Physical activity and fitness for healthy ageing

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List of abbreviations

25-OHD – 25-hydroxyvitamin D

AHBA – Allen Human Brain Atlas

ASCVD Risk Score – Assessment of Cardiovascular Disease Risk Score

ASEG – Automatically subcortical segmentation of a brain volume

ATC – Anatomical Therapeutic Chemical code

Bas – Basophils

B memory – B memory cells

BMI – Body mass index

B naïve – Naïve B cells

BP – Biological process

CC – Cellular component

CD4 memory – Memory CD4T cells

CD8 naïve – Naïve CD8T cells

CD8 memory – Memory CD8T cells

Chr – Chromosome

(95%) CI – Confidence interval

COVID-19 – Coronavirus disease - 2019

CpG – Cytosine-phosphate-Guanine

CSF – Cerebrospinal fluid

DBP – Diastolic blood pressure

DNA – Deoxyribonucleic acid

DUAC – Data use and access committee

DZNE – German Center for Neurodegenerative Diseases

Eos – Eosinophils

ESC SCORE2 – European Society of Cardiology Score

eTIV – Estimated total intracranial volume

EWAS – Epigenome-wide association analysis

FAMD – Factor analysis for mixed data

FDR – False discovery rate

FWHM – Full-width-half-maximum

GCP – Good Clinical Practice

GO – Gene Ontology

GWAS – Genome-wide association studies

HDL – High-density lipoprotein

ICH – International Council for Harmonization

ISCED – International Standard Classification of Education

KEGG – Kyoto Encyclopaedia of Genes and Genomes

LDL – Low-density lipoprotein

MET-Hours – Metabolic equivalent hours

MF – Molecular function

MNI – Montreal Neurological Institute

Mono – Monocytes

mQTL – Methylation quantitative trait loci

MRI – Magnetic resonance imaging

ms – Milliseconds

MVPA – Moderate-to-vigorous physical activity

NADH – Nicotinamide adenine dinucleotide + hydrogen

NAM – National Academy of Medicine

Neu – Neutrophils

NIH – National Institute of Health

NK – Natural killer cells

PCA – Principle component analysis

Pos – Position

ref – Reference group

RNA – Ribonucleic acid

SEM – Structural equation modelling

SBP – Systolic blood pressure

SD – Standard deviation

SPM – Statistical Parametric Mapping

TA – Acquisition time

TI – Inversion time

TIA – Transient ischemic attack

TE – Echo time

TR – Repetition time

Treg – Regulatory T cells

UCL – University College London

WHO – World Health Organization

WHR – Waist-to-hip ratio

1. Abstract

The proportion of individuals suffering from age-associated noncommunicable diseases is expected to rise rapidly in the next decades. To maintain health, well-being and socioeconomic stability in an ageing society, identifying and targeting protective and risk factors to support mobility, quality of life and functional independence is imperative. While there is growing evidence that physical activity promotes physiological health, the required dose and intensity for health benefits is unknown and the underlying mechanisms of the beneficial effects of physical activity remain unclear and are likely to be complex.

In this dissertation project, I examined whether physical activity and reduced sedentary behaviours are associated with physiological markers of health, including (1) detailed morphological markers of brain health, and (2) epigenome-wide methylation changes as well as epigenetic ageing, a proxy of biological ageing linked to functional impairment and mortality risk. Lastly, I examined (3) whether vitamin D could aid in the prevention of sarcopenia, the age-associated exacerbated loss of muscle mass and function.

In the Rhineland Study, a large ongoing community-based, prospective cohort study of adults across a wide age range, I demonstrated that higher physical activity levels are associated with greater brain volumes, grey matter density and cortical thickness. I observed the most pronounced effects in motor regions and regions with a high susceptibility to neurodegenerative diseases. Further, I found that physical activity is linked to slower epigenetic ageing and identified methylation changes of several CpGs associated with physical activity levels across the epigenome. Using detailed accelerometer-based physical activity measures, I could show that moderate-to-vigorous intensity activities have comparatively the strongest effect on physiological markers of health. Across all modalities, the effects of physical activity were most pronounced at the lower spectrum and weakened at higher levels. Moreover, I demonstrated that vitamin D levels are associated with greater grip strength, a measure of muscle mass and function. However, at potentially adverse levels, I observed the opposite effect.

