729

II Kankaanpää, A., Tolvanen, A., Saikkonen, P., Heikkinen, A., Laakkonen, E.K., Kaprio, J., Ollikainen, M., & Sillanpää, E. (2022). Do epigenetic clocks provide explanations for sex differences in life span? A cross-sectional twin study. *The Journals of Gerontology - Series A: Biological Sciences and Medical Sciences*, 77(9), 1898–1906. https://doi.org/10.1093/gerona/glab337

III Kankaanpää, A., Tolvanen, A., Bollepalli, S., Leskinen T., Kujala, U.M., Kaprio, J., Ollikainen, M. & Sillanpää, E. (2021). Leisure-time and occupational physical activity associates differently with epigenetic ageing. *Medicine & Science in Sports & Exercise*, 53(3), 487–495. https://doi.org/10.1249/MSS.0000000000002498

IV Kankaanpää, A., Tolvanen, A., Joensuu, L., Waller, K., Heikkinen, A., Kaprio, J., Ollikainen, M., & Sillanpää, E. The associations of long-term physical activity in adulthood with later biological ageing and all-cause mortality – A prospective twin study. Submitted for publication.

In all the studies included in this thesis, I was privileged to use pre-existing data from the Finnish Twin Cohort. As the first author of all four publications, I considered comments from supervisors and co-authors, and I was mainly responsible for statistical analysis, data interpretation, writing the manuscripts and all steps of the publication process. However, Associate Professor Elina Sillanpää had a significant impact on the writing, especially in *Study II*. The preprocessing of DNA methylation data was conducted in collaboration with researchers from the Institute for Molecular Medicine Finland.

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ABBREVIATIONS

A	Additive genetic influences/variance component
ACE	Biometrical twin model containing additive genetic (A), common environmental (C) and unique environmental (E) variance components
ADE	Biometrical twin model containing additive genetic (A), dominant genetic (D) and unique environmental (E) variance components
ADM	Adrenomedullin
AFAR	The American Federation of Aging Research
ApoA1	Apolipoprotein A-1
ApoB100	Apolipoprotein B-100
AA	Age acceleration
AIC	Akaike's information criterion
AvePP	Average posterior probabilities for most likely latent class membership
B2M	Beta-2-microglobulin
BCH	Bolck–Croon–Hagenaars
BIC	Bayesian information criterion
BMI	Body mass index
C	Shared (common) environmental influences
CI	Confidence interval
CpG	Cytosine-phosphate-guanine dinucleotide
CRP	C-reactive protein
CVD	Cardiovascular disease
D	Dominant genetic influences
DNA	Deoxyribonucleic acid
DNAm	DNA methylation
DZ	Dizygotic
E	Unique (specific, non-shared) environmental influences
EBP	Epigenetic biomarker proxy
EM	Expectation maximisation
FEV_1	Forced expiratory volume in the first second
FVC	Forced vital capacity
GDF-15	Growth differentiation factor 15
GWAS	Genome-wide association study
HOR	Hazard odds ratio
HR	Hazard ratio
HDL	High-density lipoprotein
HbA1C	Haemoglobin A1C
ICC	Intraclass correlation coefficient
LCA	Latent class analysis
LPA	Latent profile analysis
LTPA	Leisure-time physical activity
MAR	Missing at random

MET	Metabolic equivalent
MLR	Maximum likelihood with robust standard errors
MZ	Monozygotic
OPA	Occupational physical activity
PA	Physical activity
PAI-1	Plasminogen activator inhibitor-1
PC	Principal component
RCT	Randomised controlled trial
SEM	Structural equation modelling
SMD	Standardised mean difference
SNP	Single-nucleotide polymorphisms
TIMP-1	Tissue inhibitor of metalloproteinases
WHO	World Health Organization

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ORIGINAL PUBLICATIONS

1 INTRODUCTION

Globally, life expectancy has increased in recent decades, except for a temporary decline due to the impact of the COVID-19 pandemic (Cao et al., 2023). In Finland, the average life expectancy at birth was 78.6 years for boys and 83.8 years for girls in 2022, whereas a century ago, the corresponding figures were 65.6 and 74.2 years, respectively (Official Statistics of Finland, 2022). As the population ages, there is a growing burden of chronic disease and disability (Kennedy et al., 2014). However, the increased life expectancy appears to be accompanied by better physical functioning (Koivunen et al., 2021), suggesting that the extended average lifespan probably includes increased disease-free years, not just years of survival. The rapid increases in lifespans suggest that the ageing process is not regulated only by genetic factors; modifiable environmental factors may also play a role.

Gerosciense is a research field that aims to discover the biological mechanisms that make ageing a common risk factor and driver of multiple chronic diseases (Ferrucci et al., 2020). At the biological level, ageing results from the impact of the accumulation of a wide variety of molecular and cellular damage over time (López-Otín et al., 2013, 2023). Biological ageing can be defined as a gradual and progressive decline in system integrity that occurs with advancing age, resulting in increased risks of morbidity and mortality (Kirkwood, 2005; López-Otín et al., 2013). According to the 'geroscience hypothesis', by directly targeting the biological ageing process itself, the onset and progression of age-related diseases can be delayed (Kennedy et al., 2014; Kritchevsky & Justice, 2020). Consequently, health spans – numbers of disease-free years – could be extended.

Epidemiological studies of life course have suggested that the biological ageing process and age-related diseases may have their origins in earlier life (Ben-Shlomo et al., 2016; Ben-Shlomo & Kuh, 2002; Kuh et al., 2003). Modifiable risk factors, such as poor nutrition and low levels of physical activity, may have both direct long-term effects on biological ageing during sensitive periods of growth and cumulative effects across a life course. To investigate these effects, a

measure of biological ageing is needed (Gaylord et al., 2023). Ideally, this measure would predict between-individual differences in the timing of disease onset, functional decline and death in younger populations. Currently, there is no gold-standard measure of biological ageing, but DNA methylation (DNAm)-based estimators, including 'epigenetic clocks', are probably the most promising candidates (Ferrucci et al., 2020; Jylhävä et al., 2017).

DNAm is an epigenetic modification that regulates gene expression without altering the underlying DNA sequence itself (Greenberg & Bourc'his, 2019). Epigenetic modifications are prone to environmental influences and thus act as interfaces between a genome and a dynamically changing environment (Cavalli & Heard, 2019). Alterations in DNAm patterns are one of the primary hallmarks of ageing at the cellular and molecular levels (López-Otín et al., 2023). These age-related changes in DNAm levels begin to accumulate already in early life, and an individual's biological age can be evaluated based on these patterns (Horvath, 2013; Knight et al., 2016). DNAm-based measures potentially express the accumulated weight of the genetic and environmental influences on biological ageing during a life course (Horvath & Raj, 2018) and may thus serve as valuable tools for understanding the ageing process and development of age-related multi-morbidity.

In this PhD thesis, I present our findings on the genetic and environmental influences on biological ageing as measured by DNAm-based estimators at different ages. We evaluated heritability, i.e. the extent to which genetic factors can explain variation in biological ageing, using twin data. We investigated the role of adolescent lifestyles in the later biological ageing process. Moreover, we studied whether epigenetic clocks can explain the narrowing sex gap in life expectancy by studying sex differences in biological ageing and assessing whether lifestyle-related factors explain these differences in different age cohorts. We investigated the role of leisure-time physical activity in the biological ageing process and the risk of all-cause mortality. Using twin data offer valuable approach to strengthening causal inferences (McGue et al., 2010; Neale & Cardon, 1992). Based on our findings, obtained using twin data, I assess the plausibility of the causal link between lifestyle-related factors and biological ageing.

2 REVIEW OF THE LITERATURE

2.1 Biological ageing

2.1.1 Definition of biological ageing

Generally, ageing is defined as a progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age (Kirkwood & Austad, 2000). It encompasses a range of physical, psychological and social changes that accumulate over time. Ageing is considered to begin after reproductive maturation, when growth and development are complete and the individual is fertile (Hayflick, 2000). Age-related changes, such as reductions in muscle mass and strength (Porter et al., 1995) and bone density (Boskey & Coleman, 2010), start manifesting between the ages of 20 and 40 years, and the rate of changes accelerates in subsequent decades. As a result, ageing becomes evident through a decline in physical function, the onset of disabilities and an increased incidence of chronic non-communicable diseases in later life (Kirkwood, 2005).

At the biological level, ageing results from the impact of the accumulation of a wide variety of molecular and cellular damage over time (López-Otín et al., 2013, 2023; Schmauck-Medina et al., 2022). Biological ageing can be defined as a gradual and progressive decline in system integrity that occurs with advancing age, resulting in increased risks of morbidity and mortality (Kirkwood, 2005; López-Otín et al., 2013). At the cellular and molecular levels, age-related changes begin to accumulate in early life – indeed, as early as conception (Knight et al., 2016).

2.1.2 Evolutionary theories of ageing

Theories of ageing aim to explain why we age. More than 300 theories have attempted to explain ageing at the biological level (Medvedev, 1990). According to Darwin's theory of natural selection, individuals who are best adapted to their environments are likelier to survive and reproduce. A phenotype is an observed characteristic, such as a physiological or behavioural trait. Put simply, a phenotype is heritable if it varies in the population and is more similar in parents and offspring than in randomly selected individuals in the population. Whenever there is variation in a certain phenotype within a population and that variation is heritable, there will be selection for individuals with the most advantageous variations. 'Darwinian fitness' refers to an individual's capability to contribute to future generations. Differences in fitness lead to the evolution of phenotypes.

By definition, the ageing phenotype impairs survival and fertility; therefore, it is not clear why it has evolved (Kirkwood & Austad, 2000). In the evolutionary perspective, such a phenotype reduces fitness, so selection should not favour it. The first theories of ageing stated that ageing evolved to remove older members of a species and thereby free up resources for the next generation (Weismann, 1889). These kinds of adaptive programmed-ageing theories state that ageing is a genetically determined process. Therefore, these theories predict the existence of 'ageing genes'. However, if such ageing genes existed, natural selection would select against them (Kowald & Kirkwood, 2016). Moreover, these theories are circular because they often explain ageing by referring to the fact that organisms age.

Many widely accepted theories of ageing rely on the declining force of natural selection with age (Kirkwood & Austad, 2000; Mc Auley, 2021). Natural selection obviously benefits survival to maturity and reproduction. After reproduction, phenotypes that exert harmful effects later in life have already been passed on to the next generation. The concept of the selection shadow denotes the time during which reproduction probability and selective pressure are low (Kirkwood & Austad, 2000; Medawar, 1952). Non-programmed-ageing theories based on the declining force of natural selection and the selection shadow are currently considered the best explanations for the evolution of the ageing process (Kowald & Kirkwood, 2016). These include three main theories: mutation accumulation, antagonist pleiotropy and disposable soma.

Mutation accumulation theory states that ageing is due to the accumulation of detrimental gene mutations (i.e. alterations in the nucleic acid sequence of the genome; Medawar, 1952). Detrimental mutations expressed later in life, within the selection shadow, are relatively unaffected by selective pressure, as their carriers have already passed on their genes. These mutations will remain as long as the probability of reproduction within that age range remains low. The theory predicts that ageing is due to a loss of genetic information. However, later experiments have shown that mammals can be cloned from old somatic (non-reproductive) cells to produce new individuals with normal lifespans (Burgstaller & Brem, 2017). Thus, it has been stated that gene mutations are probably not the main drivers of the ageing process (Yang et al., 2023).

Pleiotropy is a phenomenon in which one gene controls more than one phenotype in an organism. Antagonist pleiotropy states that ageing is an unfortunate byproduct of selection; genes that favourably affect reproductive success early in life may have negative effects on survival, for example, later in life (Williams, 1957). This negative genetic correlation between the phenotypes does not allow them to evolve independently. Mutation accumulation and antagonist pleiotropy provide a mechanistic explanation for why the ageing phenotype can have a genetic component.

The theory of disposable soma is based on the observation that there is a trade-off between lifespan and reproduction: species that are targets for predation, such as wild mice, tend to invest in fast maturation and reproduction to maximise the genes they pass on to the next generation (Kirkwood & Austad, 2000). Limited resources must be allocated to growth, maintenance, repair, and reproduction. Eventually, the costs of maintenance and repair exceed the benefits. According to this theory, ageing is caused by insufficient investment in the maintenance and repair of somatic cells and tissues. The failure of repair mechanisms leads to the accumulation of molecular and cellular damage. The mechanisms underlying cellular and molecular ageing are inherently stochastic – that is, they are strongly influenced by chance. Such damage accumulates throughout life, from the moment somatic cells and tissue first begin to form, and is regulated by genetic mechanisms for maintenance and repair. As cell damage accumulates, the effects on the body eventually lead to age-related frailty, disability and disease.

2.1.3 Mechanisms of ageing

López-Otín et al. (2013) identified nine candidate hallmarks of ageing, i.e. the cellular- and molecular-level mechanisms that underlie the ageing process. The identified hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. Compromised macroautophagy, chronic inflammation and dysbiosis were recently identified as additional hallmarks of ageing in a subsequent work by the same authors (López-Otín et al., 2023).

These authors have proposed classifying the hallmarks into three categories: primary, antagonistic and integrative (López-Otín et al., 2013, 2023). The primary hallmarks of ageing, considered the causes of cellular damage, include genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis. They are potentially the main drivers of cellular damage and accelerated biological ageing. Antagonistic hallmarks are part of compensatory or antagonistic responses to damage, and they include deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence. Integrative hallmarks are the end results of the previous two classes of hallmarks and are responsible for the functional decline associated with ageing (López-Otín et al., 2013). The hallmarks of ageing are interdependent; changes in one hallmark likely affect the others as well (López-Otín et al., 2023).

2.1.4 Markers of biological ageing

Clearly, there is considerable between-person variation in rates of biological ageing, susceptibility to disease and premature death. Identifying individuals at the highest risk would help tailor prevention and intervention approaches to delay the incidence of multi-morbidities and other harmful ageing phenotypes. The American Federation of Ageing Research (AFAR) has formulated criteria for biomarkers of ageing (Johnson, 2006). The ideal measure of biological ageing would predict one's rate of ageing. It should be a better predictor of lifespan than chronological age and reflect the normal ageing process, not the effects of disease. Moreover, it must be testable repeatedly without harming subjects and applicable to both humans and laboratory animals. Currently, there is no gold-standard measure of biological ageing, but the search for one is underway (Ferrucci et al., 2020; Jylhävä et al., 2017).

The hallmarks of ageing can collectively serve as a framework for the processes that should be quantified and measured to capture an individual's biological ageing rate (Ferrucci et al., 2020; Hägg et al., 2019). For example, telomere attrition is one of the primary hallmarks of ageing (López-Otín et al., 2013, 2023). This refers to the gradual shortening of the protective repetitive DNA sequences that cap chromosomes (Blackburn et al., 2006). Telomeres shorten every time cells divide, limiting the number of divisions cells can undergo. Telomere length has been widely studied and was once considered a promising biomarker of ageing because it mainly fulfilled the criteria proposed by the AFAR (Ferrucci et al., 2020; Jylhävä et al., 2017). However, the techniques used to measure telomere length involve large measurement errors, and observational studies have indicated irregular longitudinal changes in telomere length (Ferrucci et al., 2020). For these reasons, telomere length is not recommended in its current form for use as a measure of biological ageing.

2.1.4.1 DNA methylation – one type of epigenetic alteration

Epigenetic alterations are another primary hallmark of ageing (López-Otín et al., 2013). Epigenetic clocks, which estimate an individual's biological age based on DNA methylation (DNAm), are currently among the most promising biomarkers of ageing and are ready for use (Ferrucci et al., 2020; Jylhävä et al., 2017).

Epigenetic alterations are chemical modifications that regulate gene expression without altering the underlying DNA sequence itself. The epigenome, the collection of genome-wide patterns of epigenetic modifications, is dynamic and can be impacted by environmental factors, such as lifestyle-related influences (Alegría-Torres et al., 2011; Feil & Fraga, 2012). Epigenetic modifications are mitotically heritable; they can be transferred from cell generation to daughter cells. Epigenetic drift occurs when small changes gradually accumulate after cell replication over time.

DNAm, histone modifications and non-coding RNAs are the main epigenetic modifications. Of these, DNAm is the most extensively studied (Greenberg & Bourc'his, 2019). DNAm refers to the attachment of a methyl group

to C-5 of a cytosine base in the context of the cytosine-phosphate-guanine (CpG) dinucleotide in a DNA strand. The presence of a methyl group, especially at a promoter site, silences a gene by blocking transcription factors from binding to promoter sequences. DNAm has a profound role in cell differentiation, the process in which genetically identical stem cells develop into different types of cells, such as muscle, bone or nervous cells.

Environmental influences, such as lifestyle-related factors, can affect DNAm through various routes. For example, nutrition can directly influence DNAm, as some micronutrients, such as the folates found in dark-green leafy vegetables, can act as methyl group donors needed for the maintenance of DNAm patterns (Ideraabdullah & Zeisel, 2018). Moreover, lifestyle-related factors may induce or repress activity in enzymes involved in epigenetic regulation, but the most complex routes through which the environment can affect the epigenome are not yet fully understood.

In age-related epigenetic drift, the DNAm changes associated with chronological age within an individual include age-related changes in the epigenome that are induced both environmentally and stochastically (Jones et al., 2015). Many studies have provided evidence of age-related hypomethylation or hypermethylation within specific CpG sites; in other words, DNAm levels within these CpG sites correlate with chronological age (Christensen et al., 2009; Jones et al., 2015). Epigenetic clocks are markers of biological ageing that aim to capture these specific CpG sites, whose age-related changes in DNAm are progressive and common across individuals (Horvath, 2013; Jones et al., 2015). They are algorithms that quantify biological ageing using DNAm levels within specific CpG sites. A summary of the DNAm-based measures of biological ageing used in this research is provided in TABLE 1.

2.1.4.2 First-generation epigenetic clocks

First-generation epigenetic clocks are DNAm-based estimators of chronological age (Bocklandt et al., 2011; Hannum et al., 2013; Horvath, 2013). The corresponding genome-wide DNAm datasets consist of large multivariate data, where the number of variables representing DNAm within different CpG sites typically exceeds the number of samples analysed. Penalised regression (or shrinkage or regularisation methods) allows us to create a linear regression model that is penalised for containing too many variables (James et al., 2021). This is done by adding a constraint – a penalty term – in the regression equation. Implementing the penalty term in the regression model reduces or shrinks the regression coefficient values of the less contributory variables towards zero. The three most common methods that use different penalty terms are ridge, lasso (least absolute shrinkage and selection operator) and their combination, elastic net regression (James et al., 2021; Zou & Hastie, 2005). In the case of epigenetic clocks, a penalised regression model automatically selects the most informative CpG sites for the age prediction or estimation model (Horvath & Raj, 2018). As a result, the set of CpG sites and a corresponding mathematical algorithm that

incorporates the DNAm levels into an age estimate are obtained (Horvath & Raj, 2018).

First-generation clocks, including those of Horvath and Hannum, were developed by predicting chronological age based on genome-wide DNAm data using penalised (elastic net) regression (Hannum et al., 2013; Horvath, 2013). Both clocks provide highly accurate estimates of chronological age (Horvath & Raj, 2018). The rate of changes in DNAm levels at age-dependent CpG sites corresponds to the 'ticking rate' of the clock. Horvath devised a multi-tissue clock that uses 353 CpG sites to predict chronological age (Horvath, 2013), whereas Hannum's clock uses DNAm levels within 71 CpG sites derived from whole blood (Hannum et al., 2013).

Epigenetic clocks provide an estimate of an individual's biological (or epigenetic) age in years. Age acceleration (AA) describes the discrepancy between estimated biological age and chronological age. Positive AA is observed in individuals whose biological age is older than expected based on their chronological age, while the reverse situation is described as negative AA. AA based on both first-generation clocks has been shown to be associated with an increased risk of all-cause mortality, but the effect sizes have been relatively small (Fransquet et al., 2019). According to a meta-analysis, each 5-year increase in biological age is associated with an 8%–15% higher risk of mortality (Fransquet et al., 2019), which is equivalent to a 1-year increase in AA being associated with a 1%–3% increased risk of mortality. Higher AA has also been shown to be cross-sectionally associated with age-related phenotypes, such as diminished physical function as measured by grip strength and walking speed, but not with longitudinal changes (Sillanpää et al., 2018). It has been suggested that approaches to predicting chronological age may omit CpG sites whose DNAm levels would rather predict deviation of biological age from chronological age than solely chronological age (Levine et al., 2018).

2.1.4.3 Second-generation epigenetic clocks

Second-generation clocks are designed to capture the CpG sites whose DNAm levels may better reflect health. They are trained to predict age-related biology and health-related phenotypes. Levine and colleagues developed the biological age estimator DNAm PhenoAge by replacing chronological age in the prediction model with 'phenotypic age' and using DNAm levels determined from whole blood (Levine et al., 2018). The estimator was developed in two stages. First, phenotypic age, a composite of clinical biomarkers shown to predict all-cause and cause-specific mortality, was developed using a penalised survival model (penalised Cox). Chronological age and 9 out of 42 clinical biomarkers that best predicted time to death were selected for use in phenotypic age prediction (see TABLE 1). The biomarkers included reflect, for example, liver function (albumin), kidney function (creatinine), metabolic health (glucose), inflammation (C-reactive protein [CRP]) and immune system activity (alkaline phosphatase). Second, genome-wide DNAm data and chronological age were used to predict phenotypic age, again using penalised regression (elastic net). As a result, an

estimator was obtained that uses DNAm levels within 513 CpG sites and chronological age to estimate biological age.

Another widely used second-generation epigenetic clock, DNAm GrimAge, was also a product of a two-stage development method (Lu et al., 2019). It first used blood-based DNAm data to develop DNAm-based surrogates for a set of biomarkers, including plasma proteins and self-reported smoking pack years, which are known to be associated with morbidity and mortality. This was done using elastic net regression. Second, these DNAm-based biomarkers were used to predict time to death using an elastic net Cox survival model. In both steps, information on participants' sex and chronological age was used. The authors were able to develop DNAm-based biomarkers for 12 out of 88 plasma proteins that performed relatively well. For the DNAm GrimAge estimator, the following DNAm-based surrogates were selected in addition to sex and chronological age: DNAm-based smoking pack-years, DNAm adrenomedullin (ADM), DNAm beta-2-microglobulin (B2M), DNAm cystatin C, DNAm growth differentiation factor 15 (GDF15), DNAm leptin, DNAm plasminogen activator inhibitor 1 (PAI-1) and DNAm tissue inhibitor metalloproteinases 1 (TIMP-1). These DNAm-based biomarkers comprised 1,030 CpG sites. Many of the target plasma proteins (ADM, B2M, GDF15, TIMP-1) have been shown to predict cardiovascular event incidence (Ho et al., 2018), and PAI-1 plays a role in the progression of atherosclerosis (Schneiderman et al., 1992). B2M and cystatin-C indicate kidney function (Astor et al., 2012).

Both second-generation epigenetic clocks estimate biological age in years. The AA values based on these measures appear to be better predictors of mortality than the values based on the first-generation clocks: a 1-year increase in AA based on DNAm PhenoAge was shown to be associated with a 4.5% higher risk of mortality (Levine et al., 2018), and an AA increase based on DNAm GrimAge was linked with a 10% higher risk (Lu et al., 2019). Later studies have confirmed that DNAm GrimAge outperforms other clocks in predicting age-related phenotype and mortality (Li et al., 2020; McCrory et al., 2020). Notably, there is some evidence that AA based on DNAm GrimAge predicts age-related decline in physical function (as measured by walking speed and the timed up and go [TUG] test; Föhr et al., 2021).

2.1.4.4 Further advances

Although DNAm and AA measures are widely used in ageing research at this point, their reliability issues are often overlooked (Higgins-Chen et al., 2022; Sugden et al., 2020). AA measures are attributable to technical variation (or noise), which is not biologically meaningful, although preprocessing methods (e.g. normalisation methods) are used to reduce this noise in DNAm data. To improve AA measures by removing this variation, Higgins-Chen and colleagues developed principal component (PC)-based epigenetic clocks (Higgins-Chen et al., 2022). PC analysis is a statistical method used to reduce the dimensions of a multivariate dataset while preserving maximal information within the PCs (James et al., 2021). The authors trained the clocks using PCs instead of individual

CpG sites; this approach undermines the noise from any single CpG site (Higgins-Chen et al., 2022). They showed, for example, that PC-based clocks demonstrated greatly improved agreement between technical replicates. Moreover, the correlations between the AA measures obtained using different PC-based clocks were considerably stronger than those linking AAs based on the original clocks. A recent study compared PC-based clocks with the original clocks and concluded that replacing AA values calculated using original clocks with PC-based AA does not significantly alter the relationship of AA with health outcomes or mortality (Faul et al., 2023).

2.1.4.5 DNAm-based biomarkers of pace of ageing

The DunedinPoAm estimator and its refined version, DunedinPACE, differ from preceding epigenetic clocks in that they were developed to predict the pace of ageing based on longitudinal data (Belsky et al., 2020, 2022). In other words, these estimators were trained to predict within-individual age-related changes. In the first stage, pace of ageing was constructed to describe changes over time in several biomarkers of organ-system integrity among same-aged adults (born in 1972–1973) (Belsky et al., 2015; Elliott et al., 2021). These changes (slopes) were estimated for each individual using a linear growth curve model. An individual's pace of ageing was calculated as the sum of the slopes, and the resulting composite measure was scaled in units representing the mean trend in the cohort – that is, the average change occurring during one calendar year. In the second stage, the authors used an elastic net regression model in developing the DNAm-based estimator of pace of ageing.

DunedinPoAm was trained to predict pace of ageing based on 18 biomarkers at 26, 32 and 38 years of age (Belsky et al., 2015, 2020), while for DunedinPACE, data were used from extended follow-up of 19 biomarkers at 26, 32, 38 and 45 years of age (Belsky et al., 2022; Elliott et al., 2021). The biomarkers included reflect the functionality of multiple organ systems, such as cardiovascular, metabolic, pulmonary and immune function (TABLE 1). The biomarkers used mainly overlap for DunedinPoAm and DunedinPACE. For DunedinPACE, two new biomarkers, leptin and caries, were included, whereas telomere length was omitted because of emerging criticisms of its measurement techniques (Belsky et al., 2022; Nettle et al., 2021). Moreover, DunedinPACE was developed using only CpG sites proven to be reliable in blood. These improvements resulted in higher test–retest reliability of the measure.

The DunedinPoAm and DunedinPACE estimators estimate pace of ageing in years per calendar year (Belsky et al., 2020, 2022). These estimators are sometimes referred to as third-generation epigenetic clocks (Crimmins et al., 2021). However, the authors call these measures DNAm-based biomarkers of pace of ageing because they are conceptually and empirically distinct from the epigenetic clocks (Belsky et al., 2020, 2022). Because these measures were developed using longitudinal data, they (particularly DunedinPACE) may better capture within-person changes and therefore prove useful for clinical trials (Belsky et al., 2022). Moreover, it has been suggested that these measures may be

more appropriate for young adult and middle-aged populations than preceding epigenetic clocks, as they were developed using data from younger individuals (Gaylord et al., 2023). DunedinPACE was shown to be associated with morbidity, disability and mortality, with effect sizes similar to those observed with AA based on DNAm GrimAge (Belsky et al., 2022).

TABLE 1 Summary of the selected DNAm-based measures of biological ageing.

		Training phenotype	Variables used in biological age prediction	Number of CpG sites	Reference	Measure of biological ageing
1.	Horvath's clock	Chronological age	DNAm levels	353	Horvath, 2013	AA (in years)
2.	Hannum's clock	Chronological age	DNAm levels	71	Hannum et al., 2013	AA (in years)
3.	DNAm PhenoAge	Phenotypic age: a composite of clinical biomarkers that best predict time to death: albumin (neg), creatinine, glucose, CRP (log), lymphocyte percent (neg), mean cell volume, red cell distribution width, alkaline phosphatase, white blood cell count and chronological age	Chronological age and DNAm levels	513	Levine et al., 2018	AA (in years)
4.	DNAm GrimAge	Time to death	Sex, chronological age, DNAm-based surrogates for plasma proteins (ADM, B2M, cystatin C, GDF15, leptin [neg], PAI-1 and TIMP-1) and smoking pack-years	1,030	Lu et al., 2019	AA (in years)
5.	PC-based clocks	See the corresponding original clocks (1–4) above	Principal components (PCs) from CpG-level data		Higgins-Chen et al., 2022	AA (in years)

		Training phenotype	Variables used in biological age prediction	Number of CpG sites	Reference	Measure of biological ageing
6.	DunedinPoAm	Pace of ageing: the sum of longitudinal changes over 12 years (from age 26 to 38) in 18 biomarkers of organ-system integrity among same-aged individuals: HbA1C, cardiorespiratory fitness (rev), waist–hip ratio, FEV1/FVC (rev), FEV1 (rev), mean arterial pressure, BMI, leukocyte telomere length (rev), creatine clearance (rev), urea nitrogen, lipoprotein, triglycerides, gum health, total cholesterol, white blood cell count, high sensitivity CRP, HDL cholesterol (rev) and ApoB100/ApoA1	DNAm levels	46	Belsky et al., 2020	Pace of ageing (years/calendar year)
7.	DunedinPACE	Pace of ageing: sum of longitudinal changes over 20 years (from age 26 to 46) in 19 biomarkers of organ-system integrity among same-aged individuals (see DunedinPoAm above). Leukocyte telomere length was omitted, while leptin and caries were included.	DNAm levels	173	Belsky et al., 2022	Pace of ageing (years/calendar year)

DNAm, DNA methylation; CpG, cytosine-phosphate-guanine dinucleotide; AA, age acceleration; neg, negative weight; CRP, C-reactive protein; log, log-transformed; ADM, adrenomedullin; B2M, beta-2-microglobulin; GDF15, growth differentiation factor 15; PAI-1, plasminogen activator inhibitor 1; TIMP-1, tissue inhibitor metalloproteinases 1; HbA1C, haemoglobin A1C; rev, reversed; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; BMI, body mass index; HDL, high-density lipoprotein; ApoB100, apolipoprotein B-100; ApoA1, apolipoprotein A-1.

2.1.4.6 Epigenetic clocks and evolutionary theories of ageing

It remains unclear whether age-related DNAm is a cause or a consequence of ageing. Evolutionary theories of ageing provide a framework that can be used to illuminate the link between DNAm and age-related health (de Magalhães, 2023; Mc Auley, 2021, 2023).

Epigenetic clocks are often thought to provide evidence for programmed theories of ageing since the 'ticking rate' of the epigenetic clock appears to be programmed (Mc Auley, 2021). DNAm has been suggested as the mechanism that controls programmed ageing. DNAm may also provide a framework for antagonistic pleiotropy by enabling the expression of genes to change throughout life (Mc Auley, 2021; Vijayakumar & Cho, 2022). Moreover, the theory of disposable soma aligns well with age-related DNAm. Studies have identified several genes involved in maintenance and repair processes, and these genes' promoters become methylated, and subsequently silenced, during the ageing process (Mc Auley, 2021). However, for DNAm to play a role in the evolution of ageing, transgenerational epigenetic inheritance would have to occur – that is, the phenomenon in which epigenetic modifications are passed from one generation to the next. Currently, there is no conclusive evidence for transgenerational epigenetic inheritance in humans (Fitz-James & Cavalli, 2022). Instead, DNAm appears to be genetically heritable (van Dongen et al., 2016), and research has suggested that underlying genetic variants probably fully explain the observed heritability (Villicaña & Bell, 2021). Therefore, it is probable that the mechanisms that establish and maintain DNAm patterns are genetically heritable, and the patterns are not directly transferred from one generation to the next.

A generally accepted explanation for the evolution of ageing is still lacking, and new insights are needed. Horvath and Raj (2018) formulated the 'epigenetic clock theory of ageing'. This is a quasi-programmed theory proposal that ageing is the result of a combination of both genetic programming and stochastic events that occur over time. In other words, while there is a genetic component that influences the ageing process, random events also play a role. According to this theory, ageing begins soon after conception and continues throughout life until death. The theory contends that ageing is an unintended consequence of both developmental and maintenance processes, which separates it from adaptive programmed-ageing theories (Mc Auley, 2021). Another new framework, the information theory of ageing, states that the loss of epigenetic information is a reversible cause of ageing (Yang et al., 2023). According to this view, cells with an accelerated epigenetic age lose the ability to maintain cellular identity, and ageing is characterised by changes in cellular identity and function over time.

2.1.5 The life course perspective on biological ageing

Life course epidemiology is a research area that investigates the long-term consequences of physical or social exposure during gestation, childhood, adolescence, young adulthood and later adult life on later health or disease risk

(Gluckman & Hanson, 2004; Kuh et al., 2003). Many age-related changes may have their origins in earlier life (Ben-Shlomo et al., 2016; Ben-Shlomo & Kuh, 2002).

Life course epidemiology originated in the 1980s with David Barker's observation that mortality from cardiovascular disease (CVD) was higher in poorer areas, where the birth weight of children was also lower (Barker & Osmond, 1986). Barker considered low birth weight an indicator of poor foetal nutrition and suggested that early-life conditions could have an impact on the development of CVD and other chronic diseases. According to Barker's hypothesis, when food intake is limited, the foetus adapts to the prevailing conditions and prepares for life in an environment where resources are scarce. When the postnatal environment does not correspond to the clues received during the foetal period, it exposes the individual to harmful consequences. Barker's hypothesis is also known as the theory of early programming (or the foetal origins hypothesis) and has been supported by later studies (Risnes et al., 2011; Zanetti et al., 2018).

There are several processes through which early-life exposure may be linked to later health (Kuh et al., 2003). For example, there are critical and sensitive periods during development in which risk factors may have lasting effects on health, independent of subsequent exposure. Epigenetics, particularly DNAm, is a plausible mechanism through which early exposure alters biological function in a long-term manner and thereby shapes later health (Demetriou et al., 2015). Moreover, early-life exposure can set individuals on distinct trajectories, such as different lifestyles during their life course, which influence their health outcomes in later life. There may also be cumulative effects; the accumulation of life events, depending on their duration and intensity, can lead to various health outcomes.

Epigenetic clocks link developmental and maintenance processes to biological ageing, providing a unified theory of life course (Horvath & Raj, 2018; Ryan, 2021). Moreover, DNAm-based measures of biological ageing can indicate disease susceptibility (Ferrucci et al., 2020) and may detect 'preclinical ageing' even in younger and healthier populations (Belsky et al., 2022; Levine et al., 2018). From a life-course perspective, DNAm-based measures may serve as valuable tools for understanding the ageing process and the development of age-related multi-morbidity.

2.2 The classical twin study design

2.2.1 Heritability of phenotypes

Ageing is a complex phenotype influenced by multiple genes, environmental factors and probably their interactions. The classical twin study is a powerful design for detecting genetic and shared environmental influences that underlie complex phenotypes (Neale & Cardon, 1992; Posthuma et al., 2003). The classical

twin study design focuses on monozygotic (MZ) twin pairs, known as identical twins, and dizygotic (DZ) twin pairs, known as fraternal or nonidentical twins, that have grown up in the same home. MZ twins in a pair are genetically identical, sharing 100% of their genes. DZ twins in a pair are as genetically similar as ordinary siblings, and they share, on average, 50% of their genes. By comparing the resemblance in a specific phenotype of these two types of twin pairs, it is possible to evaluate the genetic and environmental influences on that phenotype.

Heritability is the estimated degree of intra-population variation in a phenotype that results from genetic variation between individuals in that population (Neale & Cardon, 1992). It is expressed as the genetic variation relative to all the causes of variation. The total genetic influence on a phenotype is a composite of additive genetic influences (A) and dominant genetic influences (D). The genetic locus is the site of a gene on a chromosome. Alleles are alternative forms of a gene that occupy the same locus on a chromosome. Component A corresponds to the influences of alleles, which act in an additive manner; that is, two copies of an allele at the same locus have twice the influence of one copy on a phenotype. Individuals with two different alleles at a specific genetic locus (heterozygotes) do not always have expression levels that are exactly in the middle of those with two identical alleles (homozygotes). Component D refers to the extent to which genotypes differ from the additive genetic value, i.e. interaction effects between alleles at the same locus.

Any variation in a phenotype not explained by genetic influences is assumed to be due to common (or shared) environmental influences (C) and specific (unique, non-shared or random) environmental influences (E). C refers to all environmental influences that make siblings within a family similar, and E to all environmental influences that make individuals within a family differ with respect to a given phenotype. The decomposition of the variation of a phenotype into components A, D, C and E can be done using quantitative genetic modelling within the structural equation modelling (SEM) framework.

2.2.2 Twin study designs and causal inference

In observational studies, the association between two phenotypes may arise from true causation but also from confounding and reverse causation (outcome leads to exposure). For a variable to be considered a confounder, it must be a cause of the outcome, be correlated with the exposure and not be influenced by the exposure. Confounders generate a spurious association between exposure and outcome. In observational studies, confounding is generally addressed by adjusting the statistical models by adding a measured set of variables as covariates. However, the possibility that unmeasured confounding produces bias in the results can rarely be ruled out.

Randomised controlled trials (RCTs) are considered the gold standard for assessing causality. One of the primary advantages of this study design is that random assignment to the treatment and control groups effectively accounts for both measured and unmeasured confounding factors. However, RCTs are

expensive to implement, and especially in studies of biological ageing, long follow-up times are required to verify the effects of interventions.

Twin studies offer a valuable means of strengthening causal inferences in observational studies (de Moor et al., 2008; McAdams et al., 2021; McGue et al., 2010; Neale & Cardon, 1992). Co-twin control designs, such as the use of exposure-discordant twin pairs, provide a setting for examining whether the relationship between an exposure and an outcome is confounded by familial factors, including genetic and common environmental influences (Kaprio & Koskenvuo, 1989; McGue et al., 2010). Adopting a twin study design and using siblings as controls will automatically match with respect to several unmeasured confounders, such as family characteristics and parenting practices. Moreover, especially in MZ twin pairs, this approach fully controls for genetic confounding. If the exposure of interest is a cause of the outcome, the association between the exposure and outcome should be observed at the individual level (in the general population) as well as within DZ and MZ twin pairs (McGue et al., 2010). If the association is attenuated in DZ twin pairs and further in MZ twin pairs, the association is confounded by genetics and the common environment.

Quantitative genetic models (particularly bivariate models) can be applied to data obtained from a classical twin study design to assess the plausibility of a causal link between two phenotypes. The purpose of bivariate genetic analysis is to decompose covariation between two phenotypes – in this case, exposure and outcome – into genetic and environmental influences (Neale & Cardon, 1992). Using bivariate models, the proportions expressing the extent to which the genetic and environmental factors contribute to the association can be evaluated. These proportions correspond to the strength of the genetic and environmental correlations between the phenotypes. Under the causal hypothesis, genetic and environmental correlations between exposures and outcomes should be significant, whereas a significant genetic correlation in the absence of environmental correlation falsifies the hypothesised causal effect of exposure on outcome (de Moor et al., 2008). Using a co-twin control design to investigate the association between exposure and outcome within MZ twin pairs (while controlling for common environments and fully genetic influences) is, in fact, analogous to focusing on the non-shared environmental correlation between exposure and outcome in bivariate twin models (McAdams et al., 2021).

2.3 Correlates of biological ageing

2.3.1 Genetic factors

According to evolutionary theory, 'ageing genes' probably do not exist because natural selection would have selected against them (Kowald & Kirkwood, 2016). However, theories of mutation accumulation and antagonist pleiotropy provide an evolutionary explanation for why biological ageing may have a genetic component (Kirkwood, 2008). Moreover, novel quasi-programmed theories of

ageing (Horvath & Raj, 2018; Yang et al., 2023) predict that genetic factors have a significant role in the ageing process.

Twin studies have suggested that genetic factors explain about 20%–30% of the variation in longevity (Herskind et al., 1996). Only a few studies have reported on the heritability of biological ageing as measured with epigenetic clocks and twin data. According to studies that have used Horvath's clock, genetic factors explain 51% to 73% of the variance in biological ageing (Jylhävä et al., 2019; Sillanpää et al., 2019). Heritability estimates of 34% to 41% have been reported for DNAm PhenoAge (Jylhävä et al., 2019) and 58% for DNAm GrimAge (Lundgren et al., 2022). Previous research using longitudinal data on biological ageing suggests that genetic factors account for most of the stability in AA over time (Jylhävä et al., 2019).

2.3.2 Sex

In our usage, sex refers to biological sex as determined by sex chromosomes: women have two X chromosomes in their cells, while men have one X and one Y (XX and XY karyotypes, respectively). Globally, women tend to live longer than men, but the sex gap in life expectancy varies by time and country (Dicker et al., 2018). In Finland, the sex gap increased in the first half of the twentieth century. This gap was greatest in the mid-1970s, at 9 years; since then, it has narrowed to 5.2 years (Official Statistics of Finland, 2022). Several reasons for the sex gap have been proposed, including differences in lifestyle-related factors, such as smoking behaviour (Östergren & Martikainen, 2020; Rogers et al., 2010) and societal influences (Rogers et al., 2010).

There is clear evidence that the cellular and molecular mechanisms of ageing are better maintained in women (Hägg & Jylhävä, 2021). According to research conducted using first-generation epigenetic clocks, sex differences in biological ageing seem to appear in adolescence (Simpkin et al., 2017). In adulthood, men are biologically 1–2 years older than women (Crimmins et al., 2021; Horvath et al., 2016). The first published results on biological age determination using second-generation clocks have also indicated that men are biologically older than women (Crimmins et al., 2021; Li et al., 2020). The sex difference was 1.1 years for DNAm PhenoAge and 3 years for GrimAge independent of ethnicity, education, body mass index (BMI) and smoking (Crimmins et al., 2021). Similarly, men appear to exhibit faster biological ageing according to the DunedinPoAm estimator (0.01 years/calendar year; Crimmins et al., 2021).

Changes in hormonal levels during menopause have a detrimental effect on women's health (Mauvais-Jarvis et al., 2013); thus, sex differences in biological ageing would be expected to diminish around and after the age of 50. After menopause, women may reach the same levels of biological ageing as men (Hägg & Jylhävä, 2021). However, a study using longitudinal data on biological ageing assessed with epigenetic clocks found that men were biologically older than women between the ages of 50 and 90 years and that biological ageing proceeded at a similar rate during the follow-up period in both sexes (Li et al., 2020).

2.3.3 Pubertal development

From an evolutionary point of view, an individual who matures and becomes fertile at an early age may have greater reproductive potential than an individual who matures late. Therefore, based on disposable soma theory, early pubertal development can be seen as an investment in reproduction at the expense of somatic maintenance and can be hypothesised to predict faster biological ageing. Previous studies have linked early pubertal development to worse health conditions in adulthood, such as obesity and cardiometabolic risk factors (Prentice & Viner, 2013). However, the first studies to investigate the associations between pubertal timing and biological ageing measured with epigenetic clocks provided inconsistent results (Hamlat et al., 2021; Maddock et al., 2021).

2.3.4 Body size

BMI (weight [kg]/height [m^2]) is commonly used as a measure of body size and degree of obesity. It is easy to measure, but it does not reflect body composition; a high BMI can indicate high muscle mass or high fat mass. In adults, cutoff points of 25 and 30 are generally used for overweight and obesity, respectively, whereas a BMI lower than 18 is indicative of thinness (underweight). BMI changes substantially in childhood and adolescence; therefore, age-specific cutoff points for child overweight, obesity and thinness have been determined (Cole et al., 2000, 2007).

Being overweight and obese in adulthood is associated with an increased risk of all-cause mortality, while the lowest risk of all-cause mortality is observed in the BMI range of normal weight (20.0–24.9; Berrington de Gonzalez et al., 2010). Twin studies have shown that the effect of genetic factors on BMI is lowest during infancy, when it explains about 40% of the variation (Silventoinen et al., 2016). However, genetic influences increase with age, reaching a maximum level of 75% in young adulthood and then slightly decreasing to 60% in older adulthood (Silventoinen et al., 2016, 2017).

A systematic review conducted by Oblak and colleagues (2021) concluded that BMI correlates with accelerated biological ageing when measured with Horvath's clock, DNAm PhenoAge and GrimAge, but not with the Hannum clock. The association between BMI and biological ageing (as assessed with DNAm GrimAge and DunedinPoAm) appears to already be in place in childhood (Etzel et al., 2022). Moreover, a recent study indicated that a higher BMI in adulthood associates with accelerated biological ageing as measured with DNAm GrimAge, even after accounting for genetic factors (Lundgren et al., 2022).

2.3.5 Education

Education is often used as a marker of socioeconomic status. Studies have suggested that a lower socioeconomic position correlates with a higher risk of all-cause mortality and that these socioeconomic differences are largely explained

by health behaviours, such as smoking, nutrition and physical activity (Laaksonen et al., 2008; Stringhini et al., 2010).

Many studies have indicated that a higher education level correlates with decelerated biological ageing as measured with epigenetic clocks (Oblak et al., 2021). However, an epigenome-wide association study identified nine CpGs whose DNAm levels were associated with education, but all these associations were confounded by exposure to smoking (Karlsson Linnér et al., 2017).

2.3.6 Physical activity

Physical activity (PA) refers to 'any bodily movement produced by skeletal muscles that result in energy expenditure' (Caspersen et al., 1985). PA can be categorised in various ways – for example, based on the mode, namely aerobic activity or muscle strengthening. In this study, our primary focus was on aerobic PA, as in most of the existing literature. PA can also be categorised by the proportion of daily life during which the activity occurs, such as at work, as occupational PA (OPA), and during leisure time, as leisure-time PA (LTPA). PA can be performed at different intensities as well. PA intensity is often reported in metabolic equivalent (MET) values, defined as the ratio of the working metabolic rate to the standard resting metabolic rate (Ainsworth et al., 2015). One MET is the rate of energy expended while at rest, and it is equal to an oxygen uptake of 3.5 ml per 1 kg of body weight per minute. PA intensities can be classified as follows: 1–1.5 METs are considered to indicate sedentary behaviour, while 1.6–2.9, 3.0–5.9 and 6 or more METs indicate light, moderate and vigorous physical activity, respectively (Ainsworth et al., 2011). PA can be measured using questionnaires or devices such as accelerometers. With accelerometers, both the intensity and duration of PA can be assessed, but they cannot accurately evaluate some activities, such as swimming and cycling.

The health benefits of LTPA are well documented (World Health Organization, 2020). For example, increased LTPA associates with a lower risk of several diseases, such as CVD (Li et al., 2013), and a lower risk of premature mortality (Löllgen et al., 2009). The dose-response associations of LTPA and device-based PA with all-cause mortality appear to be curvilinear rather than linear (Arem et al., 2015; Ekelund et al., 2019). According to the newest World Health Organization (WHO) guidelines, adults should undertake 150–300 minutes of moderate-intensity or 75–150 minutes of vigorous PA (or some equivalent combination) per week to obtain significant health benefits and mitigate health risks (Bull et al., 2020). However, the benefits of OPA are more controversial. There is some evidence that OPA may even adversely affect health outcomes, such as risk of all-cause mortality, CVD and long-term sickness absence, whereas LTPA correlates with a low risk (Coenen et al., 2018; Holtermann et al., 2011; J. Li et al., 2013). This conflicting association of OPA with health outcomes is referred to as 'the physical activity health paradox' (Holtermann et al., 2011).

Although an association between LTPA and a lower risk of premature mortality is frequently reported in observational studies, evidence based on

RCTs has failed to confirm that LTPA prevents premature mortality (Ballin & Nordström, 2021). Moreover, genetically informed studies have suggested that genetic pleiotropy may partly account for these associations, as the same genes may regulate both LTPA and health-related outcomes (Karvinen et al., 2015; Sillanpää et al., 2022). In fact, the association between LTPA and mortality is susceptible to reverse-causality bias: an underlying suboptimal physiological or predisease state may negatively affect LTPA, which means that the observed association may be due to a causal relationship between the covert disease state and subsequent premature death (Strain et al., 2019). There is evidence that increased control for reverse causality results in weaker associations between LTPA and mortality (Lee et al., 2021; Rezende et al., 2022; Strain et al., 2019).

Only a few studies have investigated the association between PA and biological ageing as measured with epigenetic clocks. Previous studies, mostly based on cross-sectional data, have indicated that whether an association is observed depends on the type of epigenetic clock used (Fox et al., 2023; Kresovich et al., 2021; Sillanpää et al., 2019; Spartano et al., 2023). Increased LTPA and device-based PA appear to be most consistently associated with slower biological ageing, according to the DNAm GrimAge estimator.

2.3.7 Smoking

Smoking is the most detrimental lifestyle habit in terms of all-cause mortality (Kaprio & Koskenvuo, 1989; Kujala et al., 2002; Stanaway et al., 2018). Twin studies have indicated that many dimensions of smoking behaviour are regulated by genetic factors; for example, heritability estimates of 44% for smoking initiation and 75% for nicotine dependence have been reported (Vink et al., 2005).

According to systematic reviews, smoking is consistently associated with biological ageing when assessed with second-generation clocks (Oblak et al., 2021), but not when first-generation epigenetic clocks are used (Oblak et al., 2021; Ryan et al., 2019). Smoking has been shown to affect DNAm levels; a recent twin study using discordant MZ twin pairs for smoking behaviours found 13 differentially methylated CpG sites associated with smoking, independent of genetic factors (van Dongen et al., 2023). The DNAm levels within these CpG sites were largely reversible upon smoking cessation. Moreover, several DNAm-based scales for assessing smoking behaviour have been developed (Heikkinen et al., 2022). DNAm GrimAge includes DNAm-based smoking pack years as a component (Lu et al., 2019). DunedinPoAm uses a specific CpG site (located within gene AHRR) whose methylation level is strongly affected by tobacco exposure (Belsky et al., 2020). Therefore, these two estimators of biological ageing are especially sensitive to smoking exposure. Interestingly, DNAm-based smoking included in the DNAm GrimAge estimator, outperformed self-reported pack years in predicting lifespan (Lu et al., 2019). There is some evidence that biological ageing is one mechanism that links lifetime exposure to smoking with the development of disease and premature death in later life (Klopack et al., 2022).

2.3.8 Alcohol use

Excessive alcohol use is a major risk factor for several age-related diseases, such as cancer and liver diseases (Rehm et al., 2017), and all-cause mortality (Xi et al., 2017). However, the association between alcohol use and biological ageing appears to be more inconsistent in comparison to smoking behaviour (Oblak et al., 2021; Ryan et al., 2019). Alcohol use and smoking behaviour are strongly clustered (Noble et al., 2015); therefore, their independent associations with biological ageing might be difficult to disentangle. However, DNAm changes due to alcohol use appear to be much rarer and weaker compared to the effects of smoking exposure (Stephenson et al., 2021).

2.4 Study framework

The framework of this study is illustrated in FIGURE 1. From a life course perspective, biological ageing can be viewed as a continuum from early-life development to morbidity and mortality (Ben-Shlomo et al., 2016; Horvath & Raj, 2018; C. P. Ryan, 2021). The assessment of biological ageing with DNAm-based estimators is considered to capture the lifetime burden of genetic and environmental influences associated with mortality. Consequently, biological ageing as measured by DNAm-based estimators may act as a mediator of these lifetime influences on mortality. Environmental factors, such as lifestyle-related factors during sensitive or critical periods in early life, can have direct long-term effects on biological ageing. Moreover, lifelong exposure to unhealthy lifestyle can have cumulative effects on the biological ageing process. Both lifestyle-related factors and biological ageing are largely heritable; therefore, genetic factors may confound the association of lifestyle-related factors with biological ageing and mortality.

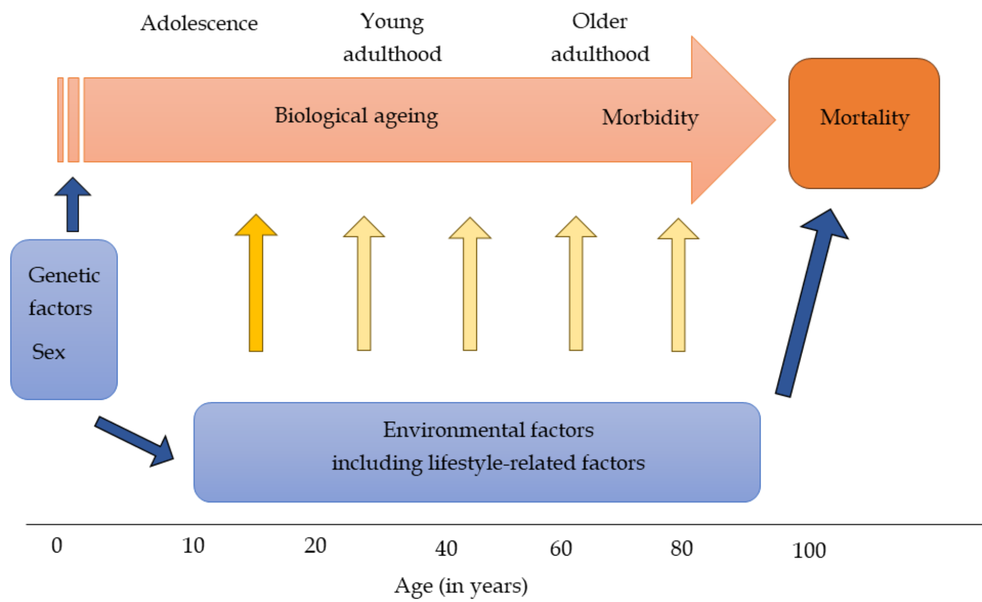


FIGURE 1 The framework of this study. Biological ageing is considered a continuum from early-life development to morbidity and mortality. The dark-yellow arrow indicates the direct long-term effects of environmental factors during sensitive or critical periods of early life, and the light-yellow arrows denote cumulative effects across the life course.

3 PURPOSE OF THE STUDY

The main purpose of this thesis was to investigate the genetic and environmental influences on biological ageing as measured with epigenetic clocks. Specifically, the research questions were as follows:

1. How heritable is biological ageing? (*Studies I* and *III*)

2. What kinds of lifestyle-related patterns can be identified in adolescence? What is the role of adolescent lifestyle in biological ageing? What is the role of underlying familial factors in the potential associations between these phenomena? (*Study I*)

3. Is there a sex difference in biological ageing, and is the difference dependent on familial factors? Do lifestyle-related factors, including education, BMI, PA, smoking and alcohol use, explain the potential sex differences in biological ageing? (*Study II*)

4. Is the association of different types of PA (LTPA and OPA) similar with biological ageing? What is the role of the underlying familial factors in the association? (*Study III*)

5. What kinds of long-term patterns of LTPA can be identified in adulthood? Does long-term LTPA associate with later biological ageing (*Studies III* and *IV*) and all-cause mortality (*Study IV*)? Does biological ageing mediate the potential differences in all-cause mortality (*Study IV*)? Is long-term LTPA associated with all-cause mortality independent of familial factors, and is this association susceptible to reverse causality bias (*Study IV*)?

4 MATERIALS AND METHODS

4.1 Study designs and participants

Data for *Studies I–IV* were retrieved from the Finnish Twin Cohort (FTC), which consists of three large cohort studies. The oldest FTC study included twins born before 1958, FinnTwin16 studied twins born from 1975–1979 and FinnTwin12 included twins born from 1983–1987 (Kaprio, 2013; Kaprio et al., 2019; Rose et al., 2019). All three cohorts included MZ and DZ twin pairs reared together and identified through the Central Finnish Population Register. A more detailed description of each cohort study is provided in the following chapters. The data resources, study designs, outcomes and statistical modelling approaches of the studies are summarised in TABLE 2.

TABLE 2 Summary of the data sources, designs, outcomes, and statistical modelling approaches of *Studies I–IV*.

	Data source	Design	Outcomes	Statistical modelling
I	FinnTwin12	Prospective twin study design	Biological ageing [a,b,c,d,e,f,g+]	LCA, BCH approach, ACDE model
II	FinnTwin12 FinnTwin16 Older cohort	Cross-sectional twin study design, Exposure-discordant twin pairs	Biological ageing [a,b,c,d]	Path analysis, Multilevel modelling
III	FinnTwin12 Older cohort TWINACTIVE	Cross-sectional twin study design, Exposure-discordant twin pairs	Biological ageing [a,c,d]	ACDE model
IV	Older cohort	Prospective twin study design	Biological ageing [f,g], All-cause mortality	LPA, BCH approach, Survival modelling, Multilevel modelling

[a] $AA_{Horvath}$, [b] AA_{Hannum}, [c] AA_{Pheno}, [d] AA_{Grim}, [e] DunedinPoAm, [f] DunedinPACE, [g] $AA_{PC\text{-}Grim}$
+ The estimator was used in the sensitivity analysis.
LCA, latent class analysis; BCH, Bolck–Croon–Hagenaars; ACDE, a biometrical twin model; LPA, latent profile analysis.

4.1.1 FinnTwin12

The longitudinal FinnTwin12 study included twins born from 1983–1987 (Kaprio, 2013; Rose et al., 2019). A total of 5,600 twins and their families initially enrolled in the study. Originally, the main aim of the FinnTwin12 study was to investigate adolescent behavioural development and health habits. At baseline, the twins filled out the questionnaires on their lifestyle-related habits at 11–12 years of age, and follow-up assessments were conducted at ages 14 and 17.5 years, on average. The response rates were high for each assessment (85%–90%). Initially, the study included 810 same-sex and 802 opposite-sex twin pairs.

In young adulthood, at an average age of 22 years, blood samples for DNA analyses were collected from a subsample of twins during in-person clinical studies. Data on health-related behaviours were collected through questionnaires and interviews. A total of 1,295 twins from the FinnTwin12 cohort were examined and measured, either in person or through telephone interviews.

4.1.2 FinnTwin16

The FinnTwin16 study was a nationwide cohort study of the health behaviours of Finnish twins and their families (Kaidesoja et al., 2019; Kaprio, 2006, 2013). It included twin pairs born in 1975–79. The first survey wave took place in 1991–1995, and the questionnaires were sent to 16-year-old twins. For the first wave of data collection, 3,215 twin pairs with both co-twins alive were contacted. Follow-up assessments were conducted at ages 17 and 18.5 years, on average, and in young adulthood (at an average age of 24 years). The response rates were high for each assessment (85%–95%). The fifth and latest survey wave was conducted through a web-based questionnaire in the subjects' mid-thirties (at an average age of 35 years) between 2010 and 2012, with a response rate of 72%.

A subsample of these twins participated in onsite visits, where blood samples were collected for DNA analysis (N = 1,539). Blood-based DNAm was used to assess biological ageing at ages ranging from 23 to 42 years (*Study II*, n = 630).

4.1.3 The older cohort

The oldest FTC study was established 45 years ago, and the data collected have been extensively described (Kaprio et al., 2019). Originally, the FTC consisted of same-sex twins born in Finland before 1958, where both co-twins were alive in 1967. Initially, 36,922 twin individuals were invited to participate. Questionnaires were mailed in 1975 and 1981 to all twins born before 1958 and living in Finland, with a follow-up questionnaire conducted in 1990 with twins born from 1930–1957. The response rates were high (77%–89%) at each measurement. The fourth wave of data collection was conducted in three parts according to birth year from 1999–2017.

Blood samples were taken during the 1990–2020 period, and blood-based DNAm was used to assess biological ageing at ages ranging from 37 to 81 years.

4.1.4 The TWINACTIVE study

PA-discordant twin pairs (TWINACTIVE) were drawn from the older cohort of the FTC. The selection process, which included multiple measurements of PA recorded since 1975, has been described in detail by Leskinen et al. (2009, 2013). LTPA was examined with standardised questionnaires, where scores were quantified as metabolic equivalent (MET; intensity × duration × frequency) and expressed as a sum score of leisure-time MET hours per day (h/day). Twin pairs whose differences in PA volumes were greater than 3 MET h/day were invited to retrospective follow-up interviews on LTPA (covering the years from 1980 to 2005 in 5-year intervals; Leskinen et al., 2009). The interviews were carried out from 2005–2007. Of the 5,663 originally healthy same-sex twin pairs, 16 fulfilled the LTPA discordance criteria and participated in the TWINACTIVE study. For more than 30 years, the mean within-pair difference in LTPA was 8.8 MET h/day.

The study included detailed health-related clinical examinations and DNA sample collections in 2007 (Leskinen et al., 2009).

4.2 Ethics of the study

The data collection and all studies were conducted in accordance with the Declaration of Helsinki. The FTC data collection was approved by Indiana University's institutional review board and the ethics committees of the University of Helsinki (113/E3/01 and 346/E0/05) and Helsinki University Central Hospital (270/13/03/01/2008 and 154/13/03/00/2011). The ethics committee of the Central Finland Health Care District approved the TWINACTIVE study design (August 15, 2006). Blood samples for DNA analyses were collected after written informed consent forms were signed.

4.3 Measurements

4.3.1 Biological ageing

Blood-based DNAm was measured using bisulphite treatment. Bisulphite treatment protects methylated cytosines by converting unmethylated cytosines to uracil residues, which are later converted to thymine during polymerase chain reaction (PCR) amplification. In our study, high molecular weight white blood cell DNA was converted to bisulphite using an EZ-96 DNA Methylation Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Co-twin samples were converted on the same plate to minimise potential batch effects. Genome-wide DNAm was measured using Illumina's Infinium HumanMethylation450 BeadChip and the Infinium MethylationEPIC BeadChip according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The Illumina BeadChip measures single-CpG-resolution DNAm levels across the human genome.

For *Studies I–III*, DNAm data were preprocessed using the R package *minfi* (Aryee et al., 2014). We also used beta-mixture quantile (BMIQ) normalisation implemented in the R package *wateRmelon* (Pidsley et al., 2013) to correct type 2 probe bias (Teschendorff et al., 2013). Detection P values comparing the total signals for each probe to the background signal levels were calculated to evaluate the quality of the sample (or signal; Maksimovic et al., 2017). Samples of poor quality (mean detection $P > 0.01$) were excluded from further analysis. Because the data included samples from different platforms, the data were normalised using the single-sample Noob normalisation method (Fortin et al., 2017). Beta values representing CpG methylation levels were calculated as the ratio of methylated intensities (M) to overall intensities (beta value = $M/(M + U + 100)$, where U is an unmethylated probe intensity). For *Study IV*, the analysis pipeline

was updated, and methylation data were preprocessed using the R package *meffil*, which has been shown to perform preprocessing flexibly and with an efficient use of memory (Min et al., 2018). The pipeline for preprocessing was recently described in detail (Sehovic et al., 2023).

The choice of the DNAm-based measures of biological ageing used for each substudy was primarily based on their availability at the time of the study. For *Study IV*, only the measures that have proven to perform better than their predecessors were chosen (Belsky et al., 2022; Higgins-Chen et al., 2022). A publicly available online calculator (https://dnamage.genetics.ucla.edu/home) was used to generate the estimates of biological age using Horvath's clock (*Studies I–III*) and Hannum's clock (*Studies I–II*) as well as the DNAm PhenoAge (*Studies I–III*) and DNAm GrimAge (*Studies I–III*) estimators. R packages were used to estimate pace of ageing using DunedinPoAm (*Study I*) (Belsky et al., 2020; https://github.com/danbelsky/DunedinPoAm38) and DunedinPACE (*Studies I* and *IV*) (Belsky et al., 2022; https://github.com/danbelsky/DunedinPACE). Moreover, we used R packages to generate biological age estimates using PC-based clocks (*Studies I* and *IV*) (Higgins-Chen et al., 2022; https://github.com/MorganLevineLab/PC-Clocks).

Preprocessed beta values were used as input to estimate biological age. Epigenetic age acceleration (AA), which describes the difference between chronological and biological age estimates in years, was calculated as the residual of a linear regression model of estimated epigenetic age on chronological age. DunedinPoAm and DunedinPACE provide direct estimations of the pace of ageing in years per calendar year. The DNAm-based measures of biological ageing (AA and pace of ageing) were screened for outliers. Estimates more than five standard deviations away from the mean were recoded as missing values.

4.3.2 All-cause mortality

Dates of death were retrieved from the Population Register Centre of Finland and Statistics Finland. The follow-up continued until December 31, 2020.

4.3.3 Zygosity

For the large cohort data, zygosity was based on self-reports (Sarna et al., 1978). Whenever possible, the zygosity of the twin pairs was confirmed using DNA extracted from blood or saliva samples. In particular, zygosity was confirmed for the whole subsample of twins for which information on DNAm was available.

4.3.4 Lifestyle-related factors

Lifestyle-related factors had different roles across the substudies. In *Study I*, lifestyle-related factors in adolescence were treated as indicators in latent class analysis (LCA) to consider adolescent lifestyle as a whole. In *Study II*, they were treated as mediating variables of sex differences in biological ageing. In *Studies III* and *IV*, the main focus was on the associations of PA with biological ageing

(*Studies III* and *IV)* and mortality (*Study IV*). In these studies, other lifestyle-related factors were considered potential confounding variables.

For the main analyses of *Study I*, the lifestyle-related factors measured in adolescence at 12, 14 and 17 years of age were used. In *Studies II* and *III*, lifestyle-related factors measured in adulthood at the time of blood draw were used. In *Study IV,* using the data from the older cohort, LTPA in adulthood was assessed in 1975, 1981 and 1990, but for other lifestyle-related factors, measurements taken in 1981 were used. The twins were 18–50 years old at baseline in 1975. In all four substudies (*Studies I–IV*), lifestyle-related factors were self-reported, except for BMI, which was measured at the time of blood draw.

4.3.4.1 Physical activity

LTPA in adolescence (at ages 12, 14 and 17 years) was assessed with a single question about the frequency of LTPA. At the age of 12 years, LTPA was assessed with the question, 'How often do you engage in sports (i.e. team sports and training)?' The answers were classified as 0 = less than once a week, 1 = once a week and 2 = every day. At 14 and 17 years of age, the question differed slightly: 'How often do you engage in physical activity or sports during your leisure time (excluding physical education)?' The answers were classified as 0 = less than once a week, 1 = once a week, 2 = 2–5 times a week and 3 = every day.

PA measured at the time of blood draw was assessed using the Baecke Questionnaire (Baecke et al., 1982). It includes four questions on sports activity and leisure-time activity (excluding sports) and eight questions on work-related PA scored on a 5-point scale. Activities are scored as 1, 3 or 5 according to how physically demanding they are. A sport index, non-sport leisure-time (leisure) index and work index, respectively, were derived from the mean scores of each section, as earlier described by Baecke et al. (1982) and Mustelin et al. (2012) for the FinnTwin12 study.

LTPA in adulthood was assessed in MET h/day with a structured, validated questionnaire in 1975, 1981 and 1990 (Kujala et al., 1998; Lahti et al., 2010; Waller et al., 2008). In 1975 and 1981, questions concerned the monthly frequency of LTPA and mean session duration and intensity. The MET index was calculated as the product of the frequency, intensity and duration of leisure activities as well as commuting activities, with the resulting values added together (Kujala et al., 1998; Waller et al., 2008). In 1990, the questionnaire differed slightly, and participants reported their time spent on LTPA (including commuting PA) at different intensity levels. The MET index was calculated by multiplying the time spent on LTPA by the estimated MET value of each intensity level and adding up the values (Lahti et al., 2010).

4.3.4.2 Other lifestyle-related factors

Body mass index (BMI; kg/m^2). Adolescent BMI was assessed at ages 12, 14 and 17, and adult BMI in 1981; they were based on self-reported weight and height. BMI at the time of blood draw was calculated based on the measured weight and

height. Self-reports have been shown to agree well with BMI based on measured values (Tuomela et al., 2019).

Smoking status. Smoking status in adolescence (at ages 14 and 17 years) was determined using self-reported frequency of smoking and classified as 0 = never a smoker, 1 = former smoker, 2 = occasional smoker and 3 = daily smoker. Smoking status at the time of blood draw was classified as 0 = never a smoker, 1 = current smoker and 2 = former smoker. Smoking status in 1981 was self-reported based on an extensive smoking history (Kaprio & Koskenvuo, 1988) and classified as 0 = never, 1 = occasional, 2 = former and 3 = current light (1–9 cigarettes per day [CPD]), 4 = current medium (10–19 CPD) and current heavy (≥ 20 CPD) smokers.

Alcohol use. Alcohol use in adolescence (at ages 14 and 17 years) was assessed using the self-reported frequency of drinking to intoxication with the following classes: 'How often do you get very drunk?' The available responses were 0 = never, 1 = less than once a month, 2 = approximately once or twice a month and 3 = once a week or more. Alcohol use (100% alcohol grams/day) at the time of blood draw was derived from the Semi-Structured Assessment for the Genetics of Alcoholism (Bucholz et al., 1994) and based on the quantity and frequency of use and content of alcoholic beverages as assessed by trained interviewers. Alcohol use in 1981 was based on average consumption (g/day) of beer, wine and spirits (Sipilä et al., 2016) and classified as never, former, occasional (> 0.1 and < 1.3 g), low (≥1.3 and < 25 g), medium (≥ 25 and < 45 g), high (≥ 45 and < 65 g) and very high (≥ 65 g) (Zhao et al., 2023).

4.3.5 Potential confounding factors

Pubertal development. The participants' baseline pubertal development at age 12 was assessed using the five-item Pubertal Development Scale (PDS) questionnaire (Petersen et al., 1988). Both sexes answered three questions each concerning growth in height, body hair and skin changes. Moreover, boys were asked questions about their development of facial hair and voice changes, while girls were asked about breast development and menarche. Each question had response categories of 1 = growth/change has not begun, 2 = growth/change has barely started and 3 = growth/change is definitely underway, except for menarche, which was dichotomous, with 1 = has not occurred and 3 = has occurred (see also Wehkalampi et al., 2008). The highest response category on the original questionnaire (development completed) was omitted for all items except menarche because complete development was assumed to be very rare by the age of 12. PDS scores were calculated as the mean score of the five items, and higher values indicated more advanced pubertal development at 12 years of age.

Education. In *Studies II – III*, education in years at the time of blood draw was self-reported. In *Study IV*, education (in years) in 1981 was based on self-reported latest education and converted into years of education as follows: less than primary school (4), primary school (6), junior high school (9), high school graduate (12), university degree (17) and ≥ 1 year of education, such as vocational

training in addition to primary school (8), junior high school (11) or high school (14) (Silventoinen et al., 2004).

Health status. In *Study IV*, an indicator of specific physician-diagnosed somatic diseases (angina pectoris, myocardial infarction and diabetes) based on self-reports from 1975 and 1981 was used.

4.4 Statistical analysis

Statistical analyses were conducted using the Mplus statistical package (version 8.2; Muthén & Muthén, 1998—2017).

4.4.1 Analyses at the individual level (in the general population)

4.4.1.1 Within-twin pair dependence

The family structure of the twin data causes dependencies in the data, as siblings within a family are likely more similar than randomly selected individuals from different families. We used intraclass correlation coefficients (ICCs) to assess this within-family resemblance in the phenotypes measured. Here, ICC was defined as the ratio of between-family variance to total phenotypic variance. ICC values close to 1 reflect a high resemblance of a phenotype within families. Moreover, a higher ICC in MZ twins than in DZ twins indicates genetic influences on a phenotype.

When the associations are estimated at the individual level (in the general population), these dependencies can be viewed as a nuisance, given that basic statistical analysis methods assume independent samples. In *Studies I, II* and *IV*, the analyses conducted in the general population were corrected for nested sampling within families using the 'type = complex' option in Mplus. This approach produces, for example, estimates equal to the basic regression coefficients, but the standard errors (SEs) of the parameters are corrected for non-independence due to family structure using a sandwich estimator (Asparouhov, 2005; Muthén & Muthén, 1998—2017).

4.4.1.2 Latent class analysis

Latent class analysis (LCA) was conducted to identify different adolescent lifestyle behaviours (*Study I*) and long-term LTPA patterns (*Study IV*) at the individual level. LCA belongs to a family of finite mixture models. These techniques involve one or more latent class variables. They are data-driven statistical methods used to identify latent or 'hidden' subgroups in a heterogenous population (Nylund-Gibson & Choi, 2018). The analysis is based on a chosen set of observed variables – latent class indicators – that can be measured at different scales (continuous, categorical, etc.). LCA with categorical variables involves estimating conditional item probabilities across classes. Latent profile analysis (LPA) is a special type of LCA in which all indicators are

continuous. With continuous indicators, conditional means and variances are estimated. Indicator variables are assumed to be conditionally independent within each class; the latent class variable is supposed to capture the associations between indicator variables. The modelling is conducted stepwise: at each step, an additional class is included in the model, and the model fit and classification quality are assessed.

We used several indices to evaluate the goodness of fit of the latent class models (see the original publications for details, *Studies I* and *IV*). In addition to the model fit and classification quality, the final model for further analyses was chosen based on the parsimony (i.e. a simpler model with fewer parameters was favoured over more complex models) and interpretability of the classes.

4.4.1.3 Latent class analysis and distal outcomes

The main goal of S*tudies I* and *IV* was to investigate the mean differences in biological ageing between the identified latent classes. For these purposes, we used the Bolck–Croon–Hagenaars (BCH) approach (a bias-adjusted 3-step approach; Asparouhov & Muthén, 2021). The BCH approach accounts for classification errors in the further analysis, and at the same time, changes in latent class membership are avoided (Asparouhov & Muthén, 2021). The class-specific BCH weights for each participant were computed and saved during the estimation of LCA (or LPA). After that, a secondary model conditional on the latent class variable was specified using weights as training data: DNAm-based measures of biological ageing were treated as distal outcomes one at a time, and the mean differences across classes were studied while adjusting for potential confounding variables. The Wald chi-square test of parameter equalities was used to retrieve the overall values of mean differences in distal outcome between the latent classes. If the Wald test indicated statistically significant ($p < 0.05$) differences, pairwise comparisons were conducted. We calculated 95% confidence intervals (CIs) for the differences. In *Study I*, we further calculated 99% CIs for the mean differences to reduce the risk of Type I errors associated with multiple testing.

4.4.1.4 Mediation models

In S*tudy II*, mediation models were used to test whether the association between sex and AA was direct or mediated by lifestyle-related factors, thereby assessing whether these factors act as potential mechanisms for explaining sex differences in biological ageing. The modelling involved testing the significance of the indirect effects at the 0.05 level quantified as products of path (or regression) coefficients from the independent variable (sex) to the mediators and from the mediators to the outcome (AA) (Preacher et al., 2007).

First, the single-mediator models were fitted. These models included indirect paths from sex to AA through one lifestyle-related factor at a time as well as the direct effect of sex on AA. We further studied whether these associations differed according to age group, that is, whether the age group moderated the associations. For these purposes, interaction terms were included in the models.

Second, a multiple mediation model was fitted to simultaneously assess the mediation effect of the different lifestyle-related factors. The age-specific indirect effects of sex (male) on AA through each mediator variable were calculated using the parameters of the models (see the original publication for details, *Study II*).

4.4.1.5 Survival models

In *Study IV*, a discrete-time survival model (Muthén & Masyn, 2005; Pratschke et al., 2016) was used to investigate the differences in all-cause mortality between the classes with different patterns of long-term LTPA as determined using LCA. A discrete-time survival model offers the advantage of flexible modelling within an SEM framework. For example, it enables the mediation model to be fitted and meaningful indirect effects to be estimated with time-to-event outcomes (Pratschke et al., 2016). We used the year as the unit of discrete-time survival from 1991 to 2020 and constructed a latent variable describing propensity for death. The latent variable was regressed on the latent class membership of long-term LTPA and the potential confounding variables. We report the hazard odds ratios (hORs), which converge on the hazard ratios (HRs) provided by the traditionally used Cox proportional hazards model, as the time units used become more fine-grained (Pratschke et al., 2016).

The best method for relating LCA to time-to-event distal outcomes is still under study (Bakk & Kuha, 2021; Lythgoe et al., 2019). Because the BCH approach was not available for the survival models, we used posterior classification probabilities as sampling weights to control for measurement errors in the classification. In the literature, this approach is referred to as the three-step approach with proportional assignment (Bakk & Kuha, 2021).

4.4.2 Analyses at the within-twin pair level

4.4.2.1 Comparison of exposure-discordant twin pairs

Studies II and *III* involved exposure-discordant twin pairs, where one twin within a pair had the trait while the other did not, indicating discordance for a specific exposure. Exposure-discordant twin pairs provide a setting for examining whether the relationship between an exposure and an outcome is confounded by familial factors, including genetic and common environmental influences (Kaprio & Koskenvuo, 1989; McGue et al., 2010). Especially, it can be hypothesized that any within-twin pair difference in outcome among MZ pairs is due to non-shared environmental factors.

In *Study II,* the within-twin pair differences in biological ageing in opposite-sex twin pairs were tested for significance using a paired samples t-test. In *Study III*, for the same-sex PA-discordant twin pairs (TWINACTIVE), within-twin pair differences and their 95% CIs were calculated for all pairs, DZ pairs and MZ pairs. Among MZ twin pairs, the within-twin pair differences were controlled for shared environmental and (in practice) all genetic factors, whereas among DZ twin pairs, they were controlled for shared environmental factors and partly for

genetic factors. The study design controlled for chronological age and sex, as twins in a pair were of the same sex and age.

4.4.2.2 Multilevel models

In *Studies II* and *IV*, we used multilevel modelling to account for familial confounding. Using multilevel modelling, the models within the SEM framework can be specified for each level of the hierarchical data (Hox, 2012) – in our case, for the between-twin pair level (or between-family level) and the within-twin pair level. In the multilevel SEM, the random intercepts were between-twin pair-level latent variables, which captured the variation in the means of the observed within-twin pair-level variables. For example, for continuous outcome Y, (fixed effect) linear regression would be specified as follows:

$$\text{Within-twin pair (level 1): } Y_{ij} = \beta_{0j} + \beta_1 X_{ij} + e_{ij},$$
$$\text{Between-twin pair (level 2): } \beta_{0j} = \gamma_{00} + u_{0j},$$

where i denotes twin i, i = 1, 2; j denotes family number; Y_{ij} is individual-level outcome, X_{ij} individual-level covariate, β_{0j} random intercept, β_1 fixed effect, e_{ij} individual-level random residual, γ_{00} fixed (average) intercept for family level, u_{0j} random residual for family level.

In *Study II*, mediation models were fitted at the within-twin pair level among DZ opposite-sex twin pairs. In *Study IV*, survival models were fitted at the within-twin pair level in all pairs, DZ pairs and MZ pairs. The within-twin pair estimates were controlled for shared environmental and genetic factors.

4.4.3 Quantitative genetic modelling

4.4.3.1 The basic biometrical twin model (univariate model)

In S*tudies I* and *III,* we used quantitative genetic modelling within the SEM framework to assess the genetic and environmental influences on biological ageing. The basic biometric ADCE model (univariate model) decompose the total variation in a phenotype into additive genetic (A), dominant genetic (D), common environmental (C) and non-shared environmental components (E) (Neale & Cardon, 1992). The observed phenotype (P) is considered the linear function of the following components:

$$P_i = aA_i + dD_i + cC_i + eE_i$$

where i denotes twin i, i = 1, 2. A path diagram of the univariate model we applied is presented in FIGURE 2. Genetic and environmental factors (components A, D, C and E) are specified as latent variables. They are not measured directly; rather, their influence is inferred through their effects on the

covariances of the corresponding twins' siblings. Measurement error is modelled as part of component E.

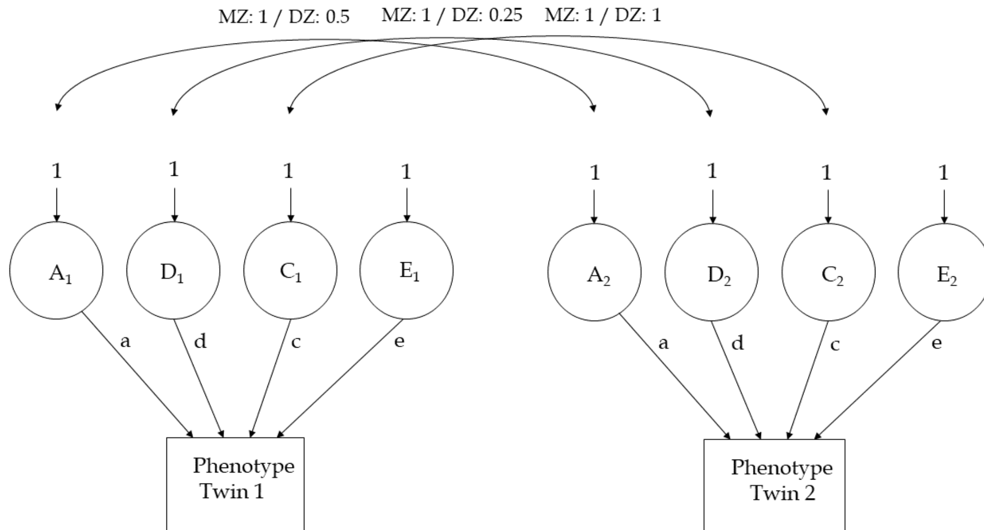


FIGURE 2 Path diagram of the basic univariate genetic model, including additive genetic (A), dominant genetic (D), common environmental (C) and non-shared environmental (E) components. MZ, monozygotic; DZ, dizygotic.

The empirical data can be summarised as observed covariance matrices. In order to test the hypotheses, the expected covariance matrices need to be derived from the model (TABLE 3). When using data in the classical twin study design, where MZ and DZ pairs are reared together, the model that includes all four components – A, C, D and E – is not identifiable (Neale & McArdle, 2000). This means that the number of free parameters in the model (a, d, c and e) exceeds the number of equations in the expected covariance matrix. Therefore, the parameters of the model cannot be uniquely determined from the available data. Unless we have information on either twin reared apart or other relatives, it is necessary to assume that one of the free parameters is zero. Moreover, components C and D cannot be simultaneously estimated.

TABLE 3 Expected covariance matrices for monozygotic and dizygotic twin pairs as derived from the model, including additive genetic (A), common environmental (C) and non-shared environmental (E) components.

Monozygotic twin pairs			Dizygotic twin pairs		
	Twin 1	Twin 2		Twin 1	Twin 2
Twin 1	$a^2 + d^2 + c^2 + e^2$	$a^2 + d^2 + c^2$	Twin 1	$a^2 + d^2 + c^2 + e^2$	$0.5 * a^2 + 0.25*d^2 + c^2$
Twin 2	$a^2 + d^2 + c^2$	$a^2 + d^2 + c^2 + e^2$	Twin 2	$0.5 * a^2 + 0.25*d^2 + c^2$	$a^2 + d^2 + c^2 + e^2$

In *Studies I* and *III*, the sequences of the models were fitted (ACE, ADE, AE, CE and E), and these submodels were compared. Models that contain dominant genetic influences (D) in the absence of additive genetic influences (A) are biologically implausible (Posthuma et al., 2003), and the DE model was therefore omitted. We used several model fit indices to evaluate the goodness of fit between the hypothesised model and the observed data (see the original publications for details, *Studies I* and *III*) as recommended by Hu & Bentler (1999). The most parsimonious model with an adequate model fit was selected. Using the parameters of the model, (narrow-sense) heritability was evaluated as the additive genetic variation (a^2) relative to the total variation ($a^2 + c^2 + e^2$).

4.4.3.2 Assumptions of twin models

There are several assumptions on which quantitative genetic models rely, and violations of these premises may bias estimates of genetic and environmental influences. These assumptions include an equivalence of means and variances for twin 1 and twin 2 as well as for MZ and DZ twin pairs. There are also assumptions that usually cannot be directly tested unless an appropriate study design is used. These include assumptions about an equal environment, random mating and a lack of gene–gene (epistasis) and gene–environment interactions (Posthuma et al., 2003). The equal environment assumption means that the same amount of phenotype-relevant environmental variation affects MZ and DZ twin pairs. For example, MZ twins in a pair may be treated more similarly as children than DZ twins in a pair, which may violate the assumption and lead to an overestimation of genetic influences. The classical twin design assumes random mating in the parents of twins. This assumption is known to be violated for several phenotypes, such as body height and weight (Silventoinen et al., 2003), as individuals select partners phenotypically similar to themselves (positive assortative mating). If the phenotype is heritable, assortative mating results in increased genetic correlation in the next generation. In twin studies, this leads to increased within-pair similarity in DZ twins relative to MZ twins and, subsequently, underestimation of genetic effects and overestimation of shared environmental effects.

4.4.3.3 Bivariate twin model

In *Study III*, the genetic and environmental effects shared by PA and biological ageing as measured by AAs were studied. Accordingly, Cholesky's decomposition (de Jong, 1999) was applied to each PA index (phenotype 1) and AA measure (phenotype 2) while controlling for potential confounding variables as covariates (FIGURE 3). The latent variables representing PA (CH1) and the residual of AA after the effect of PA has been taken into account (CH2) were specified. The variation in latent variables CH1 and CH2 was partitioned into A, C/D and E components. The covariates were added sequentially to the model. The parameters of the model were used to calculate the relative proportions in the total variation of AA $[(a_1^2 + c_1^2 + e_1^2) * b + (a_2^2 + c_2^2 + e_2^2)]$ explained by the genetic and environmental effects shared with PA ($a_1^2 * b^2$, $c_1^2 * b^2$ and $e_1^2 * b^2$,

respectively). These proportions reflect the extent to which the same genetic or environmental factors contribute to the association between PA and biological ageing (i.e. the size of the genetic and environmental correlations between the phenotypes).

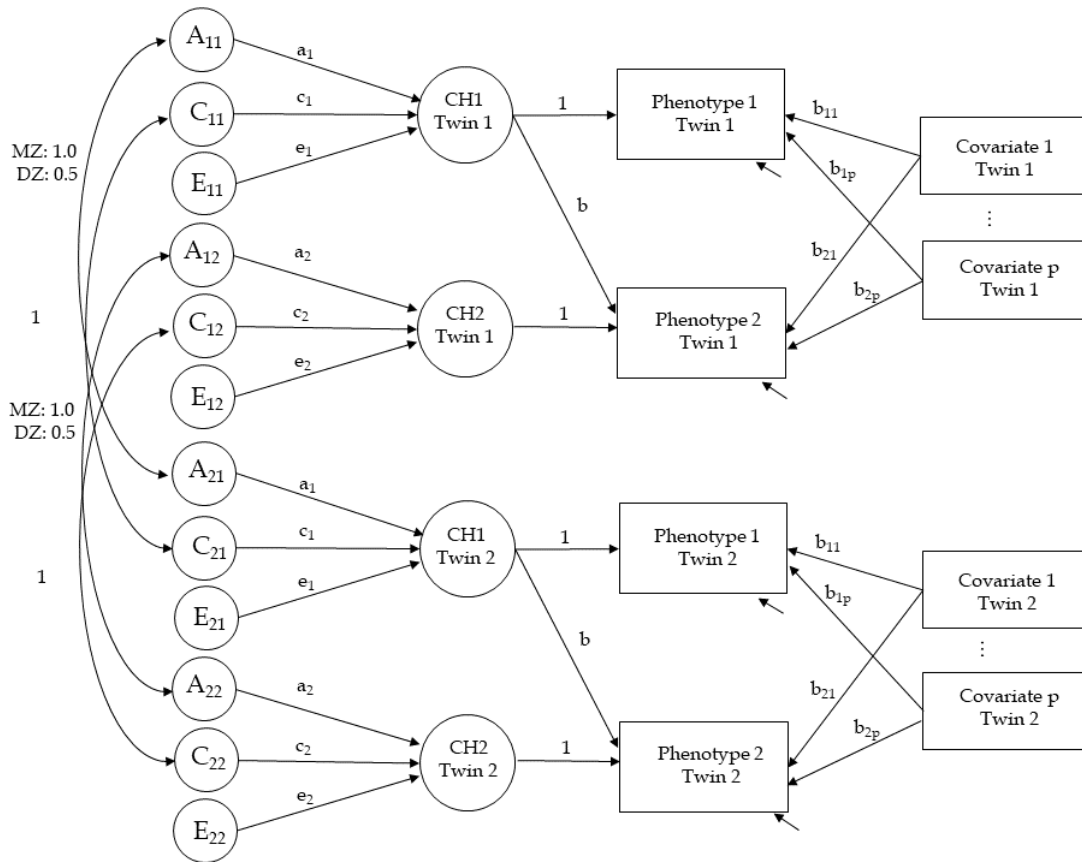


FIGURE 3 Path diagram of the bivariate structural equation model. Circles denote latent variables; rectangles denote observed variables. CH1 represents Phenotype 1, and CH2 represents the residual of Phenotype 1 after the effect of Phenotype 2 has been taken into account. A, additive genetic; C, shared environmental; E, non-shared environmental components; MZ, monozygotic; DZ, dizygotic.

In *Study I,* we studied the shared genetic and environmental influences underlying adolescent lifestyle and biological ageing. Due to the nominal nature of the latent class variable of adolescent lifestyle, it was not appropriate to conduct bivariate modelling to decompose the covariation between these two phenotypes. Instead, we conducted the modelling stepwise. First, the total variance in biological ageing was decomposed into A, C/D and E components. Second, we conducted univariate modelling for the residual term of biological ageing retrieved from the BCH model, which corresponds to the variation in biological ageing not explained by adolescent lifestyle behaviour patterns. Finally, the proportion of variation in biological ageing explained by the genetic and environmental factors shared with adolescent lifestyle patterns was evaluated using the estimated parameters of these two models. The technical details of our approach can be found in the original publication (*Study I*) and the

codes in its additional files (https://cdn.elifesciences.org/articles/80729/elife-80729-supp1-v1.txt).

4.4.4 Effect sizes

Standardised mean differences (SMD) were used as effect size measures of the mean differences between the groups. In *Studies II* and *III*, standardised regression coefficients were reported to provide information on the magnitude of the effect. For SMD, effect sizes of 0.2, 0.5 and 0.8 represent small, medium and large effects, respectively, and for correlations (and standardised regression coefficients), the corresponding levels are 0.1, 0.3 and 0.5 (Cohen, 1992). Moreover, in *Studies I* and *III*, we explored the proportions of total variation in biological ageing explained by genetic and environmental factors, reflecting the effect sizes of these influences.

4.4.5 Handling missing data and estimation methods

In all studies, missing data were assumed to be missing at random (MAR); that is, the absence of data for particular observation depends only on the observed data (covariates and outcome) and not the missing data itself (Little et al., 2014). In other words, the probability of missing data is a function of the values observed in the data. The model parameters were estimated using the full information maximum likelihood (FIML) method with robust standard error (MLR). Under the MAR assumption, the FIML method produced unbiased parameter estimates. When applied to incomplete data, FIML uses all the available information, maximising the statistical power, and produces estimates that correctly describe the entire sample (Little et al., 2014). The expectation-maximisation (EM) algorithm was used to perform estimations in the presence of latent variables. The MLR estimator is robust against slight deviations from the normality assumption. In *Study II*, in models including the ordinal mediator variable smoking status, the estimation was conducted using a robust, weighted least-squares estimator (WLSMV). In this case, the mediator was assumed to be the continuous latent variable underlying ordinal smoking status.

5 RESULTS

5.1 Participant characteristics

The samples used in the studies are briefly summarised in TABLE 4. In *Study I*, data from the full FinnTwin12 cohort were retrieved to identify adolescent lifestyle-related patterns. Twins for whom there was at least some information on lifestyle-related habits in adolescence were included in the LCA. Twins for whom there was also information on biological ageing in young adulthood were included in the subsample. In *Studies II* and *III,* twins for whom there was information on biological ageing were included. In *Study II*, data were retrieved from all three cohorts of the FTC (FinnTwin12, FinnTwin16 and older cohort), but the data were analysed according to age group, namely, 21–42-year-old twins (comprising data on FinnTwin12 and FinnTwin16 twins) and twins over 50 years old (comprising data from the older cohort). Moreover, the younger age group included 151 opposite-sex twin pairs (aged 21–30 years). In *Study III,* data were retrieved from FinnTwin12 and the older cohort, and only same-sex twin pairs for both of whom there was information on biological ageing were included. The older cohort also included 16 LTPA discordant twin pairs (TWINACTIVE; aged 50–74 years). Out of the total pairs, 7 were MZ and 5 were female pairs. The mean (SD) age of the PA-discordant twin pairs was 60.4 (6.2) years. In *Study IV*, data were retrieved from the older cohort. Data from the full cohort were used to identify long-term patterns of LTPA and their associations with all-cause mortality. Participants aged 18–50 years at baseline in 1975 for whom there was at least one measurement of LTPA and who were alive in 1990 were included in the analysis. The subsample consisted of participants for whom information was available on both LTPA and biological ageing.

Biological age estimates for the subsamples are also presented in TABLE 4. *Study III* was published first, and since then, more DNAm array data have been generated, increasing the sample size in subsequent studies. In the subsample of

older twins for whom information on biological ageing was available, women and non-smokers were overrepresented in comparison to the full cohort data (see the original publication for details, *Study IV*). Otherwise, the subsamples appeared to represent the larger cohorts (*Study I* and *Study IV*) very well. More detailed characteristics of the study samples can be found in the original publications (*Studies I–IV*).

TABLE 4 Samples used in *Studies I–IV*.

	Study I		**Study II**		**Study III**		**Study IV**	
	Full cohort	Sub-sample (21–25 y)	Younger adults (21–42 y) [a]	Older (50–76 y)	Young adults (21–25 y)	Older (55–76 y) [b]	Full cohort	Sub-sample (37–81 y)
N	5,114	824	1,477	763	577	470	22,750	1,153
Zygosity (% of MZ)	34.0	40.7	42.2	53.5	57.2	62.6	28.4	52.9
Sex (% of women)	50.5	57.0	53.6	81.4	61.4	60.0	50.3	80.9
At the time of blood draw								
Age in years, mean (SD)		22.4 (0.7)	24.6 (3.4)	65.7 (4.9)	22.4 (0.7)	62.4 (4.1)		63.5 (9.1)
Body mass index in kg/m^2, mean (SD)		23.4 (3.9)	23.8 (4.4)	27.7 (4.8)	23.3 (3.9)	27.1 (4.8)		27.0 (4.8) [c]
Smoking (% of current smokers)		36.7	32.1	10.1	35.8	15.6		23.1 [c]
Biological age in years as assessed with								
Horvath's clock, mean (SD)		28.9 (3.6)	31.8 (5.4)	65.2 (6.0)	25.0 (3.5)	61.0 (6.8)		-
Hannum's clock, mean (SD)		18.2 (3.3)	20.2 (4.4)	55.5 (5.8)	-	-		-
DNAm PhenoAge, mean (SD)		13.0 (5.3)	15.1 (6.6)	55.9 (7.6)	15.0 (4.6)	55.3 (7.0)		-
DNAm GrimAge, mean (SD)		25.2 (3.3)	27.0 (4.6)	58.7 (5.1)	28.6 (3.1)	60.2 (5.3)		70.9 (7.3) [d]
Pace of ageing in years per calendar year								
DunedinPoAm, mean (SD)		1.00 (0.07)	-	-	-	-		-
DunedinPACE, mean (SD)		0.88 (0.10)	-	-	-	-		0.98 (0.12)

MZ, monozygotic; DNAm, DNA methylation.