In conclusion, physical activity and in particular moderate-to-high intensity activities could be essential in the prevention of neurodegenerative diseases and slow the biological ageing process. Furthermore, a sufficient vitamin D supply could protect against age-associated loss of muscle mass and optimize muscle function across the adult lifespan.

2. Introduction and aims with references

> *"All parts of the body, if used in moderation and exercised in labors to which each is accustomed, become thereby healthy and well developed and age slowly; but if they are unused and left idle, they become liable to disease, defective in growth and age quickly."*
> ***Hippocrates*** (460 – 370 BC)

This quote has been attributed to Hippocrates, one of the most famous ancient Greek figures, who is often referred to as the founder of modern Medicine. The ancient Greeks considered physical activity a civic duty and believed that physical activity is tightly interlinked with physiological and mental health. Since then, this conviction has been further supported by scientific evidence.

Physical activity has been defined as any kind of skeletal muscle movement, which causes energy expenditure. Thus, physical activity is not limited to recreational activities, such as exercise, but also encompasses work-related activities, transport and household chores (World Health Organization, 2022). Physical activity has been associated with a myriad of health benefits including a higher quality of life, better cognitive performance, improved physical function and a reduced risk of developing age-associated chronic and neurodegenerative diseases such as cardiovascular diseases, cancer and Alzheimer's and Parkinson's disease (Ascherio & Schwarzschild, 2016; Hamer & Chida, 2008; World Health Organization, 2020). Based on consistent findings of the beneficial effects of physical activity, the WHO recently published updated public health guidelines on physical activity and sedentary behaviour (World Health Organization, 2020). Under the headline "*Every move counts.*", the WHO emphasizes that any kind of additional physical activity is beneficial for health, aiming to increase public awareness and to bring about behavioural change. The WHO advises adults (aged 18-64 years) to engage in at least 150 minutes of moderate-intensity or 75 minutes of vigorous-intensity activities per week, to limit sedentary behaviour and to replace sedentary time with any kind of physical activity (World Health Organization, 2020). However, the optimal frequency, duration and intensity has not been studied extensively so far and remains unknown.

Several techniques have been employed to study physical activity including pedometers (i.e., step counters), accelerometers, observer ratings, diaries and questionnaires. While questionnaires are the most commonly employed method to estimate physical activity as they are easy to administer and cost-effective, they are also prone to suffer from recall and social desirability bias, which can lead to overreporting active periods and underreporting sedentary time. Moreover, they only allow a gross estimation of physical activity components and are often restricted to only capturing leisure-time exercise rather than recording all the activities performed throughout the day. This is also one of the main reasons why accelerometers have recently gained popularity as they allow a more precise and accurate quantification of everyday activities recorded on a second-by-second basis and can accurately differentiate body position (e.g., sitting, standing, lying) and activity intensity depending on the sensor placement (Sylvia, Bernstein, Hubbard, Keating, & Anderson, 2014).

The physiological effects of physical activity are likely multifaceted and complex and the underlying mechanisms remain largely undetermined. Animal studies suggest that physical activity promotes neuronal health by stimulating cerebral blood flow, neurogenesis, neuroplasticity and angiogenesis (Voss, Nagamatsu, Liu-Ambrose, & Kramer, 2011; Voss, Vivar, Kramer, & van Praag, 2013). Physical activity may lead to enhanced cell proliferation, survival and differentiation, particularly in the hippocampus, a structure involved in learning and memory function (Voss et al., 2013). However, studies in humans are inconsistent - with some studies reporting an association between physical activity and hippocampal volume (Erickson et al., 2010; Hamer, Sharma, & Batty, 2018), whereas other studies could not confirm these findings (Benedict et al., 2013; Spartano et al., 2019). It remains unclear whether (1) the effects of physical activity on brain morphology are similar across the entire brain or restricted to certain brain regions, and (2) whether they are bound to a certain physical activity dose or intensity threshold. Therefore, we examined the relation between accelerometer-based physical activity with detailed brain volumetric, cortical thickness and grey matter density measures in a large prospective cohort study (Chapter 3.1). Specifically, we assessed the association of step counts, energy expenditure and time spent in light intensity, moderate-to-vigorous intensity and sedentary activities with global and local brain volume, vertex-based cortical thickness and voxel-based grey matter density. In addition, we explored the molecular

mechanisms underlying the effects of physical activity on brain structure and examined the gene expression profiles of the brain regions showing an effect of physical activity.