[a] The sample included 151 opposite-sex twin pairs.

[b] The sample included 16 twin pairs who were discordant in leisure-time physical activity (TWINACTIVE).

[c] N = 1,101.

[d] The principal component (PC)-based DNAm GrimAge estimator was used.

5.2 Heritability of biological ageing (*Studies I* and *III*)

In *Studies I* and *III*, the heritability of biological ageing was investigated using univariate models. In both studies, the ICCs for DNAm-based measures of biological ageing were considerably higher in MZ twin pairs (0.62–0.72) than in DZ pairs (0.16–0.49), suggesting the existence of a genetic component in biological ageing. For the most part, the assumptions of genetic twin modelling were met. There were no systematic differences between twin 1 and twin 2 within a pair or between MZ and DZ twins in the means or variances of the DNAm-based measures of biological ageing. An AE model, which included additive genetic and non-shared environmental components, was considered optimal for all measures used.

The heritability estimates we derived were 69% for $AA_{Horvath}$ (*Study III*), 64%–65% for AA_{Pheno} (*Studies I* and *III*), 62%–73% for AA_{Grim} (*Studies I* and *III*), 62% for DunedinPoAm (*Study I*) and 68% for DunedinPACE (*Study I*) in younger twins (FinnTwin12). The corresponding figures were 61% for $AA_{Horvath}$, 60% for AA_{Pheno}, and 58% for AA_{Grim} in older cohort (*Study III*). There were no statistically significant differences at 0.05 level in the heritability estimates between the age cohorts. Correspondingly, non-shared environmental factors accounted for the remainder (27%–42%) of the variation in biological ageing.

5.3 Adolescent lifestyle and biological ageing (*Study I*)

In *Study I*, adolescent lifestyle was treated as a whole, integrating longitudinal information on several lifestyle-related factors during childhood and adolescence. The classification was conducted using LCA and based on continuous BMI; categorical LTPA at 12, 14 and 17 years of age; and smoking status and alcohol use at ages 14 and 17 years (10 indicator variables). We identified five classes with different adolescent lifestyle patterns (FIGURE 4). Of the participants, 32% fell into the class of healthiest lifestyle habits (C1). Classes C2–C4 were mainly extracted based on BMI levels. About a tenth (9.5%) of the participants belonged to the fourth class (C4), in which the mean BMI exceeded the cutoff points for overweight in children at each measurement point (Cole et al., 2000). Of the participants, 15.9% were classified into the subgroup characterised by the unhealthiest lifestyle behaviours (C5). Most were daily smokers and regular alcohol users at the age of 17. They were also less likely to engage in regular LTPA than the other groups.

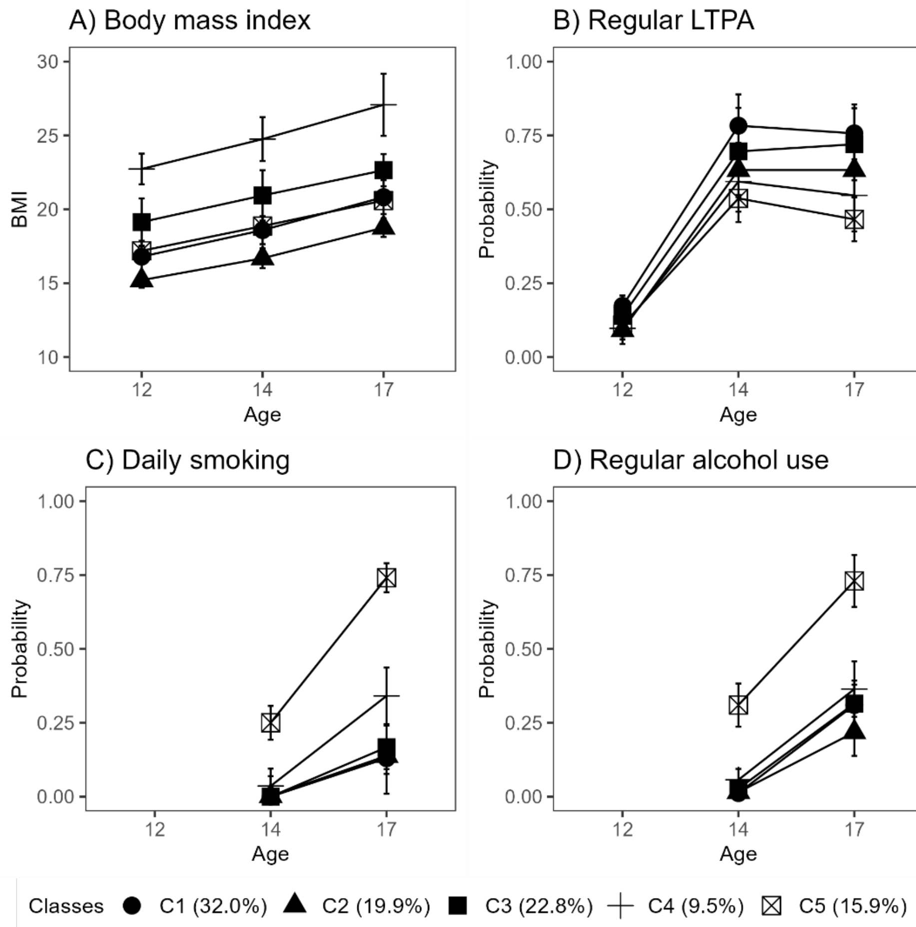


FIGURE 4 Classes C1–C5 with different lifestyle patterns (n = 5,114). The means and probability profiles (95% confidence intervals) of the indicator variables used in the classification are presented: A) body mass index, B) regular leisure-time physical activity (LTPA; several times a week), C) daily smoking and D) regular alcohol use (once a month or more). For categorical variables, the probabilities of belonging to the highest categories are presented.

There were differences across classes in biological ageing as measured with AA_{Pheno}, AA_{Grim}, DunedinPoAm and DunedinPACE (FIGURE 5), but not when first-generation clocks were used (Wald test: p = 0.49–0.55). The model was adjusted for sex, age and baseline pubertal development. After applying 99% CIs for mean differences, the group with the unhealthiest lifestyle pattern (C5) and the group with a high BMI (C4) differed quite consistently from the groups with healthier patterns. The group with the unhealthiest lifestyle pattern (C5) was, on average, biologically older than the classes with healthier lifestyle patterns and normal weight (C1–C3) when DNAm GrimAge and DunedinPoAm were used to assess biological ageing (SMD = 0.53–1.01). When DunedinPACE was used, the group differed only from the class with a low–normal BMI (C2; SMD = 0.60). The group with a high BMI (C4) was biologically older than the groups with healthier lifestyle patterns (C1–C2) when DNAm GrimAge, DunedinPoAm and DunedinPACE were used (SMD = 0.56–1.00). When DNAm PhenoAge was used to assess biological ageing, only the group with a high BMI stood out. This group

was biologically older than the groups with lower BMIs (C2, C5; SMD = 0.44–0.48).

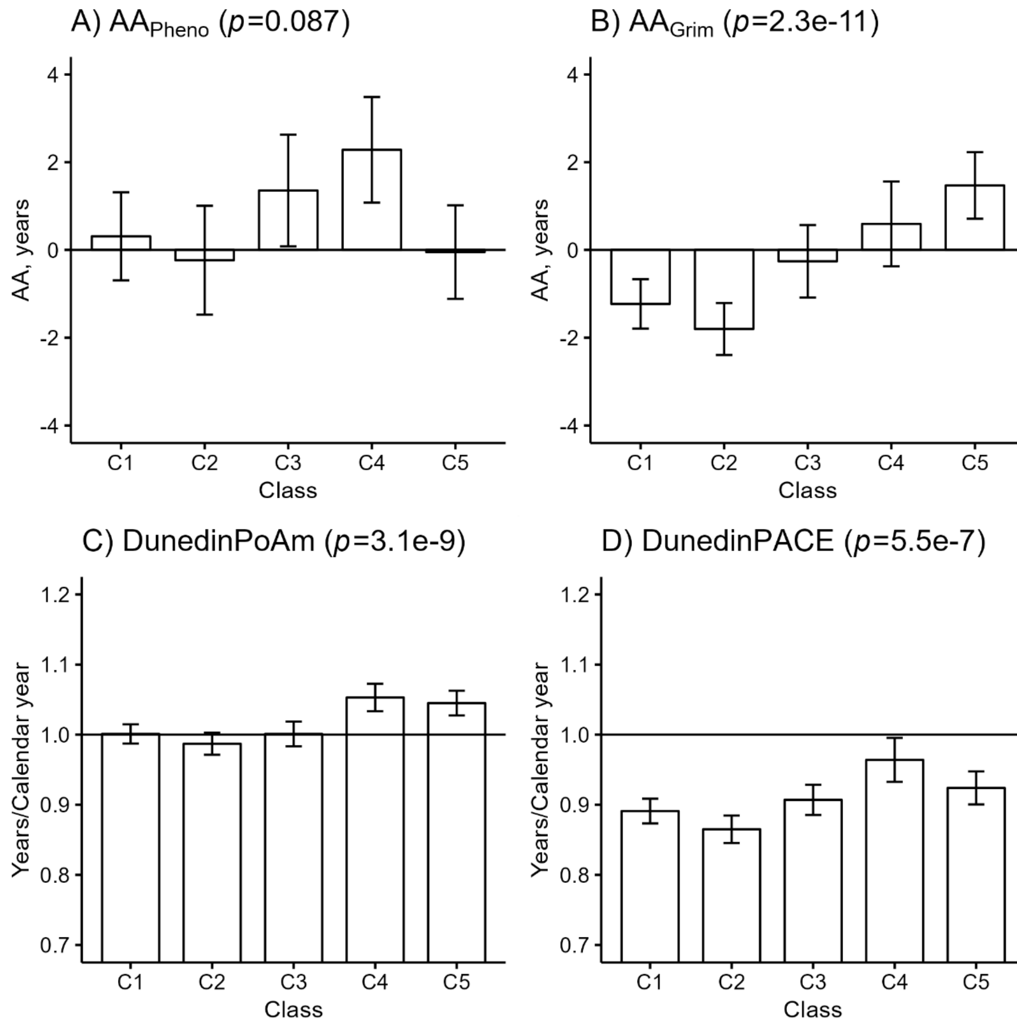


FIGURE 5 Mean differences between adolescent lifestyle behaviour patterns in biological ageing as measured with (A) DNAm PhenoAge, (B) DNAm GrimAge, (C) DunedinPoAm and (D) DunedinPACE estimators (n = 824). The model was adjusted for sex (female), age and pubertal development. Means and 95% confidence intervals are presented. C1 was the class with the healthiest lifestyle pattern. C2 was the class with a low-normal body mass index (BMI). C3 was the class with a healthy lifestyle and a high-normal BMI. C4 was the class with a high BMI. C5 was the class with the unhealthiest lifestyle patterns. AA represents age acceleration. P-values were determined using the Wald test.

The proportion of the total variation in biological ageing in early adulthood explained by adolescent lifestyle behaviour patterns was 3.7% for AA_{Pheno}, 16.8% for AA_{Grim}, 15.4% for DunedinPoAm and 10.5% for DunedinPACE (FIGURE 6). The genetic factors shared with adolescent lifestyle explained 3.7%, 13.1%, 12.6% and 10.5%, respectively, of the total variation in biological ageing. Therefore, the

association between adolescent lifestyle patterns and biological ageing in early adulthood was largely explained by shared genetic influences.

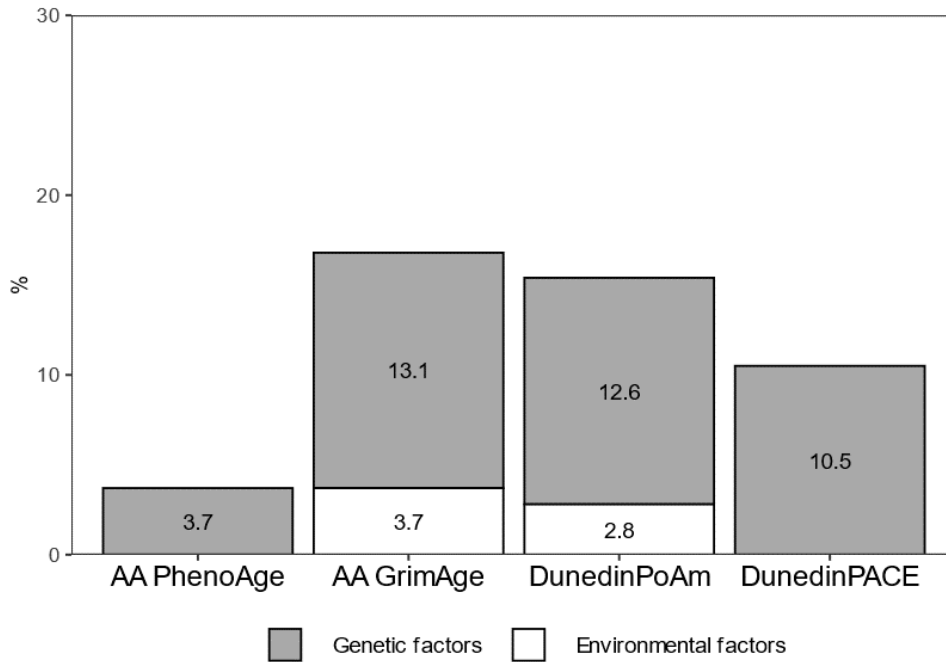


FIGURE 6 Proportions of total variation in biological ageing explained by genetic and specific environmental factors shared with adolescent lifestyle patterns among young adult twin pairs.

5.4 Sex differences in biological ageing (*Study II*)

In *Study II*, sex differences in biological ageing were investigated. Overall, the men had higher AA than the women (except for AA_{Pheno}), and the sex difference in AA tended to increase with age (FIGURE 7, A–B). The effect sizes for sex differences were small in younger adults (SMD = 0.29–0.30) and large in older adults (SMD = 0.67–0.95). When the difference was controlled for shared childhood environment and partly for genetic factors among the opposite-sex twin pairs (aged 21–30 years), the male twins had higher AA_{Hannum} and AA_{Grim} than their sisters (SMD = 0.41–0.42), but there were no significant sex differences at the 0.05 level in $AA_{Horvath}$ and AA_{Pheno} (FIGURE 7C).

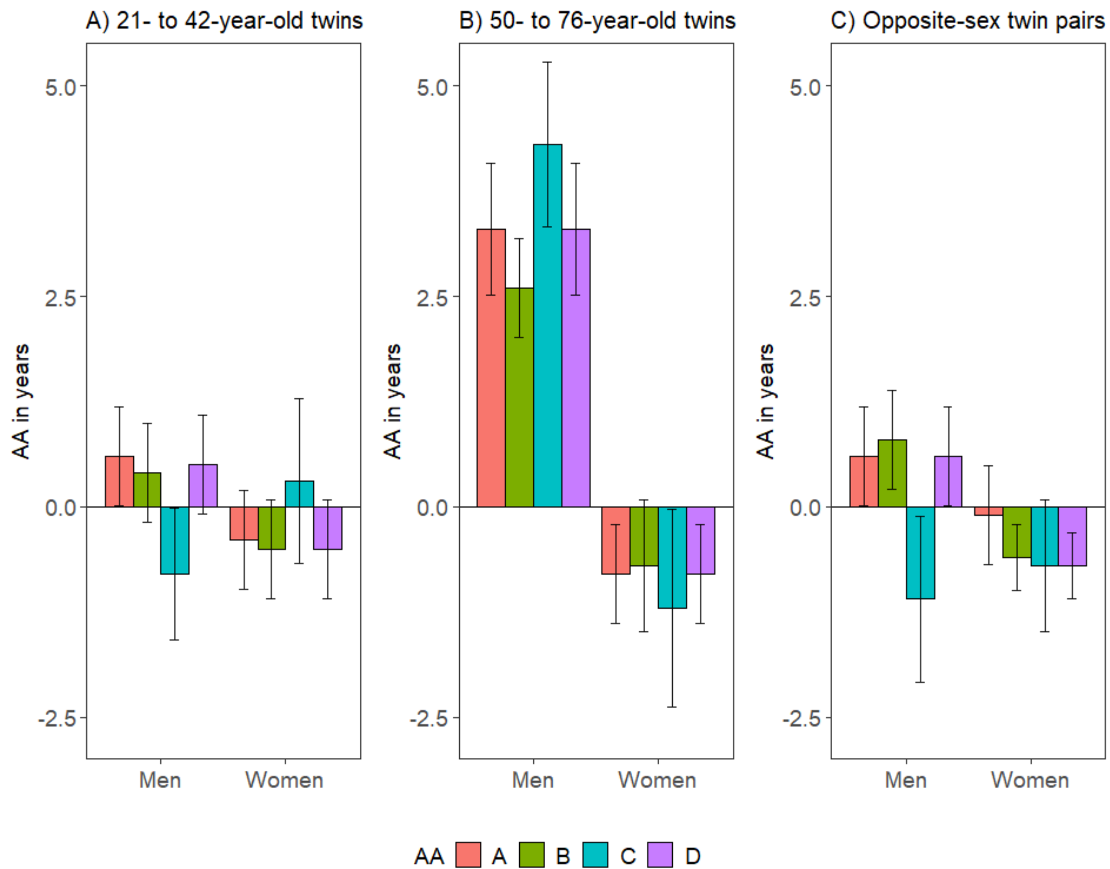


FIGURE 7 Sex differences in biological ageing in A) younger, B) older and C) opposite-sex twin pairs. Means and 95% confidence intervals are presented. AA, age acceleration. (A) $AA_{Horvath}$, (B) AA_{Hannum}, (C) AA_{Pheno} and (D) AA_{Grim}.

According to the results of the single-mediator models, several lifestyle-related factors mediated the sex differences in biological ageing (see the original publication for details, *Study II*). Based on these results, the final multiple mediator model included education, BMI, smoking, alcohol use, and leisure index as mediators (FIGURE 8). When the lifestyle factors were controlled for each other, male sex was associated with lower AA_{Grim} through a higher level of education in older twins (FIGURE 9). BMI partly mediated the association between sex and AA in both cohorts ($AA_{Horvath}$, AA_{Pheno} and AA_{Grim}). Moreover, smoking partially mediated the association between sex and AA (AA_{Hannum}, AA_{Pheno} and AA_{Grim}), and alcohol use partially mediated the association between sex and AA_{Pheno}, albeit only in the older twins. Overall, male sex still had a direct positive effect on biological ageing after all adjustments, and the association was stronger in the older cohort (FIGURE 8).

In the opposite-sex twin pairs, lifestyle-related factors did not mediate the differences in AA between the men and their female twin sisters. The only exception was observed when Horvath's clock was used: BMI partially mediated the sex difference in $AA_{Horvath}$.

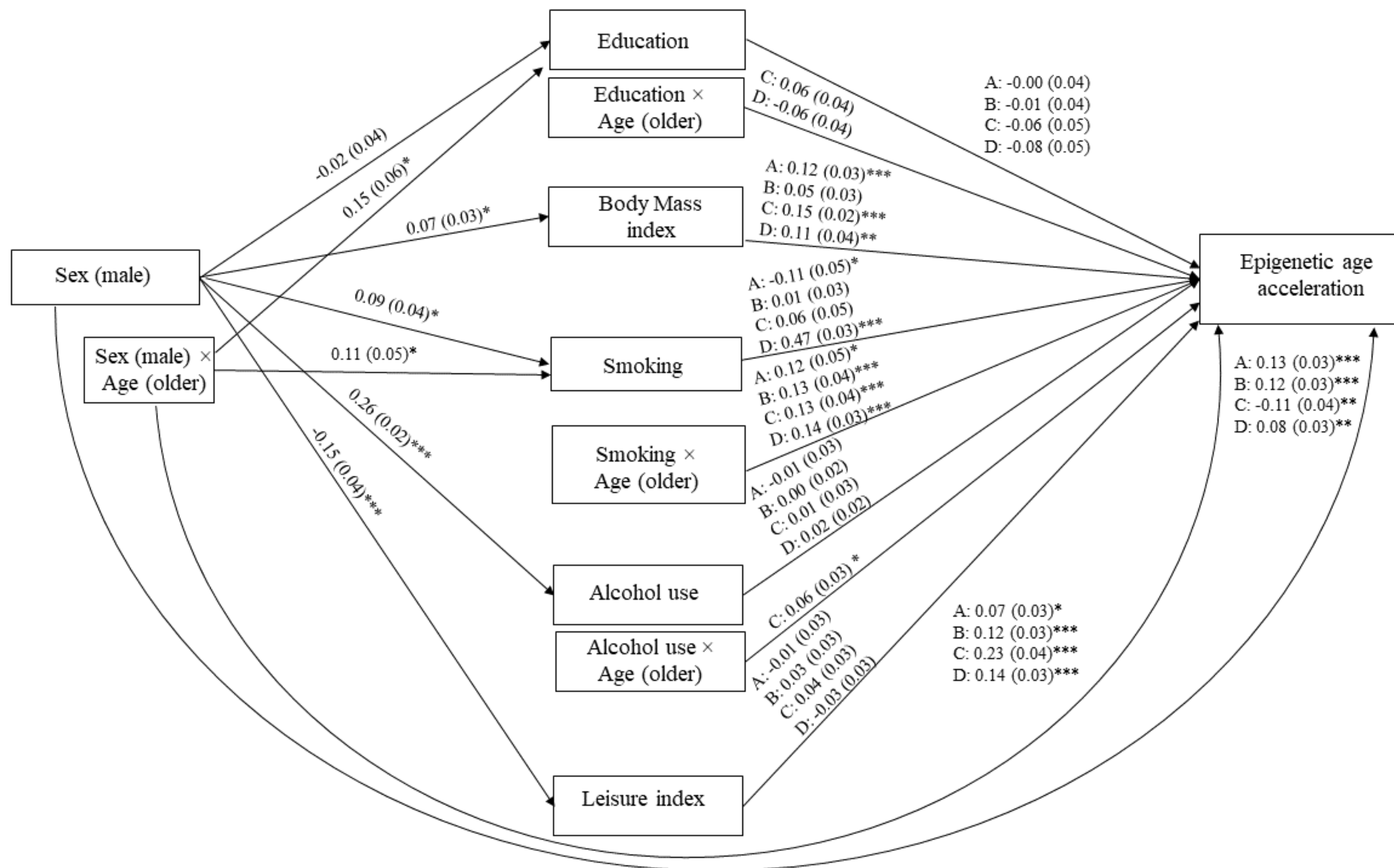
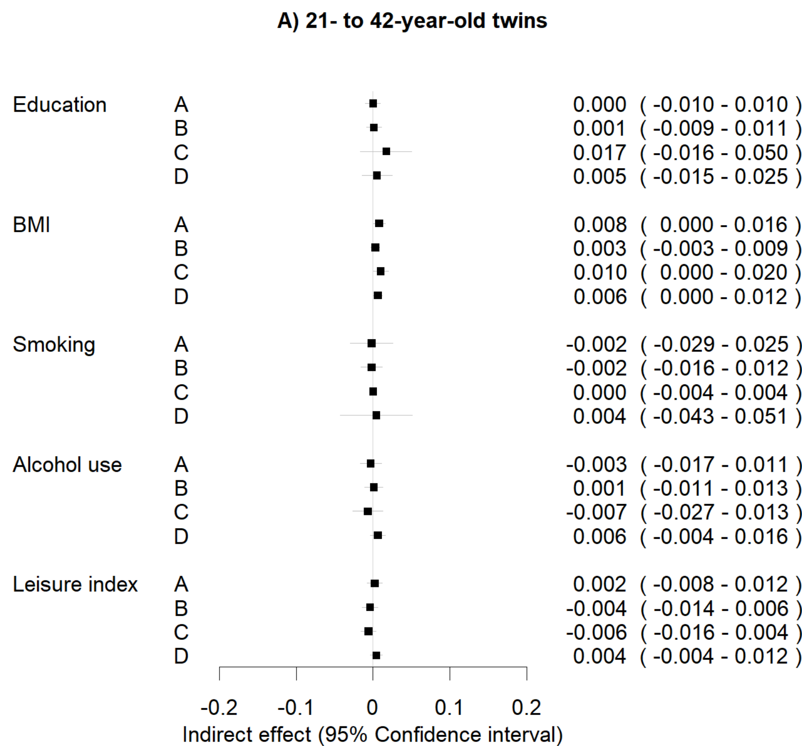


FIGURE 8 Path diagram of the multiple mediator model encompassing all twins (n = 2,240). Standardised regression coefficients (standard errors) are presented. The modelling was conducted separately for each epigenetic age acceleration (AA) measure: (A) $AA_{Horvath}$, (B) AA_{Hannum}, (C) AA_{Pheno} and (D) AA_{Grim}. ****p* < .001, ***p* < .01, **p* < .05.



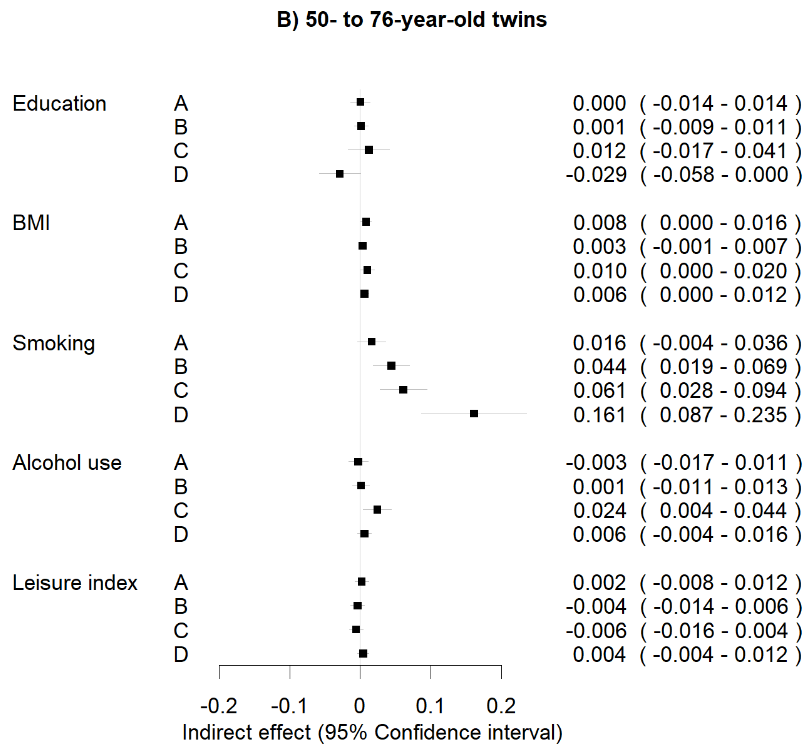


FIGURE 9 Standardised indirect effects (with 95% confidence intervals) of sex (male) on biological ageing via potential mediator variables in A) 21- to 42-year-old twins and B) 50- to 76-year-old twins. BMI, body mass index; A, $AA_{Horvath}$; B, AA_{Hannum}; C, AA_{Pheno}; D, AA_{Grim}.

5.5 Associations between physical activity and biological ageing (*Studies III* and *IV*)

In *Study III*, cross-sectional associations between different forms of PA and biological ageing were studied. Sport index and leisure index correlated with slower and work index with faster AA_{Grim} in both young adult and older twins, but not with $AA_{Horvath}$ and AA_{Pheno}. Therefore, further bivariate twin modelling was conducted only for AA_{Grim}.

In young twin pairs, genetic and environmental factors shared with sport index explained 2.7% and 1.9%, respectively, of the variation in AA_{Grim} after adjusting the model for sex (FIGURE 10, A—C). The corresponding proportions were 0.6% and 0.7% for leisure index and 1.8% and 1.7% for work index, respectively. In older twin pairs, genetic and environmental factors shared with sport index explained 1.5% and 3.5%, respectively, of the variation in AA_{Grim} (FIGURE 10, D—F). The corresponding proportions were 0.8% and 2.4% for leisure index and 0.4% and 0.7% for work index. In both cohorts, the proportions of the variation in AA_{Grim} explained by genetic and environmental factors shared with PA indices were minor (< 0.5%) after including smoking status in the models.

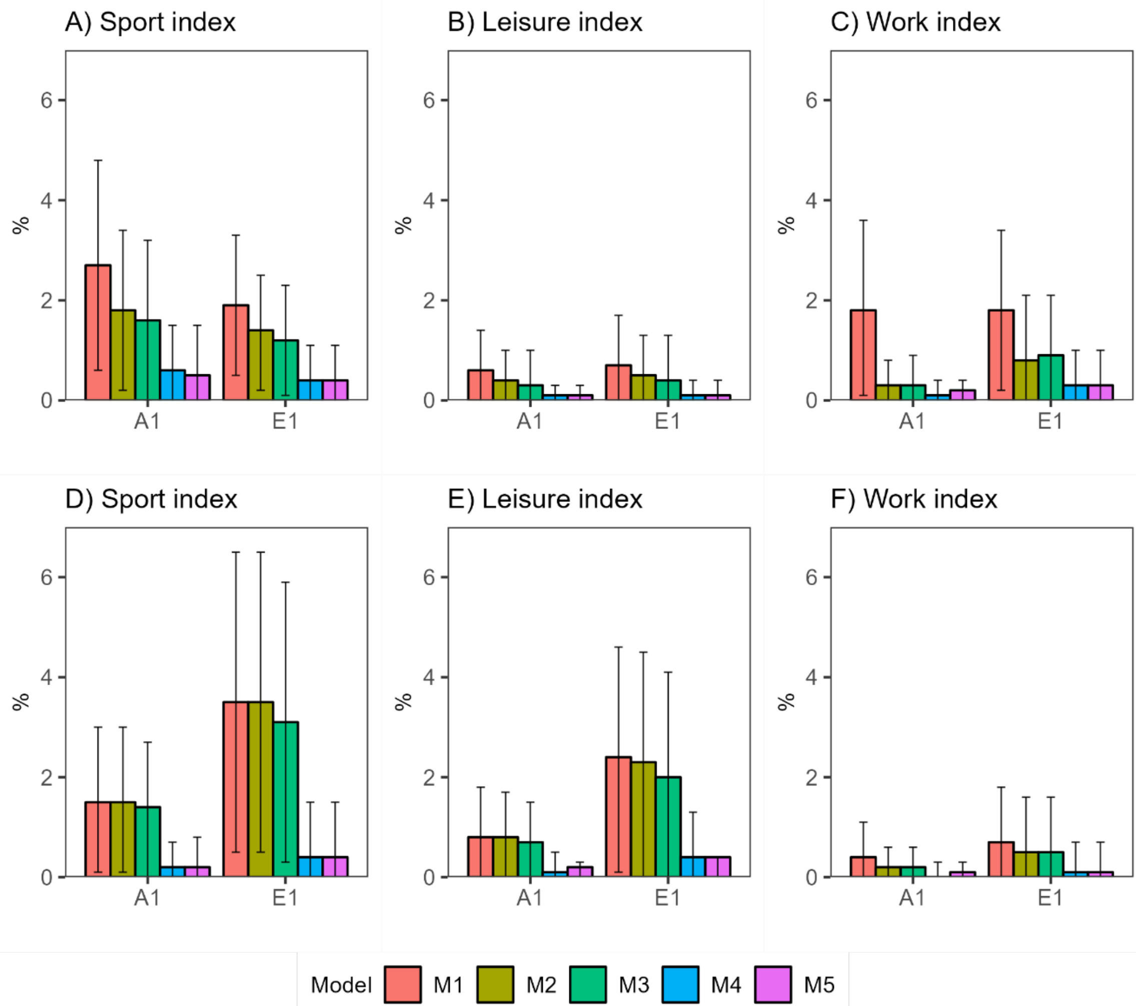


FIGURE 10 Proportion of the variation (%) in biological ageing (AA_{Grim}) explained by genetic and environmental effects shared with physical activity index in young adult (A—C) and older twin pairs (D—F). A1, additive genetic influences; E1, non-shared environmental influences; M1, adjusted for sex; M2, M1 + education; M3, M2 + body mass index; M4, M3 + smoking status; M5, M4 + alcohol use.

In *Studies III* and *IV*, we investigated the association between long-term LTPA and biological ageing. In *Study III*, the within-pair differences in biological age estimates between LTPA-discordant twin pairs were studied. When biological age was assessed using Horvath's clock, there were no differences between the active and inactive co-twins among the LTPA-discordant twin pairs, as previously reported (Sillanpää et al., 2019). However, active twins were biologically younger than their inactive co-twins when DNAm PhenoAge and DNAm GrimAge were used in all pairs (SMD: 0.37−0.56; FIGURE 11). The differences were only slightly attenuated in MZ pairs but did not reach statistically significant degree at the 0.05 level.

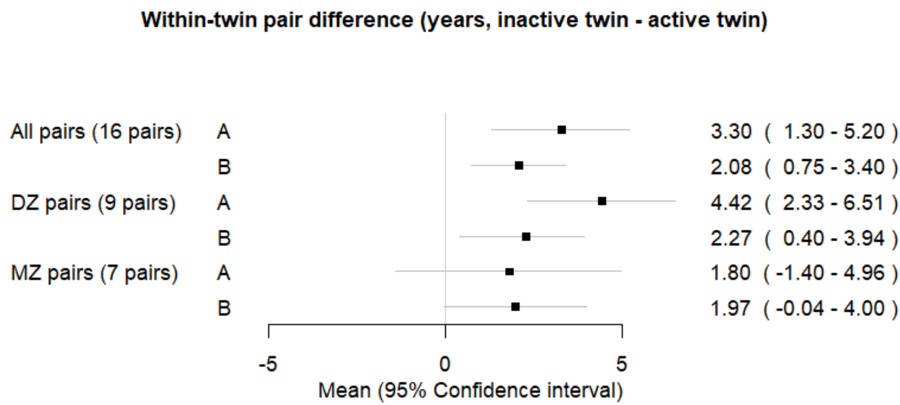


FIGURE 11. Within-twin pair differences in biological age estimates between inactive and active co-twins. A, DNAm PhenoAge; B, DNAm GrimAge; DZ, dizygotic; MZ, monozygotic.

In *Study IV*, we investigated the association between long-term LTPA patterns and later biological ageing using a prospective twin design. The patterns of long-term LTPA were identified using LPA with MET indices measured in 1970, 1981 and 1990. Four classes of long-term LTPA were identified (FIGURE 12).

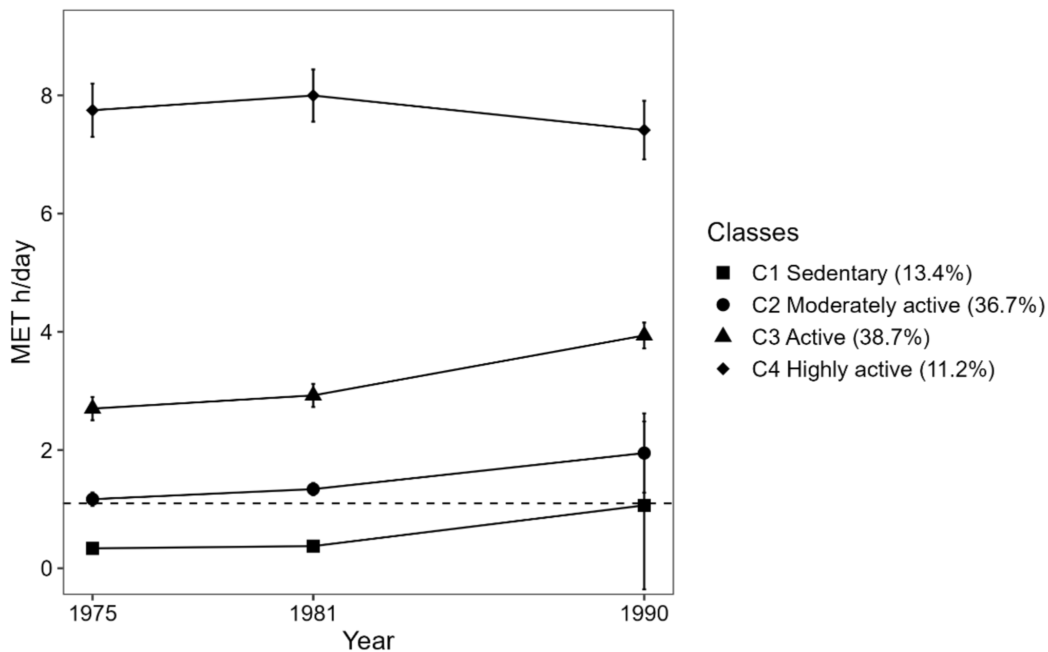


FIGURE 12 Patterns of long-term leisure-time physical activity (n = 22,750). Means of metabolic equivalent (MET) in h/day and 95% confidence intervals are presented. The dashed line indicates the World Health Organization's guidelines for the recommended minimum amount of physical activity for adults (150 min of moderate intensity physical activity per week at ~1.1 MET h/day) (Bull et al., 2020).

The association between long-term LTPA and biological ageing follows a U-shaped pattern: participants in the sedentary and highly active classes were biologically older than those who were moderately active and active (FIGURE 13). After adjusting for other lifestyle-related factors, most differences were attenuated, but based on $AA_{PC\text{-}Grim}$, the highly active class remained biologically older than the moderately active class (SMD = 0.30) and the active class (SMD = 0.42). As no significant beneficial association at the 0.05 level was observed between long-term LTPA and biological ageing, biological ageing is unlikely to mediate the association between long-term LTPA and a lower risk of all-cause mortality. No further path modelling was conducted.

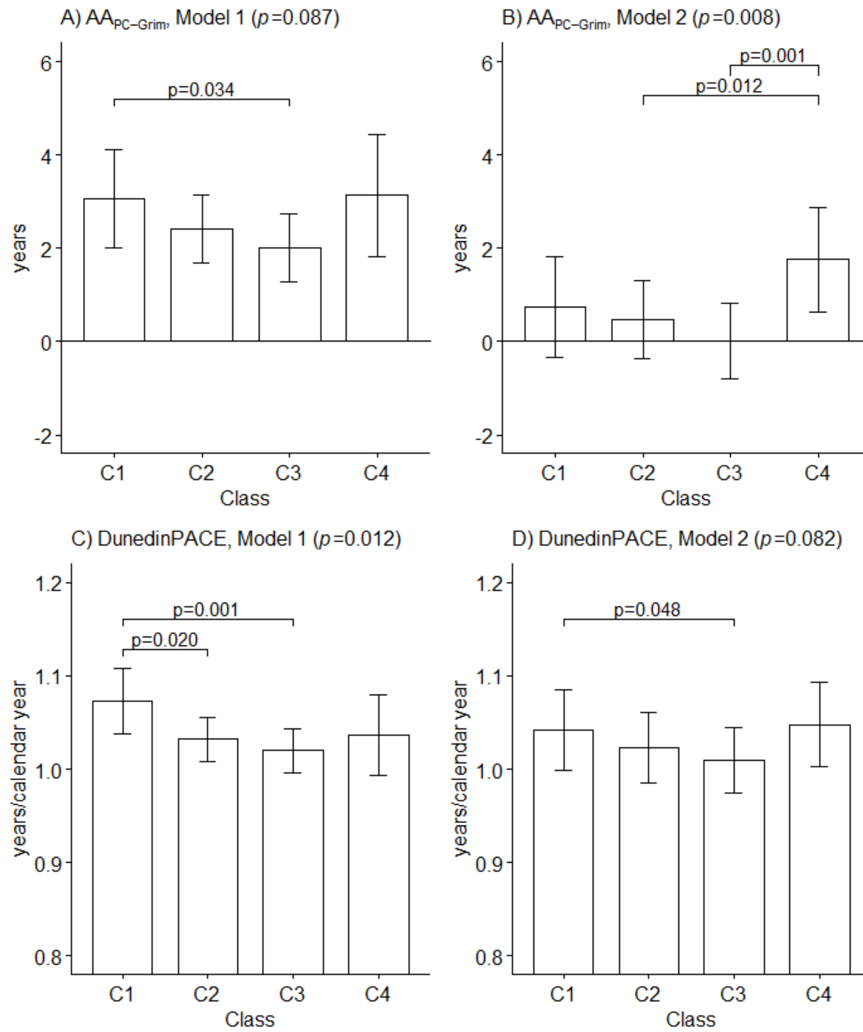


FIGURE 13 Mean differences between long-term leisure-time physical activity classes in terms of biological ageing as measured using A)–B) PC-based DNAm GrimAge and C)–D) DunedinPACE. Means and 95% confidence intervals are presented. Model 1 was adjusted for sex, age, timing of blood draw and health status, and Model 2 was further adjusted for education, body mass index, smoking and alcohol use. C1: Sedentary (8.8%); C2: moderately active (38.4%); C3: active (45.5%); C4: highly active (7.3%); AA, age acceleration. P-values were determined using the Wald test.

5.6 Long-term leisure-time physical activity and all-cause mortality (*Study IV*)

In *Study IV*, we investigated the differences in all-cause mortality between the patterns of long-term LTPA. At the within-pair level, the analyses showed that the moderately active, active and highly active classes exhibited lower risks of all-cause mortality than the sedentary class within all pairs (FIGURE 14 A, Model 1). After additionally adjusting the model for other lifestyle-related factors, the differences were considerably attenuated, but the moderately active and active classes exhibited lower risks of all-cause mortality compared to the sedentary class (FIGURE 14 A, Model 2). After excluding twin pairs who reported specific diseases in the early stages of the study (in 1981 or earlier), the differences in all-cause mortality were almost fully attenuated and were no longer significant at the 0.05 level after adjusting for other lifestyle-related factors (FIGURE 14B, Model 2).

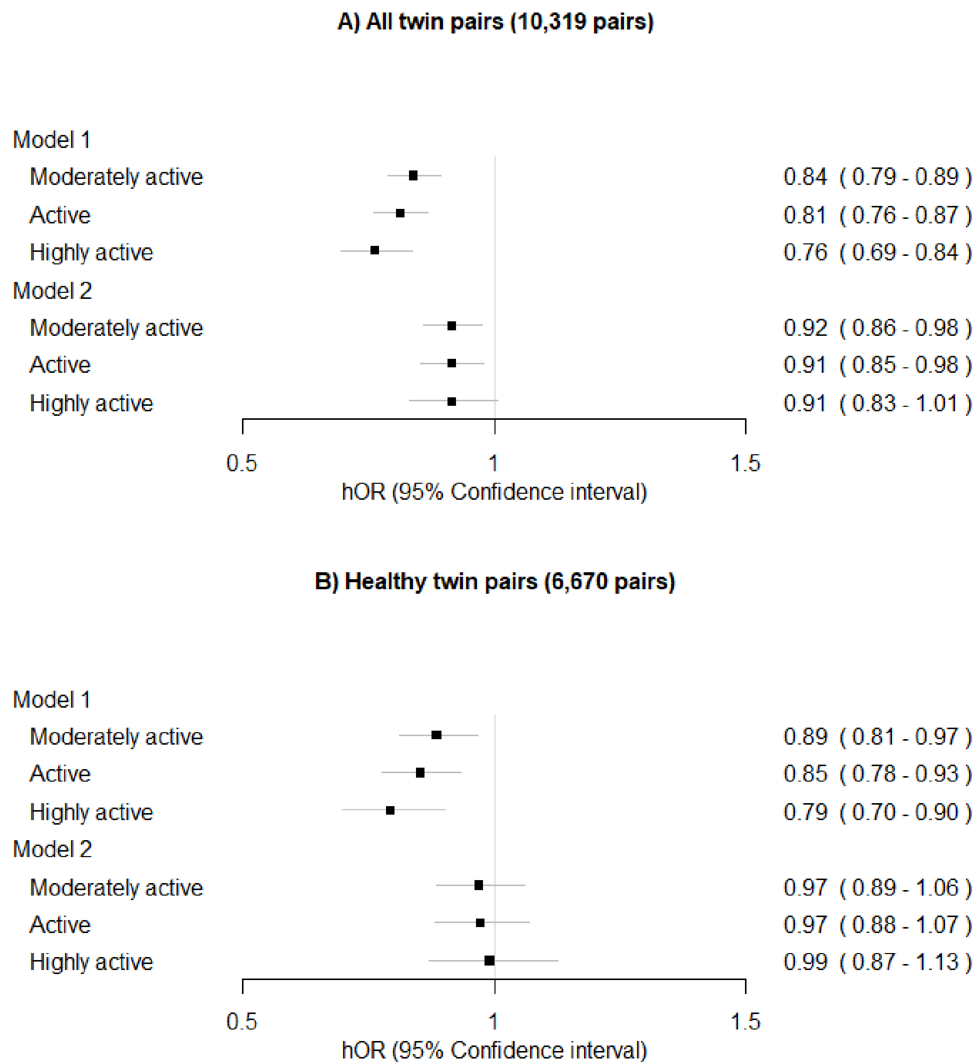


FIGURE 14 Within-twin-pair differences in all-cause mortality between the long-term leisure-time physical activity classes for A) all twin pairs and B) healthy twin pairs (i.e. pairs who did not report specific diseases: angina pectoris, myocardial infarction and diabetes). The sedentary class was treated as the reference. Only twin pairs who were alive in 1990 and for whom there was information on LTPA were included in the analysis. Model 1 was adjusted for sex (female) and age at the between-twin pair level and for health status at the within-twin pair level in all twin pairs (A). Model 2 was additionally adjusted for education, body mass index, smoking and alcohol use at the within-twin pair level. hOR, hazard odds ratio.

6 DISCUSSION

6.1 Summary of the main findings

The findings of this study suggest that DNAm-based measures of biological ageing are valuable tools for investigating epidemiological research questions traditionally addressed using time-to-event data, with the risks of morbidity and mortality taken as outcomes. They appear to capture both genetic and environmental influences on the biological ageing process during a life course. Genetic factors explained ~60% of the variation in biological ageing regardless of the measure used. However, the associations established between lifestyle-related factors and biological ageing depended on the estimator used. The newer second-generation clocks and DNAm-based biomarkers of the pace of ageing better captured the influences of lifestyle-related factors on biological ageing than the first-generation clocks developed to predict chronological age. Of the lifestyle-related factors we studied, smoking and high BMI emerged as the most important drivers of biological ageing. Already in adolescence, these phenotypes appeared to have a remarkable role in the subsequent biological ageing process. However, genetic factors explained most of these associations. Smoking and a larger body size also partly explained why men were biologically older than women. Our findings indicate that LTPA may also be associated with biological ageing, but its role is complex and probably minor. The association between LTPA and biological ageing may be susceptible to bias from multiple sources, including residual confounding, reverse causality and genetic confounding.

6.2 Genetic and environmental influences on biological ageing

We found that biological ageing as assessed with DNAm-based measures was highly heritable regardless of the measure used. The heritability of biological ageing as measured by Horvath's clock (61%–71%) was about the same as reported in previous studies (Jylhävä et al., 2019; Sillanpää et al., 2019). Only a few twin studies have reported on the heritability of biological ageing as measured by second-generation clocks (Jylhävä et al., 2019; Lundgren et al., 2022). Our heritability estimates (64%–65%) of AA based on DNAm PhenoAge were somewhat higher than those reported in a previous twin study (34%–41%; Jylhävä et al., 2019). These differences may stem from selection issues, differences in demographics and the varying methods, including the choice of the final biometrical twin model. To our knowledge, this is the first study to report on the heritability of biological ageing using the DNAm-based measures of pace of ageing developed by Belsky et al. (2020, 2022). The heritability based on these measures (62%–68%) was at about the same level as the heritability of AA measures. The remaining variation in biological ageing was explained by non-shared environmental factors.

Compared to estimates obtained from genetically informed studies that assessed heritability based on single-nucleotide polymorphisms (SNPs) (10%–17%) (McCartney et al., 2021), our estimates were considerably higher. For almost all complex phenotypes, there is a substantial gap between heritability estimates derived from twin models and those based on SNPs, a phenomenon commonly referred to as 'missing heritability' (Génin, 2020). Several reasons for missing heritability have been proposed. Most importantly, methods that estimate SNP-based heritability based on genome-wide association studies (GWAS) have underestimated heritability because, for example, previous GWAS analyses were not powerful enough to capture genetic variants with small effect sizes (Kaprio, 2012; Zhu & Zhou, 2020). Missing heritability may also be partly due to gene–environment interactions, which are assumed to be absent when calculating heritability estimates using twin data (Kaprio, 2012). Gene–environment interaction refers to the interplay between an individual's genetic makeup and the environment in which they live. In other words, the impact of genetic factors on a complex phenotype, such as biological ageing, is influenced by environmental factors, and vice versa. It has been suggested that a substantial proportion of missing heritability may result from these interactions, inflating the estimates derived from twin data (Génin, 2020; Kaprio, 2012). DNAm itself may play a role in these interactions, as it is prone to environmental exposure; at the same time, it regulates gene expression, which can influence both environmental exposure and health outcomes of interest (Villicaña & Bell, 2021). It can be hypothesised that DNAm mediates the interaction effects of the environment and genotype on the phenotype, but the extent to which it does so requires further study (Law & Holland, 2019). DNAm may also have another way of contributing to missing heritability; it has been speculated that genetically

heritable epigenetic modifications, including DNAm, could account for a remarkable part of the missing heritability observed in phenotypes (Génin, 2020). For these reasons, it is not surprising that a substantial missing heritability gap was observed in biological ageing when assessed with DNAm-based estimators.

6.3 Sex differences in biological ageing

Our findings suggest that previously reported sex differences in life expectancy are reflected in biological ageing when measured using epigenetic clocks. Men are already biologically older than women in young adulthood, and the sex difference increases with age.

The sources of sex differences in biological ageing are probably complex and multifactorial (Hägg & Jylhävä, 2021). They may be hormonal, genetic and due to differences in karyotypes but also influenced by gender identity, which is associated with societal norms, or sex-specific environmental exposure, such as lifestyle-related factors. In our study, we did not find evidence for genetic influences, as the within-pair sex difference in opposite-sex twin pairs who shared 50% of their genes was similar to that observed in the larger cohort of same-aged twins. We found that the sex difference in biological ageing was partly explained by the higher BMI and more frequent smoking observed in men relative to women.

Smoking appeared to be a plausible explanation for the narrowing sex gap in life expectancy, as smoking mediated the association only in older age group. This was mainly due to two factors. First, the association between smoking and accelerated biological ageing was stronger in older twins, suggesting a cumulative effect of smoking on DNAm-based biological ageing. Second, the sex difference in smoking behaviour was greater in older twins. A previous population-based study that investigated long-term trends in smoking in Finland showed that the prevalence of daily smoking has steadily decreased among men since the late 1970s, while the current prevalence among women is about the same (Ruokolainen et al., 2019). Although we were not able to tease apart age and cohort effects, together, these findings support recent studies suggesting that the narrowing of sex differences in smoking probably partly explains the declining sex gap in lifespan (Janssen, 2020; Östergren & Martikainen, 2020).

In accordance with the findings of Crimmins and colleagues (2021), we found a sex difference in biological ageing, which was not explained by differences in the lifestyle-related factors we studied. In our study, this observed sex gap increased with age. This finding contradicts the expectation that the sex differences in biological ageing would diminish around the age of 50 due to changes in hormonal levels during menopause and their harmful effects on women's health.

6.4 Lifestyle-related factors and biological ageing

The findings of this study indicate that lifestyle-related influences already active in adolescence may play a role in the biological ageing process. Unhealthy lifestyle-related habits in adolescence appeared to accumulate for the same individuals. The classes with the unhealthiest lifestyles and highest BMI in adolescence were biologically older in young adulthood than the classes with healthier lifestyle patterns. In addition, the path models of *Study II* highlighted the role of BMI and smoking in biological ageing at different ages. Consistent with previous literature (Oblak et al., 2021), these findings indicate that of the lifestyle-related factors studied, smoking and a high BMI are the main drivers of accelerated biological ageing as measured by DNAm-based estimators.

Our study suggests that LTPA may also impact biological ageing, but its role is complex and probably minor. Our cross-sectional results suggest the existence of a 'physical activity health paradox', as correlations with biological ageing of different types of PA, namely LTPA and OPA, appeared to differ. However, adjusting the models for smoking attenuated the associations, especially with the link between OPA and accelerated biological ageing, which may reflect an accumulation of unhealthy lifestyle habits among individuals in the lower socioeconomic classes who perform physically demanding work (Thebault et al., 2018). Moreover, the favourable associations between long-term LTPA and later biological ageing (*Study IV*) were largely explained by other lifestyle-related factors, including BMI, smoking, alcohol use and education. These factors are closely intertwined with LTPA. Thus, the independent association of LTPA with biological ageing may be difficult to disentangle. However, after all the adjustments, biological ageing appeared to be accelerated in the highly active class.

Our findings suggest that the associations of LTPA and OPA with biological ageing, particularly when assessed with DNAm GrimAge, were confounded by smoking behaviour. This measure included a DNAm-based biomarker for smoking pack years as one component, and this component appears to better capture smoking exposure than self-rated smoking status (Lu et al., 2019). Moreover, smoking behaviour is less common among physically active participants, and if they do smoke, they usually consume smaller quantities (Salin et al., 2019). For these reasons, the associations between PA and biological ageing are susceptible to residual confounding due to insufficient adjustment for smoking. Both smoking status and quantity should be considered in analyses.

6.5 Assessing the plausibility of causal links between lifestyle-related factors and biological ageing

Our findings reveal that the association established between adolescent lifestyle and biological ageing was mainly due to genetic influences. Therefore, individuals who are genetically prone to unhealthy lifestyles or overweight in adolescence are also susceptible to faster biological ageing later in young adulthood. The shared genetic influences on two phenotypes (i.e. genetic correlation) may be due to several scenarios (Solovieff et al., 2013). They may arise from genetic pleiotropy; in this case, the genes may be a common cause for both adolescent lifestyle and biological aging (horizontal pleiotropy). Another possible reason is causal relation between the phenotypes. In this case, genetic factors may affect adolescent lifestyle, which lies on the causal path to biological aging or even vice versa. Practically all variance in AA_{Pheno} and DunedinPACE shared with adolescent lifestyle was explained by shared genetic factors. Therefore, the associations between adolescent lifestyle and later biological ageing based on these measures were solely explained by genetic confounding, suggesting that the observed associations were likely not causal. For the relationship to be causal, there would have to be shared specific environmental influences on the phenotypes (de Moor et al., 2008). In our study, specific environmental influences shared with adolescent lifestyle and biological ageing were observed when the DNAm GrimAge and DunedinPoAm estimators were used. Therefore, these associations may also include a causal component, likely attributed to smoking exposure, as both estimators are sensitive to smoking.

Assessing the plausibility of the causal link between long-term LTPA and biological ageing yielded somewhat contradictory results within different study designs. When biological ageing was investigated among twin pairs who had been discordant in LTPA for 32 years (TWINACTIVE), we observed that inactive twins were biologically older than active co-twins. These differences did not notably attenuate when restricted to DZ and MZ twin pairs, which suggests that the association was independent of genetic factors. However, when a prospective study design was used, we observed a U-shaped association between long-term LTPA and biological ageing. Biological ageing was accelerated at both the highest and lowest levels of long-term LTPA when LTPA was measured earlier in adulthood. This discrepancy between the results may reflect selection and reverse causality bias. Previous studies have indicated that the occurrence of chronic diseases correlates with low levels of PA in older age (Kujala et al., 2019) and may therefore also affect the within-pair discordances in the TWINACTIVE study (Kujala et al., 2022). Restricting the analysis only to twin pairs discordant for LTPA over 30 years means that the active twins included in the study sample managed to sustain very high levels of LTPA into old age. Active twins who reduced their activity due to chronic diseases during the long-term follow-up were omitted from the study sample. Individuals healthy enough to sustain high LTPA levels will manifest a reduced risk of adverse health outcomes regardless

of their LTPA level, leading to a form of selection bias known as 'healthy exerciser bias' (Kujala, 2018). For these reasons, detecting any harmful effects of LTPA on biological ageing using LTPA-discordant twins or a cross-sectional study design, especially for older participants, may be an impossible task. Moreover, our survival analyses, conducted within a prospective study design, provided evidence for reverse causality bias; in particular, interpreting the results based on within-twin pair comparisons drastically changed after excluding twin pairs afflicted with specific diseases.

Only a few RCTs have attempted to assess the causal link between lifestyle-related factors and biological ageing as measured by DNAm-based biomarkers (Moqri et al., 2023). Perhaps the most promising findings have been obtained from lifestyle interventions targeting healthy postmenopausal women (Fiorito et al., 2021). Women assigned to the dietary intervention were advised to follow a plant-based diet with a low glycaemic load, high antioxidants and low saturated fat, trans fat and alcohol; they exhibited a significant slowing in AA based on DNAm GrimAge during the two-year intervention. However, the exercise-based intervention had no significant effect on biological ageing as assessed with DNAm GrimAge (Fiorito et al., 2021).

In addition to RCTs, modern methods based on human genetics, such as Mendelian randomisation (MR), can be used to test causality between modifiable lifestyle factors and outcomes (Davies et al., 2018). MR studies are described as naturally occurring randomised trials in which genetic factors are randomly assigned by nature. Therefore, there is no need for covariate adjustments, and the results are less likely to be affected by confounding and reverse causation than those reached using the traditional observational methods. MR uses genetic variants as instrumental variables to infer causality. Two-sample MR is a method of estimating causal effects using only GWAS summary statistics. A recent GWAS identified 137 genetic loci associated with AA measures (McCartney et al., 2021). Few studies have used these genetic variants within two-sample MR frameworks to infer causality between lifestyle-related factors and biological ageing (Kong et al., 2023; McCartney et al., 2021). These studies have suggested causal effects of adiposity and smoking on biological ageing, especially when measured with the DNAm GrimAge estimator, which is consistent with our findings. Kong et al. (2023) also investigated the association between PA and AA measures but did not find evidence for causality. Currently, there is no available GWAS on the DNAm-based biomarkers of pace of ageing developed by Belsky et al. (2020, 2022). For this reason, no two-sample MR studies have assessed causality between lifestyle-related factors and biological ageing as measured using these estimators.

6.6 Methodological considerations

The major strengths of the present research are the twin study designs, longitudinal measurements of lifestyle-related factors and relatively large sample

sizes of twins for whom information on biological ageing was available. Moreover, the newest DNAm-based biomarkers of biological ageing were used. We used large, population-based cohort data to identify patterns of lifestyle behaviour in adolescence and investigate the associations between long-term LTPA and mortality. The older cohort was followed over 45 years, and follow-up on mortality was exceptionally long-lasting, at over 30 years. Using a twin study design allowed us to efficiently take into account the shared genetic and environmental factors underlying the associations of lifestyle-related factors with biological ageing and all-cause mortality. We were also able to conduct sophisticated statistical analyses within the SEM framework. When the studies were conducted, there was no readily available method for decomposing the covariation between latent class variables and distal outcomes into shared genetic and environmental influences, but we were able to indirectly evaluate these proportions.

Our study also has limitations. Health-related factors were questionnaire-based and self-rated. Therefore, potential recall bias and the effect of social desirability cannot be excluded. Since pre-existing data were used, with measurements dating back as far as 45 years, there was no opportunity to contribute to these measurements. In addition, a twin sample might not always be fully representative of the broader population, which may limit the generalisability of the study findings. For example, twins may have reduced intrauterine growth and shorter gestation in comparison to singletons, potentially leading to adverse health outcomes later in life (Barker & Osmond, 1986; Christensen & McGue, 2020). However, studies comparing mortality and morbidity in twin registers to those in the general population have not indicated remarkable differences, supporting the generalisability of findings from twin studies (Skytthe et al., 2019). In particular, mortality in the FTC was similar to that in the general population, albeit slightly lower at the beginning of the follow-up period (Skytthe et al., 2019). This slight deviation may indicate left truncation or selection bias, as only pairs with both co-twins alive (in 1967) were included in the cohort (Skytthe et al., 2019). The subsample of twins for whom information on biological ageing was available represented the general population relatively well. However, women and non-smokers were overrepresented in the older cohort.

In all the substudies included in this thesis, biological ageing was assessed at a single time point. From a life course perspective, the use of longitudinal data on biological ageing would be helpful when investigating the dynamic development of the biological ageing process and the role of modifiable health-related factors in trajectories of biological ageing (Ben-Shlomo & Kuh, 2002; Kuh et al., 2007). However, longitudinal data on DNAm data are still rare, mainly due to the high costs of methylation analyses. Yet the prices are continuously decreasing, and in the near future, large longitudinal DNAm datasets with appropriately measured phenotypes will be available.

Although DNAm is considered one primary hallmark of ageing (López-Otín et al., 2013, 2023), the question of whether it is a cause or consequence of

ageing remains uncertain. Additionally, when blood samples are used to assess biological ageing, it is assumed that DNAm levels from leukocytes reflect the overall ageing of the body. DNAm profiles differ among various tissues, suggesting that the biological ageing rate may be tissue-specific. For example, there is some evidence that the association between LTPA and biological ageing rate differs in muscle and blood (Sillanpää et al., 2021). However, although muscle may be a more relevant tissue in terms of PA, no association was detected between PA and biological ageing using an epigenetic clock – namely, the MEAT clock for human skeletal muscle, developed by Voisin and colleagues (2020) (Sillanpää et al., 2021).

Generally, the use of the classical twin study design leads to upwardly biased estimates of additive genetic variance and downwardly biased estimates of dominant genetic variance and shared environmental variance. This is mainly due to the reduced power to detect underlying common environmental factors (McAdams et al., 2021; Verhulst et al., 2019). Our twin models did not require the inclusion of common environmental effects, as genetic models with (ACE) or without (AE) common environmental effects fit the data adequately. The heritability estimates were derived from the AE models, which may have led to overestimation of the additive genetic effects.

The classical twin study design, and particularly within-twin pair comparisons, effectively controls for several common confounders that are usually unknown. However, it should be noted that the design does not control for non-shared confounding factors, and it is therefore important to carefully adjust within-pair models for these factors. There may be non-shared, unmeasured confounders (e.g. dietary exposures) that were not controlled for in our analyses. Moreover, our results on cross-sectional associations between lifestyle-related factors and biological ageing may be susceptible to reverse causality bias, as individuals experiencing accelerated biological ageing may have altered their lifestyles due to declining health.

6.7 Future directions

DNAm-based measures of biological ageing are ready to use, but some fine-tuning is still needed (Ferrucci et al., 2020). Better-performing measures are continuously being developed. For example, an improved version of the DNAm GrimAge estimator was recently published (Lu et al., 2022). The new estimator consists of the same DNAm-based biomarkers for plasma proteins as the original version but introduces two additional ones: DNAm-based high-sensitivity CRP and haemoglobin A1C. The new version was shown to outperform the original DNAm GrimAge with respect to the strength of its association with mortality risk and age-related conditions, such as coronary heart disease (Lu et al., 2022).

The newest strategy of developing a better-performing DNAm-based measure of biological ageing is to integrate multiomic information into a single OMICmAge estimator (Chen et al., 2023). First, the authors developed a

biological ageing phenotype, EMRAge, which consists of selected clinical biomarkers that best predict mortality. Second, they developed DNAm-based predictors for proteins, metabolites and clinical outcomes and used these 'epigenetic biomarker proxies' (EBPs) to predict EMRAge. OMICmAge is the combination of the EBPs that best predicted EMRAge. The results regarding the associations between OMICmAge and lifestyle-related factors are promising, but at the time of writing, only a non-peer-reviewed preprint is available.

DNAm is a reversible epigenetic modification. Therefore, there is attractive potential for anti-ageing interventions. However, it has been stated that the molecular mechanisms underlying DNAm biomarkers need to be better understood before their potential can be fully realised (Horvath & Raj, 2018; López-Otín et al., 2023). It has been suggested that studying the associations not only at the clock level but also at the level of smaller subsets of CpG sites may provide a better, more detailed understanding of the role of lifestyle-related factors in ageing (Oblak et al., 2021). In particular, exploring DNAm-based scores for proteins, which are the primary effectors of disease (Gadd et al., 2022; Lu et al., 2019, 2022), may provide new insights into the development of age-related multi-morbidity.

To disentangle the independent associations between lifestyle-related factors and biological ageing, improved methods of measuring these factors are needed. In particular, methods of assessing PA have advanced in recent decades. The use of device-based methods, such as accelerometers, may offer more detailed information on PA behaviour and should be used in future studies.

Our studies revealed that the associations between adolescent lifestyle-related factors and biological ageing are particularly susceptible to genetic confounding. Recently, genetic sensitivity analysis was proposed as a method of assessing the extent to which genetic confounding can account for associations in observational studies (Pingault et al., 2021). This approach is applicable whenever the study sample is genotyped, which is now quite common when epidemiological cohorts are used. Future observational studies should consider genetic confounding whenever possible, especially when studying the early-life risk factors of biological ageing. In addition, greater attention should be given to the mediating role of age-related DNAm in the complex interplay of genetics and environments. Future studies should determine the extent to which DNAm can account for the effects of gene–environment interactions on adverse health outcomes associated with ageing.

7 MAIN FINDINGS AND CONCLUSIONS

The main findings and conclusions of the present study can be summarised as follows:

1. Biological ageing as measured with DNAm-based estimators is highly heritable. However, specific environmental factors also contribute to the variation in biological ageing. Therefore, these measures may capture both genetic and environmental influences on biological ageing during a life course.
2. The DNAm GrimAge, PC-based DNAm GrimAge and DunedinPACE estimators appear to be the most promising measures for use in studies of the associations between lifestyle-related factors and biological ageing.
3. Biological ageing appears to begin early in life. Unhealthy lifestyles and overweight during adolescence have a role in later biological ageing, although in this study, genetic factors largely explained the associations.
4. Of the lifestyle-related factors studied, BMI and smoking appear to be the most important drivers of biological ageing and the related sex differences.
5. PA is associated with biological ageing, but its role is complex and probably minor. The association may be susceptible to bias from multiple sources, including residual confounding, reverse causality and genetic confounding.

YHTEENVETO (SUMMARY IN FINNISH)

Biologinen vanheneminen on ikääntymisen myötä tapahtuvaa elimistön toiminnan heikkenemistä, joka johtaa kasvaneeseen sairastavuuteen ja ennenaikaisen kuoleman riskiin. Elämänkulun epidemiologisten tutkimusten perusteella biologisella vanhenemisella voi olla alkuperä jo varhaisissa elämän vaiheissa. Vanhenemista säätelevät läpi elämän sekä perintö- että ympäristötekijät, kuten muokattavissa olevat elintavat.

Solu- ja molekyylitasolla epigeneettiset muutokset ovat yksi vanhenemisen tunnuspiirteistä. Epigeneettiset muutokset säätelevät geenien ilmenemistä DNA:n emäsjärjestystä muuttamatta. Toisin kuin perintötekijät, epigenomi eli epigeneettisten merkkien kokoelma muokkautuu läpi elämän. Epigeneettisen säätelyn kautta ympäristötekijät, kuten epäterveelliset elintavat, voivat vaikuttaa geenien ilmenemiseen. DNA:n metylaatio on tutkituin epigeneettinen mekanismi ja se viittaa metyyliryhmän sitoutumiseen DNA:n sytosiini-fosfaatti-guaniini-dinukleotideihin eli CpG-kohtiin. On havaittu, että DNA:n metyloituminen tietyissä CpG-kohdissa on yhteydessä kalenteri-ikään. Tämä havainto on johtanut DNA:n metylaatioon perustuvien biologisen vanhenemisen mittareiden, mukaan lukien "epigeneettisten kellojen", kehitystyöhön.

Tämän väitöskirjatutkimuksen tarkoituksena oli tutkia perintö- ja ympäristötekijöiden vaikutuksia biologiseen vanhenemiseen eri ikävaiheissa. Tutkimuksessa hyödynsimme kaksosasetelmia, joiden avulla esimerkiksi biologisen vanhenemisen periytyvyyttä voidaan arvioida. Lisäksi elintapojen ja biologisen vanhenemisen yhteyksiä voitiin tarkastella huomioiden yhteisten ympäristötekijöiden ja perintötekijöiden sekoittavat vaikutukset.

Tämä väitöskirja koostuu neljästä alkuperäistutkimuksesta, jotka perustuvat suomalaisen kaksoskohortin aineistoon. Kaksoskohortti koostuu kolmesta suuresta kohorttitutkimuksesta: vanhempi kaksoskohortti (ennen vuotta 1958 syntyneet kaksoset), Nuorten kaksosten terveystutkimus (FinnTwin16, vuosina 1975–79 syntyneet kaksoset) ja Kaksosten kehitys ja terveys -tutkimus (FinnTwin12, vuosina 1983–1987 syntyneet kaksoset). DNA:n metylaatiotasot määritettiin osajoukolle verinäytteistä (N:~2 600) ja biologisen vanhenemisen arvioimiseen aikuisiässä käytettiin useita DNA:n metylaatioon perustuvia mittareita: Horvathin ja Hannumin kelloja sekä DNAm PhenoAge-, DNAm GrimAge-, DunedinPoAm- ja DunedinPACE-estimaattoreita. Elintapoihin liittyviä tekijöitä, mukaan lukien koulutus, painoindeksi, tupakointi, alkoholin käyttö ja fyysinen aktiivisuus, mitattiin validoiduilla kyselylomakkeilla useissa mittauspisteissä.

Tutkimuksessa biologisen vanhenemisen havaittiin olevan voimakkaasti periytyvää. Tulosten perusteella yksilöiden väliset erot biologisessa vanhenemisessa näkyivät jo varhaisessa aikuisuudessa. Epäterveelliset elämäntavat ja ylipaino nuoruudessa liittyivät nopeampaan biologiseen vanhenemiseen nuoressa aikuisuudessa. Havaitut erot biologisessa vanhenemisessa selittyivät suurelta osin nuoruuden elintapojen ja biologisen vanhenemisen taustalla vaikuttavalla samalla perimän vaihtelulla.

Miehet olivat biologisesti vanhempia kuin naiset, ja sukupuoliero kasvoi iän myötä. Sukupuoliero selittyi osittain miesten korkeammalla painoindeksillä sekä yleisemmällä tupakoinnilla vanhemmissa ikäluokissa.

Vapaa-ajan fyysinen aktiivisuus oli yhteydessä hidastuneeseen biologiseen vanhenemiseen ja työajan fyysinen aktiivisuus kiihtyneeseen biologiseen vanhenemiseen, kun yhteyksiä tarkasteltiin poikkileikkausasetelmaa hyödyntäen. Yhteydet heikkenivät voimakkaasti, kun tupakoinnin vaikutus huomioitiin. Kun vapaa-ajan fyysisen aktiivisuuden yhteyttä myöhempään biologiseen vanhenemiseen tarkasteltiin pitkittäisaineistoa hyödyntäen, yhteys oli U:n muotoinen: biologinen vanheneminen oli kiihtynyttä vähiten ja eniten liikkuvilla. Vapaa-ajan fyysisen aktiivisuuden suotuisat yhteydet katosivat, kun muut elintapoihin liittyvät tekijät huomioitiin mallinnuksessa.

Tutkimuksen tulokset viittaavat siihen, että DNA:n metylaatiotasoihin perustuvat biologisen vanhenemisen mittarit ovat hyödyllisiä elämänkulun epidemiologisissa tutkimuksissa, koska niiden avulla voidaan mitata elämän aikana kertyneiden elintapatekijöiden vaikutuksia. Tulokset vaihtelivat käytetyn biologisen vanhenemisen mittarin mukaan. Elintapojen ja biologisen vanhenemisen yhteydet näkyivät selvimmin, kun biologista vanhenemista mitattiin DNAm GrimAge ja DunedinPACE-estimaattoreilla. Tupakointi ja ylipaino näyttäisivät olevan biologisen vanhenemisen kannalta haitallisimpia elintapoja ja osasyy sille, miksi miehet vanhenevat biologisesti nopeammin kuin naiset. Fyysinen aktiivisuus oli yhteydessä biologiseen vanhenemiseen, mutta yhteys näyttää olevan altis monesta lähteestä johtuvalle harhalle. Esimerkiksi käänteinen kausaliteetti voi sekoittaa yhteyttä, sillä henkilöt, jotka pystyvät jatkamaan liikuntaharrastustaan vanhempaan aikuisuuteen, ovat usein myös terveempiä. Epäterveelliset elintavat näyttävät kasaantuvan nuoruudesta lähtien samoille yksilöille, jonka vuoksi elintapojen itsenäisiä vaikutuksia voi olla vaikea erottaa. Perintötekijöiden ja tupakoinnin sekoittavat vaikutukset tulisi ottaa mahdollisimman tarkasti huomioon tulevissa tutkimuksissa, joissa hyödynnetään DNA:n metylaatioon perustuvia mittareita.

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ORIGINAL PAPERS

I

THE ROLE OF ADOLESCENT LIFESTYLE HABITS IN BIOLOGICAL AGING: A PROSPECTIVE TWIN STUDY

by

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RESEARCH ARTICLE

The role of adolescent lifestyle habits in biological aging: A prospective twin study

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Abstract

Background: Adolescence is a stage of fast growth and development. Exposures during puberty can have long-term effects on health in later life. This study aims to investigate the role of adolescent lifestyle in biological aging.

Methods: The study participants originated from the longitudinal FinnTwin12 study (n = 5114). Adolescent lifestyle-related factors, including body mass index (BMI), leisure-time physical activity, smoking, and alcohol use, were based on self-reports and measured at ages 12, 14, and 17 years. For a subsample, blood-based DNA methylation (DNAm) was used to assess biological aging with six epigenetic aging measures in young adulthood (21–25 years, n = 824). A latent class analysis was conducted to identify patterns of lifestyle behaviors in adolescence, and differences between the subgroups in later biological aging were studied. Genetic and environmental influences on biological aging shared with lifestyle behavior patterns were estimated using quantitative genetic modeling.

Results: We identified five subgroups of participants with different adolescent lifestyle behavior patterns. When DNAm GrimAge, DunedinPoAm, and DunedinPACE estimators were used, the class with the unhealthiest lifestyle and the class of participants with high BMI were biologically older than the classes with healthier lifestyle habits. The differences in lifestyle-related factors were maintained into young adulthood. Most of the variation in biological aging shared with adolescent lifestyle was explained by common genetic factors.

Conclusions: These findings suggest that an unhealthy lifestyle during pubertal years is associated with accelerated biological aging in young adulthood. Genetic pleiotropy may largely explain the observed associations.

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Editor's evaluation

This is an important article that is methodologically compelling that provides evidence that an unhealthy lifestyle during adolescence accelerates epigenetic age in adulthood and that these associations are largely explained by the effect of shared genetic influences. The main strengths of this

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article are the relatively large sample size, longitudinal assessment of lifestyle factors, and sophisticated statistical analyses. The article will be of interest to a broad audience, including individuals working on methylation, epidemiology, and/or aging.

Introduction

Epidemiological studies of life course have indicated that exposures during early life have long-term effects on later health (***Kuh et al., 2003***). Unhealthy environments and lifestyle habits during rapid cell division can affect the structure or functions of organs, tissues, or body systems, and these changes can subsequently affect health and disease in later life (***Biro and Deardorff, 2013***; ***Power et al., 2013***). For example, lower birth weight and fast growth during childhood predispose individuals to coronary heart disease and increased blood pressure in adulthood (***Osmond and Barker, 2000***). In addition to infancy and childhood, adolescence is also a critical period of growth.

Adolescence is characterized by pubertal maturation and growth spurts. Early pubertal development is linked to worse health conditions, such as obesity and cardiometabolic risk factors in adulthood (***Prentice and Viner, 2013***). However, childhood obesity can lead to early onset of puberty, especially among girls (***Li et al., 2017***; ***Richardson et al., 2020***), and, therefore, can confound the observed associations between early pubertal development and worse later health. Moreover, early pubertal development is linked to substance use and other risky behaviors in adolescence (***Hartman et al., 2017***; ***Savage et al., 2018***), but the associations are partly explained by familial factors (***Savage et al., 2018***).

Many unhealthy lifestyle choices, such as smoking initiation, alcohol use, and a physically inactive lifestyle, are already made in adolescence and increase the risk of developing several noncommunicable diseases over the following decades (***Lopez et al., 2006***). Once initiated, unhealthy habits are likely to persist into adulthood (***Latvala et al., 2014***; ***Maggs and Schulenberg, 2005***; ***Rovio et al., 2018***; ***Salin et al., 2019***). A recent systematic review showed that healthy habits tend to cluster during childhood and adolescence, and typically, about half of the adolescents fall into subgroups characterized by healthy lifestyle habits (***Whitaker et al., 2021***). However, small minorities of adolescents are classified as heavy substance users or as having multiple other risk behaviors (***Whitaker et al., 2021***). The long-term consequences of the accumulation of unhealthy adolescent behaviors on health in later life have been rarely studied.

An unhealthy lifestyle in adolescence can affect biological mechanisms of aging at the molecular level and, subsequently, morbidity. Epigenetic alterations, including age-related changes in DNA methylation (DNAm), constitute a primary hallmark of biological aging (***López-Otín et al., 2013***). Epigenetic clocks are algorithms that aim to quantify biological aging using DNAm levels within specific CpG sites. The first-generation clocks, Horvath's and Hannum's clocks, were trained to predict chronological age (***Hannum et al., 2013***; ***Horvath, 2013***), whereas the second-generation clocks, such as DNAm PhenoAge and GrimAge, are better predictors of health span and lifespan (***Levine et al., 2018***; ***Lu et al., 2019***). For epigenetic clocks, the difference between an individual's epigenetic age estimate and chronological age provides a measure of age acceleration (AA). The DunedinPoAm estimator differs from its predecessors in that it has been developed to predict the pace of aging (***Belsky et al., 2020***). The pace of aging describes longitudinal changes over 12 years in several biomarkers of organ-system integrity among same-aged individuals. Recently, the DunedinPACE estimator, which constitutes an advance on the original DunedinPoAm, was published (***Belsky et al., 2022***). DunedinPACE was trained to predict pace of aging measured over 20-year follow-up, and only the reliable probes were used in the prediction. From the life-course perspective, epigenetic aging measures are useful tools to assess biological aging at all ages and detect changes induced by lifetime exposures.

Previous studies have linked several lifestyle-related factors, such as higher body mass index (BMI), smoking, alcohol use, and lower leisure-time physical activity (LTPA), with accelerated biological aging measured using epigenetic clocks (***Oblak et al., 2021***; ***Quach et al., 2017***). However, most of these studies were based on cross-sectional data on older adults. The first studies on the associations of adolescent lifestyle-related exposures with biological aging assessed with epigenetic aging measures indicated that advanced pubertal development, higher BMI, and smoking are associated with accelerated biological aging in adolescence (***Etzel et al., 2022***; ***Raffington et al., 2021***; ***Simpkin et al., 2017***).

eLife digest For most animals, events that occur early in life can have a lasting impact on individuals' health. In humans, adolescence is a particularly vulnerable time when rapid growth and development collide with growing independence and experimentation. An unhealthy lifestyle during this period of rapid cell growth can contribute to later health problems like heart disease, lung disease, and premature death. This is due partly to accelerated biological aging, where the body deteriorates faster than what would be expected for an individual's chronological age.

One way to track the effects of lifestyle on biological aging is by measuring epigenetic changes. Epigenetic changes consist on adding or removing chemical 'tags' on genes. These tags can switch the genes on or off without changing their sequences. Scientists can measure certain epigenetic changes by measuring the levels of methylated DNA – DNA with a chemical 'tag' known as a methyl group – in blood samples. Several algorithms – known as 'epigenetic clocks' – are available that estimate how fast an individual is aging biologically based on DNA methylation.

Kankaanpää et al. show that unhealthy lifestyles during adolescence may lead to accelerated aging in early adulthood. For their analysis, Kankaanpää et al. used data on the levels of DNA methylation in blood samples from 824 twins between 21 and 25 years old. The twins were participants in the FinnTwin12 study and had completed a survey about their lifestyles at ages 12, 14, and 17.

Kankaanpää et al. classified individuals into five groups depending on their lifestyles. The first three groups, which included most of the twins, contained individuals that led relatively healthy lives. The fourth group contained individuals with a higher body mass index based on their height and weight. Finally, the last group included individuals with unhealthy lifestyles who binge drank, smoked and did not exercise.

After estimating the biological ages for all of the participants, Kankaanpää et al. found that both the individuals with higher body mass indices and those in the group with unhealthy lifestyles aged faster than those who reported healthier lifestyles. However, the results varied depending on which epigenetic clock Kankaanpää et al. used to measure biological aging: clocks that had been developed earlier showed fewer differences in aging between groups; while newer clocks consistently found that individuals in the higher body mass index and unhealthy groups were older. Kankaanpää et al. also showed that shared genetic factors explained both unhealthy lifestyles and accelerated biological aging.

The experiments performed by Kankaanpää et al. provide new insights into the vital role of an individual's genetics in unhealthy lifestyles and cellular aging. These insights might help scientists identify at risk individuals early in life and try to prevent accelerated aging.

The few previous studies conducted on this topic have focused on single lifestyle factors, and a comprehensive understanding of the role of adolescent lifestyle in later biological aging remains unclear. Our first aim is to define the types of lifestyle behavior patterns that can be identified in adolescence using data-driven latent class analysis (LCA). The second aim is to investigate whether the identified behavioral subgroups differ in biological aging in young adulthood and whether the associations are independent of baseline pubertal development. The third aim is to assess the genetic and environmental influences shared between biological aging and adolescent lifestyle behavior patterns.

Methods

The participants were Finnish twins and members of the longitudinal FinnTwin12 study (born during 1983–1987) (***Kaprio, 2013***; ***Rose et al., 2019***). A total of 5600 twins and their families initially enrolled in the study. At the baseline, the twins filled out the questionnaires regarding their lifestyle-related habits at 11–12 years of age, and follow-up assessments were conducted at ages 14 and 17.5 years. The response rates were high for each assessment (85–90%). In young adulthood, at an average age of 22 years, blood samples for DNA analyses were collected during in-person clinical studies after written informed consent was signed. The data on health-related behaviors were collected with questionnaires and interviews. A total of 1295 twins of the FinnTwin12 cohort were examined and measured either in-person or through telephonic interviews. DNAm was determined and biological

aging was assessed for 847 twins, out of which 824 twins had also information on lifestyle-related habits in adolescence. Data collection was conducted in accordance with the Declaration of Helsinki. The Indiana University IRB and the ethics committees of the University of Helsinki and Helsinki University Central Hospital approved the study protocol (113/E3/2001and 346/E0/05).

DNAm and assessment of biological age

Genomic DNA was extracted from peripheral blood samples using commercial kits. High molecular weight DNA samples (1 µg) were bisulfite converted using EZ-96 DNA methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The twins and co-twins were randomly distributed across plates, with both twins from a pair on the same plate. DNAm profiles were obtained using Illumina's Infinium HumanMethylation450 BeadChip or the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA). The Illumina BeadChips measure single-CpG resolution DNAm levels across the human genome. With these assays, it is possible to interrogate over 450,000 (450k) or 850,000 (EPIC) methylation sites quantitatively across the genome at single-nucleotide resolution. Of the samples included in this study, 744 were assayed using 450k and 80 samples using EPIC arrays. Methylation data from different platforms was combined and preprocessed together using R package minfi (***Aryee et al., 2014***). We calculated detection p-values comparing total signal for each probe to the background signal level to evaluate the quality of the samples (***Maksimovic et al., 2016***). Samples of poor quality (mean detection p>0.01) were excluded from further analysis. Data were normalized by using the single-sample Noob normalization method, which is suitable for datasets originating from different platforms (***Fortin et al., 2017***). We also used Beta-Mixture Quantile (BMIQ) normalization (***Teschendorff et al., 2013***). Beta values representing CpG methylation levels were calculated as the ratio of methylated intensities (M) to the overall intensities (beta value = M/(M + U + 100), where U is unmethylated probe intensity). These preprocessed beta values were used as input in the calculations of the estimates of epigenetic aging.

We utilized six epigenetic clocks. The first four clocks, namely, Horvath's and Hannum's epigenetic clocks (***Hannum et al., 2013***; ***Horvath, 2013***) and DNAm PhenoAge and DNAm GrimAge estimators (***Levine et al., 2018***; ***Lu et al., 2019***), produced DNAm-based epigenetic age estimates in years by using a publicly available online calculator (https://dnamage.genetics.ucla.edu/new) (normalization method implemented in the calculator was utilized, as well). For these measures, AA was defined as the residual obtained from regressing the estimated epigenetic age on chronological age ($AA_{Horvath}$, AA_{Hannum}, AA_{Pheno}, and AA_{Grim}, respectively). The fifth and sixth clocks, namely, DunedinPoAm and DunedinPACE estimators, provided an estimate for the pace of biological aging in years per calendar year (***Belsky et al., 2020***; ***Belsky et al., 2022***). DunedinPoAm and DunedinPACE were calculated using publicly available R packages (https://github.com/danbelsky/DunedinPoAm38; ***Belsky et al., 2020*** and https://github.com/danbelsky/DunedinPACE; ***Belsky et al., 2022***, respectively). The epigenetic aging measures were screened for outliers (>5 standard deviations away from mean). One outlier was detected according to DunedinPACE and was recoded as a missing value.

The components of DNAm GrimAge (adjusted for age) were also obtained, including DNAm-based smoking pack-years and the surrogates for plasma proteins (DNAm-based plasma proteins): DNAm adrenomedullin (ADM), DNAm beta-2-microglobulin (B2M), DNAm cystatin C, DNAm growth differentiation factor 15 (GDF15), DNAm leptin, DNAm plasminogen activator inhibitor 1 (PAI-1), and DNAm tissue inhibitor metalloproteinases 1 (TIMP-1).

Lifestyle-related factors in adolescence

BMI at ages 12, 14, and 17 years

BMI (kg/m^2) was calculated based on self-reported height and weight.

LTPA at ages 12, 14, and 17 years

The frequency of LTPA at the age of 12 years was assessed with the question 'How often do you engage in sports (i.e., team sports and training)?' The answers were classified as 0 = less than once a week, 1 = once a week, and 2 = every day. At ages 14 and 17 years, the question differed slightly: 'How often do you engage in physical activity or sports during your leisure time (excluding physical education)?' The answers were classified as 0 = less than once a week, 1 = once a week, 2 = 2–5 times a week, and 3 = every day.

Smoking status at ages 14 and 17 years

Smoking status was determined using the self-reported frequency of smoking and classified as 0 = never smoker, 1 = former smoker, 2 = occasional smoker, and 3 = daily smoker.

Alcohol use (binge drinking) at ages 14 and 17 years

The frequency of drinking to intoxication had the following classes: 'How often do you get really drunk?' 0 = never, 1 = less than once a month, 2 = approximately once or twice a month, and 3 = once a week or more.

Pubertal development at age 12 years

Baseline pubertal development was assessed using a modified five-item Pubertal Development Scale (PDS) questionnaire (***Petersen et al., 1988***; ***Wehkalampi et al., 2008***). Both sexes answered three questions each concerning growth in height, body hair, and skin changes. Moreover, boys were asked questions about the development of facial hair and voice change, while girls were asked about breast development and menarche. Each question had response categories 1 = growth/change has not begun, 2 = growth/change has barely started, and 3 = growth/change is definitely underway, except for menarche, which was dichotomous, 1 = has not occurred or 3 = has occurred (see also ***Wehkalampi et al., 2008***). PDS was calculated as the mean score of the five items, and higher values indicated more advanced pubertal development at age 12 years.

Lifestyle-related factors in young adulthood at age 21–25 years

BMI (kg/m^2) was calculated based on the measured height and weight.

LTPA was assessed using the Baecke questionnaire (***Baecke et al., 1982***). A sport index was based on the mean scores of four questions on sports activity described by ***Baecke et al., 1982*** and ***Mustelin et al., 2012*** for the FinnTwin12 study. The sport index is a reliable and valid instrument to measure high-intensity physical activity (***Richardson et al., 1995***).

Smoking was self-reported and classified as never, former, or current smoker.

Alcohol use (100% alcohol grams/day) was derived from the Semi-Structured Assessment for the Genetics of Alcoholism (***Bucholz et al., 1994***) and based on quantity and frequency of use and the content of alcoholic beverages, assessed by trained interviewers.

Statistical analysis

Patterns of lifestyle behaviors in adolescence

To identify the patterns of lifestyle behaviors in adolescence, an LCA was conducted, which is a data-driven approach to identify homogenous subgroups in a heterogeneous population. The classification was based on BMI and LTPA at ages 12, 14, and 17 years and smoking status and alcohol use at ages 14 and 17 years (10 indicator variables). All variables were treated as ordinal variables, except for continuous BMI. The classification was based on the thresholds of the ordinal variables and the means and variances of BMI.

An LCA model with 1–8 classes was fitted. The following fit indices were used to evaluate the goodness of fit: Akaike's information criterion, Bayesian information criterion (BIC), and sample size-adjusted BIC. The lower values of the information criteria indicated a better fit for the model. Moreover, we used the Vuong–Lo–Mendell–Rubin likelihood ratio (VLMR) test and the Lo–Mendell–Rubin (LMR) test to determine the optimal number of classes. The estimated model was compared with the model with one class less, and the low p-value suggested that the model with one class less should be rejected. At each step, the classification quality was assessed using the average posterior probabilities for most likely latent class membership (AvePP). AvePP values close to 1 indicate a clear classification. In addition to the model fit, the final model for further analyses was chosen based on the parsimony and interpretability of the classes.

Differences in biological aging

The mean differences in biological aging between the lifestyle behavior patterns were studied using the Bolck–Croon–Hagenaars approach (***Asparouhov and Muthén, 2021***). The class-specific weights for each participant were computed and saved during the latent class model estimation. After that,

a secondary model conditional on the latent lifestyle behavior patterns was specified using weights as training data: Epigenetic aging measures were treated as distal outcome one at a time, and the mean differences across classes were studied while adjusting for sex, age and baseline pubertal development. Similarly, the mean differences in the components of DNAm GrimAge and lifestyle-related factors in young adulthood were studied. The models of epigenetic aging measures were additionally adjusted for BMI in adulthood. To evaluate the effect sizes, standardized mean differences (SMDs) were calculated.

Genetic and environmental influences

Genetic and environmental influences on biological aging in common with lifestyle behavior patterns were studied using quantitative genetic modeling. For simplicity, we adjusted the epigenetic aging variables for sex, age, and baseline pubertal development prior to the analysis.

We first carried out univariate modeling to study genetic and environmental influences on epigenetic aging measures (***Neale and Cardon, 1992***). The variance in the epigenetic aging measures was decomposed into the latent variables representing additive genetic (A), dominant genetic (D), or shared environmental (C) and non-shared environmental (E) components (ACE model or ADE model). The sequences of the models were fitted (ACE, ADE, AE, CE, and E). Because dominance in the absence of additive effects is rare, the model including D and E components (DE-model) was omitted. We used Satorra–Bentler scaled chi-squared (χ^2) test, comparative fit index (CFI), Tucker–Lewis index (TLI), root mean square error of approximation (RMSEA), and standardized root-mean-square residual (SRMR) to evaluate the goodness of fit of the models. The model fits the data well if the χ^2 test is not statistically significant (p>0.05), CFI and TLI values are close to 0.95, the RMSEA value is below 0.06, and the SRMR value is below 0.08 (***Hu and Bentler, 1999***). Moreover, BIC was used to compare non-nested models. A lower BIC value indicates a better model fit. The most parsimonious model with the sufficient fit to the data was considered optimal.

On the one hand, as described above, total variance in biological aging was decomposed in the components explained by genetic, shared, and unshared environmental factors $\left(a^2_{Tot} + c^2_{Tot} + e^2_{Tot}\right)$ $(= Var_{Tot})$ (***Figure 1A***). On the other hand, we can use the secondary model to study the differences in biological

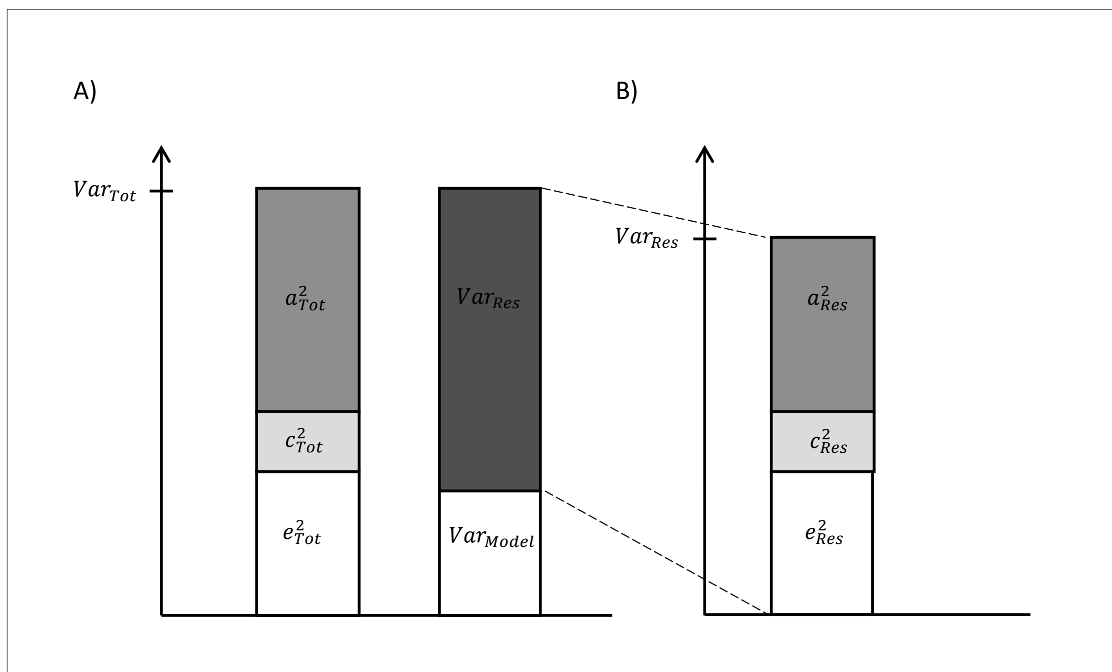

Figure 1. Decomposition of (**A**) total variation in biological aging and (**B**) the variation of the residual term.

aging between the adolescent lifestyle behavior patterns, as described above, and decompose the variance in biological aging into the variance explained by the adolescent lifestyle behavior patterns $Var_{(Model)}$ and the variance of the residual term $Var_{(Res)}$. We also conducted univariate modeling for the residual term of biological aging, which corresponds to the variation in biological aging not explained by the adolescent lifestyle behavior patterns $\left(a^2_{Res} + c^2_{Res} + e^2_{Res}\right)$ (= Var_{Res}) (***Figure 1B***). The residual terms were obtained by specifying a latent variable corresponding to the residuals of the secondary model described above (without including covariates), and the factor scores were saved. Finally, the proportion of variation in biological aging explained by the genetic factors shared with adolescent lifestyle patterns was evaluated as follows: $\left(a^2_{Tot} - a^2_{Res}\right)$ / Var_{Tot}. The proportion of variation in epigenetic aging explained by the environmental factors was evaluated similarly. These proportions reflect the extent to which the same genetic/environmental factors contribute to the association between the adolescent lifestyle patterns and biological aging (i.e., size of the genetic and environmental correlations between the phenotypes).