Physical activity has also been linked to widespread modification of gene expression as reflected in the methylation status of the DNA. DNA methylation levels at various Cytosine-phosphate-Guanine (CpG) sites have been found to reliably mirror the chronological ageing process but also to reflect accumulative effects of exposure to protective and risk factors across the lifespan. The sum of the epigenetic changes at these specific CpGs sites has been hypothesized to reflect the biological ageing process and has been termed *epigenetic clock* (Hannum et al., 2013; Horvath, 2013; Levine et al., 2018; Lu et al., 2019). Next to its effects on gene expression levels, physical activity also has a profound effect on the cardiovascular system and immune function. It leads to beneficial changes in plasma lipid levels, improved vasculature, lower blood pressure, higher insulin sensitivity and greater oxygen consumption (Myers, 2003). Simultaneously, it stimulates antipathogen activity of tissue macrophages, T-cell proliferation, circulation of leukocytes, anti-inflammatory cytokines and immunoglobins as well as NK cell cytotoxic activity (Nieman & Wentz, 2019). It has been found to be highly effective in preventing, delaying and treating symptoms of hypertension and diabetes type 2 (Kokkinos & Myers, 2010) and may slow the progression of immunosenescence, the progressive age-related loss of immune function leading to an increased vulnerability to pathogens, cancer cells and autoimmune dysfunction (Nieman & Wentz, 2019).

Previous studies suggest that physical activity may slow epigenetic ageing (Kresovich et al., 2021; Oblak, van der Zaag, Higgins-Chen, Levine, & Boks, 2021; Quach et al., 2017), but it has not been elucidated whether there might be an interplay between the beneficial effects of physical activity on DNA methylation and its effect on cardiovascular health and immune function. In chapter 3.2 we present our study examining whether accelerometer-based physical activity is associated with epigenetic ageing. In addition, we investigated whether a wide range of markers of cardiovascular health and immune function mediate the association between physical activity and epigenetic ageing. To further explore the effects of physical activity across the entire epigenome, we also assessed the effects of physical activity across 850,000 CpGs in an epigenome-wide association study (EWAS).

Despite widespread coverage on the positive physiological effects of physical activity in the public press and media, the global physical activity levels have been showing a downward trend (Kohl et al., 2012). This trend has further aggravated, with a marked global decrease of physical activity during the COVID-19 pandemic (Stockwell et al., 2021). In particular older adults were observed to engage more often in sedentary behaviour, to show reduced physical fitness and to suffer from muscle atrophy (M. R. Oliveira et al., 2022).

Physical inactivity has been linked to the loss of muscle mass and function - a process that is further exacerbated in 6% to 22% of individuals aged 65 years and above, who suffer from sarcopenia (Dent et al., 2018). Excessive age-associated muscle atrophy has been linked to diminished mobility, higher healthcare costs, higher risk of falls and mortality (Cruz-Jentoft et al., 2010; J. S. Oliveira et al., 2020). Interestingly, vitamin D receptors have been found to be expressed in muscle tissue and their expression levels have been linked to skeletal muscle mass, function and regeneration (Bass et al., 2020). Therefore, it has been suggested that vitamin D intake could be an effective preventative and curative measure against sarcopenia and age-associated muscle decline. While circulating vitamin D levels have been linked to greater muscle strength in older adults (Houston et al., 2007; Visser, Deeg, & Lips, 2003), the relation between vitamin D and muscle strength in younger adults is largely unexplored. Therefore, we examined the association between the biologically active form of vitamin D, 25-hydroxyvitamin D, and handgrip strength as proxy of muscle strength in adults across a wide age range (Chapter 3.3).