Missing data were assumed to be missing at random (MAR). The model parameters were estimated using the full information maximum likelihood (FIML) method with robust standard errors. Under the MAR assumption, the FIML method produced unbiased parameter estimates. The standard errors of the latent class models and secondary models were corrected for nested sampling (TYPE = COMPLEX). Descriptive statistics were calculated using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp, Armonk, NY), and further modeling was conducted using Mplus, version 8.2 (***Muthén and Muthén, 1998***).

Results

The descriptive statistics of the study variables are presented in ***Table 1***. A total of 5114 twins answered questionnaires on lifestyle-related behaviors during their adolescent years at least once. For 824 twins, epigenetic aging estimates were obtained. The mean age (SD) of the twins having information on biological aging was 22.4 (0.7) years. The means of the epigenetic age estimates were estimated as follows: Horvath's clock 28.9 (3.6), Hannum's clock 18.2 (3.3), DNAm PhenoAge 13.0 (5.3), and DNAm GrimAge 25.2 (3.3) years. The intraclass correlation coefficients (ICCs) of epigenetic aging measures were consistently higher in MZ twin pairs than in DZ twin pairs (***Table 2***). This suggests an underlying genetic component in biological aging. The correlations between the different epigenetic aging measures ranged from –0.12 to 0.73. The lowest correlation was observed between $AA_{Horvath}$ and DunedinPoAm and between $AA_{Horvath}$ and DunedinPACE. All other correlations were positive. The highest correlations (>0.5) were observed between AA_{Hannum} and AA_{Pheno}, AA_{Grim} and DunedinPoAm, and DunedinPoAm and DunedinPACE.

Patterns of lifestyle behaviors

Increasing the number of classes continued to improve AIC, BIC, and ABIC (***Table 3***). However, the VLMR and LMR tests indicated that even a solution with four classes would be sufficient. In the fifth step, a class of participants with high BMI was extracted. Previous studies have shown the role of being overweight or obese in biological aging (***Lundgren et al., 2022***). After including the sixth class, the information criteria still showed considerable improvement, but the AvePPs for several classes were below 0.8. For these reasons, and to have adequate statistical power for subsequent analyses, a five-class solution was considered optimal. The AvePPs ranged from 0.78 to 0.91 for the five-class solution, indicating reasonable classification quality.

Of the participants, 32% fell into the class of healthiest lifestyle habits (C1) (see ***Figure 2***, and the distributions of indicator variables according to the adolescent lifestyle behavior patterns in ***Table 4***). They had normal weight, on average, and were more likely to engage in regular LTPA compared to the other groups; most of them were non-smokers and did not use alcohol regularly. Every fifth (19.9%) participant belonged to the second class (C2), characterized by the low mean level of BMI in the range of normal weight for children (low-normal BMI) (***Cole et al., 2007***). They also had healthy lifestyle habits, but they were not as physically active as the participants in class C1. The participants placed in the third class (C3, 22.8%) had lifestyle habits similar to those of the participants in class C1; however, they had a higher level of BMI in the range of normal weight for children (high-normal BMI). About every tenth (9.5%) of the participants belonged to the fourth class (C4), with the highest

Table 1. Descriptive statistics of the adolescent lifestyle-related variables in all twins and in the subsample of twins with information on biological aging.

	All twins (n = 5114)		Subsample (n = 824)	
	n	Mean (SD) or %	n	Mean (SD) or %
Zygosity	4852		824	
MZ	1650	34.0	335	40.7
Same-sex DZ	1603	33.0	262	31.8
Opposite-sex DZ	1599	33.0	227	27.5
Sex	5114		824	
Female	2584	50.5	470	57.0
Male	2530	49.5	354	43.0
At age 12				
Pubertal development (1–3)	5111	1.6 (0.5)	823	1.6 (0.5)
Body mass index	4913	17.6 (2.6)	793	17.7 (2.6)
Leisure-time physical activity	5038		813	
Less than once a week	1877	37.3	295	35.3
Once a week	2499	49.6	416	51.2
Every day	662	13.1	102	12.5
At age 14				
Body mass index	4473	19.3 (2.7)	787	19.5 (2.6)
Leisure-time physical activity	4590		799	
Less than once a week	688	15.0	110	13.8
Once a week	796	17.3	149	18.6
2–5 times a week	2182	47.5	370	46.3
Every day	924	20.1	170	21.3
Smoking status	4570		800	
Never	3954	86.5	687	85.9
Former	296	6.5	57	7.1
Occasional	122	2.7	24	3.0
Daily smoker	198	4.3	32	4.0
Alcohol use (binge drinking)	4565		796	
Never	3501	76.7	602	75.6
Less than once a month	756	16.6	135	17
Once or twice a month	275	6.0	50	6.3
Once a week or more	33	0.7	9	1.1
At age 17				
Body mass index	4158	21.4 (3.0)	760	21.4 (2.7)
Leisure-time physical activity	4208		766	
Less than once a week	748	17.8	132	17.2
Once a week	686	16.3	130	17.0
2–5 times a week	1977	47.0	363	47.4

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Table 1 continued

	All twins (n = 5114)		Subsample (n = 824)	
	n	Mean (SD) or %	n	Mean (SD) or %
Every day	797	18.9	141	18.4
Smoking status	4190		762	
Never	2419	57.7	454	59.7
Former	493	11.8	83	10.9
Occasional	213	5.1	48	6.3
Daily smoker	1065	25.4	176	23.1
Alcohol use (binge drinking)	4217		766	
Never	881	20.9	152	19.8
Less than once a month	1807	42.9	340	44.4
Once or twice a month	1240	29.4	222	29.0
Once a week or more	289	6.9	52	6.8

MZ, monozygotic twins; DZ, dizygotic twins; SD, standard deviation.

level of BMI (high BMI). At each measurement point, the mean BMI level exceeded the cutoff points for overweight in children (***Cole et al., 2007***). The prevalence of daily smoking was slightly higher in C4 compared to classes C1, C2, and C3. Of the participants, 15.9% were classified into the subgroup characterized by the unhealthiest lifestyle behaviors (C5). Most of them were daily smokers and used alcohol regularly at the age of 17. They also had a lower probability of engaging in regular LTPA compared to the other groups; however, they were of normal weight, on average.

Boys were slightly over-represented in the classes that were most physically active (C1, C3) and had the highest levels of BMI (C3, C4) (percentage of boys: C1: 57.2%; C3: 51.5%; and C4: 52.7%), and under-represented in the classes with lowest levels of BMI (C2) and the unhealthiest lifestyle behavior pattern (C5) (C2: 42.7%; C5: 44.1%). There were also differences in pubertal development at baseline between the groups. The subgroups with the highest levels of BMI (C3, C4) and the class with

Table 2. The intraclass correlation coefficients (ICCs) of epigenetic aging measures by zygosity and correlation coefficients between the measures (n = 824).

	ICCs (95% CI)		Correlation coefficients (95% CI) off-diagonal and means (standard deviations) on the diagonal					
	MZ twin pairs	DZ twin pairs	$AA_{Horvath}$	AA_{Hannum}	AA_{Pheno}	AA_{Grim}	DunedinPoAm	DunedinPACE
$AA_{Horvath}$	0.71 (0.63, 0.79)	0.40 (0.24, 0.55)	0.00 (3.51)					
AA_{Hannum}	0.66 (0.56, 0.76)	0.32 (0.16, 0.48)	0.40 (0.33, 0.48)	0.00 (3.27)				
AA_{Pheno}	0.69 (0.60, 0.78)	0.16 (0.00, 0.33)	0.36 (0.29, 0.44)	0.61 (0.56, 0.66)	0.00 (5.25)			
AA_{Grim}	0.72 (0.63, 0.80)	0.35 (0.15, 0.55)	0.08 (0.01, 0.16)	0.32 (0.24, 0.40)	0.39 (0.33, 0.46)	0.00 (3.24)		
DunedinPoAm	0.62 (0.52, 0.71)	0.42 (0.24, 0.60)	–0.05 (-0.12, 0.03)	0.20 (0.13, 0.27)	0.41 (0.35, 0.47)	0.57 (0.52, 0.63)	1.00 (0.07)	
DunedinPACE	0.71 (0.64, 0.78)	0.46 (0.31, 0.61)	–0.04 (–0.11, 0.04)	0.30 (0.22, 0.38)	0.49 (0.43, 0.55)	0.55 (0.49, 0.61)	0.62 (0.57, 0.67)	0.88 (0.10)

CIs were corrected for nested sampling.
CI, confidence interval; AA, age acceleration; MZ, monozygotic; DZ, dizygotic.

Table 3. Model fit of the latent class models (n = 5114).

AIC	BIC	ABIC	VLMR	LMR	Class sizes	AvePP
128842	129012	128929				
122533	122880	122711	<0.001	<0.001	74.0%, 26.0%	0.95, 0.92
119937	120460	120206	<0.001	<0.001	44.9%, 40.5%, 14.6%	0.88, 0.89, 0.93
118030	118729	118389	<0.001	<0.001	36.4%, 32.7%, 16.7%, 14.2%	0.83, 0.86, 0.87, 0.92
117167	118043	117617	0.529	0.530	32.0%, 22.8%, 19.9%, 15.9%, 9.5%	0.78, 0.82, 0.85, 0.88, 0.91
116526	117578	117076	0.169	0.170	31.5%, 18.5%, 15.7%, 14.0%, 12.7%, 7.7%	0.77, 0.84, 0.83, 0.78, 0.78, 0.90
116099	117328	116731	0.043	0.044	21.0%, 17.5%, 15.2%, 13.8%, 12.9%, 12.8%, 6.9%	0.73, 0.82, 0.70, 0.77, 0.83, 0.83, 0.91
115695	117101	116418	0.407	0.408	20.3%, 16.2%, 13.6%, 13.5%, 12.3%, 11.3%, 9.3%, 3.4%	0.72, 0.75, 0.82, 0.71, 0.83, 0.80, 0.82, 0.89

AIC, Akaike's information criterion; BIC, Bayesian information criterion; ABIC, sample size-adjusted Bayesian information criterion; VLMR, Vuong–Lo–Mendell–Rubin likelihood ratio test; LMR, Lo–Mendell–Rubin-adjusted likelihood ratio test; AvePP, average posterior probabilities for most likely latent class membership.

unhealthiest lifestyle habits (C5) were, on average, the most advanced in pubertal development (mean PDS, C3: 1.67 95% CI: [1.63–1.71], C4: 1.69 [1.64–1.74]; and C5: 1.68 [1.63–1.72]), while the class with the healthiest lifestyle pattern (C1) and that with the lowest level of BMI (C2) were less advanced in pubertal development (C1: 1.53 [1.50–1.56]; C2: 1.44 [1.41–1.47]).

The distribution of lifestyle behavior patterns in the subsample of participants having information on biological aging was very similar to that in the large cohort data (C1: 33.0%; C2: 16.6%; C3: 20.6%; C4: 10.1%; C5: 19.7%). In the subsample, the differences in lifestyle-related factors were maintained well over the transition from adolescence to young adulthood (***Figure 2—figure supplement 1***).

Differences in biological aging

There were differences among the classes in AA_{Pheno} (Wald test: p=0.006), AA_{Grim} (p=2.3e-11), DunedinPoAm (p=3.1e-9), and DunedinPACE (p=5.5e-7) in the models adjusted for sex, age, and baseline pubertal development. There were no differences in biological aging when Horvath's clock (p=0.550) and Hannum's clock (p=0.487) were used. The overall results considering AA_{Grim}, DunedinPoAm, and DunedinPACE were very similar (***Figure 3*** and ***Table 5***).

The group with the unhealthiest lifestyle pattern (C5) was, on average, 1.7–3.3 years biologically older than the groups with healthier lifestyle patterns and normal weight (C1–C3) when DNAm GrimAge was used to assess biological aging (***Table 5***, M1). Moreover, the unhealthiest group had, an average, 2–3 weeks/calendar year faster pace of biological aging, as measured with DunedinPoAm. The differences in DunedinPACE were very similar to those observed in DunedinPoAm, but there was no difference between the unhealthiest class (C5) and the class with a healthy lifestyle and high-normal BMI (C3) and, moreover, the difference between the healthiest class (C1) was not significant at 0.01 level.

When DNAm GrimAge was used, the group with a high BMI (C4) was, on average, 1.8–2.4 years biologically older than the two groups with healthier lifestyle patterns (C1 and C2) (***Table 5***, M1). When measured with the DunedinPoAm estimator, the class had, on average, 3–4 weeks/calendar year faster pace of aging, and when measured with the DunedinPACE estimator, it had 4–5 weeks/calendar year faster pace of aging. Moreover, when DunedinPoAm and DunedinPACE were used, the class had approximately 3 weeks/calendar year faster pace of aging compared to the group with healthy lifestyle with normal-high BMI (C3), and when DunedinPACE was used, the class had 2 weeks/calendar year faster pace of aging compared to the group with unhealthiest lifestyle pattern (C5). When DNAm PhenoAge was used to assess biological aging, only the group with a high BMI stood out. The group was biologically 2.0–2.5 years older than the groups with lower mean levels of BMI (C1–C2, C5). Based on the estimation results of the models, baseline pubertal development was associated with advanced biological aging only when Hannum's clock was used to derive biological AA (standardized regression coefficient B = 0.10 [0.01–0.18]).

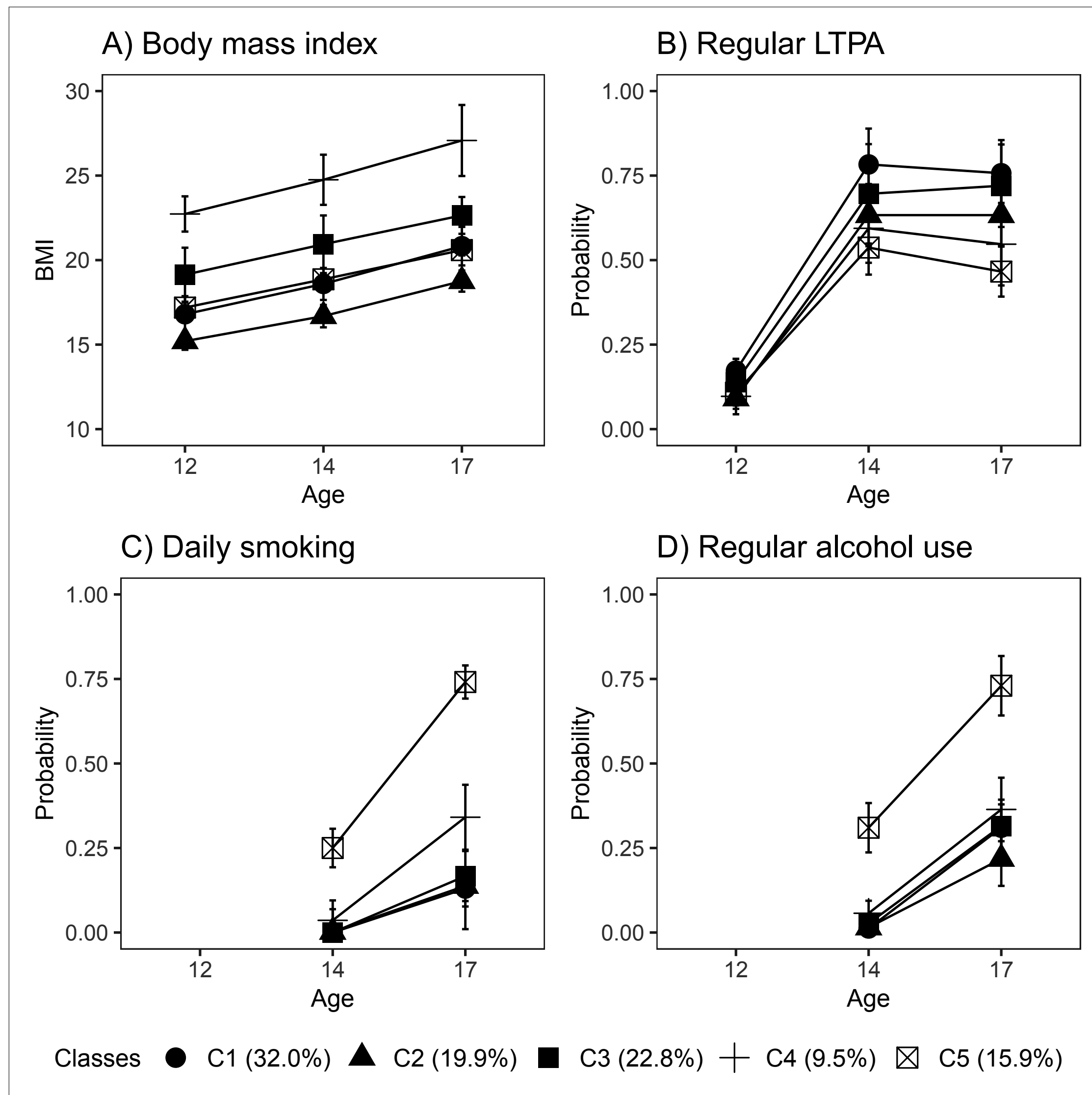

Figure 2. Classes with different lifestyle patterns (n = 5114). Mean and probability profiles (95% confidence intervals) of the indicator variables utilized in the classification: (**A**) body mass index, (**B**) regular leisure-time physical activity (LTPA) (several times a week), (**C**) daily smoking, and (**D**) regular alcohol use (once a month or more). For categorical variables, the probabilities of belonging to the highest categories are presented.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. The estimation results of a latent class analysis (LCA) model with five classes.

Figure supplement 1. Lifestyle-related factors in adulthood (21–25 years) according to the adolescent lifestyle behavior classes in the subsample of participants with information on biological aging (n = 824).

Figure supplement 1—source data 1. Means and 95% confidence intervals of the lifestyle-related factors in adulthood according to the adolescent lifestyle behavior classes (BCH approach).

Table 4. The classes with different adolescent lifestyle behavior patterns (n = 5114).

	C1 (32.0%)		C2 (19.9%)		C3 (22.8%)		C4 (9.5%)		C5 (15.9%)	
	Est	**95% CI**	**Est**	**95% CI**	**Est**	**95% CI**	**Est**	**95% CI**	**Est**	**95% CI**
Body mass index										
At age of 12 years	16.8	15.7, 17.9	15.2	14.7, 15.7	19.1	17.5, 20.7	22.7	21.7, 23.8	17.2	16.9, 17.5
At age of 14 years	18.6	17.6, 19.5	16.7	16.0, 17.3	20.9	19.2, 22.6	24.8	23.3, 26.2	18.9	18.6, 19.2
At age of 17 years	20.8	19.7, 22.0	18.8	18.1, 19.4	22.6	21.6, 23.7	27.1	25.0, 29.2	20.6	20.3, 20.9
Leisure-time physical activity										
At age of 12 years										
Less than once a week	0.29	0.22, 0.37	0.45	0.39, 0.51	0.35	0.26, 0.43	0.44	0.37, 0.50	0.44	0.39, 0.48
Once a week	0.54	0.48, 0.59	0.46	0.41, 0.50	0.52	0.47, 0.56	0.47	0.39, 0.54	0.46	0.41, 0.50
Every day	0.17	0.14, 0.21	0.09	0.04, 0.14	0.14	0.07, 0.21	0.10	0.06, 0.13	0.11	0.08, 0.14
At age of 14 years										
Less than once a week	0.08	0.05, 0.11	0.17	0.12, 0.22	0.14	0.07, 0.22	0.18	0.13, 0.23	0.27	0.22, 0.31
Once a week	0.14	0.07, 0.20	0.20	0.17, 0.24	0.16	0.10, 0.23	0.23	0.17, 0.28	0.20	0.16, 0.23
2–5 times a week	0.52	0.45, 0.59	0.45	0.41, 0.49	0.51	0.43, 0.59	0.43	0.37, 0.49	0.40	0.35, 0.45
Every day	0.27	0.23, 0.30	0.18	0.13, 0.23	0.19	0.12, 0.25	0.17	0.12, 0.21	0.14	0.10, 0.17
At age of 17 years										
Less than once a week	0.10	0.05, 0.14	0.19	0.14, 0.23	0.13	0.06, 0.20	0.27	0.19, 0.35	0.35	0.29, 0.40
Once a week	0.15	0.11, 0.18	0.18	0.15, 0.21	0.15	0.11, 0.19	0.18	0.14, 0.23	0.19	0.15, 0.23
2–5 times a week	0.50	0.44, 0.56	0.45	0.41, 0.49	0.53	0.48, 0.57	0.44	0.36, 0.52	0.36	0.32, 0.41
Every day	0.26	0.22, 0.29	0.18	0.13, 0.23	0.20	0.12, 0.27	0.11	0.07, 0.15	0.10	0.07, 0.13
Smoking status										
At age of 14 years										
Never	0.99	0.98, 1.00	0.98	0.95, 1.00	0.97	0.95, 1.00	0.83	0.74, 0.93	0.33	0.24, 0.43
Former	0.01	0.00, 0.02	0.02	0.00, 0.03	0.02	0.00, 0.04	0.09	0.04, 0.14	0.29	0.24, 0.34
Occasional	0.00		0.01	–0.01, 0.02	0.00	0.00, 0.01	0.04	0.01, 0.07	0.13	0.10, 0.16
Daily smoker	0.00		0.00	0.00, 0.01	0.00		0.04	0.00, 0.07	0.25	0.19, 0.31
At age of 17 years										
Never	0.69	0.61, 0.77	0.73	0.65, 0.81	0.68	0.59, 0.78	0.50	0.41, 0.59	0.03	0.00, 0.06
Former	0.12	0.09, 0.15	0.09	0.05, 0.13	0.12	0.07, 0.16	0.11	0.06, 0.16	0.15	0.12, 0.19
Occasional	0.06	0.04, 0.07	0.04	0.02, 0.06	0.04	0.01, 0.06	0.05	0.02, 0.07	0.07	0.05, 0.10
Daily smoker	0.13	0.08, 0.18	0.14	0.09, 0.18	0.17	0.09, 0.24	0.34	0.24, 0.44	0.74	0.69, 0.79
Alcohol use (binge drinking)										
At age of 14 years										
Never	0.88	0.85, 0.91	0.94	0.90, 0.97	0.84	0.79, 0.89	0.76	0.69, 0.83	0.23	0.15, 0.31
Less than once a month	0.11	0.08, 0.14	0.05	0.02, 0.08	0.13	0.09, 0.17	0.18	0.12, 0.24	0.46	0.41, 0.51
Once or twice a month	0.01	0.00, 0.02	0.02	0.00, 0.03	0.03	0.01, 0.04	0.05	0.02, 0.08	0.27	0.22, 0.32
Once a week or more	0.00		0.00		0.00		0.00	0.00, 0.01	0.04	0.02, 0.06
At age of 17 years										
Never	0.21	0.18, 0.25	0.33	0.26, 0.41	0.22	0.16, 0.28	0.23	0.15, 0.30	0.01	0.00, 0.02

Table 4 continued on next page

Table 4 continued

	C1 (32.0%)		C2 (19.9%)		C3 (22.8%)		C4 (9.5%)		C5 (15.9%)	
	Est	**95% CI**	**Est**	**95% CI**	**Est**	**95% CI**	**Est**	**95% CI**	**Est**	**95% CI**
Less than once a month	0.48	0.43, 0.52	0.45	0.40, 0.49	0.46	0.41, 0.52	0.41	0.35, 0.47	0.26	0.22, 0.31
Once or twice a month	0.28	0.24, 0.32	0.18	0.12, 0.24	0.28	0.23, 0.33	0.29	0.23, 0.35	0.51	0.46, 0.55
Once a week or more	0.03	0.00, 0.06	0.04	0.02, 0.06	0.03	0.00, 0.06	0.08	0.04, 0.11	0.22	0.18, 0.26

Mean and probability profiles of the indicator variables utilized in the classification.

BMI, body mass index; Est, estimated mean or probability; CI, confidence interval; C1, the class with the healthiest lifestyle pattern; C2, the class with low-normal BMI; C3, the class with healthy lifestyle and high-normal BMI; C4, the class with high BMI; C5, the class with the unhealthiest lifestyle pattern.

According to the previous literature, it is controversial whether childhood obesity has a direct effect on later health or whether the association is fully mediated by BMI in adulthood (***Park et al., 2012***). The role of adult BMI may depend on which disease outcome is studied (***Richardson et al., 2020***). After additionally adjusting the model for BMI in adulthood, the differences in AA_{Pheno} and DunedinPACE between the class of participants with high BMI (C4) and those with lower BMI (C1, C2, C5) were attenuated (***Table 5***, M2). This finding suggests that the observed differences in biological aging probably are fully mediated by BMI in adulthood. However, the differences in biological aging were only slightly attenuated when the DNAm GrimAge and DunedinPoAm estimators were used, suggesting that childhood overweight may leave permanent imprint on biological aging assessed with these measures. However, when DNAm GrimAge was used, the difference between the classes C4 and C1 was not significant at 0.01 level.

In our study, high standard deviations of epigenetic age estimates were observed. Therefore, variation in AA measures may largely be attributable to technical variation, which is not biologically meaningful. Recently developed principal component (PC)-based clocks are shown to improve the reliability and validity of epigenetic clocks (***Higgins-Chen et al., 2022***). We therefore replicated our main analyses using PC-based epigenetic clocks (data not shown). The standard deviations of epigenetic age estimates were similar or even higher compared with those obtained with the original clocks, but the correlations between AA measures assessed with different clocks were consistently higher when PC-based epigenetic clocks were used. Importantly, the observed associations with the adolescent lifestyle behavior patterns did not substantially change.

Differences in DNAm-based plasma proteins and smoking pack-years

Overall, after controlling for sex, age, and baseline pubertal development, there were differences in DNAm-based ADM (Wald test: p=0.010), B2M (p=0.014), and Packyrs (p=1.3e-5), but not in DNAm-based cystatin C (p=0.140), GDF15 (p=0.228), Leptin (p=0.228), PAI-1 (p=0.055), and TIMP-1 (p=0.089) between the adolescent lifestyle behavior patterns. The class with the unhealthiest lifestyle habits (C5) differed unfavorably from the other classes only by DNAm smoking pack-years while the class of participants with high BMI (C4) stood out by several DNAm-based plasma proteins including DNAm ADM, PAI-1, and TIMP-1 (***Figure 3—figure supplement 1***).

Genetic and environmental effects

Twin pairs with biological aging data on both members of the pair were used in the quantitative genetic modeling to estimate the genetic and environmental components of variance for biological aging (n = 154 monozygotic and 211 dizygotic pairs). The model including additive genetic and non-shared environmental component (AE model) was considered optimal for all the epigenetic aging measures (***Table 6***). Generally, ACE and ADE fit the data about as well, and models without genetic component (CE model) provided significantly worse fit. Based on these results, AE model was also chosen for the further modeling of the residual term of biological aging. Genetic factors explained 62–73% of the total variation in biological aging depending on the estimator. The rest of the variation (27–38%) was explained by unshared environmental factors.

The proportion of the total variation in biological aging in early adulthood explained by adolescent lifestyle behavior patterns was 3.7% for AA_{Pheno}, 16.8% for AA_{Grim}, 15.4% for DunedinPoAm,

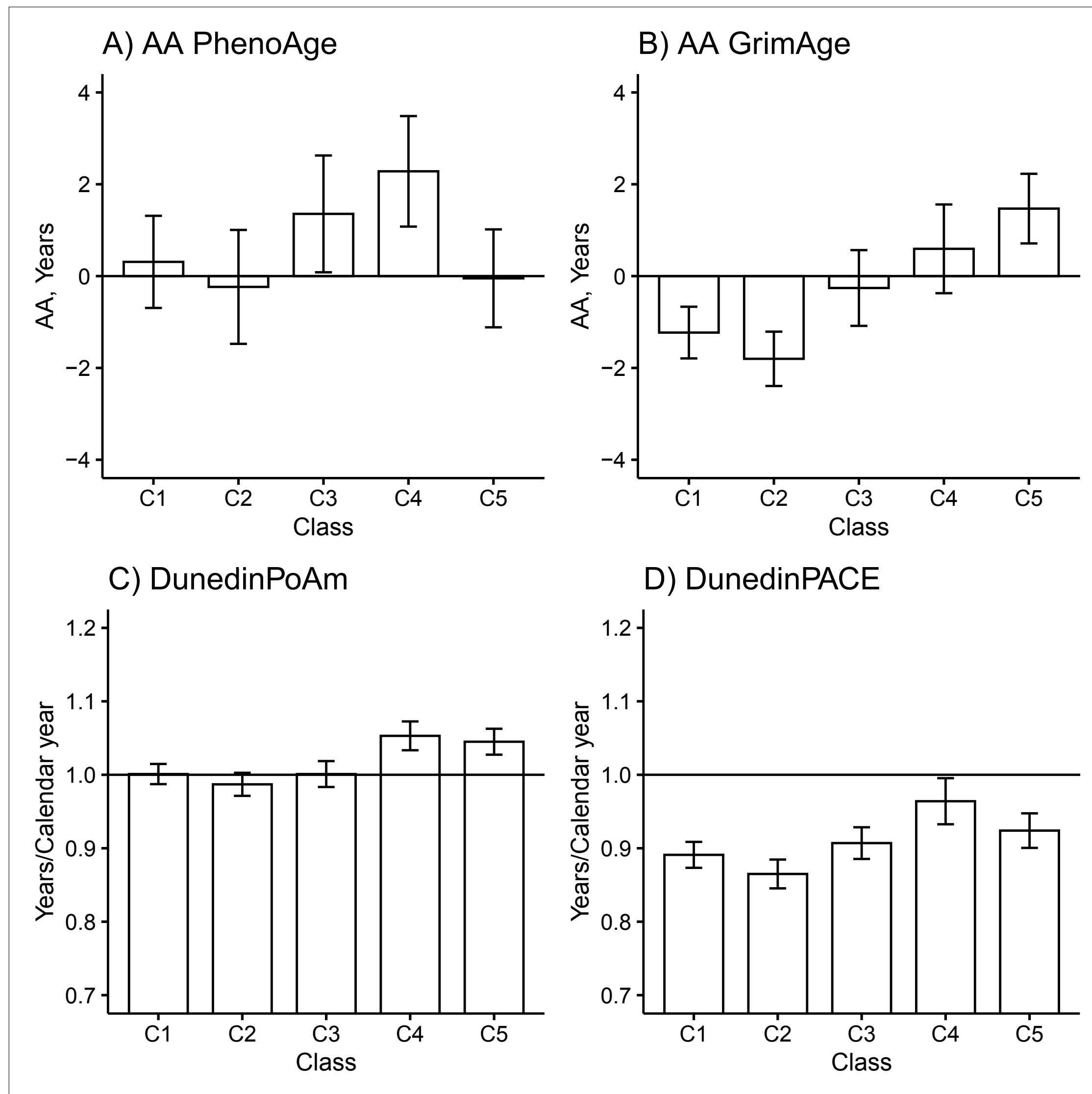

Figure 3. Mean differences between the adolescent lifestyle behavior patterns in biological aging measured with (**A**) DNAm PhenoAge, (**B**) DNAm GrimAge, (**C**) DunedinPoAm, and (**D**) DunedinPACE estimators (n = 824). The analysis was adjusted for sex (female), standardized age, and baseline pubertal development. Means and 95% confidence intervals are presented. C1, the class with the healthiest lifestyle pattern; C2, the class with low-normal body mass index (BMI); C3, the class with a healthy lifestyle and high-normal BMI; C4, the class with high BMI; C5, the class with the unhealthiest lifestyle pattern; AA, age acceleration.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Means and 95% confidence intervals of biological aging according to the adolescent lifestyle behavior patterns (BCH approach).

Figure supplement 1. DNA methylation (DNAm)-based plasma proteins and smoking pack-years according to the adolescent lifestyle behavior patterns (n = 824).

Figure supplement 1—source data 1. Means and 95% confidence intervals of DNA methylation (DNAm)-based plasma proteins and smoking pack-years according to the adolescent lifestyle behavior patterns (BCH approach).

Table 5. Differences in biological aging between classes with different adolescent lifestyle behavior patterns.

	AA_{Pheno}			AA_{Grim}			DunedinPoAm			DunedinPACE		
	Diff	**95% CI**	**SMD**	**Diff**	**95% CI**	**SMD**	**Diff**	**95% CI**	**SMD**	**Diff**	**95% CI**	**SMD**
C2 vs. C1												
M1	–0.55	–2.15, 1.06	–0.10	–0.57	–1.37, 0.23	–0.18	–0.01	–0.03, 0.01	–0.14	–0.03	–0.05, 0.00	–0.30
M2	–0.13	–1.79, 1.54	–0.02	–0.54	–1.38, 0.29	–0.17	–0.01	–0.03, 0.01	–0.14	–0.01	–0.04, 0.02	–0.10
C3 vs. C1												
M1	1.04	–0.54, 2.63	0.20	0.97	–0.01, 1.95	0.30	0.00	–0.02, 0.02	0.00	0.02	–0.01, 0.04	0.20
M2	0.60	–1.01, 2.21	0.11	0.94	–0.10, 1.97	0.29	0.00	–0.02, 0.02	0.00	0.00	–0.03, 0.03	0.00
C4 vs. C1												
M1	1.97	0.44, 3.50	0.38	1.83	0.74, 2.91*	0.56	0.05	0.03, 0.07*	0.71	0.07	0.04, 0.11*	0.70
M2	0.66	–1.31, 2.63	0.13	1.73	0.26, 3.21	0.53	0.04	0.01, 0.07*	0.57	0.02	–0.02, 0.07	0.20
C5 vs. C1												
M1	–0.36	–1.76, 1.04	–0.07	2.70	1.74, 3.66*	0.83	0.04	0.02, 0.07*	0.57	0.03	0.00, 0.06	0.30
M2	–0.45	–1.82, 0.93	–0.09	2.69	1.73, 3.66*	0.83	0.04	0.02, 0.06*	0.57	0.03	0.00, 0.06	0.30
C3 vs. C2												
M1	1.59	–0.07, 3.25	0.30	1.54	0.58, 2.50*	0.48	0.01	–0.01, 0.04	0.14	0.04	0.01, 0.07*	0.50
M2	0.73	–1.10, 2.55	0.14	1.48	0.36, 2.60*	0.46	0.01	–0.02, 0.03	0.14	0.01	–0.03, 0.04	0.10
C4 vs. C2												
M1	2.52	0.85, 4.18*	0.48	2.40	1.28, 3.51*	0.74	0.07	0.04, 0.09*	1.00	0.10	0.06, 0.14*	1.00
M2	0.79	–1.59, 3.16	0.15	2.27	0.59, 3.95*	0.70	0.05	0.02, 0.09*	0.71	0.03	–0.02, 0.08	0.30
C5 vs. C2												
M1	0.19	–1.40, 1.77	0.04	3.27	2.32, 4.23*	1.01	0.06	0.03, 0.08*	0.86	0.06	0.03, 0.09*	0.60
M2	–0.32	–1.97, 1.33	–0.06	3.24	2.21, 4.27*	1.00	0.05	0.03, 0.08*	0.71	0.04	0.01, 0.07	0.40
C4 vs. C3												
M1	0.93	–0.82, 2.67	0.18	0.85	–0.45, 2.16	0.26	0.05	0.03, 0.08*	0.71	0.06	0.02, 0.10*	0.60
M2	0.06	–1.91, 2.03	0.01	0.79	–0.68, 2.26	0.24	0.05	0.02, 0.08*	0.71	0.02	–0.02, 0.07	0.20
C5 vs. C3												
M1	–1.40	–2.99, 0.18	–0.27	1.73	0.62, 2.84*	0.53	0.04	0.02, 0.07*	0.57	0.02	–0.02, 0.05	0.20
M2	–1.05	–2.63, 0.54	–0.20	1.76	0.63, 2.88*	0.54	0.05	0.02, 0.07*	0.71	0.03	0.00, 0.06	0.30
C5 vs. C4												

Table 5 continued on next page

Table 5 continued

	AA_{Pheno}			AA_{Grim}			DunedinPoAm			DunedinPACE		
	Diff	**95% CI**	**SMD**	**Diff**	**95% CI**	**SMD**	**Diff**	**95% CI**	**SMD**	**Diff**	**95% CI**	**SMD**
M1	–2.33	−3.84, –0.82*	–0.44	0.88	–0.32, 2.07	0.27	–0.01	–0.03, 0.02	–0.14	–0.04	–0.08, 0.00	–0.40
M2	–1.10	–3.01, 0.80	–0.21	0.96	–0.51, 2.44	0.30	0.00	–0.03, 0.03	0.00	0.01	–0.04, 0.05	0.10

AA, age acceleration; BMI, body mass index; Diff, difference; CI, confidence interval; SMD, standardized mean difference; C1, the class with the healthiest lifestyle pattern; C2, the class with low-normal BMI; C3, the class with healthy lifestyle and high-normal BMI; C4, the class with high BMI; C5, the class with the unhealthiest lifestyle pattern; M1, model was adjusted for sex, age, and pubertal status at age 12; M2, model was additionally adjusted for BMI in adulthood.

*The corresponding 99% confidence interval did not overlap zero.

and 10.5% for DunedinPACE (***Figure 4***). The association between adolescent lifestyle patterns and biological aging in early adulthood was largely explained by shared genetic influences; the genetic factors shared with adolescent lifestyle explained 3.7, 13.1, 12.6, and 10.5%, respectively, of the total variation in biological aging. Depending on the biological aging estimate, only 0–3.7% of the total variation in biological aging was explained by (unshared) environmental factors shared with adolescent lifestyle patterns. The rest of the total variation in biological aging was explained by genetic and (unshared) environmental factors unique to biological aging.

Discussion

We conducted a twin study with a longitudinal lifestyle follow-up during the adolescent years and measured biological aging from genome-wide DNAm data using the most recent epigenetic aging clocks. Our findings supported previous studies, which showed that lifestyle-related behaviors tend to cluster in adolescence. In our study, most participants generally followed healthy lifestyle patterns, but we could also identify a group of young adults characterized by higher BMI (10% of all participants) in adolescence, as well as a group (16% of all participants) with more frequent co-occurrence of smoking, binge drinking, and low levels of physical activity in adolescence. We observed differences in biological aging between the classes characterized by adolescent lifestyle patterns in young adulthood, but the differences depended on the utilized epigenetic aging measure. Both the class with the overall unhealthiest lifestyle and that with a high BMI were biologically 1.7–3.3 years older than the classes with healthier lifestyle patterns when DNAm GrimAge was used to assess biological aging (AA_{Grim}). Moreover, they had 2–5 weeks/calendar year faster pace of biological aging (DunedinPoAm). The class with high BMI was biologically the oldest one when and DNAm PhenoAge and DunedinPACE were used. There were no differences when Horvath's and Hannum's clocks were used to estimate biological aging. The differences in lifestyle-related factors were maintained well over the transition from adolescence to young adulthood. However, genetic factors shared with adolescent lifestyle explained most of the observed differences in biological aging.

In our study, when the most recently published epigenetic aging measures were used, the class with the unhealthiest lifestyle was biologically 1.7–3.3 years older (AA_{Grim}) and had 2–3 weeks/calendar year faster pace of biological aging (DunedinPoAm) than the classes with healthier patterns. These measures can predict mortality and morbidity, especially cardiometabolic and lung diseases (***Belsky et al., 2020***; ***Belsky et al., 2022***; ***Lu et al., 2019***). A previous meta-analysis focusing on adults in a wide age range (17–99 years) showed that the number of healthy lifestyle behaviors is inversely associated with all-cause mortality risk (***Loef and Walach, 2012***). The mortality risk was up to 66% lower for individuals having multiple healthy behaviors compared to those adhering to an unhealthy lifestyle (smoking, low or high levels of alcohol use, unhealthy diet, no physical activity, and overweight). The accumulation of multiple unhealthy lifestyle habits during lifetime probably has a more detrimental effect on biological aging as well than any single lifestyle habit. However, our approach did not allow us to disentangle the effects of single lifestyle habits on biological aging. Our results suggest that the unhealthy lifestyle-induced changes in biological aging begin to accumulate in early life. These changes might predispose individuals to premature death in later life.

Table 6. The estimation results of the univariate model for biological aging among young adult twin pairs (MZ n = 154, DZ n = 211).

	Model fit									**Parameter estimates and their 95% confidence intervals**							
	X^2	**df**	**SC**	**p**	**CFI**	**TLI**	**RMSEA**	**SRMR**	**BIC**	**a^2/total**		**c^2 or d^2/total**		**e^2/total**		**Total**	
AA_{Pheno}																	
ACE	5.2	3	1.27	0.155	0.98	0.99	0.06	0.06	2009	0.65	0.56, 0.74	0.00		0.35	0.26, 0.45	1.00	0.89, 1.12
ADE	0.6	3	0.99	0.904	1.00	1.02	0.00	0.02	2003	0.03	–0.46, 0.51	0.65	0.15, 1.15	0.33	0.25, 0.41	0.99	0.88, 1.09
AE	7.0	4	0.96	0.136	0.97	0.99	0.06	0.06	2003	0.65	0.56, 0.74	-		0.35	0.26, 0.45	1.00	0.89, 1.12
CE	43.5	4	0.96	<0.001	0.60	0.80	0.23	0.11	2038	-		0.39	0.30, 0.48	0.61	0.52, 0.70	0.99	0.88, 1.10
E	107	5	0.96	<0.001	0.00	0.59	0.33	0.21	2093	-		-		1.00		0.99	0.88, 1.10
AA_{Grim}																	
ACE	4.3	3	2.05	0.231	0.99	0.99	0.05	0.09	1989	0.73	0.66, 0.80	0.00		0.27	0.20, 0.34	1.03	0.87, 1.20
ADE	5.6	3	1.55	0.133	0.98	0.98	0.07	0.09	1989	0.64	0.09, 1.19	0.09	–0.48, 0.66	0.27	0.20, 0.34	1.03	0.87, 1.19
AE	5.7	4	1.54	0.220	0.98	0.99	0.05	0.09	1983	0.73	0.66, 0.80	-		0.27	0.20, 0.34	1.03	0.87, 1.19
CE	33.0	4	0.87	<0.001	0.72	0.86	0.20	0.12	2018	-		0.50	0.40, 0.60	0.50	0.41, 0.60	1.02	0.87, 1.17
E	104	5	1.41	<0.001	0.06	0.62	0.33	0.26	2115	-		-		1.00		1.02	0.87, 1.17
DunedinPoAm																	
ACE	1.3	3	1.12	0.722	1.00	1.02	0.00	0.04	2003	0.52	0.20, 0.85	0.09	–0.20, 0.37	0.39	0.30, 0.48	0.98	0.86, 1.11
ADE	1.2	3	1.60	0.746	1.00	1.02	0.00	0.04	2003	0.62	0.53, 0.70	0.00		0.38	0.30, 0.47	0.98	0.86, 1.10
AE	1.6	4	1.20	0.802	1.00	1.02	0.00	0.04	1997	0.62	0.53, 0.70	-		0.38	0.30, 0.47	0.98	0.86, 1.10
CE	12.7	4	1.10	0.013	0.88	0.94	0.11	0.07	2009	-		0.45	0.36, 0.55	0.55	0.45, 0.64	0.98	0.86, 1.10
E	85.1	5	1.15	<0.001	0.00	0.55	0.30	0.22	2087	-		-		1.00		0.98	0.86, 1.10
DunedinPACE																	
ACE	2.0	3	1.08	0.582	1.00	1.00	0.00	0.05	1998	0.54	0.20, 0.87	0.08	–0.21, 0.37	0.39	0.30, 0.48	0.99	0.87, 1.11
ADE	1.3	3	1.68	0.740	0.99	1.00	0.00	0.05	1981	0.42	–0.13, 0.97	0.27	–0.31, 0.84	0.32	0.24, 0.39	0.98	0.84, 1.13

Table 6 continued on next page

Table 6 continued

	Model fit									Parameter estimates and their 95% confidence intervals							
	X^2	df	SC	p	CFI	TLI	RMSEA	SRMR	BIC	a^2/total		c^2 or d^2/total		e^2/total		Total	
AE	2.1	4	1.58	0.724	1.00	1.10	0.00	0.05	1976	0.68	0.52, 0.82	-		0.32	0.24, 0.40	0.99	0.84, 1.15
CE	23.7	4	1.45	<0.001	0.76	0.88	0.16	0.10	2007	-		0.45	0.35, 0.54	0.55	0.46, 0.65	0.98	0.84, 1.13
E	78.5	5	1.47	<0.001	0.09	0.64	0.28	0.23	2118	-		-		1.00		0.98	0.84, 1.13

The epigenetic aging measures were adjusted for sex, age, and baseline pubertal development prior to analysis.

SC, scaling correction; CFI, comparative fit index; RMSEA, root mean square error of approximation; SRMR, standardized root-mean-square residual; BIC, Bayesian information criterion; MZ, monozygotic; DZ, dizygotic.

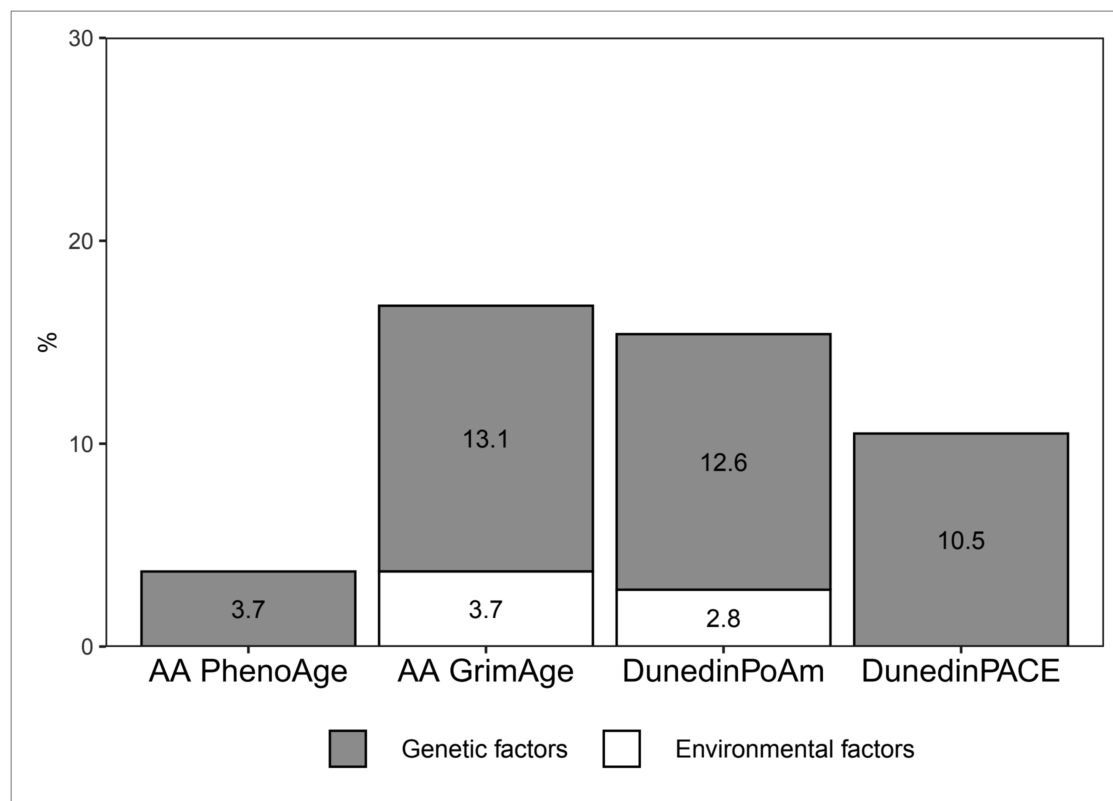

Figure 4. Proportions of the total variation in biological aging explained by genetic and (unshared) environmental factors shared with adolescent lifestyle patterns among young adult twin pairs (MZ n = 154, DZ n = 211). The results are based on the model including additive genetic and non-shared environmental component (AE model). AA, age acceleration.

The online version of this article includes the following source data for figure 4:

Source data 1. Genetic and environmental factors underlying the association between adolescent lifestyle patterns and biological aging.

To the best of our knowledge, this is the first study to investigate common genetic influences underlying lifestyle clusters and biological aging. Our results suggest genetic correlation between adolescent lifestyle and biological aging; individuals who are genetically prone to unhealthy lifestyles or overweight in adolescence are also susceptible to faster biological aging later in young adulthood. The shared genetic influences on two phenotypes may be due to several scenarios (***Solovieff et al., 2013***). They may arise from genetic pleiotropy; in this case, the genes may be a common cause for both adolescent lifestyle and biological aging. Another possible reason is causal relation between the phenotypes. In this case, genetic factors may affect adolescent lifestyle, which lies on the causal path to biological aging (or vice versa). However, for the relationship to be causal, it is necessary that there are shared environmental influences on the phenotypes (***De Moor et al., 2008***). In our study, environmental influences shared with adolescent lifestyle on biological aging were observed only when DNAm GrimAge and DunedinPoAm estimators were used. In line with our study, ***McCartney et al., 2021*** showed that there are shared underlying genetic contributions between single lifestyle factors and biological aging (AA_{Grim}, AA_{Pheno}) using polygenetic risk scores for epigenetic AA. Their Mendelian randomization analysis also suggested causal influences of BMI and smoking on biological aging, but only when DNAm GrimAge was used.

To the best of our knowledge, this is also the first study reporting the association between adolescent BMI (relative weight) and biological aging in later life. Previous systematic reviews have concluded that being overweight or obese in childhood and adolescence has a consistent impact on mortality

and morbidity in later life (**Park et al., 2012**; **Reilly and Kelly, 2011**). In particular, the associations with cardiometabolic morbidity are well-established, but the results of the studies investigating the associations independent of adult BMI are inconclusive (**Park et al., 2012**). A more recent study showed that early-life body size indirectly predisposes coronary artery disease and type 2 diabetes through body size in adulthood rather than having a direct effect (**Richardson et al., 2020**). Our results considering biological aging are in line with the existing literature but depend on the epigenetic clock utilized. In our study, the participants assigned to the class that was, on average, overweight in adolescence were biologically older (based on AA_{Pheno}, AA_{Grim}, DunedinPoAm, and DunedinPACE) in young adulthood compared to the classes of normal weight and healthy lifestyle habits. The group stood out, especially when AA_{Pheno} and DunedinPACE were used to measure biological aging, but adult BMI explained the observed differences in these measures. Practically all variance of AA_{Pheno} and DunedinPACE shared with adolescent lifestyle was explained by shared genetic factors. Therefore, these measures probably capture aspects of biological aging that are attributed to genetic factors shared with BMI. Mainly, the differences in AA_{Grim} and DunedinPoAm did not attenuate after additionally controlling for adult BMI, suggesting that higher BMI in adolescence has a direct long-term effect on biological aging measured with these epigenetic clocks.

LTPA is associated with a lower risk of mortality and cardiovascular diseases (**Li et al., 2013**; **Löllgen et al., 2009**). Twin studies and genetically informed studies have suggested that genetic pleiotropy can partly explain these frequently observed associations (**Karvinen et al., 2015**; **Sillanpää et al., 2022**). Previous studies have shown that LTPA is also associated with slower biological aging (**Kankaanpää et al., 2021**). In this study, lower levels of physical activity in adolescence were closely intertwined with other unhealthy behaviors. To fully understand the role of adolescence physical activity in later biological aging would require a more comprehensive analysis of activity patterns, intensities, and modes, as well as subgroup analyses that account for other lifestyle factors, such as diet.

Adolescent smoking behavior and alcohol use appeared to be strongly clustered, in line with the findings of a recent systematic review (**Whitaker et al., 2021**). For this reason, the associations of smoking and alcohol use with biological aging might be difficult to disentangle. Smoking is the most detrimental lifestyle factor, and its association with accelerated biological aging has been frequently reported (**Oblak et al., 2021**). However, the results obtained for the association between alcohol use and biological aging remain unclear (**Oblak et al., 2021**). A recent study showed that smoking has a causal effect on AA_{Grim}, whereas alcohol use did not exhibit such effect (**McCartney et al., 2021**). Epigenetic methylation changes due to alcohol seem to be much fewer in number and magnitude compared to smoking exposure (**Stephenson et al., 2021**). In our study, the unhealthiest lifestyle class, in which smoking and alcohol use co-occurred, exhibited accelerated biological aging, especially when GrimAge and DunedinPoAm were used. These epigenetic aging measures are highly sensitive to tobacco exposure (**Belsky et al., 2020**; **Lu et al., 2019**). DNAm GrimAge is a composite biomarker comprising seven DNAm surrogates for plasma markers and smoking pack-years, which can predict the time to death (**Lu et al., 2019**). DunedinPoAm utilizes a specific CpG site (located within the gene AHRR), the methylation of which is strongly affected by tobacco exposure (**Belsky et al., 2020**). For these reasons, most of the variation in biological aging, which is explained by environmental factors shared with adolescent lifestyle, is probably due to smoking exposure.

To better understand the observed differences in biological aging, we also studied differences in DNAm-based surrogates included in the DNAm GrimAge estimator (***Figure 3—figure supplement 1***). Surprisingly, the class with the unhealthiest lifestyle pattern differed unfavorably from those with healthier habits only in DNAm-based smoking pack-years. The class with a high BMI had increased levels of several DNAm-based plasma markers, including DNAm PAI-1 and TIMP-1, which are associated with markers of inflammation and metabolic conditions (**Lu et al., 2019**). These findings support the suggestions that AA_{Grim} is a useful biomarker for cardiovascular health and a potential predictor of cardiovascular disease already in young adulthood (**Joyce et al., 2021**).

Recent studies have yielded inconsistent results regarding the association between pubertal timing and biological aging (**Hamlat et al., 2021**; **Maddock et al., 2021**). In our models studying the differences in biological aging across adolescent lifestyle patterns, pubertal development at the age of 12 was not associated with accelerated biological aging in young adulthood (except for AA_{Hannum}). Moreover, the class with a high BMI included participants with advanced pubertal development, which might reflect the common genetic background underlying BMI and age at menarche (**Kaprio et al.,**

1995). All these findings support the studies showing that childhood obesity, which tracks forward into adulthood, explains the observed associations between advanced pubertal status and worse cardiovascular health (***Bell et al., 2018***) and can further reflect the genetic architecture underlying BMI, pubertal development, and worse health (***Day et al., 2015***).

Our study has the following major strengths. Adolescent lifestyle-related patterns were identified using population-based large cohort data (N ~ 5000), with longitudinal measurements of lifestyle-related factors assessed using validated questionnaires. Response rates were high and the distribution of the lifestyle-related patterns in the subsample of twins with information on biological aging was similar to the distribution in large cohort data, supporting the generalizability of our findings. Moreover, adolescent lifestyle behavior patterns were identified using data-driven LCA. This approach enabled us to use all available data on adolescent lifestyle-related behaviors and identify the patterns without using artificial cutoff points for the variables. The reciprocal associations between different lifestyle-related factors, as well as their joint association with biological aging, are complex, and individual associations are difficult to interpret. However, our approach produced results with easy interpretation. The data were prospective, and biological aging was assessed with novel epigenetic aging measures, including a recently published DunedinPACE estimator. Furthermore, for the first time, we could evaluate the proportions of genetic and environmental influences underlying adolescent lifestyle as a whole in relation to biological aging by using quantitative genetic modeling. However, our study also has some limitations. Adolescent lifestyle-related behaviors were self-reported and, therefore, might be susceptible to recall bias and bias through social desirability.

In conclusion, later biological aging reflects adolescent lifestyle behavior. Our findings advance research on biological aging by showing that a shared genetic background can underlie both adolescent lifestyle and biological aging measured with epigenetic clocks.

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Author contributions

Anna Kankaanpää, Conceptualization, Formal analysis, Methodology, Writing – original draft; Asko Tolvanen, Supervision, Methodology, Writing – review and editing; Aino Heikkinen, Data curation, Writing – review and editing, Preprocessing of the DNAm data; Jaakko Kaprio, Elina Sillanpää, Conceptualization, Resources, Supervision, Funding acquisition, Project administration, Writing – review and editing; Miina Ollikainen, Conceptualization, Resources, Supervision, Funding acquisition, Writing – review and editing

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Ethics

Human subjects: Data collection was conducted in accordance with the Declaration of Helsinki. The Indiana University IRB and the ethics committees of the University of Helsinki and Helsinki University Central Hospital approved the study protocol (113/E3/2001and 346/E0/05). The blood samples for DNA analyses were collected during in-person clinical studies after written informed consent was signed.

Decision letter and Author response

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Additional files

Supplementary files

- Supplementary file 1. The codes used to analyse the data.
- MDAR checklist

Data availability

A subsample of the FTC with DNA methylation age estimates, phenotypes, and information on the adolescent lifestyle behaviour patterns (BCH weights) will be located in the Biobank of the National Institute for Health and Welfare. All these data will be publicly available for use by qualified researchers following a standardised application procedure (see the website https://thl.fi/en/web/thl-biobank/for-researchers for details on the application process). Because of the consent given by study participants and the high degree of identifiability of the twin siblings in Finland, the full cohort data cannot be made publicly available. The full cohort data are available through the Institute for Molecular Medicine Finland (FIMM) Data Access Committee (DAC) for authorized researchers who have IRB/ethics approval and an institutionally approved study plan. For more details, please contact the FIMM DAC (fimm-dac@helsinki.fi). The codes used to analyse the data are provided in the supplementary file 1 and the processed data used to generate figures have been uploaded as the source data files.

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II

DO EPIGENETIC CLOCKS PROVIDE EXPLANATIONS FOR SEX DIFFERENCES IN LIFE SPAN? A CROSS-SECTIONAL TWIN STUDY

by

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Research Article

Do Epigenetic Clocks Provide Explanations for Sex Differences in Life Span? A Cross-Sectional Twin Study

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Abstract

Background: The sex gap in life expectancy has been narrowing in Finland over the past 4–5 decades; however, on average, women still live longer than men. Epigenetic clocks are markers for biological aging which predict life span. In this study, we examined the mediating role of lifestyle factors on the association between sex and biological aging in younger and older adults.
Methods: Our sample consists of younger and older twins (21–42 years, n = 1 477; 50–76 years, n = 763) including 151 complete younger opposite-sex twin pairs (21–30 years). Blood-based DNA methylation was used to compute epigenetic age acceleration by 4 epigenetic clocks as a measure of biological aging. Path modeling was used to study whether the association between sex and biological aging is mediated through lifestyle-related factors, that is, education, body mass index, smoking, alcohol use, and physical activity.
Results: In comparison to women, men were biologically older and, in general, they had unhealthier life habits. The effect of sex on biological aging was partly mediated by body mass index and, in older twins, by smoking. Sex was directly associated with biological aging and the association was stronger in older twins.
Conclusions: Previously reported sex differences in life span are also evident in biological aging. Declining smoking prevalence among men is a plausible explanation for the narrowing of the difference in life expectancy between the sexes. Data generated by the epigenetic clocks may help in estimating the effects of lifestyle and environmental factors on aging and in predicting aging in future generations.

Keywords: Biological age, DNA methylation, Life span, Lifestyle, Sex gap

Both sexes have experienced tremendous increases in life expectancy over the twentieth century. However, through all historical periods, women have had a longer life expectancy than men. The sex gap in life expectancy varies across time and country (1). In Finland, the sex gap increased greatly in the first half of the twentieth century. That gap was greatest in the mid-1970s (9 years); since then, it has narrowed to 5.4 years (2).

It has been suggested that sex differences in life span are caused by a complex combination of biological (genetic, hormonal) and nonbiological (behavioral, economic, social, environmental, and cultural) factors (3). Investigating sex differences in cause-specific mortality increases the understanding of the mechanisms underlying the sex differences in overall mortality. In comparison to women, men experience a higher risk of death from almost all causes (4). External causes of deaths, such as traffic accidents, trauma, alcohol intoxication, illicit drug overdoses, and suicides, are more common among men. However, at most, these factors typically explain a modest fraction of all premature deaths. The majority of premature deaths are caused by noncommunicable diseases (eg, cardiometabolic diseases, lung diseases, cancers, mental disorders, and dementia) (4). The biological and behavioral factors predisposing an individual to these diseases are predominantly the most important drivers of male-to-female differences in mortality.

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Overweight and obesity are dramatically increasing worldwide, predisposing both men and women to several noncommunicable diseases (5). While total body fat is lower in men, accumulation of harmful ectopic fat seems to be higher in men than women (6). Data regarding how obesity trends affect the sex gap in life expectancy are limited. Of the health-hazardous behavioral factors, tobacco smoking has been seen as the predominant driver of both the trend and the extent of sex differences in life expectancy. A recent study suggested that increasing smoking-related mortality among women and decreasing smoking-related mortality among men may account for as much as 40% of the narrowing sex gap in life expectancy over the last 2 decades (7). In general, men consume more alcohol than women. In Finland, the risk for alcohol-related death is 3 times higher in men than women (2). Globally, men tend to be more physically active than women at all ages (8), and leisure-time physical activity is known to be associated with a lower risk of premature death (9). Therefore, leisure-time physical activity is expected to diminish the sex gap in life expectancy. Socioeconomic factors might also affect the sex gap, such as differences in education, income, and physical demands of work between the sexes. For example, the sex gap is probably diminishing because many deaths related to trauma and toxication that previously occurred in male-dominated occupations are much rarer nowadays (5).

In addition to societal factors, differences in innate biology may also have a role in the survival gap between the sexes. Genetic and physiological differences between the sexes include progressive skewing of X chromosome inactivation, telomere attrition, maternally inherited mitochondrial inheritance, and hormonal and cellular differences in inflammatory and immunological responses and in substrate metabolism (10). The biological longevity advantage of women may also result from estrogen-associated greater resistance to oxidative damage (10). However, in women, sex hormone levels change drastically during menopause, potentially contributing to the reduction of age-related sex differences in health outcomes such as cardiovascular risk factors. The presentation of many diseases is directly influenced by biological factors as well as by gender identity associated with societal norms (3); through multiple routes, these are likely to contribute to the observed sex gaps in mortality and life expectancy.

Life expectancy may not always be a reliable proxy for how fast the population is aging, as it is the most distal outcome of aging processes. To better monitor population health, more sensitive methods are needed to track changes in aging. Novel biological clocks, that is, epigenetic clocks, may help track and understand the individual aging process and offer insights into sex differences in biological age and how lifestyle may counteract the aging process (11–15). These composite measures have been developed to quantify an individual's biological age, and they may enable accurate estimation of the pace of aging in all age groups. The first published results on biological age determined by epigenetic clocks have shown that men tend to be biologically older than women (16–20).

This study aimed to examine sex differences in biological age measured by novel epigenetic clocks in age groups younger and older than 50 years, with 50 being a proxy for menopausal age (21). Moreover, we aimed to assess whether the potential difference in biological aging between the sexes is mediated by different lifestyle factors, and whether age modifies these associations.

Method

Study Population

The Finnish Twin Cohort (FTC) includes 3 large cohort studies: (a) The older FTC includes twins born before 1958, (b) Finntwin16 includes twins born in 1975–1979, and (c) Finntwin12 includes twins born in 1983–1987 (22–24). The older FTC was established 45 years ago, and data collection has been extensively described recently (24). Finntwin16 was initiated in 1991 and to date, it includes 5 waves of completed data collections (22). The main scope of the project is to identify the genetic and environmental determinants of various health-related behaviors and diseases in different stages of life. Finntwin12 is the youngest of the 3 FTC cohorts (23). All eligible twins born in Finland during 1983–1987 along with their biological parents were enrolled to participate in 4 waves of questionnaires. Selected twins took part in laboratory studies with repeated interviews, neuropsychological tests, and collection of DNA were made as part of Wave 4 in early adulthood (23).

Twins from all 3 cohorts (age range from 21 to 76 years) who had taken part in clinical in-person studies with sampling for whole-blood DNA and subsequent DNA methylation (DNAm) analyses and who had the relevant phenotype data were included in the current study. The analysis sample included monozygotic (MZ) and dizygotic (DZ) same-sex twins (N = 1 893, 54% MZ) as well as opposite-sex twins (347 twin individuals, 151 complete twin pairs). Zygosity of same-sex pairs was confirmed by multiple genetic markers from genome-wide array data.

The FTC data collections were approved by the ethics committees of the University of Helsinki (113/E3/01 and 346/E0/05) and Helsinki University Central Hospital (270/13/03/01/2008 and 154/13/03/00/2011).

Main Variables

DNAm and assessment of biological age

DNAm profiles were obtained using Illumina's Infinium HumanMethylation450 BeadChip or the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA). A more detailed description of the preprocessing and normalizing of the DNAm data is provided in Supplementary Material. We utilized 4 epigenetic clocks to produce biological age estimates. Horvath's and Hannum's versions incorporate methylation levels of 353 and 71 age-related CpGs, respectively, and were trained via regressing on chronological age through a penalized regression model (12,13). The third epigenetic age estimator, DNAm PhenoAge, was trained on a composite clinical measure of phenotypic age and includes 513 CpG sites (14). The newest epigenetic clock, DNAm GrimAge, includes 1 030 CpG sites and was a product of the 2-step development method (15). It first utilized DNAm data to predict a set of biomarkers (plasma proteins and smoking pack-year) and then these developed DNAm-based biomarkers were used to predict mortality. In both steps, information on participants' sex and chronological age was used as well.

DNAm-based epigenetic age estimates, obtained by Horvath's and Hannum's clocks and by PhenoAge and GrimAge estimators, were calculated using a publicly available online calculator (https://dnamage.genetics.ucla.edu/new). The age acceleration (AA) of each clock was defined as the residual from regressing the estimated biological age on chronological age ($AA_{Horvath}$, AA_{Hannum}, AA_{Pheno}, and AA_{Grim}, respectively).

The components of DNAm GrimAge (adjusted for age) were obtained as well, including DNAm-based smoking pack-years and the surrogates for plasma proteins (DNAm-based plasma proteins):

DNAm adrenomedullin (ADM), DNAm beta-2-microglobulin (B2M), DNAm cystatin C, DNAm growth differentiation factor 15 (GDF15), DNAm leptin, DNAm plasminogen activator inhibitor 1 (PAI-1), and DNAm tissue inhibitor metalloproteinases 1 (TIMP-1).

Potential Mediating Variables

We surmised that differences in the covariates between the sex groups are more likely the factors that underlie the sex differences rather than being confounders. To study the factors underlying the sex differences, we chose lifestyle correlates that theoretically can be part of the mechanism leading to differences in cardiovascular diseases as well as in the length of the life span. The potential mediators included body mass index (BMI), smoking, alcohol use, physical activity, and educational attainment, which is a key component of socioeconomic status.

Educational attainment was assessed as the number of years of full-time education.

BMI, measured as kg/m^2, can be used as an estimate of healthy diet and sufficient energy intake. A high BMI describes excess fat in the body; thus, it is a consequence of a long-term imbalance between energy intake and expenditure. We measured height in cm using a stadiometer and body mass in kg using a beam scale in kg.

Smoking was self-reported and classified as never, former, and current smokers.

Alcohol use was measured based on self-reported quantity and frequency of use and the content of the alcoholic beverages. These data were transformed into 100% alcohol grams per day.

Physical activity was assessed using the Baecke Questionnaire (25). The questionnaire has 3 sections: sports participation, leisure-time physical activity excluding sports, and work- or school-related physical activity. The questionnaire includes 4 questions on sports activity and leisure-time activity, excluding sports, and 8 questions on occupational physical load scored on a 5-point scale. A sport index, a nonsport leisure-time (leisure) index, and a work index, respectively, were based on the mean scores of each section as described by Baecke et al. (25) and Mustelin et al. for the FinnTwin12 study (26).

Statistical Analysis

To compare differences in the study variables between men and women, we used linear regression analysis for the continuous variables and (multinomial) logistic regression for the categorical variables. In the models, the within-pair dependency of twin individuals was taken into account using the cluster option in the analysis.

Correlation coefficients between age and epigenetic age (DNAmAge) estimates and between AA measures were studied. The shape of the association between age and AA was studied using polynomial models of age as the continuous variable. To study whether sex differences in AA varied by age, the interaction effects of sex and age were also included in the regression models.

Mediation models were used to test whether the association between sex and AA is direct or mediated through lifestyle factors in all twins and opposite-sex twin pairs. First, the single mediation models were fitted. These models included indirect paths from sex to AA through one lifestyle factor at a time as well as the direct effect of sex on AA. In all twins, we further studied whether these associations differed according to age group, that is, whether age moderated the associations (Figure 1). The single mediation models included the interaction effect of sex and age group on the mediator variable (*i*1) and directly on AA (*i*2). Furthermore, the interaction effect between the mediator variable and age group on AA (*i*3) was tested for significance. Second, a multiple mediation model was fitted to assess the mediation effect of the different lifestyle factors simultaneously. The mediators as well as the interactions were included in the final multiple mediation model based on the results of the single mediator models.

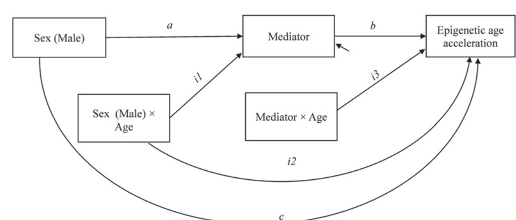

Figure 1. The path diagram of the single mediator model in all twins. The model included also the direct effect of age on the mediator and epigenetic age acceleration.

The standard errors were corrected for nested sampling using the special option in Mplus (TYPE = COMPLEX). The models for the opposite-sex twin pairs were fitted using multilevel modeling, and the mediation models were specified at the within-twin pairs level. The approach controls for shared childhood environmental factors and partly for genetic factors.

The age-specific indirect effects of sex (male) on AA through a mediator variable were calculated using the parameters of the models and the following formula: $(a + i1 \times \text{Age}) \times (b + i3 \times \text{Age})$ (27). The standardized indirect effects were reported as an effect size measure. These coefficients reflect the sex difference in AA explained by a certain mediator variable on a standardized scale. In the main analysis, age was treated as a dichotomous variable (0 = younger, 1 = older). As a sensitivity analysis, we reanalyzed the data using polynomial functions of age in the modeling to confirm that observed associations are not due to the differences in age distribution between sexes. For opposite-sex twin pairs, the indirect effects were calculated as the product of the within-twin pair level regression coefficients ($a \times b$).

The parameters of the models were estimated using the full information maximum likelihood method with robust standard errors. For the models including the ordinal mediator variable smoking status, the estimation was conducted using a robust weighted least squares estimator. In that case, the mediator is assumed to be the continuous latent variable underlying ordinal smoking status. Descriptive statistics and differences in the study variables were calculated and tested using Stata 16 software (StataCorp LLC, College Station, TX), and further modeling was conducted with the Mplus statistical package (version 8.2) (28).

Results

Sex Differences in Lifestyle Factors

The characteristics of the younger and older twins and the opposite-sex twin pairs included in this study are presented in Table 1. In both younger and older groups, there were fewer men than women. Among the older twins, the men were younger and better educated than the women. The men belonging to an opposite-sex twin pair had a lower level of education in comparison to their twin sisters. The men had a higher BMI in young adulthood than the women. Among all twins, there were more current smokers among the

Table 1. Sex Differences in Lifestyle-Related Factors, DNA Methylation Age, and Age Acceleration (AA) Estimates According to Age Group in All Twins (n = 2 240) and in Opposite-Sex Twin Pairs (151 pairs)

	All Twins								Opposite-Sex Twin Pairs			
	21- to 42-Year-Old Twins (N = 1 477)				Over 50-Year-Old Twins (N = 763)				21- to 30-Year-Old Twin Pairs (N =151)			
	Women	Men	Sex Difference		Women	Men	Sex Difference		Women	Men	Sex Difference	
			Mean	p			Mean	p			Mean	p
N	792[a]	685[b]			621[c]	142[d]			151[e]	151[e]		
Zygosity, mz/dz	349/443	274/411			322/299	86/56						
Education, years	16.8 (3.6)	16.6 (3.6)	−0.2	.266	9.6 (3.3)	12.3 (4.1)	2.6	<.001	17.3 (0.3)	16.3 (0.3)	−1.0	.003
Age, years	24.4 (3.5)	24.8 (3.3)	0.3	.166	66.6 (4.7)	62.0 (3.8)	−4.5	<.001	23.9 (2.2)			
DNAmAge, est. years												
Horvath	31.1 (5.5)	32.5 (5.1)	1.3	<.001	65.5 (6.1)	64.2 (5.4)	−1.2	.057	31.1 (4.6)	31.7 (4.9)	0.6	.076
Hannum	19.7 (4.5)	20.9 (4.3)	1.2	.001	55.7 (5.9)	55.0 (5.4)	−0.7	.311	19.1 (3.4)	20.6 (4.0)	1.4	<.001
PhenoAge	15.5 (6.9)	14.7 (6.2)	−0.8	.057	55.7 (7.7)	56.7 (7.2)	1.0	.231	14.2 (5.4)	13.8 (5.8)	−0.4	.459
GrimAge	26.4 (4.5)	27.7 (4.5)	1.3	<.001	58.6 (5.0)	59.2 (5.8)	0.6	.348	25.8 (3.1)	27.1 (3.7)	1.3	<.001
DNAmAge acceleration												
$AA_{Horvath}$	−0.4 (3.8)	0.6 (3.7)	1.1	<.001	−0.8 (3.6)	3.3 (5.2)	4.1	<.001	−0.1 (3.5)	0.6 (3.9)	0.6	.076
AA_{Hannum}	−0.5 (3.4)	0.4 (3.2)	1.0	<.001	−0.7 (4.7)	2.6 (4.2)	3.2	<.001	−0.6 (3.0)	0.8 (3.6)	1.4	<.001
AA_{Pheno}	0.3 (5.8)	−0.8 (5.1)	−1.1	<.001	−1.2 (7.2)	4.3 (6.0)	5.5	<.001	−0.7 (5.4)	−1.1 (5.7)	−0.4	.458
AA_{Grim}	−0.5 (3.5)	0.5 (3.5)	1.0	<.001	−0.8 (3.6)	3.3 (5.2)	4.1	<.001	−0.7 (2.9)	0.6 (3.4)	1.3	<.001
Lifestyle-related variables												
BMI, kg/m²	23.4 (4.8)	24.3 (3.8)	0.9	.001	27.6 (4.9)	28.0 (4.5)	0.4	.408	22.5 (3.5)	23.9 (3.5)	1.4	<.001
Smoking, n (%)												
Never	406 (51.3)	300 (43.8)			480 (77.3)	63 (44.4)		<.001	66 (43.7)	61 (40.4)		.646
Former	155 (19.6)	141 (20.6)	1.2[f]	.214	90 (14.5)	53 (37.3)	4.5[f]	.002	50 (33.1)	48 (31.8)	1.0[f]	.882
Current	230 (29.1)	244 (35.6)	1.4[f]	.028	51 (8.2)	26 (18.3)	3.9[f]	.023	35 (23.2)	42 (27.8)	1.3[f]	.323
Alcohol, g/day	7.4(9.4)	15.2 (18.0)	7.9	<.001	4..1 (7.4)	11.3 (19.7)	7.2	<.001	7.8 (9.3)	16.4 (19.9)	8.5	<.001
Physical activity												
Work index	2.7 (0.7)	2.7 (0.7)	0.0	.757	2.3 (1.0)	2.5 (0.98)	0.2	.161	2.7 (0.7)	2.8 (0.8)	0.1	.379
Sport index	2.9 (0.8)	3.0 (0.8)	0.1	.013	3.1 (0.8)	3.0 (0.83)	−0.1	.386	3.0 (0.8)	2.9 (0.8)	0.0	.942
Leisure index	3.0 (0.6)	2.8 (0.6)	−0.2	.001	2.9 (0.6)	2.7 (0.61)	−0.2	.026	3.0 (0.6)	2.8 (0.6)	−0.3	.001

Notes: DNAmAge = DNA methylation age; BMI = body mass index. Values are means and standard deviations (BMI, alcohol, physical activity indexes) or numbers and percentages (smoking). Between sex difference (linear or multinomial logistic regression analysis adjusted with family relatedness) is significant when p < .050. In physical activity indices *N*: [a]544–556, [b]440–446, [c]177–184, [d]139–141, and [e]91–93. [f]The odds ratio, never smokers were the reference category.

men in comparison to the women; there was no sex difference in smoking among the opposite-sex twin pairs. In all the groups, the men consumed more alcohol than the women. Moreover, the men had a lower level of leisure index in all the groups in comparison to the women, but the men in the younger same-sex twin group had a higher level of sport index than women.

Sex Differences in Epigenetic Aging

The correlation coefficients between chronological age and DNAmAge estimates ranged from 0.54 to 0.76 in younger twins (Figure 2), from 0.41 to 0.69 in older twins (Figure 3), and from 0.23 to 0.69 in opposite-sex twins (Supplementary Figure 1). The correlation coefficients between AA measures ranged from 0.08 to 0.58 in younger twins, from 0.25 to 0.68 in older twins, and from −0.06 to 0.63 in opposite-sex twins (Supplementary Figure 2). The lowest correlation coefficients were observed between $AA_{Horvath}$ and AA_{Grim}, and the highest between AA_{Hannum} and AA_{Pheno}.

Overall, the men had higher AA than the women, and the sex difference in AA tended to increase with age (Table 1 and Supplementary Table 1 and Supplementary Figure 3). Interestingly, when DNAm PhenoAge was used to assess AA, the men were epigenetically younger than the women in the younger age, which was in contrast with the AA estimates derived from the other clocks. When controlling for shared childhood environment and partly for

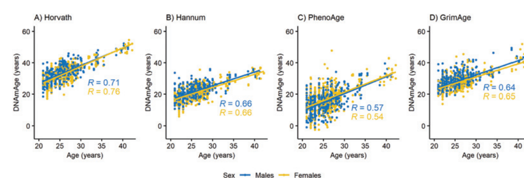

Figure 2. Association between chronological age and DNA methylation age (DNAmAge) estimates obtained by (A) Horvath's clock, (B) Hannum's clock, (C) PhenoAge, and (D) GrimAge estimators in younger (21- to 42-year-old) twins. R = Pearson's correlation coefficient.

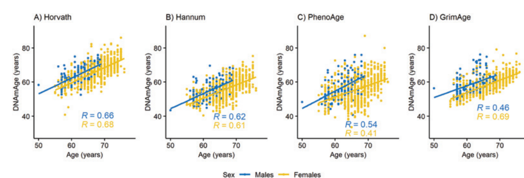

Figure 3. Association between chronological age and DNA methylation age (DNAmAge) estimates obtained by (A) Horvath's clock, (B) Hannum's clock, (C) PhenoAge, and (D) GrimAge estimators in older (older than 50 years old) twins. R = Pearson's correlation coefficient.

genetic factors among the opposite-sex twin pairs, the male twins had higher AA_{Hannum} and AA_{Grim} in comparison to their sisters, but there were no significant differences in $AA_{Horvath}$ and AA_{Pheno} between the sexes (Table 1).

Mediation Models in All Twins

Education, BMI, smoking, alcohol use, sport index, leisure index, and work index were considered to be the potential mediator variables. Here, smoking was assumed to be a continuous latent variable underlying ordinal smoking status. The estimation results of the single mediator models revealed that the association of sex with education and smoking differed between the age groups (Figure 1, *i*1 and Supplementary Table 2). There were also some differences in the associations of the potential mediator variables (including education, smoking, and alcohol use) with AA between the age groups (*i*3). The results of the associations between lifestyle-related factors and AA are presented in Supplementary Material and Supplementary Table 2.

The estimated age-specific indirect associations of male sex on AA through the potential mediator variables are given in Table 2. Male sex was associated with higher AA_{Pheno} and AA_{Grim} through lower level of education in younger twins and with lower AA_{Grim} through higher level of education in older twins. Male sex was associated with higher $AA_{Horvath}$ and AA_{Pheno} through higher BMI, but only in the younger twins. Smoking partly mediated the association of male sex with higher AA_{Hannum}, AA_{Pheno}, and AA_{Grim} in the older twins. Greater alcohol use partly mediated the association of male sex with higher AA_{Pheno} in the older twins and with AA_{Grim} in both age groups. Moreover, in younger twins, male sex was associated with lower AA_{Grim} through a higher sport index, but in older twins, with higher AA_{Grim} through lower leisure index. Work index did not mediate the sex difference in AA. Leisure index was chosen as an indicator of physical activity in further modeling.

Based on the results of the single mediator models, the final multiple mediator model included education, BMI, smoking, alcohol use, and leisure index as the mediators (Figure 4). The model also included the interaction effect between sex and age on education, the interaction effect between sex and age on smoking, and the interaction effect between smoking and age on AA. Furthermore, the interaction effect between education and age on AA_{Pheno} and AA_{Grim} and the interaction effect between alcohol use and age on AA_{Pheno} were included in the model. When the lifestyle factors were controlled for each other, male sex was associated with lower AA_{Grim} through higher level of education in older twins (Table 2). BMI partly mediated the association between sex and AA ($AA_{Horvath}$, AA_{Pheno}, and AA_{Grim}). Moreover, smoking partly mediated the association between sex and AA (AA_{Hannum}, AA_{Pheno}, and AA_{Grim}) but only in the older twins. Alcohol use partly mediated the sex difference in AA_{Pheno} in the older twins. Male sex was still found to have a direct positive effect on $AA_{Horvath}$, AA_{Hannum}, and AA_{Grim} after all the adjustments, and the association was stronger in the older cohort (Figure 4). Moreover, male sex was found to have a positive direct effect on AA_{Pheno}, but only in the older cohort.

Sensitivity Analysis

Finally, we reanalyzed the data using polynomial functions of age. The results were very similar to the ones obtained in the main analysis, but there were few exceptions (Supplementary Material, Supplementary Tables 3 and 4, and Supplementary Figures 4–17). Importantly, based on the multiple mediator models, the indirect effects of male sex on AA through BMI, smoking, and alcohol use were consistent with ones observed in the main analysis.

Mediation Models in the Opposite-Sex Twin Pairs

Information on the association between the lifestyle-related factors and AA is provided in Supplementary Text and Supplementary Table 5. Based on the estimation results of the single mediator models, male sex was associated with accelerated $AA_{Horvath}$ through higher BMI in the opposite-sex twin pairs (Table 3). Otherwise, there were no significant indirect effects. Similar to the models for all twins, education, BMI, smoking, alcohol use, and leisure index were included in the multiple mediator model as the mediator variables (Figure 5). A significant indirect association of male sex on $AA_{Horvath}$ through higher BMI was also observed after controlling for other lifestyle factors (Table 3). Otherwise, lifestyle factors did not mediate the differences in AA between the men and their female twin sisters. A direct effect of male sex on higher AA_{Hannum} and AA_{Grim} was also observed among the opposite-sex twin pairs (Figure 5).

Sex Differences in the DNAm-Based Plasma Proteins and Smoking Pack-Years

Information on sex differences in DNAm-based surrogates included in the DNAm GrimAge estimator is given in Supplementary Text, Supplementary Table 6, and Supplementary Figures 18 and 19.

Discussion

Our findings suggest that previously reported sex differences in life expectancy can be seen in biological aging when measured with epigenetic clocks (namely Horvath's clock, Hannum's clock, DNAm PhenoAge, and GrimAge). Sex difference was already evident in young adulthood, and it increased with age; on average, the men were 1.2–1.3 years older than women in the younger twins (21–42 years of age) and 3.2–4.3 years older in the older twins (50–76 years of age). The only exception was observed in the younger twins when PhenoAge was used to assess biological aging; the men were epigenetically 1.1 years younger than the women, but the sex difference in biological age reversed in the older adult twins. According to previous studies, sex difference in biological aging measured with epigenetic clocks seems to appear in adolescence (29), and men are epigenetically 1–2 years older than women in adulthood (30).

Opposite-sex twins provide a natural setting for studying sex differences while maximally controlling for genetic factors and shared childhood environmental factors. Epigenetic aging is highly heritable (31). Although the share of genes is 50% in male–female dizygotic twins, the mean difference by sex of epigenetic aging within these twin pairs (21–30 years of age) was comparable to the sex differences observed in the larger cohort of younger twins.

The observed increase in sex difference with age was mainly due to the fact that epigenetic aging accelerated with age among men. Changes in the hormonal levels during menopause have a detrimental effect on women's health (32); thus, sex differences in biological aging would be expected to diminish around and after the age of 50. Our analysis studying the shape of the association between chronological age and epigenetic aging did not find any evidence that this is the case in biological aging (Supplementary Material, Supplementary Table 1, and Supplementary Figure 3). This is in line with a recently published study investigating sex differences in the longitudinal trajectories of epigenetic aging from midlife onward (50–90 years) (18). According to the study, men were biologically

Table 2. Standardized Indirect Effects of Sex (male) on Epigenetic Age Acceleration (AA) Through the Potential Mediator Variables in All Twins

	Mediator[*,†,‡]													
	Education		BMI		Smoking		Alcohol Use		Sport Index		Leisure Index		Work Index	
	B (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*
Single mediator models														
Indirect associations in the younger twins														
$AA_{Horvath}$	−0.007 (0.008)	.347	**0.014 (0.007)**	**.049**	−0.002 (0.013)	.895	−0.011 (0.012)	.372	0.005 (0.007)	.528	−0.003 (0.007)	.618	0.004 (0.009)	.699
AA_{Hannum}	0.007 (0.007)	.358	0.002 (0.005)	.732	−0.001 (0.006)	.855	−0.011 (0.011)	.280	0.001 (0.007)	.899	−0.008 (0.008)	.321	−0.008 (0.011)	.435
AA_{Pheno}	**0.020 (0.009)**	**.028**	**0.020 (0.009)**	**.020**	0.000 (0.001)	.965	−0.009 (0.011)	.386	−0.011 (0.009)	.227	−0.003 (0.008)	.700	−0.003 (0.009)	.787
AA_{Grim}	**0.053 (0.016)**	**.001**	0.013 (0.007)	.057	0.000 (0.030)	.902	**0.045 (0.008)**	**<.001**	**−0.023 (0.011)**	**.044**	0.006 (0.007)	.358	−0.029 (0.023)	.199
Indirect associations in the older twins														
$AA_{Horvath}$	−0.001 (0.009)	.947	0.005 (0.004)	.182	0.013 (0.010)	.170	−0.005 (0.010)	.589	0.000 (0.001)	.852	0.004 (0.006)	.534	0.01 (0.013)	.425
AA_{Hannum}	0.008 (0.010)	.417	0.003 (0.002)	.224	**0.042 (0.013)**	**.001**	0.014 (0.009)	.109	0.000 (0.001)	.910	0.000 (0.007)	.980	−0.007 (0.011)	.495
AA_{Pheno}	0.006 (0.010)	.517	0.006 (0.004)	.182	**0.063 (0.017)**	**<.001**	**0.037 (0.01)**	**<.001**	0.001 (0.002)	.734	−0.003 (0.008)	.655	−0.002 (0.01)	.881
AA_{Grim}	**−0.025 (0.011)**	**.020**	0.006 (0.004)	.167	**0.160 (0.040)**	**<.001**	**0.048 (0.01)**	**<.001**	0.004 (0.008)	.609	**0.018 (0.009)**	**.042**	−0.005 (0.012)	.677
Multiple mediator models[§]														
Indirect associations in the younger twins														
$AA_{Horvath}$	0.000 (0.005)	.958	**0.008 (0.004)**	**.030**	−0.002 (0.014)	.874	−0.003 (0.007)	.663	—[ǁ]		0.002 (0.005)	.672	—[ǁ]	
AA_{Hannum}	0.001 (0.005)	.787	0.003 (0.003)	.328	−0.002 (0.007)	.825	0.001 (0.006)	.895			−0.004 (0.005)	.421		
AA_{Pheno}	0.017 (0.017)	.322	**0.010(0.005)**	**.029**	0.000 (0.002)	.885	−0.007 (0.010)	.501			−0.006 (0.005)	.164		
AA_{Grim}	0.005 (0.010)	.610	**0.006 (0.003)**	**.037**	0.004 (0.024)	.866	0.006 (0.005)	.181			0.004 (0.004)	.247		
Indirect associations in the older twins														
$AA_{Horvath}$	0.000 (0.007)	.958	**0.008 (0.004)**	**.030**	0.016 (0.010)	.131	−0.003 (0.007)	.663	—[ǁ]		0.002 (0.005)	.672	—[ǁ]	
AA_{Hannum}	0.001 (0.005)	.787	0.003 (0.002)	.139	**0.044 (0.013)**	**.001**	0.001 (0.006)	.895			−0.004 (0.005)	.421		
AA_{Pheno}	0.012 (0.015)	.403	**0.010 (0.005)**	**.029**	**0.061 (0.017)**	**<.001**	**0.024 (0.01)**	**.013**			−0.006 (0.005)	.164		
AA_{Grim}	**−0.029 (0.015)**	**.046**	**0.006 (0.003)**	**.037**	**0.161 (0.038)**	**<.001**	0.006 (0.005)	.181			0.004 (0.004)	.247		

Notes: BMI = body mass index; *B* = standardized (STDYX) indirect effect; *SE* = standard error.

[*]The model was controlled for zygosity.

[†]*SE*s were corrected for nested sampling.

[‡]Significant regression coefficients at the level 0.05 are presented in bold.

[§]The indirect effects were estimated as equal in the younger and older twins whenever interaction terms were not needed.

[ǁ]Based on the results of the single mediator models, the corresponding mediator variable was dropped out from the final multiple mediator model.

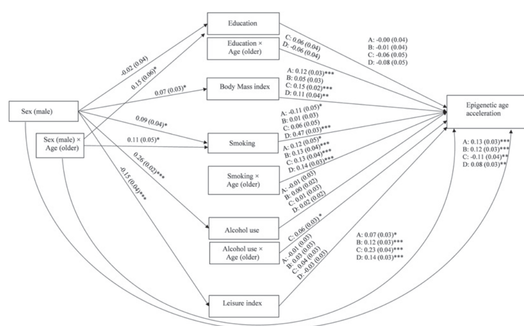

Figure 4. The path diagram of the multiple mediator model in all twins (n = 2 240). Standardized regression coefficients (standard errors) are presented. The modeling was conducted separately for each epigenetic age acceleration (AA) measure: (A) $AA_{Horvath}$, (B) AA_{Hannum}, (C) AA_{Pheno}, and (D) AA_{Grim}. ***p < .001, **p < .01, *p < .05.

older than women when Horvath's and Hannum's epigenetic clocks and GrimAge were used, and the difference remained constant across the age span (18).

To the best of our knowledge, this is the first study that has tested the mechanisms underlying sex differences in biological aging measured with epigenetic clocks. We found that several lifestyle-related factors partly mediated the association of sex with biological aging in all twins when the mediation of these factors was assessed one at a time. However, after controlling for each of the health-related behaviors in the multiple mediator models, BMI consistently mediated the sex difference in all twins and smoking in the older twins. Smoking was associated with accelerated biological aging, and the association was stronger in the older twins; this suggests the cumulative effect of smoking on biological aging. Moreover, the sex difference in smoking behavior was larger in the older twins; in fact, the difference in the proportion of never smokers between men and women was wider in the older twins (45% vs 73%) in comparison to the younger twins (44% vs 51%). A previous population-based study investigating long-term trends in smoking in Finland has shown that the prevalence of daily smoking has steadily decreased among men since the late 1970s (37%–17%); in contrast, the current prevalence among women is about the same as it was 4 decades ago (~15%) (33). Together, these findings support recent studies suggesting that the narrowing of the sex differences in smoking probably partly explains the declining sex gap in life span (7,34).

We observed some differences in the associations between lifestyle-related factors and epigenetic aging across the utilized clocks. These inconsistencies are probably due to differences in the procedures used to develop these epigenetic age estimators. The first-generation clocks, namely Horvath's clock and Hannum's clock, were trained to predict chronological age. More novel estimators are supposed to also capture CpG sites whose DNAm levels correlate with the deviation of biological age from chronological age.

Of the epigenetic age estimators employed in our study, DNAm GrimAge is the one that has been most recently published, and it outperforms other estimators in terms of predicting mortality (15,18). It is a mortality predictor by design and therefore may be the most relevant epigenetic age estimator in understanding sex differences in life expectancy. DNAm GrimAge utilizes information on chronological age, sex, and 7 DNAm-based surrogates for 7 plasma proteins and for smoking pack-years. Although sex difference in GrimAge is

Table 3. Standardized Indirect Effects of Sex (male) on Epigenetic Age Acceleration (AA) Through the Potential Mediator Variables in the Opposite-Sex Twins at Within-Twin Pair Level

	Mediator*,†													
	Education		BMI		Smoking		Alcohol Use		Sport Index		Leisure Index		Work Index	
	B (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*	*B* (*SE*)	*p*
Single mediator models														
$AA_{Horvath}$	−0.019 (0.016)	.251	**0.054 (0.022)**	**.015**	−0.003 (0.009)	.770	−0.026 (0.021)	.200	0.002 (0.002)	.847	−0.001 (0.018)	.957	−0.009 (0.010)	.385
AA_{Hannum}	−0.010 (0.014)	.480	−0.012 (0.023)	.605	−0.004 (0.008)	.628	−0.032 (0.022)	.159	0.000 (0.010)	.985	−0.026 (0.020)	.186	0.001 (0.005)	.893
AA_{Pheno}	−0.017 (0.012)	.173	0.015 (0.020)	.458	0.008 (0.011)	.460	−0.018 (0.020)	.366	0.001 (0.011)	.959	−0.025 (0.016)	.131	0.003 (0.006)	.640
AA_{Grim}	0.025 (0.015)	.095	−0.006 (0.024)	.804	0.029 (0.029)	.307	0.009 (0.023)	.689	0.000 (0.001)	.939	0.022 (0.018)	.221	0.006 (0.008)	.411
Multiple mediator models														
$AA_{Horvath}$	−0.022 (0.014)	.116	0.060 (0.025)	.019	0 (0.009)	.955	−0.028 (0.025)	.263	—‡		0.013 (0.028)	.646	—‡	
AA_{Hannum}	0.006 (0.013)	.665	−0.018 (0.016)	.255	0.003 (0.006)	.677	−0.023 (0.019)	.222			−0.042 (0.025)	.096		
AA_{Pheno}	−0.019 (0.028)	.503	0.035 (0.027)	.192	0.018 (0.022)	.394	−0.034 (0.041)	.403			−0.040 (0.05)	.416		
AA_{Grim}	0.023 (0.016)	.161	0.007 (0.013)	.589	0.023 (0.023)	.306	−0.005 (0.021)	.816			0.001 (0.025)	.977		

Notes: BMI = body mass index; *B* = standardized (STDYX) indirect effect; *SE* = standard error.

*The model was controlled for age as twin pairs participated in measurements at slightly different ages (±1 year).

†Significant indirect effects at the level 0.05 are presented in bold.

‡Based on the results of the single mediator models in the same-sex and the opposite-sex twin pairs, the corresponding mediator variable was dropped out from the final multiple mediator model.

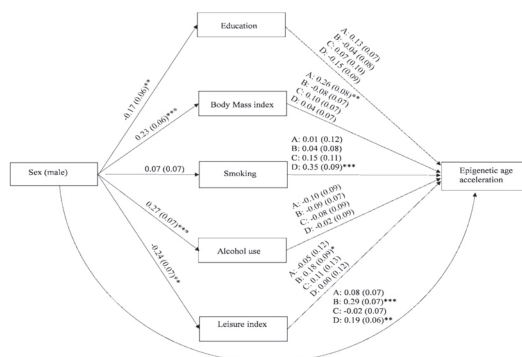

Figure 5. The path diagram of the multiple mediator model in the opposite-sex twin pairs (151 twin pairs). The standardized regression coefficients (standard errors) at the within-twin pair level are presented. The modeling was conducted separately for each epigenetic age acceleration (AA) measure: (A) $AA_{Horvath}$, (B) AA_{Hannum}, (C) AA_{Pheno}, and (D) AA_{Grim}. ***$p < .001$, **$p < .01$, *$p < .05$.

in-built, reflecting differences in mortality, the observed sex differences were very similar to the corresponding ones measured with Hannum's clock, which is purely based on CpG sites with their DNAm levels correlating with chronological age. To further understand the sex differences in biological aging, we studied the DNAm-based surrogates included in the GrimAge estimator (Supplementary Material, Supplementary Table 6, and Supplementary Figures 18 and 19). We observed a significantly higher level of DNAm-based PAI-1 among men in comparison to women. Moreover, in men, the level of DNAm PAI-1 drastically increased with age. This DNAm-based surrogate predicts morbidity better than DNAm GrimAge, and it associates with hypertension, type 2 diabetes, and coronary heart disease (15). Therefore, higher levels of DNAm PAI-1 in men may play a role in the sex differences in cardiovascular mortality observed in previous studies (4).

Our study has several strengths. We utilized recently published epigenetic clocks that are shown to predict mortality (18,35). The large sample size of our study enabled us to use complex mediator models. Because data from opposite-sex twin pairs were available, we were also able to control the analyses for shared childhood environmental factors and partly for genetic factors. This study also had some limitations. Most of the studied lifestyle-related factors were self-reported. Furthermore, our data were cross-sectional, and our analysis did not rule out the possibility of reversed causality when studying the associations between lifestyle-related factors and epigenetic aging.

Our results deepen the understanding of the association between sex-dependent lifestyle factors and the aging process. The results suggest that sex difference in life span is narrowing among future aging generations, and the main reason for this is that at the mean level women and men are approaching each other in life habits, especially in smoking, which is rapidly declining in men. Progress in the methodology of biological aging measurements may enable us to determine individual trajectories in aging already in early adulthood. This makes it possible to investigate the effects of environmental and societal changes and lifestyle interventions on biological aging. Produced knowledge would help in preparing our societies for the aging of future generations.

Supplementary Material

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

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Conflict of Interest

The authors declare no conflicts of interest.

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Author Contributions

E.S. conceived the idea for the study. A.H. and A.K. preprocessed the DNAm data. A.K., E.S., and A.H. accessed and verified the data set. A.K., A.T., and E.S. designed the statistical analysis, and A.K. and E.S. performed the analyses. J.K. and M.O. have designed and collected the FTC data set and participated in designing the analysis of this manuscript. A.K. and E.S. drafted the first version of the manuscript. A.K., A.T., P.S., A.H., E.K.L., J.K., M.O., and E.S. contributed to the interpretation of results and drafting or revising the manuscript.

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III

LEISURE-TIME AND OCCUPATIONAL PHYSICAL ACTIVITY ASSOCIATES DIFFERENTLY WITH EPIGENETIC AGEING

by

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OPEN

Leisure-Time and Occupational Physical Activity Associates Differently with Epigenetic Aging

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ABSTRACT

KANKAANPÄÄ, A., A. TOLVANEN, S. BOLLEPALLI, T. LESKINEN, U. M. KUJALA, J. KAPRIO, M. OLLIKAINEN, and E. SILLANPÄÄ. Leisure-Time and Occupational Physical Activity Associates Differently with Epigenetic Aging. *Med. Sci. Sports Exerc.*, Vol. 53, No. 3, pp. 487–495, 2021. **Purpose:** Greater leisure-time physical activity (LTPA) associates with healthier lives, but knowledge regarding occupational physical activity (OPA) is more inconsistent. DNA methylation (DNAm) patterns capture age-related changes in different tissues. We aimed to assess how LTPA and OPA are associated with three DNAm-based epigenetic age estimates, namely, DNAm age, PhenoAge, and GrimAge. **Methods:** The participants were young adult (21–25 yr, $n = 285$) and older (55–74 yr, $n = 235$) twin pairs, including 16 pairs with documented long-term LTPA discordance. Genome-wide DNAm from blood samples was used to compute DNAm age, PhenoAge, and GrimAge Age acceleration (Acc), which describes the difference between chronological and epigenetic ages. Physical activity was assessed with sport, leisure-time, and work indices based on the Baecke Questionnaire. Genetic and environmental variance components of epigenetic age Acc were estimated by quantitative genetic modeling. **Results:** Epigenetic age Acc was highly heritable in young adult and older twin pairs (~60%). Sport index was associated with slower and OPA with faster DNAm GrimAge Acc after adjusting the model for sex. Genetic factors and nonshared environmental factors in common with sport index explained 1.5%–2.7% and 1.9%–3.5%, respectively, of the variation in GrimAge Acc. The corresponding proportions considering OPA were 0.4%–1.8% and 0.7%–1.8%, respectively. However, these proportions were minor (<0.5%) after adjusting the model for smoking status. **Conclusions:** LTPA associates with slower and OPA with faster epigenetic aging. However, adjusting the models for smoking status, which may reflect the accumulation of unhealthy lifestyle habits, attenuated the associations. **Key Words:** BIOLOGICAL AGING, METHYLATION, QUANTITATIVE GENETICS, SMOKING

The health benefits of leisure-time physical activity (LTPA) are well documented. High LTPA is associated with a low risk of several diseases, such as cardiovascular disease and metabolic syndrome (1,2), and with low risk of premature death in a dose-dependent manner (3). The benefits of occupational physical activity (OPA) are more controversial. There is some evidence that OPA may even adversely affect health outcomes, such as risk of all-cause mortality, cardiovascular disease, and long-term sickness absence, whereas LTPA associates with low risk (1,4,5). This contrasting association of OPA with health outcomes is referred to as “the physical activity health paradox” (6).

Several possible reasons for the paradox have been proposed (7). LTPA and OPA may produce divergent physiological responses, as LTPA typically differs in terms of intensity, duration, and movement type from the monotonous OPA. The different physiological demands of LTPA and OPA are

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accompanied by other environmental and psychological factors regulating the response to physical activity (PA). Therefore, these two forms of activities may produce an inverse impact on levels of inflammation and autonomic imbalance (6). However, there are also many selection issues and third variables (confounders such as genetic and social factors as well as other health habits) possibly explaining some of the difference in the associations (8), but these relationships are as yet poorly understood.

Epigenetics refers to DNA or chromatin modifications that regulate gene expression without altering the underlying DNA sequence itself. DNA methylation (DNAm; attachment of a methyl group to C-5 of cytosine base in the context of CpG dinucleotide in a DNA strand) is one type of epigenetic modification. Many studies have provided evidence of age-related hypomethylation or hypermethylation within specific CpG sites or islands (9). These findings have laid the ground for the development of epigenetic biomarkers of aging, also known as epigenetic clocks. Horvath's DNAm age was the first widely used epigenetic estimate for chronological age (10). It has been argued that DNAm age may exclude CpGs whose methylation patterns may reflect a deviation of the biological age from the chronological age (11). Therefore, novel DNAm-based biomarkers for aging that capture CpGs associated with the functional stage along with the chronological age have been developed in recent years. DNAm PhenoAge was developed using "phenotypic age measure" instead of chronological age as a reference in the biological age prediction (11). DNAm GrimAge is a combination of DNAm-based surrogate biomarkers for health-related plasma proteins and smoking pack-years as well as sex and chronological age, predicting best mortality risk (12).

Epigenetic age acceleration measures the discrepancy between chronological age and epigenetic age. According to a systematic review and meta-analysis, there was no consistent association between LTPA and epigenetic aging assessed with Horvath's DNAm age (13). The first results considering the novel epigenetic age estimates seem to be more promising. LTPA has been shown to be correlated negatively with both DNAm PhenoAge and GrimAge acceleration (12,14). However, there is no conclusive evidence on this topic, as the novel epigenetic clocks were published very recently. To our knowledge, no studies are investigating the association between OPA and epigenetic aging.

Here, we investigated the relative contributions of the genetic and environmental factors predicting epigenetic aging measured by DNAm age, PhenoAge, and GrimAge estimates in young adulthood and older age. We further assessed cross-sectional associations of LTPA and OPA with epigenetic aging, as well as the genetic and environmental factors explaining the association. Finally, we studied long-term effects of LTPA on epigenetic aging by comparing co-twins that differed for LTPA for over three decades at least.

MATERIALS AND METHODS

The participants were members of the FinnTwin12 study (born in 1983–1987) and an older cohort (born before 1958) of The Finnish Twin Cohort (FTC) (15,16). Both cohorts included monozygotic (MZ) and dizygotic (DZ) twin pairs. Data on health-related behavior were collected with questionnaires and interviews. More detailed information on data collection is available in Supplemental Text Appendix (Text, Supplemental Digital Content 1, additional information on material and methods, http://links.lww.com/MSS/C106). Blood samples for DNA analyses were collected during in-person clinical studies after a written informed consent form was signed. A total of 1295 twins of the FinnTwin12 study and 447 of the older cohort were examined and measured. The same-sex twin pairs in which both had information on DNAm in a young adult (age range, 21–25 yr; 163 MZ and 122 DZ pairs) and an older cohort (age range, 55–72 yr; 140 MZ and 78 DZ pairs) were included in this study. The FTC data collection has been approved by the ethics committees of the University of Helsinki and Helsinki University Central Hospital.

PA-discordant twin pairs. The PA-discordant twin pairs (TWINACTIVE) initiated from the older cohort of the FTC (15,17). The comprehensive selection process that included multiple measurements of PA since 1975 has been described in detail by Leskinen et al. (18,19). LTPA was examined with standardized repeated questions, which were quantified as metabolic equivalent (MET; intensity × duration × frequency) and expressed as a sum score of leisure-time MET hours per day. Twin pairs whose difference in the volume of PA were >3 MET·h·d^{-1} were invited to the retrospective follow-up interviews on leisure activity (covering the years from 1980 to 2005 in 5-yr intervals), which were carried out during the years 2005–2007 (19). Of the 5663 originally healthy same-sex twin pairs, 16 twin pairs (age range, 50–74 yr; 7 MZ and 9 DZ pairs, total 5 female pairs) participated in the TWINACTIVE study. During the 30+ yr before the clinical examination and DNA sample collection, the mean intrapair difference in LTPA was 8.8 MET·h·d^{-1}. The participants representing pairs with the longest and highest discordance in LTPA were comprehensively selected from the FTC. The ethics committee of the Central Finland Health Care District has approved the TWINACTIVE study.

PA. PA was assessed by the Baecke Questionnaire (20). It includes four questions on sports activity and leisure-time activity excluding sports and eight questions on work-related PA on a 5-point scale. Activities were scored as 1, 3, or 5 according to how physically demanding they are. A sport index, a nonsport leisure-time (leisure) index and a work index, respectively, were yielded from mean scores of each section as described earlier by Baecke et al. (20) and Mustelin et al. (21) for the FinnTwin12 study.

Confounding variables. Body mass index was calculated as the ratio of measured body weight (in kilograms) to height squared (in meters per squared). Sex, education years, and smoking status (never, former, and current smoker (includes regular and occasional use)) and alcohol use (in grams per day) were assessed through interviews.

Epigenetic Age Estimates

DNAm. Generating and normalizing the DNAm data have been described in Supplementary Text Appendix (Text,

Supplemental Digital Content 1, preprocessing the DNAm data, http://links.lww.com/MSS/C106). Genome-wide DNAm from blood samples was determined on Illumina 450K and EPIC BeadChips, and the epigenetic age estimates DNAm age (10), DNAm PhenoAge (11), and DNAm GrimAge (12) were calculated by an online calculator (https://dnamage.genetics.ucla.edu/new; Text, Supplemental Digital Content 1, additional information on epigenetic age estimates, http://links.lww.com/MSS/C106). Epigenetic age acceleration (Acc), which describes the difference between chronological age and epigenetic age estimate, was calculated as the residuals from a linear regression model of epigenetic age estimate on chronological age.

The components of DNAm GrimAge (adjusted for age) were obtained using the calculator as well, including DNAm-based smoking pack-years and the surrogate biomarkers for plasma proteins (DNAm-based plasma proteins): DNAm adrenomedullin, DNAm β2-microglobulin, DNAm cystatin-C, DNAm growth differentiation factor 15, DNAm leptin, DNAm plasminogen activator inhibitor 1, and DNAm tissue inhibitor metalloproteinases 1.

Statistical Methods

Descriptive statistics were calculated using IBM SPSS statistics, and further modeling was performed by using Mplus statistical package (version 8.2) (22).

Quantitative genetic modeling. Quantitative genetic modeling was conducted using a structural equation framework. First, intraclass correlation coefficients (ICCs) and correlations between epigenetic age Acc measures and LTPA (sport index and leisure index), as well as OPA (work index) were studied. Second, univariate modeling was carried out to study the magnitude of genetic and environmental factors affecting epigenetic age Acc measures and PA indices in young adult and older twin pairs as described in Supplemental Text Appendix (Text, Supplemental Digital Content 1, modeling procedure of the univariate models, http://links.lww.com/MSS/C106) (23). The univariate models were adjusted for sex, education, and smoking status. Third, structural equation modeling was used to study the associations of PA indices on epigenetic aging. Shared genetic and environmental effects between epigenetic age Acc and PA, as well as the genetic and environmental factors unique to epigenetic age Acc were studied. For these purposes, Cholesky's decomposition was applied to PA indices and epigenetic age Acc measures after controlling for covariates (Fig. 1). The latent variables representing PA (CH1) and the residual of epigenetic age Acc after the effect of PA has been taken into account (CH2) were specified (24). The model was initially adjusted for sex only. The variation in both the latent variables CH1 and CH2 was partitioned in the genetic and environmental components as described in Supplementary Text Appendix (Text, Supplemental Digital Content 1, modeling procedure of the bivariate models, http://links.lww.com/MSS/C106).

The potential confounding variables, including sex, education, body mass index, smoking status, and alcohol use, were added sequentially to the model. At each stage, the regression coefficients between PA and epigenetic age Acc (b) were studied. In addition, the parameters of the model were used to calculate the relative proportions in the total variation of epigenetic age Acc $[(a_1^2 + c_1^2 + e_1^2)*b^2 + (a_2^2 + c_2^2 + e_2^2)]$ explained by the genetic and environmental effects in common with PA ($a_1^2*b^2$, $c_1^2*b^2$, and $e_1^2*b^2$, respectively) as well as the unique genetic and environmental factors of epigenetic age Acc (a_2^2, c_2^2, and e_2^2, respectively).

Discordant twin-pair analysis. The mean within-pair differences in epigenetic age estimates between active and inactive co-twins were calculated. Standardized mean difference (SMD; within-pair difference/SD of the variable among the members of the pairs) was used to evaluate effect size. The within-pair differences were regressed on zygosity to study whether there were differences in the effect of LTPA on epigenetic aging between MZ and DZ twins. The study design controls for chronological age and sex, as the twins are of the same sex and age.

RESULTS

Among the young adults, DZ twins had a slightly higher body mass index and leisure PA index compared with MZ twins (Table 1). The mean age of the participants was 22.4 yr, whereas the mean of the different epigenetic age estimates ranged from 15.0 to 28.6 yr, depending on the estimate utilized. In the older cohort, DZ twins were slightly older compared with MZ twins (mean, 62.9 vs 62.0 yr), and there were also differences in the means of the epigenetic age estimates. The mean of the different epigenetic age estimates ranged from 54.7 to 62.0 yr.

Heritability. In both cohorts, ICCs for epigenetic age Acc measures were consistently higher in MZ twins than in DZ twins, suggesting the influence of a genetic component (Table 2). According to univariate modeling, additive genetic factors accounted for 69% of the variation in DNAm age Acc, 64% of the variation in DNAm PhenoAge Acc, and 62% of the variation in DNAm GrimAge Acc in young adult twin pairs (Text, Supplemental Digital Content 1, http://links.lww.com/MSS/C106; Table, Supplemental Digital Content 2, the estimation results of the univariate model for epigenetic aging among young adult twin pairs, http://links.lww.com/MSS/C107). Correspondingly, nonshared environmental factors accounted for the remainder (31%–38%) of the variation in epigenetic aging. In older twin pairs, additive genetic factors explained 61% of the variation in DNAm age Acc, 60% of the variation in DNAm PhenoAge Acc, and 58% of the variation in DNAm GrimAge Acc (Text, Supplemental Digital Content 1, http://links.lww.com/MSS/C106; Table, Supplemental Digital Content 3, the estimation results of the univariate model for epigenetic aging among older twin pairs, http://links.lww.com/MSS/C108), whereas unique environmental factors accounted for the remaining variance. The proportions of the variation in epigenetic age Acc measures explained by the genetic factors did not differ between young

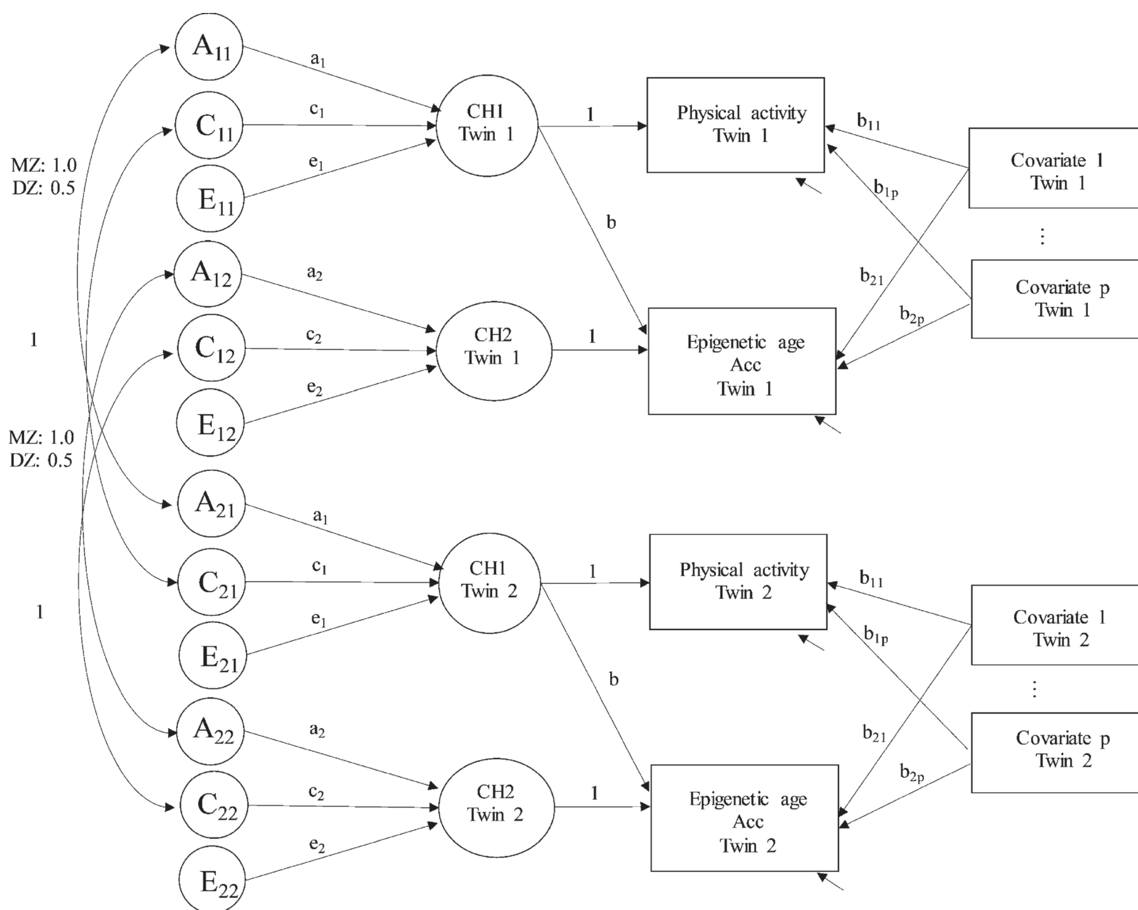

FIGURE 1—The path diagram of the structural equation model. Circles denote latent variables; rectangles denote observed variables. CH1 represents PA, and CH2 represents the residual of the epigenetic age Acc after the effect of PA has been taken into account. A, additive genetic; C, shared environmental; E, nonshared environmental components.

adult and older twin pairs (Wald test, P = 0.076–0.220). The results considering PA are presented in the supplementary material (Text, Supplemental Digital Content 1, http://links.lww.com/MSS/C106; Tables, Supplemental Digital Contents 4–5, the estimation results of the univariate model for PA indices among young adult and older twin pairs, respectively, http://links.lww.com/MSS/C109 and http://links.lww.com/MSS/C110).

Bivariate twin models. Sport index and leisure index were associated with slower and work index with faster DNAm GrimAge Acc in both young adult and older twins as shown by correlation coefficients in Table 2. Epigenetic age Acc and PA indices were not consistently correlated, when DNAm age and DNAm PhenoAge estimates were used to assess epigenetic age. Therefore, no further modeling was considered for these measures. Additional information on the model selection is available in the supplemental material (Text, Supplemental Digital Content 1, http://links.lww.com/MSS/C106; Table, Supplemental Digital Content 6, the model fit of the bivariate models, http://links.lww.com/MSS/C111).

In young adult twin pairs, sport index was associated with slower DNAm GrimAge acceleration after all the adjustments, but in an older cohort, the association was no longer significant after controlling for smoking status (Table 3). In both cohorts, leisure index was associated with slower and work index with accelerated epigenetic aging, but these associations attenuated after controlling for covariates, especially for smoking status.

In young twin pairs, genetic and environmental factors in common with sport index explained 2.7% and 1.9%, respectively, of the variation in DNAm GrimAge Acc, after adjusting the model for sex (Table 4). The corresponding proportions were 0.6% and 0.7% for leisure index and 1.8% and 1.7% for work index, respectively. In older twin pairs, genetic and environmental factors in common with sport index explained 1.5% and 3.5%, respectively, of the variation in DNAm GrimAge Acc (Table 5). The corresponding proportions for leisure index were 0.8% and 2.4% and for OPA 0.4% and 0.7%, respectively. In both cohorts, the proportions of the variation in DNAm GrimAge Acc explained by genetic and environmental factors in common with PA indices were minor (<0.5%) after including smoking status in the models.

The associations of the DNAm-based surrogates (adjusted for age) included in DNAm GrimAge with DNAm GrimAge

TABLE 1. Descriptive statistics of the study variables by zygosity among young adult (n = 570) and older (n = 470) twin individuals.

	Young Adult (21–25 yr)					Older (55–72 yr)				
	MZ Twins (n = 326)		DZ Twins (n = 244)			MZ Twins (n = 294)		DZ Twins (n = 176)		
	Mean (SD)	n	Mean (SD)	n	P^a	Mean (SD)	n	Mean (SD)	n	P^a
Women, n (%)	208 (63.8)		142 (58.2)		0.174	188 (63.9)		94 (53.4)		0.024
Chronological age, yr	22.4 (0.7)	326	22.4 (0.7)	244	0.672	62.0 (4.1)	294	62.9 (4.3)	176	0.036
DNAm age, yr	25.0 (3.5)	326	24.4 (3.4)	244	0.057	60.2 (6.2)	294	62.1 (7.6)	176	0.004
DNAm PhenoAge, yr	15.0 (4.6)	326	15.3 (4.8)	244	0.496	54.7 (6.5)	294	56.0 (8.1)	176	0.045
DNAm GrimAge, yr	28.6 (2.9)	326	28.6 (3.4)	244	0.843	59.8 (5.3)	294	60.8 (5.5)	176	0.061
Body mass index, kg·m^{-2}	23.1 (3.8)	326	23.7 (4.2)	244	0.028	27.1 (4.7)	294	27.2 (4.8)	176	0.727
Education, yr	16.7 (3.5)	325	16.6 (3.6)	244	0.429	11.9 (3.8)	252	11.4 (3.5)	96	0.266
Smoking status		325		244			292		176	
Never smokers, n (%)	182 (56.0)		116 (47.5)			148 (50.8)		88 (50.0)		
Former smokers, n (%)	37 (11.4)		30 (12.3)			97 (33.3)		63 (35.8)		
Current smokers, n (%)	106 (32.6)		98 (40.2)		0.121	47 (15.9)		25 (14.2)		0.787
Alcohol use, g·d^{-1}	12.4 (16.7)	326	13.4 (16.7)	244	0.460	6.9 (11.2)	278	8.8 (11.6)	158	0.080
LTPA										
Sport index	2.9 (0.8)	251	3.0 (0.8)	233	0.358	3.2 (0.9)	241	3.1 (0.8)	156	0.421
Nonsport leisure index	2.9 (0.6)	253	3.1 (0.6)	234	0.023	2.9 (0.7)	241	2.8 (0.7)	156	0.715
OPA										
Work index	2.7 (0.6)	256	2.8 (0.6)	234	0.315	2.4 (1.0)	234	2.3 (1.0)	150	0.935
Out of working life		322		243			241		156	
Retired or unemployed, n (%)	49 (15.2)		26 (10.7)			105 (43.6)		76 (50)		0.209

[a] P value for the difference between the groups from independent-samples t-test or x^2 test.

Acc, as well as with PA indices, were studied using correlation coefficients (Table, Supplemental Digital Content 7, the correlations among young adult and older individual twins, http://links.lww.com/MSS/C112). Sport index in young adults and PA indices in older cohort were associated with lower levels of several DNAm-based plasma proteins, whereas inconsistent associations of work index were observed in both cohorts. In both cohorts, sport index was negatively associated with DNAm-based smoking pack-years (r = −0.20 to −0.16), whereas the opposite association of work index was observed (r = 0.11 to 0.30). DNAm-based smoking pack-years and DNAm GrimAge Acc were highly correlated (r > 0.80).

Twin pairs discordant for LTPA for 32 yr: the TWINACTIVE sample. The mean (SD) age of the participants was 60.4 (6.2) yr, whereas the means of the epigenetic age estimates DNAm age, PhenoAge, and GrimAge were 56.5 (4.8), 46.8 (5.8), and 59.7 (5.7) yr, respectively. There was no difference in Horvath's DNAm age between the active and inactive co-twins among the LTPA discordant twin pairs, as we have reported previously (25). The two newer epigenetic age estimates, however, differed between active and inactive co-twins. Active twins were on average 3.27 yr (95% confidence interval (CI), 1.34 to 5.20 yr; SMD, 0.56 yr) younger compared with their inactive co-twins in DNAm PhenoAge and 2.08 yr (95% CI, 0.75 to 3.41 yr; SMD, 0.37 yr) younger in DNAm GrimAge.

Mean within-pair difference for DNAm PhenoAge was among MZ pairs 1.80 (95% CI, −1.40 to 4.96; SMD, 0.30) and among DZ pairs 4.42 (95% CI, 2.33 to 6.51; SMD, 0.77; Figure, Supplemental Digital Content 8, the epigenetic age estimates in the LTPA discordant MZ and DZ twin pairs, http://links.lww.com/MSS/C113). In DNAm GrimAge, active MZ twins were on average 1.97 yr (95% CI, −0.04 to 4.00 yr; SMD, 0.40 yr) and DZ twins 2.27 (95% CI, 0.40 to 3.94 yr; SMD, 0.36 yr) younger compared with their inactive co-twins. Among the small number of twin pairs, the difference in the association

TABLE 2. The ICCs of epigenetic age Acc measures and PA indices by zygosity, as well as the correlation coefficients between the measures among young adult (n = 570) and older (n = 470) twins individuals.

	ICCs and Their 95% CI				Correlation Coefficients and Their 95% CI[a,b]									
	MZ		DZ		1		2		3		4		5	
Young adult twins														
1. DNAm age Acc	0.70	0.61 to 0.79	0.49	0.35 to 0.64										
2. DNAm PhenoAge Acc	0.67	0.58 to 0.75	0.18	0.02 to 0.34	**0.35**	**0.28 to 0.43**								
3. DNAm GrimAge Acc	0.67	0.57 to 0.76	0.42	0.22 to 0.62	0.08	−0.01 to 0.17	**0.34**	**0.25 to 0.42**						
4. Sport index	0.61	0.48 to 0.75	0.32	0.15 to 0.50	0.09	−0.02 to 0.19	−0.09	−0.19 to 0.01	**−0.21**	**−0.30 to −0.12**				
5. Leisure index	0.43	0.27 to 0.58	0.34	0.17 to 0.51	0.05	−0.05 to 0.15	0.03	−0.07 to 0.13	**−0.12**	**−0.21 to −0.02**	**0.28**	**0.19 to 0.37**		
6. Work index	0.51	0.37 to 0.65	0.26	0.07 to 0.46	−0.01	−0.12 to 0.10	0.00	−0.10 to 0.10	**0.26**	**0.16 to 0.35**	−0.06	−0.17 to 0.04	−0.01	−0.11 to 0.10
Older twins														
1. DNAm age Acc	0.62	0.51 to 0.73	0.28	0.15 to 0.41										
2. DNAm PhenoAge Acc	0.62	0.52 to 0.72	0.32	0.13 to 0.52	**0.34**	**0.23 to 0.45**								
3. DNAm GrimAge Acc	0.64	0.54 to 0.75	0.40	0.19 to 0.62	**0.21**	**0.11 to 0.31**	**0.40**	**0.32 to 0.48**						
4. Sport index	0.32	0.15 to 0.50	0.21	−0.04 to 0.46	−0.03	−0.13 to 0.07	−0.06	−0.19 to 0.06	**−0.20**	**−0.31 to −0.08**				
5. Leisure index	0.27	0.10 to 0.44	0.26	0.03 to 0.48	**−0.12**	**−0.21 to −0.03**	0.00	−0.13 to 0.12	**−0.21**	**−0.31 to −0.12**	**0.45**	**0.36 to 0.53**		
6. Work index	0.33	0.14 to 0.51	0.34	0.12 to 0.55	0.09	−0.02 to 0.20	0.07	−0.04 to 0.17	**0.13**	**0.03 to 0.23**	**−0.13**	**−0.23 to −0.03**	−0.02	−0.13 to 0.09

[a] CIs were corrected for nested sampling.
[b] Significant correlations at the level 0.05 are presented in bold.

TABLE 3. The estimation results of the structural equation modeling: the standardized linear regression coefficients of DNAm GrimAge Acc on PA indices among young adult (MZ, *n* = 163; DZ:, *n* = 122) and older (MZ, *n* = 147; DZ, *n* = 88) twin pairs.

	Sport Index			Leisure Index			Work Index[a]		
	B	95% CI	*P*	*B*	95% CI	*P*	*B*	95% CI	*P*
Young adult twin pairs									
PA index + sex	**−0.212**	**−0.288 to −0.136**	**<0.001**	**−0.113**	**−0.192 to −0.033**	**0.006**	**0.189**	**0.105 to 0.273**	**<0.001**
+ Education years	**−0.172**	**−0.244 to −0.099**	**<0.001**	**−0.089**	**−0.167 to −0.012**	**0.024**	**0.103**	**0.027 to 0.179**	**0.008**
+ Body mass index	**−0.161**	**−0.236 to −0.087**	**<0.001**	−0.085	−0.163 to −0.007	0.108	**0.106**	**0.029 to 0.182**	**0.007**
+ Smoking status	**−0.085**	**−0.153 to −0.017**	**0.015**	−0.029	−0.104 to 0.046	0.451	0.051	−0.020 to 0.122	0.157
+ Alcohol use	**−0.082**	**−0.151 to −0.014**	**0.019**	−0.029	−0.104 to 0.046	0.451	0.052	−0.018 to 0.123	0.147
Older twin pairs									
PA index + sex	**−0.212**	**−0.298 to −0.126**	**<0.001**	**−0.169**	**−0.248 to −0.090**	**<0.001**	**0.098**	**0.014 to 0.182**	**0.023**
+ Education years	**−0.211**	**−0.296 to −0.126**	**<0.001**	**−0.165**	**−0.244 to −0.086**	**<0.001**	0.078	−0.005 to 0.160	0.066
+ Body mass index	**−0.198**	**−0.283 to −0.113**	**<0.001**	**−0.153**	**−0.232 to −0.075**	**<0.001**	0.077	−0.006 to 0.161	0.070
+ Smoking status	−0.053	−0.127 to 0.020	0.154	−0.048	−0.112 to 0.017	0.145	0.028	−0.038 to 0.094	0.410
+ Alcohol use	−0.054	−0.126 to 0.017	0.138	−0.053	−0.118 to 0.012	0.108	0.029	−0.037 to 0.095	0.388

[a]The model was additionally adjusted for indicator of being out of working life.
+, The model was additionally adjusted for the following variables; *B*, standardized regression coefficient.
Significant associations at the level 0.05 are presented in bold.

between LTPA and DNAm PhenoAge or DNAm GrimAge between MZ and DZ twin pairs was not significant ($P = 0.091$ and $P = 0.887$, respectively).

DISCUSSION

In this study, we showed that the heritability estimates of different epigenetic clocks (namely DNAm age, PhenoAge, and GrimAge) are very similar. Genetic factors accounted for about 60% of the variation in epigenetic age acceleration, whereas nonshared environmental factors explained the remainder. Models with no genetic effects showed poorer fit to the data than did the models with genetic effects. Our twin models did not require the inclusion of shared environmental effects as genetic models with (ACE) or without (AE) shared environmental effects fit the data adequately. The observed heritability estimates of the epigenetic age acceleration based on the newer epigenetic age estimates were considerably higher than those reported in previous studies, possibly because of the methodological differences in constructing epigenetic age estimates and the age differences of the target cohorts. Lu and colleagues reported low to moderate estimates of heritability for DNAm GrimAge- and DNAm PhenoAge-based epigenetic age acceleration (30% and 11%, respectively) (12). However, in other studies, moderate to high heritability estimates have been reported for the latter one (33%–51%) (11,26). The AE model was more parsimonious and therefore used as the basis for the bivariate models to explore the common genetic and environmental factors underlying both epigenetic aging and PA.

Our findings revealed that the associations between PA and epigenetic aging depended on the utilized epigenetic age estimate and the form of PA. The results supported the existence of the PA paradox: high-intensity LTPA (sport index) was related to slower epigenetic aging and OPA (work index) to faster epigenetic aging when the newest epigenetic age estimate DNAm GrimAge was used. However, the associations

TABLE 4. The proportion of the variation in DNAm GrimAge Acc explained by genetic and environmental effects in young adult twin pairs.

		Sport Index			Leisure Index			Work Index[a]		
		Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*
A_1	PA index + sex	**0.027**	**0.006 to 0.048**	**0.011**	0.006	−0.002 to 0.014	0.167	**0.018**	**0.001 to 0.036**	**0.039**
	+ Education	**0.018**	**0.002 to 0.034**	**0.029**	0.004	−0.003 to 0.010	0.253	0.003	−0.002 to 0.008	0.232
	+ Body mass index	**0.016**	**0.000 to 0.032**	**0.044**	0.003	−0.003 to 0.010	0.278	0.003	−0.002 to 0.009	0.225
	+ Smoking status	0.006	−0.004 to 0.015	0.234	0.001	−0.002 to 0.003	0.702	0.001	−0.002 to 0.004	0.499
	+ Alcohol use	0.005	−0.004 to 0.015	0.249	0.001	−0.002 to 0.003	0.703	0.002	−0.002 to 0.004	0.487
A_2	PA index +sex	**0.680**	**0.597 to 0.762**	**<0.001**	**0.706**	**0.629 to 0.782**	**<0.001**	**0.661**	**0.576 to 0.747**	**<0.001**
	+ Education	**0.689**	**0.606 to 0.771**	**<0.001**	**0.706**	**0.627 to 0.784**	**<0.001**	**0.689**	**0.606 to 0.772**	**<0.001**
	+ Body mass index	**0.685**	**0.602 to 0.769**	**<0.001**	**0.701**	**0.622 to 0.780**	**<0.001**	**0.689**	**0.606 to 0.771**	**<0.001**
	+ Smoking status	**0.617**	**0.516 to 0.718**	**<0.001**	**0.620**	**0.520 to 0.721**	**<0.001**	**0.617**	**0.515 to 0.718**	**<0.001**
	+ Alcohol use	**0.619**	**0.515 to 0.718**	**<0.001**	**0.618**	**0.518 to 0.719**	**<0.001**	**0.615**	**0.513 to 0.717**	**<0.001**
E_1	PA index + sex	**0.019**	**0.005 to 0.033**	**0.008**	0.007	−0.003 to 0.017	0.178	**0.018**	**0.002 to 0.034**	**0.030**
	+ Education	**0.014**	**0.002 to 0.025**	**0.023**	0.005	−0.004 to 0.013	0.277	0.008	−0.004 to 0.021	0.183
	+ Body mass index	**0.012**	**0.001 to 0.023**	**0.038**	0.004	−0.004 to 0.013	0.301	0.009	−0.004 to 0.021	0.176
	+ Smoking status	0.004	−0.003 to 0.011	0.220	0.001	−0.003 to 0.004	0.709	0.003	−0.005 to 0.010	0.478
	+ Alcohol use	0.004	−0.003 to 0.011	0.235	0.001	−0.003 to 0.004	0.710	0.003	−0.005 to 0.010	0.467
E_2	PA index + sex	**0.275**	**0.200 to 0.349**	**<0.001**	**0.281**	**0.203 to 0.360**	**<0.001**	**0.302**	**0.223 to 0.382**	**<0.001**
	+ Education	**0.280**	**0.204 to 0.356**	**<0.001**	**0.286**	**0.206 to 0.367**	**<0.001**	**0.299**	**0.220 to 0.379**	**<0.001**
	+ Body mass index	**0.287**	**0.210 to 0.364**	**<0.001**	**0.291**	**0.210 to 0.372**	**<0.001**	**0.299**	**0.220 to 0.379**	**<0.001**
	+ Smoking status	**0.373**	**0.274 to 0.471**	**<0.001**	**0.378**	**0.277 to 0.480**	**<0.001**	**0.380**	**0.279 to 0.480**	**<0.001**
	+ Alcohol use	**0.371**	**0.275 to 0.473**	**<0.001**	**0.381**	**0.279 to 0.482**	**<0.001**	**0.382**	**0.281 to 0.481**	**<0.001**

[a]The model was additionally adjusted for indicator of being out of working life.
+, The model was additionally adjusted for the following variables; A_1, genetic factors of DNAm GrimAge Acc in common with PA index; A_2, unique genetic factors of DNAm GrimAge Acc; E_1, environmental factors of DNAm GrimAge Acc in common with PA index; E_2, unique environmental factors of DNAm GrimAge Acc.
Significant associations at the level 0.05 are presented in bold.

TABLE 5. The proportion of the variation in DNAm GrimAge Acc explained by genetic and environmental effects in older twin pairs.

		Sport Index			Leisure Index			Work Index[a]		
		Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*
A_1	PA index + sex	**0.015**	**0.001 to 0.03**	**0.034**	0.008	−0.001 to 0.018	0.074	0.004	−0.003 to 0.011	0.256
	+ Education	**0.015**	**0.001 to 0.03**	**0.035**	0.008	−0.001 to 0.017	0.079	0.002	−0.002 to 0.006	0.380
	+ Body mass index	**0.014**	**0.000 to 0.027**	**0.047**	0.007	−0.001 to 0.015	0.099	0.002	−0.002 to 0.006	0.381
	+ Smoking status	0.002	−0.003 to 0.007	0.455	0.001	−0.002 to 0.005	0.471	0.000	−0.002 to 0.003	0.683
	+ Alcohol use	0.002	−0.003 to 0.008	0.437	0.002	−0.002 to 0.003	0.426	0.001	−0.002 to 0.003	0.669
A_2	PA index + sex	**0.627**	**0.516 to 0.738**	**<0.001**	**0.623**	**0.509 to 0.738**	**<0.001**	**0.618**	**0.493 to 0.743**	**<0.001**
	+ Education	**0.627**	**0.517 to 0.738**	**<0.001**	**0.624**	**0.509 to 0.738**	**<0.001**	**0.623**	**0.499 to 0.748**	**<0.001**
	+ Body mass index	**0.622**	**0.508 to 0.737**	**<0.001**	**0.617**	**0.497 to 0.737**	**<0.001**	**0.612**	**0.480 to 0.744**	**<0.001**
	+ Smoking status	**0.584**	**0.489 to 0.680**	**<0.001**	**0.583**	**0.486 to 0.680**	**<0.001**	**0.579**	**0.481 to 0.678**	**<0.001**
	+ Alcohol use	**0.597**	**0.505 to 0.690**	**<0.001**	**0.598**	**0.524 to 0.723**	**<0.001**	**0.595**	**0.501 to 0.690**	**<0.001**
E_1	PA index + sex	**0.035**	**0.005 to 0.065**	**0.023**	**0.024**	**0.001 to 0.046**	**0.041**	0.007	−0.005 to 0.018	0.267
	+ Education	**0.035**	**0.005 to 0.065**	**0.022**	**0.023**	**0.000 to 0.045**	**0.045**	0.005	−0.006 to 0.016	0.361
	+ Body mass index	**0.031**	**0.003 to 0.059**	**0.032**	0.020	−0.001 to 0.041	0.062	0.005	−0.006 to 0.016	0.370
	+ Smoking status	0.004	−0.007 to 0.015	0.487	0.004	−0.006 to 0.013	0.467	0.001	−0.004 to 0.007	0.678
	+ Alcohol use	0.004	−0.007 to 0.015	0.470	0.004	−0.003 to 0.004	0.423	0.001	−0.005 to 0.007	0.664
E_2	PA index + sex	**0.323**	**0.221 to 0.424**	**<0.001**	**0.345**	**0.235 to 0.455**	**<0.001**	**0.371**	**0.246 to 0.496**	**<0.001**
	+ Education	**0.322**	**0.222 to 0.422**	**<0.001**	**0.346**	**0.235 to 0.456**	**<0.001**	**0.370**	**0.245 to 0.495**	**<0.001**
	+ Body mass index	**0.333**	**0.228 to 0.438**	**<0.001**	**0.356**	**0.240 to 0.473**	**<0.001**	**0.381**	**0.248 to 0.514**	**<0.001**
	+ Smoking status	**0.410**	**0.316 to 0.503**	**<0.001**	**0.412**	**0.316 to 0.508**	**<0.001**	**0.419**	**0.322 to 0.516**	**<0.001**
	+ Alcohol use	**0.396**	**0.305 to 0.488**	**<0.001**	**0.396**	**0.275 to 0.476**	**<0.001**	**0.403**	**0.310 to 0.496**	**<0.001**

[a]The model was additionally adjusted for indicator of being out of working life.

+, The model was additionally adjusted for the following variables; A_1, genetic factors of DNAm GrimAge Acc in common with PA index; A_2, unique genetic factors of DNAm GrimAge Acc; E_1, environmental factors of DNAm GrimAge Acc in common with PA index; E_2, unique environmental factors of DNAm GrimAge Acc.

Significant proportions at the level 0.05 are presented in bold.

mainly attenuated after controlling for smoking status. Associations between other epigenetic clocks (DNAm age and PhenoAge acceleration) and PA were very minor or nonexistent. Only a few previous studies have reported on the associations between PA and epigenetic aging with the novel epigenetic clocks. Stevenson and colleagues (14) showed a cross-sectional negative association of DNAm PhenoAge acceleration with LTPA at the age of 70 yr, but the analysis was controlled only for age and childhood cognitive ability. Zhao and colleagues (27) did not observe significant associations between LTPA and DNAm PhenoAge or DNAm GrimAge acceleration in older African Americans.

In addition to the cross-sectional associations, we provided evidence for beneficial association of long-term LTPA using a discordant twin pair design. Twin pairs discordant for LTPA for 32 yr differed in epigenetic aging measured with DNAm PhenoAge and DNAm GrimAge, although this was not seen when measured with DNAm age. Active twin pairs were epigenetically 2 to 3 yr younger on average compared with their inactive co-twins, when the genetic factors were controlled for partially (DZ pairs) or fully (MZ pairs). There were no differences in the effects between MZ and DZ twin pairs, but the mean within-pair differences were not significant in MZ pairs.

All the utilized epigenetic aging acceleration measures have been shown to predict mortality and morbidity risk, but DNAm GrimAge acceleration stands out in the prediction accuracy (12,28,29). Previous studies have shown that DNAm GrimAge may capture the stimulus of a variety of health- and lifestyle-related factors (12,27). In our study, LTPA was most consistently associated with DNAm GrimAge acceleration. DNAm GrimAge is a composite biomarker based on seven DNAm surrogates for plasma markers and smoking pack-years, which strongly predict time to death (12). Whereas CpGs for the other clocks were selected based on their association with a single reference, DNAm GrimAge was developed in two stages. First, CpGs for DNAm surrogates were selected based on their associations with the corresponding plasma protein levels and self-reported smoking pack-years. Second, DNAm-based surrogates for DNAm GrimAge estimator were selected based on their ability to predict mortality risk. This approach may have efficiently captured the CpGs associated with diverse health-related factors.

In our study, both genetic and nonshared environmental factors common to PA and the DNAm GrimAge acceleration explained the observed associations, but these influences attenuated after controlling the model for smoking status. Therefore, the observed opposite associations of LTPA and OPA on DNAm GrimAge acceleration may reflect an accumulation of unhealthy lifestyle habits among individuals in the lower socioeconomic class performing physically demanding work (30). Both genetic and environmental factors regulate smoking (31). Moreover, smoking has been shown to predict lower LTPA, also independently of genetic factors (32). Smoking is one of the most detrimental lifestyle factors and has been seen not only to increase the risk for multiple diseases (33) and mortality (34) but also to accelerate cellular aging (35). Interestingly, DNAm-based smoking pack-years (a component of DNAm GrimAge) has been shown to predict mortality risk better than original self-reported measure (12) and fully mediate the effects of self-reported smoking on biological aging (36). In line with this, we observed a very high correlation between DNAm smoking pack-years and DNAm GrimAge acceleration. Moreover, the opposite association of LTPA and OPA with the components of GrimAge was most evident in the case of DNAm smoking pack-years. These findings may support the recently stated arguments that the PA paradox is probably partly explained by an insufficient adjustment for smoking (8,37).

We observed that the lower values in several DNAm-based surrogate biomarkers included in the GrimAge estimator were

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correlated with higher levels of LTPA. LTPA promotes changes in multiple mechanistic and regulatory pathways that underlie the exercise-induced adaptations in metabolic profile, fitness, and body fat and muscle distribution. Lack of these LTPA-induced adaptations may increase the risk of cardiovascular and metabolic diseases at the population level, as different metabolic profiles have also been found among LTPA-discordant twin pairs (38), although differences in life expectancy have not been observed (39). Our study suggests that benefits of the LTPA may also be seen in epigenetic aging based on DNAm levels in blood, but its role is minor. The effect size was about half or less of the magnitude of the previously reported effect of LTPA on certain other health-related traits such as body fat, liver fat, and artery structure (38) known to be associated cardiovascular and other inactivity-related diseases. LTPA induces adaptations also directly in muscle tissue, which plays an important role in age-related decline in physical functioning. Future studies utilizing recently published epigenetic clock for human skeletal muscle (40) may show whether LTPA has a more substantial effect on epigenetic aging of muscle tissue.

Strengths and limitations. To our knowledge, this is the first study investigating the association of both LTPA and OPA with epigenetic aging. Our study utilizes novel epigenetic clocks that were published very recently. Twin design and the use of quantitative genetic modeling enabled us to study the genetic and environmental effects on epigenetic aging. In addition, we were able to investigate the effects of long-term LTPA on epigenetic aging after controlling for genetic factors by comparing co-twins of pairs discordant for LTPA for 30+ yr.

We acknowledge that our results are based on self-reported measure of PA, and potential recall bias and the effect of social desirability cannot be excluded. Baecke questionnaire has been shown to be valid and reliable tool to assess high-intensity LTPA, but all the light-intensity activities may not be properly measured (41). Activities such as gardening and household, which are increasingly important determinants of physical functioning with age (42), are not directly assessed by the questionnaire. In addition, the sample size of the LTPA discordant twins is limited, and therefore, statistical power to detect small effects may be insufficient. It should be noted that recent studies have shown that biological aging may be distinct stages rather than a continuous process, and aging progression may not be linear throughout the studied age ranges (43,44).

CONCLUSIONS

We show that LTPA associates with slower epigenetic aging, whereas OPA associates with accelerated epigenetic aging. The observed associations are explained by both common genetic and environmental factors. Importantly, adjusting the models for smoking status, which may reflect the accumulation of unhealthy lifestyle habits, attenuated the negative association of LTPA and the positive association of OPA with epigenetic aging.

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The authors declare that there are no conflicts of interest. The results of the present study do not constitute endorsement by the American College of Sports Medicine. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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IV

THE ASSOCIATIONS OF LONG-TERM PHYSICAL ACTIVITY IN ADULTHOOD WITH LATER BIOLOGICAL AGEING AND ALL-CAUSE MORTALITY – A PROSPECTIVE TWIN STUDY

by

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Title

The associations of long-term physical activity in adulthood with later biological ageing and all-cause mortality – a prospective twin study

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Abstract

Objectives:

The association between leisure-time physical activity (LTPA) and a lower risk of mortality is susceptible to bias from multiple sources. We investigated the potential of biological ageing to mediate the association between long-term LTPA and mortality and whether the methods used to account for reverse causality affect the interpretation of this association.

Methods:

Study participants were twins from the older Finnish Twin Cohort (n = 22,750; 18–50 years at baseline). LTPA was assessed using questionnaires in 1975, 1981 and 1990. The mortality follow-up lasted until 2020 and biological ageing was assessed using epigenetic clocks in a subsample (n = 1,153) with blood samples taken during the follow-up. Using latent profile analysis, we identified classes with distinct longitudinal LTPA patterns and studied differences in biological ageing between these classes. We employed survival models to examine differences in total, short-term and long-term all-cause mortality, and multilevel models for twin data to control for familial factors.

Results:

We identified four classes of long-term LTPA: sedentary, moderately active, active and highly active. Although biological ageing was accelerated in sedentary and highly active classes, after adjusting for other lifestyle-related factors, the associations mainly attenuated. Physically active classes had a maximum 7% lower risk of total mortality over the sedentary class, but this association was consistent only in the short term and could largely be accounted for by familial factors. LTPA exhibited less favourable associations when prevalent diseases were exclusion criteria rather than covariate.

Conclusion:

Being active may reflect a healthy phenotype instead of causally reducing mortality.

Keywords: DNA methylation, mortality, lifespan, biological age, physical activity

INTRODUCTION

The association between leisure-time physical activity (LTPA) and a lower risk of mortality from all causes and cardiovascular diseases is frequently reported [1–4]. However, the evidence is generally based on observational studies, and LTPA is typically assessed at single time points. Studies using repeatedly measured LTPA have suggested that being both persistently and increasingly active over adulthood are associated with a reduced risk of mortality [2]. However, evidence based on randomized controlled trials has failed to confirm that LTPA prevents premature mortality [5]. This may be due to the lack of exercise trials designed with mortality as a primary outcome, carried out with high long-term compliance and with sufficient statistical power to detect the effect [6,7], but also to the absence of causal relationship between LTPA and mortality. Genetically informed studies have suggested that genetic selection may partly account for the association between LTPA and mortality, as genetically healthy participants tend to engage in LTPA [8,9]. In fact, the association is susceptible to reverse-causality bias: an underlying suboptimal physiological or predisease state may negatively affect LTPA, which means that the observed association may be due to a causal relationship between the covert disease state and subsequent premature death [10]. Previous studies have shown that increased control for reverse causality results in weaker associations between LTPA and mortality [10,11]. Moreover, researchers have proposed that, rather than LTPA *per se* reducing mortality risk, participation in LTPA and the ability to increase LTPA in later life are themselves indicators of good fitness and health [7].

Slower biological ageing is a plausible mechanism for explaining the path from an active lifestyle (or a healthy phenotype) to reduced mortality risk. Biological ageing is the gradual and progressive decline in system integrity that occurs with advancing age and results in increased risk of morbidity and mortality [12,13]. Epigenetic clocks produce estimates for biological ageing based on DNA methylation (DNAm) alterations within specific CpG (a cytosine nucleotide followed by a guanine nucleotide) sites and are one of the primary hallmarks of biological ageing [13,14]. Epigenetic clocks can sum up genetic influences and lifetime burden of lifestyles that predict time-to-death. Although previous studies have suggested that LTPA is associated with decelerated biological ageing [15,16], more research is required because reliable biological age indicators have only recently become available [17–19].

The main purpose of this study was to identify classes of long-term LTPA patterns and to examine whether these classes differ in terms of biological ageing. Furthermore, we aimed to explore class-specific differences in all-cause mortality, considering biological ageing to be a potential mediator of the favourable associations between long-term LTPA and all-cause mortality. To evaluate the influence of reverse causality, we studied whether the association between long-term LTPA and mortality is affected by how prevalent diseases are accounted for, and we investigated the association separately for short-term and long-term mortality. Finally, using a twin study design, we examined whether the associations between long-term LTPA and mortality are independent of shared genetic and environmental factors.

MATERIAL AND METHODS

Study design and participants

Participants were twins from the older Finnish Twin Cohort (FTC) [20]. The FTC consists of same-sex twins born in Finland before 1958 and with both co-twins alive in 1967. Questionnaires were mailed in 1975 and 1981 to all twins born before 1958 and living in Finland, with a follow-up questionnaire conducted in 1990 with twins born in 1930–1957. The response rates were high (77–89%). Participants aged 18–50 years at baseline in 1975, who had at least one measurement of LTPA and were alive in 1990, were included in the present study (*n*=22,750). For the subsample (*n*=1,153), a blood sample was taken during the 1993–2020 period, and blood-based DNAm was used to assess biological ageing at age range from 37 to 81 years.

Measurements

Leisure-time physical activity (LTPA)

LTPA was measured in metabolic equivalent (MET) hours per day (h/day) using a structured validated questionnaire in 1975, 1981 and 1990 [9,21–23] (see Supplementary Tables S1–S2 for details). In 1975 and 1981 questions concerned monthly frequency of LTPA, mean duration and intensity of the sessions (Table S1). The MET index was calculated by multiplying the frequency, intensity, and duration of leisure activities as well as commuting activities, and then summing up the resulting values [21,22]. In 1990 the questionnaire slightly differed and participants reported their time spent in LTPA (including commuting activity) at different intensity levels (Table S2). The MET index was calculated by

multiplying the time spent in LTPA by the estimated MET value of each intensity level and then summing up the resulting values [23].

Outcome variables

Biological ageing was assessed using blood-based epigenetic ageing measures, namely, principal component (PC)-based DNAm GrimAge [18,19] and DunedinPACE [17]. DNAm GrimAge is a mortality predictor by design, and it is composite of age, sex, DNAm-based surrogates for seven plasma proteins and smoking pack-years [19]. Recently, PC-based epigenetic clocks have been developed to bolster the reliability and validity of the clocks [18]. The DunedinPACE estimator was developed to predict pace of ageing, describing longitudinal changes in 19 age-related biomarkers [17]. It provides an estimate of the pace of ageing in years per calendar year.

Genomic DNA was extracted from peripheral blood samples using commercial kits. High molecular weight DNA samples (1 µg) were bisulfite converted using EZ-96 DNA /methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The twins and co-twins were randomly distributed across plates, with both twins from a pair on the same plate. DNAm profiles were obtained using Illumina's Infinium HumanMethylation450 BeadChip or the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA, USA). The Illumina BeadChips measure single-CpG resolution DNAm levels across the human genome. With these assays, it is possible to interrogate over 450,000 (450k) or 850,000 (EPIC) methylation sites quantitatively across the genome at single-nucleotide resolution. Of the samples included in the present study, 419 were assayed using 450k and 734 samples using EPIC arrays. Methylation data from different platforms was separately preprocessed using R package *meffil* [24], and the pipeline for preprocessing was recently described in detail [25]. Beta values representing DNAm levels were used as input in the calculations of the estimates of epigenetic ageing.

We produced PC-based GrimAge estimates using an R package [18] (https://github.com/MorganLevineLab/PC-Clocks). Age acceleration ($AA_{PC\text{-}Grim}$) in years was determined as a residual by regressing the estimated epigenetic age on chronological age. In addition, we obtained PC-based components of GrimAge (adjusted for age), including DNAm smoking pack-years, DNAm adrenomedullin (ADM), DNAm beta-2-microglobulin

(B2M), DNAm cystatin C, DNAm growth differentiation factor 15 (GDF15), DNAm leptin, DNAm plasminogen activator inhibitor 1 (PAI-1), and DNAm tissue inhibitor metalloproteinases 1 (TIMP-1). The estimates for pace of ageing were calculated using an R package [17] (https://github.com/danbelsky/DunedinPACE). The measures were screened for outliers (> 5 standard deviations away from mean), and there were no outliers.

Mortality. Dates of death were retrieved from the Population Register Centre of Finland and Statistics Finland. The mortality follow-up started with the response date to the 1990 questionnaire and continued until the date of emigration, death or 31 December 2020, whichever came first.

Confounding variables

Time-varying confounders cause bias for the studies on long-term LTPA and mortality [2]. In our study, on one hand, LTPA was measured over 15-year period and the other lifestyle-related factors may have considerably changed after baseline. On the other hand, exposure may have affected the other lifestyle-related factors measured over a long period and thus, these factors may partly mediate rather than confound the association between exposure and outcome [2]. Moreover, our sample included older participants (born in 1925–29) who had information on mortality (n=1,667) and DNAm (n=144) but were not invited to answer questionnaire in 1990. For these reasons, we considered year 1981 the optimal measurement point of confounders. Specific diseases and other lifestyle-related factors that could have affected long-term LTPA and mortality were considered as potential confounders.

Health status. An indicator of specific physician-diagnosed somatic diseases (angina pectoris, myocardial infarction, and diabetes) based on self-reports from 1975 and 1981 was used.

Education (in years) was based on the self-reported latest education in 1981 and converted into years of education as follows: less than primary school (4), primary school (6), junior high school (9), high school graduate (12), university degree (17), and ≥ 1 year of education such as vocational training in addition to primary school (8), junior high school (11) or high school (14) [26].

Body mass index (BMI) (kg/m^2) was calculated based on self-reported height and weight in 1981. BMI based on self-reports has been shown to agree well with BMI based on measured values [27].

Smoking status was self-reported based on an extensive smoking history [28] and classified as never, occasional, former and current light (1–9 cigarettes per day [CPD]), medium (10–19 CPD) and heavy (≥20 CPD) smokers.

Alcohol use was based on average alcohol consumption (g/day) in 1981 of beer, wine and spirits [29] and classified as never, former, occasional (>0.1 and <1.3 g), low (≥1.3 and <25 g), medium (≥25 and <45 g), high (≥45 and <65 g) and very high (≥65 g)[30].

Statistical analysis

The main analyses were conducted using Mplus 8.2 [31]. The parameters of the models were estimated using the full information maximum method (FIML) with robust standard errors, which uses all available data during estimation.

We conducted latent profile analysis (LPA) to identify long-term patterns of LTPA based on the means and variances of MET indices in 1975, 1981 and 1990. An LPA model with 1–6 classes was fitted. Several indices were used to evaluate the goodness of fit: Akaike's information criterion, Bayesian information criterion and sample size-adjusted Bayesian information criterion. The lower values of the information criteria indicated a better fit for the model. Furthermore, we used the Vuong–Lo–Mendell–Rubin likelihood ratio (VLMR) test and the Lo–Mendell–Rubin (LMR) test to determine the optimal number of classes. The estimated model was compared with the model with one class less, and the low p value suggested that the model with one class less should be rejected. At each step, the classification quality was assessed using the average posterior probabilities for most likely latent class membership (AvePP). AvePP values close to 1 indicate a clear classification. In addition to the model fit, the final model for further analyses was chosen based on the parsimony and interpretability of the classes.

The mean differences between the classes in later biological ageing were studied using the Bolck-Croon-Hagenaars (BCH) approach, which controls for measurement error in

classification [32]. The standard errors of the models were corrected for nested sampling within families. The analyses were adjusted for sex, age, health status and timing of the blood drawn (Model 1) as well as for education, BMI, smoking and alcohol use (Model 2). The continuous covariates were standardized before entering the model. In addition, differences in the DNAm-based plasma proteins and smoking pack-years between the classes were explored.

We investigated differences in total all-cause mortality as well as in short- and long-term all-cause mortality between the classes using a discrete-time survival model, which enables flexible modelling within the structural equation framework [33,34]. We used year as the unit of discrete-time survival indicators from 1991 to 2020 and constructed a latent variable describing propensity for death [33,34] (see Fig. 1 A). The latent variable was regressed on the latent class membership of long-term LTPA and the potential confounding variables. Moreover, we divided the follow-up time into two parts, formed latent variables representing propensity for short- and long-term death, and the associations of long-term LTPA on short- and long-term mortality were studied (Fig. 1 B). This approach relaxes strict proportionality assumption often necessary in the survival modeling, by allowing the associations of exposure with short- and long-time mortality to vary. Although the model was adjusted using a procedure like that employed for the models of biological ageing, we accounted for health status in two ways. First, we adjusted the model for health status, and second, we excluded participants who reported selected diseases (angina pectoris, myocardial infarction, and diabetes) from the analysis. The best practices in relating LPA to time-to-event distal outcomes are still under research (Bakk & Kuha, 2021; Lythgoe et al., 2019). Because BCH approach was not available for the survival models, we used posterior classification probabilities as sampling weights to control for measurement error in the classification (three-step approach with proportional assignment) (Bakk & Kuha, 2021). The standard errors of the model parameters were corrected for nested sampling within families.

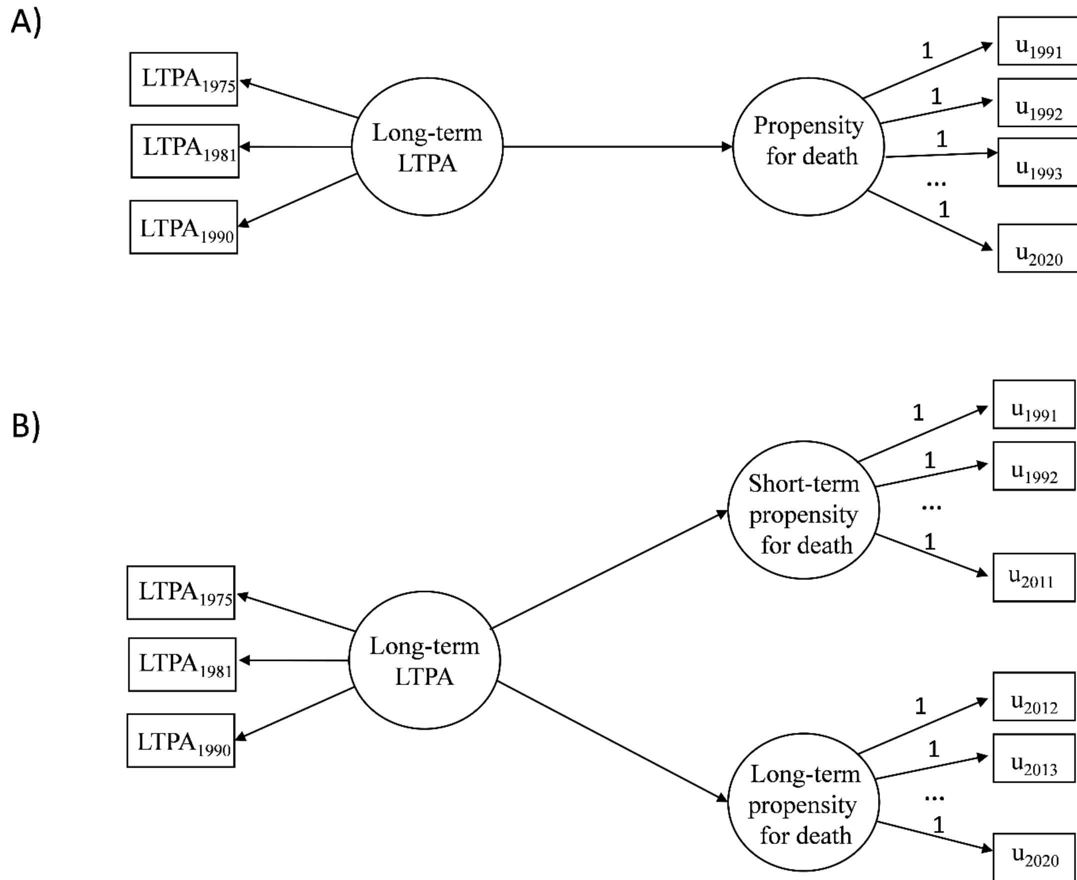

Fig. 1 Path diagram of the discrete-time survival models for A) total mortality and B) short- and long-term mortality. Follow-up time was treated as time scale in the analysis. Circles denote latent variables and rectangles observed variables.
LTPA, leisure-time physical activity; u, discrete-time survival indicators.

To account for familial factors, the associations between long-term LTPA and total all-cause mortality were studied at the within-twin-pair level using multilevel modelling. For monozygotic (MZ) twin pairs, the association was controlled for shared environmental factors and genetics, while for dizygotic (DZ) pairs, it was controlled for shared environmental factors and partially for genetics.

RESULTS

The descriptive statistics of the study variables are presented in Table 1 and are stratified by sex in Supplementary Table S3.

Table 1. Descriptive statistics of the study variables for all twins and the subsample of twins with information on biological ageing

	All twins (n = 22,750)		Subsample (n = 1,153)	
	n	Mean (SD) or %	n	Mean (SD) or %
Sex				
Female	11,308	49.7	933	80.9
Male	11,442	50.3	220	19.1
Age at baseline	22,750	30.0 (8.9)	1,153	33.2 (9.7)
Zygosity				
Unsure	1,902	8.4	-	
Monozygotic	6,462	28.4	610	52.9
Same-sex dizygotic	14,386	63.2	543	47.1
Health status				
Diseases[a]	18,113		1,055	
No	17,018	94.0	1,012	95.9
Yes	1,095	6.0	43	4.1
Latest education in 1981 (in years)	22,205	8.5 (3.2)	1,131	9.2 (3.5)
Leisure-time physical activity				
Metabolic equivalent (MET) index (h/day)				
in 1975	20,984	2.4 (3.1)	1,116	2.2 (2.5)
in 1981	20,165	2.6 (3.1)	1,098	2.4 (2.4)
in 1990	12,312	3.3 (3.4)	841	3.1 (2.8)
Lifestyle-related factors in 1981				
Body mass index (kg/m^2)	20,074	23.5 (3.4)	1,097	23.4 (3.4)
Smoking	19,850		1,153	
Never	8,932	45.0	703	64.8
Occasional	624	3.1	33	3.0
Former	4,149	20.9	182	16.8
Light	1,476	7.4	53	4.9
Medium	2,714	13.7	77	7.1
Heavy	1,955	9.8	37	3.4
Alcohol use[b]	19,818		1,091	

Never	1,768	8.9	167	15.3
Former	1,092	5.5	69	6.3
Occasional	1,045	5.3	77	7.1
Low	14,337	72.3	746	68.4
Medium	961	4.8	20	1.8
High	398	2.0	10	0.9
Very high	217	1.1	2	0.2
Outcomes				
Deaths (1991–2020)	6,949	30.6	267	23.2
Biological ageing				
Age at blood drawn	-		1,153	63.5 (9.1)
PC-based DNAm GrimAge (in years)			1,153	70.9 (7.3)
DunedinPACE (year/calendar year)	-		1,153	0.98 (0.12)

SD, standard deviation; DNAm, DNA methylation; PC, principal component.

[a]Self-reported physician-diagnosed angina pectoris, myocardial infarction, or diabetes in 1975 or 1981.

[b]High and very high classes were combined for further analysis.

Patterns of long-term LTPA

The model-fit based on the information criteria improved at each step (Supplementary Table S4). However, at the sixth step, only a small class (<5%) was extracted, and therefore, a solution with 4–5 classes was considered optimal. At the fifth step, a class with increasing LTPA pattern from sedentary to moderate level was identified. To achieve sufficient power for the further analysis, we used a four-class solution in the main analyses (Fig. 2). The level of LTPA appeared to increase slightly between years 1981 and 1990, except in the highly active class. This is probably due to the small changes in the questionnaire (see Supplementary Tables S1–S2) rather than reflecting actual increase in LTPA. For sensitivity analysis, we conducted the main analyses using a five-class solution (Supplementary Fig. S3–S5).

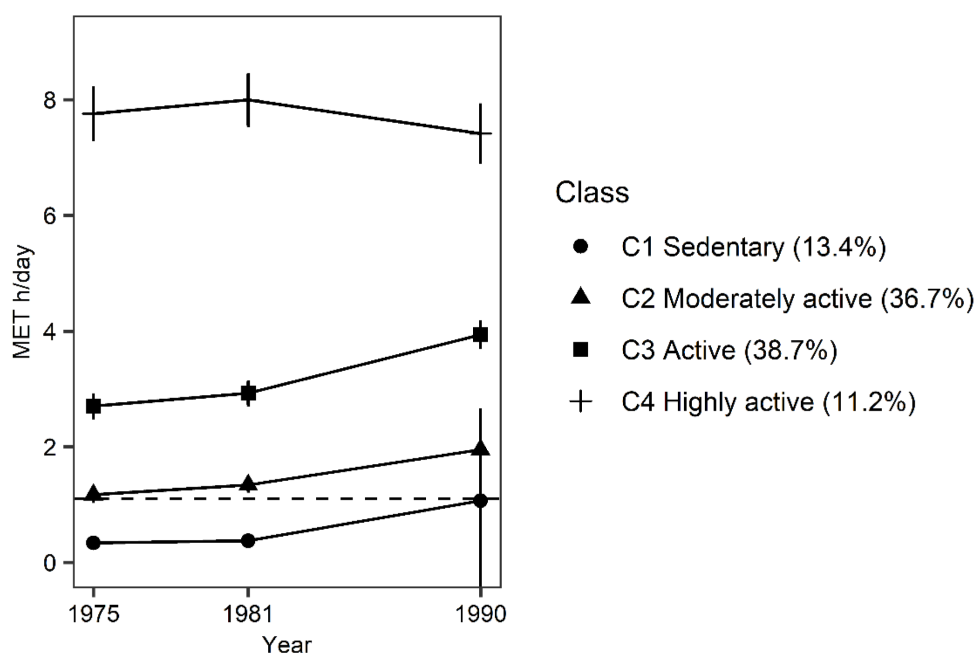

Fig. 2 Patterns of long-term leisure-time physical activity (*n* = 22,750)

Means of metabolic equivalent (MET) hours (h)/day and 95% confidence intervals are presented. The dashed line denotes World Health Organization guidelines for the recommended minimum amount of physical activity for adults (150 min of moderate intensity physical activity per week ~ 1.1 MET h/day) [35].

Differences in biological ageing between long-term LTPA classes

There were differences between the classes in terms of biological ageing, measured using $AA_{PC\text{-}Grim}$ and DunedinPACE (Fig. 3). The association between long-term LTPA and biological ageing followed a U-shaped pattern: participants in the sedentary and highly active classes were biologically older than those who were moderately active and active. After adjusting for other lifestyle-related factors, most differences were attenuated, but based on $AA_{PC\text{-}Grim}$, the highly active class remained, on average, 1.2 years (95% confidence interval: 0.2–2.2) biologically older than the moderately active class and 1.7 years (0.6–2.7) biologically older than the active class. As no significant beneficial association between long-term LTPA and slower biological ageing was observed, biological ageing was unlikely to act as a mediator for the association between long-term LTPA and lower mortality, and no further path modelling was conducted.

Of the PC-GrimAge components, the U-shaped association was most pronounced in DNAm-based cystatin C and B2M (Supplementary Fig. S1). There were also differences in DNAm-

based smoking pack-years. In the highly active class, the level was higher than in the other classes, which may indicate under-reporting in the highly active class because the models were adjusted for self-reported smoking. There were no differences in DNAm ADM, GDF15, leptin, PAI-1 or TIMP-1.

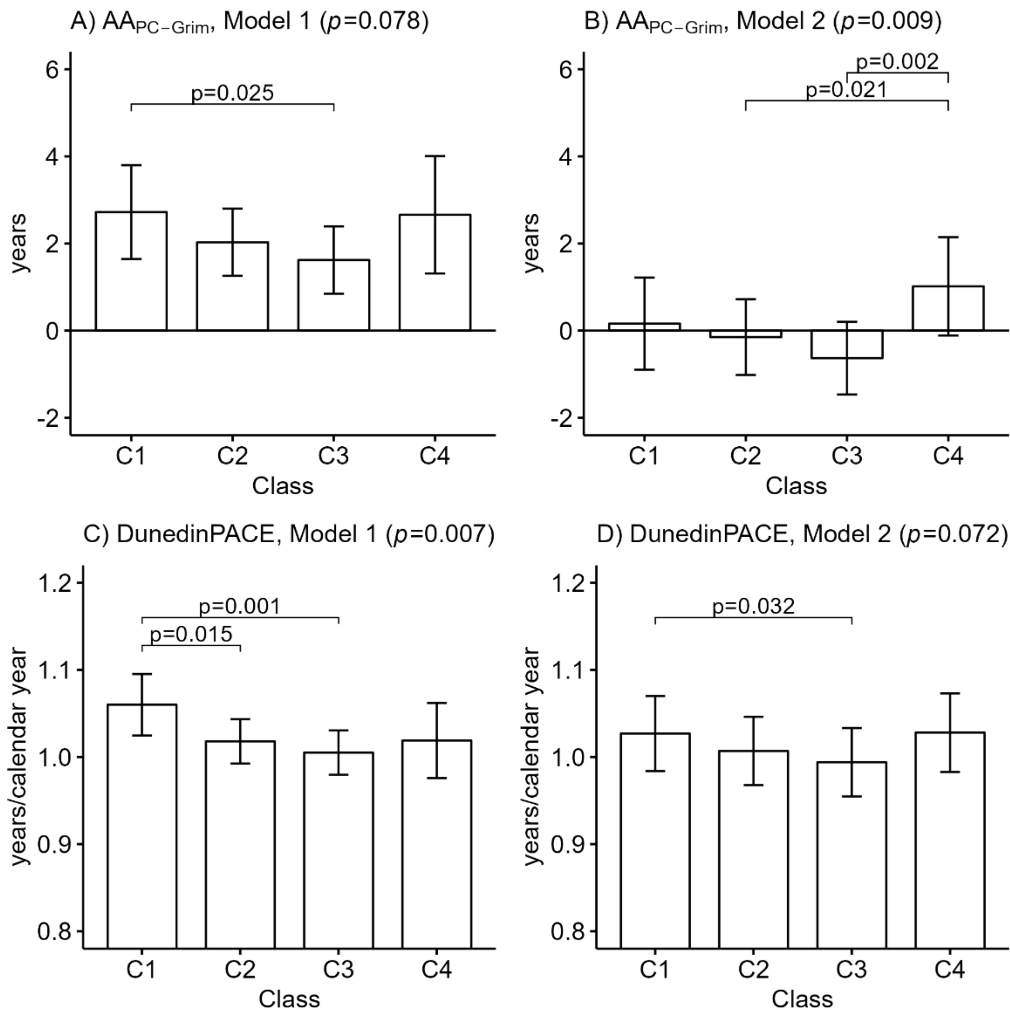

Fig. 3 Mean differences between the long-term leisure-time physical activity classes in terms of biological ageing measured using A)–B) PC-based GrimAge and C)–D) DunedinPACE (*n* = 1,153).

Means and 95% confidence intervals are presented. Model 1 was adjusted for sex, age, timing of the blood drawn and health status, and Model 2 was additionally adjusted for education, body mass index, smoking and alcohol use. C1: Sedentary (8.8%); C2: Moderately active (38.4%); C3: Active (45.5%); C4: Highly active (7.3%); AA, Age acceleration; *p*-values from the Wald test.

Differences in mortality between the latent long-term LTPA classes

Over a third (38.8%) of the participants from the sedentary class died during the mortality follow-up period, compared to 30.8%, 29.0% and 25.4% from the more active classes, respectively. Active classes had 15–23% lower all-cause mortality risk compared to the sedentary class, but after accounting for other health-related factors, the reduction in mortality risk was a maximum of 7% (Fig. 4 A). Approximately half of the deaths occurred in 2011 or earlier. Therefore, 2011 was considered the cut-off point for short- and long-term mortality (Fig. 4B–C). For sensitivity analysis, we performed the modelling using 2006 as the cut-off, when one-third of the deaths had occurred (Supplementary Fig. S6). Overall, the favourable associations of long-term LTPA were more consistent with short-term than long-term mortality. In particular, high activity was associated only with lower risk of short-term mortality. The results were similar after excluding participants with reported specific diseases (Supplementary Fig. S2).

A) Total mortality

Model 1
Moderately active 0.85 (0.80 - 0.90)
Active 0.82 (0.77 - 0.87)
Highly active 0.77 (0.70 - 0.84)
Model 2
Moderately active 0.93 (0.88 - 0.99)
Active 0.93 (0.88 - 0.99)
Highly active 0.94 (0.86 - 1.03)

0.5 1 1.5
hOR (95% Confidence interval)

B) Short-term mortality

Model 1
Moderately active 0.84 (0.78 - 0.91)
Active 0.80 (0.74 - 0.87)
Highly active 0.71 (0.63 - 0.80)
Model 2
Moderately active 0.93 (0.86 - 1.00)
Active 0.92 (0.85 - 1.00)
Highly active 0.88 (0.78 - 0.99)

0.5 1 1.5
hOR (95% Confidence interval)

C) Long-term mortality

Model 1
Moderately active 0.85 (0.78 - 0.93)
Active 0.83 (0.76 - 0.91)
Highly active 0.83 (0.73 - 0.94)
Model 2
Moderately active 0.94 (0.86 - 1.02)
Active 0.94 (0.86 - 1.03)
Highly active 1.01 (0.89 - 1.15)

0.5 1 1.5
hOR (95% Confidence interval)

Fig. 4 Associations of long-term leisure-time physical activity with A) total mortality, B) short-term mortality (1990–2011) and C) long-term mortality (2012–2020) (n = 22,750).

The sedentary class was treated as the reference. Model 1 was adjusted for sex (female), age and health status. Model 2 was additionally adjusted for education, body mass index, smoking and alcohol use. hOR, hazard odds ratio.

Within-twin-pair differences in mortality between long-term LTPA classes

In a model adjusted only for health status at the within-pair level, the analyses showed that the moderately active and active classes exhibited lower risks of all-cause mortality compared to the sedentary class within all pairs, DZ pairs and MZ pairs (Fig. 5, Model 1). After additionally adjusting the model for other lifestyle-related factors, the differences were considerably attenuated, but the moderately active and active classes exhibited lower risks of all-cause mortality compared to the sedentary class within all pairs and MZ pairs (Fig. 5, Model 2).

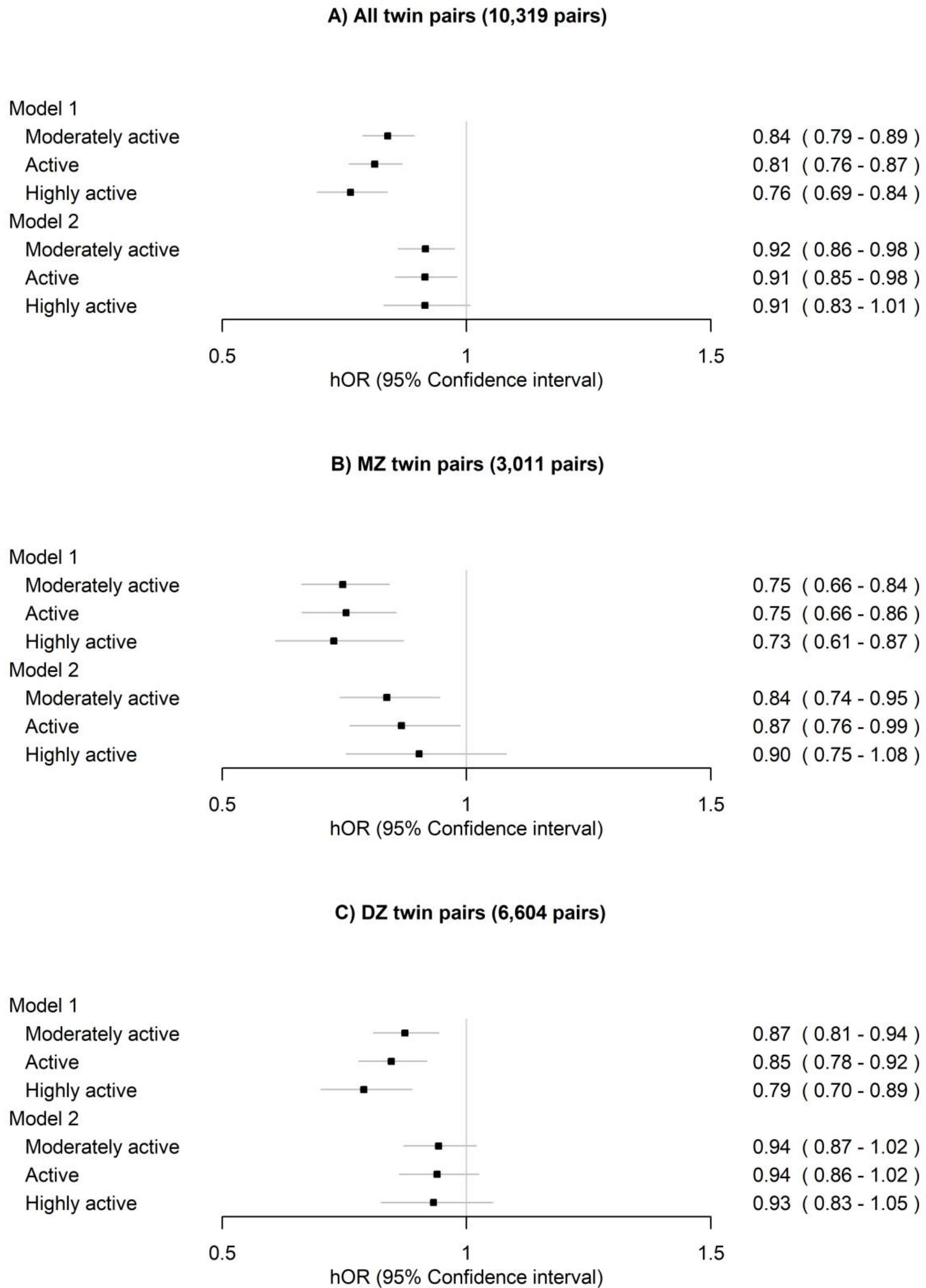

Fig. 5 Within-twin-pair differences in all-cause mortality between the long-term leisure-time physical activity classes for A) all twin pairs, B) monozygotic (MZ) pairs and C) dizygotic (DZ) pairs. The sedentary class was treated as the reference. Only twin pairs with information on LTPA and alive in 1990 were included in the analysis. Model 1 was adjusted for sex

(female) and age at the between-twin-pair level and health status at the within-twin pair level. Model 2 was additionally adjusted for education, body mass index, smoking and alcohol use at the within-twin pair level. hOR, hazard odds ratio.

After excluding the twin pairs who reported specific diseases, the differences in all-cause mortality were almost fully attenuated and were no longer significant after adjusting for other lifestyle-related factors for all twin pairs, MZ pairs and DZ pairs (Fig. 6, Model 2).

A) All twin pairs (6,670 pairs)

	hOR (95% Confidence interval)
Model 1	
Moderately active	0.89 (0.81 - 0.97)
Active	0.85 (0.78 - 0.93)
Highly active	0.79 (0.70 - 0.90)
Model 2	
Moderately active	0.97 (0.89 - 1.06)
Active	0.97 (0.88 - 1.07)
Highly active	0.99 (0.87 - 1.13)

0.5 1 1.5
hOR (95% Confidence interval)

B) MZ twin pairs (2,107 pairs)

	hOR (95% Confidence interval)
Model 1	
Moderately active	0.80 (0.68 - 0.95)
Active	0.82 (0.69 - 0.97)
Highly active	0.84 (0.67 - 1.05)
Model 2	
Moderately active	0.90 (0.76 - 1.06)
Active	0.93 (0.78 - 1.11)
Highly active	1.04 (0.83 - 1.30)

0.5 1 1.5
hOR (95% Confidence interval)

C) DZ twin pairs (4,306 pairs)

	hOR (95% Confidence interval)
Model 1	
Moderately active	0.93 (0.84 - 1.04)
Active	0.89 (0.80 - 1.00)
Highly active	0.79 (0.67 - 0.93)
Model 2	
Moderately active	1.01 (0.90 - 1.13)
Active	1.00 (0.89 - 1.13)
Highly active	0.98 (0.83 - 1.16)

0.5 1 1.5
hOR (95% Confidence interval)

Fig. 6 Within-twin-pair differences in all-cause mortality between the long-term leisure-time physical activity classes for A) all twin pairs, B) monozygotic (MZ) pairs and C) dizygotic (DZ) pairs after excluding twin pairs who reported specific diseases (angina pectoris, myocardial infarction, and diabetes in 1975 or 1981). The sedentary class was treated as the

reference. Only twin pairs with information on LTPA and alive in 1990 were included in the analysis. Model 1 was adjusted for sex (female) and age at the between-twin-pair level. Model 2 was additionally adjusted for education, body mass index, smoking and alcohol use at the within-twin-pair level.

Sensitivity Analysis using a five-class solution

After including fifth class in the LCA model, a class of increasingly active (from sedentary to moderate) participants was extracted (Supplementary Fig. S3). According to the overall test there were differences between the classes in biological ageing measured with $AA_{PC\text{-}Grim}$ but not in DunedinPACE (Supplementary Fig. S4). Biological ageing measured with $AA_{PC\text{-}Grim}$ appeared to be accelerated in highly active class. There were differences in mortality between the classes, but the differences were smaller than observed in the main analysis (Supplementary Fig. S5). Increasingly active class did not differ from sedentary class in terms of mortality risk. After accounting for other health-related factors, there were differences only in short-term mortality.

DISCUSSION

We conducted a 30-year mortality follow-up of a longitudinal study of LTPA with a large cohort of adult twins and identified classes according to long-term LTPA patterns. Our analyses were based on standard cohort approaches to individuals (adjusting for the sampling of twin pairs) and on within-pair modelling to account for familial and genetic factors. The results showed that the beneficial associations of long-term LTPA with slow biological ageing and reduced mortality were largely accounted for by other health-related factors. The most remarkable reduction of 7% in all-cause mortality was observed already when the recommended minimum amount of LTPA was, on average, achieved, with no additional benefits provided by higher levels of LTPA. This result is in line with the World Health Organization guidelines [35] and studies demonstrating that dose-response association between physical activity (PA) and all-cause mortality is curvilinear rather than linear [3,4,36]. The association between long-term LTPA and all-cause mortality was susceptible to reverse-causality bias because a consistent association was observed only in the short term. Moreover, the results of within-twin-pair comparisons depended on how health status was controlled for. An alternative explanation is a change in PA exposure as a function of time; in other words, some people may have changed their PA levels later in adulthood.

The association between long-term LTPA and mortality was susceptible to bias from multiple sources

Previous studies of long-term LTPA have reported larger differences in mortality between consistently active and inactive participants (16–36%) [37–39] than what we observed in our study. This discrepancy can be explained by differences in the length of the mortality follow-up time; longer follow-up time has been shown to be associated with weaker associations between PA and mortality [10]. In our study, participants were followed over a 30-year period. Therefore, we were able to divide the follow-up time into two parts to reflect short- and long-term survival. Our analysis revealed that the associations of long-term LTPA were more consistent with short-term than long-term mortality. Being highly active was associated with reduced mortality only in the short term and thus may not have long-term mortality benefits unless activity is maintained continuously.

Another reason for the higher effect sizes observed in previous studies may be residual confounding due to insufficient adjustments. In our study, when the models were minimally adjusted, the reduction in mortality (15–23%) was closer to the level observed in previous studies. After adjustment for smoking in terms of both status and quantity, and other lifestyle-related factors (education, BMI and alcohol use), the association was considerably attenuated. Smoking is the most harmful lifestyle habit in terms of mortality [40,41]. Often, only smoking status is adjusted for in analyses, but this may not be sufficient because current smokers who are physically active tend to smoke less than those who are sedentary (Supplementary Table S5).

Previous twin studies have provided somewhat inconsistent results regarding the association between LTPA and mortality after accounting for familial factors. Studies using data from Finnish twins have suggested that the association between LTPA and all-cause mortality is due to genetic selection, as there was no difference in mortality between MZ co-twins discordant in terms of LTPA [9,41], whereas a study with a similar twin study setting found a difference within Swedish twin pairs [42]. Several reasons for this discrepancy have been proposed, such as differences in LTPA measurement methods and in controlling for prevalent diseases [43]. Our within-twin-pair comparisons were considerably affected by how the prevalent diseases were controlled for. Excluding twin pairs with one or both co-twins

reporting diseases (as done in prior Finnish studies) attenuated the differences more strongly compared to adjusting for health status, particularly when other lifestyle-related factors were controlled for. This finding may support studies arguing that adjusting the survival model for health status may not be sufficient to mitigate reverse causation [11] and may also reflect an accumulation of unhealthy lifestyle habits and prevalent diseases in the sedentary class (Supplementary Table S5).

Long-term LTPA and biological ageing

Previous studies, mostly based on cross-sectional data, have indicated that the observed associations, or lack of associations, between LTPA and biological ageing depend on the type of epigenetic clock used [15,44–47]. This is probably because the first-generation clocks were developed to predict chronological age [48,49], while newer clocks, such as DNAm GrimAge, are better predictors of health and lifespan [19,50]. LTPA is most consistently associated with biological ageing, assessed using the DNAm GrimAge estimator. To the best of our knowledge, this is the first study to report on the association between LTPA and DunedinPACE. We found that biological ageing was, on average, slower in the active class compared to the sedentary class when these two markers of biological ageing were used. However, after adjusting the model for other lifestyle-related factors, the differences were largely attenuated, which likely reflects an accumulation of factors related to an unhealthy lifestyle in the sedentary class [51]. Moreover, the weak associations observed in the present study may be due to the prospective study design, as the beneficial influences of LTPA may diminish after a time lag of several years.

Contrary to the existing literature [15,45,47], we observed that the highly active class was, on average, biologically older than the moderately active and active classes when the DNAm (PC) GrimAge was used. However, a recent study indicated a curvilinear association between accelerometer-based PA and biological ageing [46]. Some studies have reported U-shaped associations between LTPA and mortality [52], and it has been suggested that sudden cardiac deaths after/during exercise may explain the increased mortality at high levels of LTPA [7,52]. However, a recent meta-analysis did not find evidence of increased levels of mortality at high PA levels [1].

To better understand the reasons for the observed patterns, we explored the DNAm-based

plasma proteins included in the DNAm GrimAge estimator. Interestingly, the levels of DNAm-based cystatin C and beta-2-microglobulin (B2M) were higher in both the sedentary and highly active classes. These proteins are markers of kidney function, and their higher concentrations are linked to mortality due to cardiovascular diseases [53] and sudden cardiac death [54]. Further studies are required to determine whether these DNAm-based proteins play a role in sudden cardiac death in athletes.

Strengths and limitations

Our study has major strengths. We used prospective population-based large cohort data with longitudinal measurements of LTPA over 15 years with validated questionnaires and 30 years of mortality follow-up. The LTPA patterns were studied using a data-driven method without using preselected cut-offs. Biological ageing was assessed using novel epigenetic clocks, which have been shown to perform better than their predecessors [17,18]. The twin study design enabled us to control the analyses for genetics and the shared environment.

However, our study also had limitations. In the subsample of twins having information on biological ageing, women and non-smokers were overrepresented but otherwise the subsample represented larger cohort relatively well (see Supplementary Table S3). LTPA was self-reported, and a considerable proportion of the participants did not have LTPA at all three measurement points. Self-reports may be susceptible to recall and social-desirability biases. However, the questionnaire-based MET index has been shown to be a reliable tool for measuring LTPA [22], and self-reports may better reflect long-term LTPA than device-based measures. Although twin design efficiently controls for shared confounders, there may be influences of non-shared unmeasured confounders (e.g. dietary exposures) which were not controlled in the analyses.

Conclusions

Our findings support the suggestion that, rather than LTPA per se reducing the risk of mortality, being active may be an indicator of a healthy phenotype and an overall healthy lifestyle, which co-occur with a lower mortality risk. Further research on DNAm-based surrogates may provide insights into the mechanisms behind the beneficial and detrimental health influences of LTPA.

STATEMENTS AND DECLARATIONS

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Competing Interests

The authors declare no conflicts of interest.

Author Contributions

AK, LJ, KW, MO, JK and ES conceptualised the research question. AH preprocessed the DNAm data. AK, AT and ES designed the statistical analysis, and AK performed the statistical modelling under the supervision of AT. JK and MO designed and collected the FTC dataset and participated in designing the analysis of this manuscript. AK drafted the first version of the manuscript, and ES and LJ contributed significantly to the writing. AK, AT, LJ, KW, AH, JK, MO and ES contributed to the interpretation of the results and revising the manuscript. ES, MO and JK acquired the funding for the study.

Data sharing

A subsample of the FTC with DNA methylation age estimates, phenotypes and information on the classes of long-term LTPA will be located in the Biobank of the National Institute for Health and Welfare. All these data will be publicly available for use by qualified researchers following a standardised application procedure (for details on the application process, see the following website: https://thl.fi/en/web/thl-biobank/for-researchers). Because of the consent given by the study participants and the high degree of identifiability of the twin siblings in Finland, the full cohort data cannot be made publicly available. However, the full cohort data are available through the Institute for Molecular Medicine Finland (FIMM) Data Access Committee (DAC) for authorised researchers with an IRB/ethics approval and an

institutionally approved study plan. For more details, please contact the FIMM DAC (fimm-dac@ helsinki.fi).

Ethical approval

Data collection was conducted in accordance with the Declaration of Helsinki.
The ethics committees of the University of Helsinki and Helsinki University Central Hospital approved the study protocol (113/E3/2001 and 346/E0/05).

Consent to participate

Blood samples for DNA analyses were collected during in-person clinical studies after written informed consent was signed.

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