2.1 Aim

This dissertation project aimed to examine (1) the dose-intensity-response relationship between physical activity, fitness and markers of physiological health, and (2) the potential underlying mechanisms mediating the beneficial effects of physical activity in participants of the Rhineland Study, a large community-based prospective cohort study located in Bonn, Germany. The Rhineland Study collects deep phenotyping data of participants aged 30 years and above including comprehensive anthropometric, physical activity and fitness assessments as well as self-administered health questionnaires, MRI scans and multi-omics profiling. Thus, the Rhineland Study provided the ideal setting to assess:

1. The relation between accelerometer-derived physical activity and detailed measures of brain morphometry (Chapter 3.1).
2. The association between accelerometer-based measures of physical activity and epigenetic ageing as well as epigenome-wide methylation changes (Chapter 3.2).
3. The relation between 25-hydroxyvitamin concentration and handgrip strength, a proxy for overall muscle mass and function, in adults across a wide age range (Chapter 3.3).

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3. Publications

3.1 The relation between accelerometer-derived physical activity and brain structure: A population-based cohort study

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Abstract

Background and Objectives: While there is growing evidence that physical activity promotes neuronal health, studies examining the relation between physical activity and brain morphology remain inconclusive. We therefore examined whether objectively-quantified physical activity is related to brain volume, cortical thickness and grey matter density in a large cohort study. Additionally, we assessed molecular pathways that may underlie the effects of physical activity on brain morphology.

Methods: We used cross-sectional baseline data from 2,550 eligible participants (57.6% women; mean age: 54.7 years, range: 30–94 years) of a prospective cohort study. Physical activity dose (metabolic equivalent hours and step counts) and intensity (sedentary, light-intensity and moderate-to-vigorous intensity activities) were recorded with accelerometers. Brain volumetric, grey matter density and cortical thickness measures were obtained from 3T MRI scans using FreeSurfer and Statistical Parametric Mapping. The relation of physical activity (independent variable) and brain structure (outcome) was examined with polynomial multivariable regression, while adjusting for age, sex, intracranial volume, education and smoking. Using gene expression profiles from the Allen Brain Atlas, we extracted molecular signatures associated with the effects of physical activity on brain morphology.

Results: Physical activity dose and intensity were independently associated with larger brain volumes, grey matter density and cortical thickness of several brain regions. The effects of physical activity on brain volume were most pronounced at low physical activity quantities and differed between men and women and across age. For example, more time spent in moderate-to-vigorous intensity activities was associated with greater total grey matter volume, but the relation leveled off with more activity (standardized ß [95% confidence intervals]: 1.37 [0.35, 2.39] and -0.70 [-1.25, -0.15] for the linear and quadratic terms, respectively). The strongest effects of physical activity were observed in motor regions and cortical regions enriched for genes involved in mitochondrial respiration.

Discussion: Our findings suggest that physical activity benefits brain health, with the strongest effects in motor regions and regions with a high oxidative demand. While young adults may particularly profit from additional high-intensity activities, older adults may already benefit from light-intensity activities. Physical activity and reduced

sedentary time may be critical in the prevention of age-associated brain atrophy and neurodegenerative diseases.

Introduction

Physical activity may slow the rate of age-related cognitive decline and reduce the risk of developing neurodegenerative diseases.[1,2] It is unclear, however, which dose and intensity of physical activity is required to achieve brain health benefits.[2] Determining which physical activity components are linked to brain health and whether the effects vary across demographic factors could facilitate the development of targeted physical activity regimens as lifestyle interventions against neurodegeneration.

Several mechanisms underlying the neuroprotective effects of physical activity have been proposed, including enhancement of cerebral blood flow and stimulation of neurotrophin release, neuronal growth and maturation,[3,4] but the underlying molecular pathways remain largely unknown. Moreover, previous studies examining the relation between physical activity and brain morphology, as a proxy of brain health, have reported inconsistent findings: [5–12] While some studies found both grey and white matter structures to benefit from physical activity,[5,11] other studies reported an association with either only grey matter[6,7,10] or white matter volume.[8,13] Regional changes associated with physical activity were observed in frontal, temporal, parietal, occipital and motor regions.[7,10,12,14] Physical activity has also been suggested to induce hippocampal neurogenesis,[4] but findings on the effects of physical activity on hippocampal volume are equivocal.[7,8,10] The majority of previous studies relied on physical activity questionnaires,[7,9,14] which are a cost-efficient way to gather information on physical activity, but come with potential pitfalls such as social desirability and recall bias and cannot distinguish between different physical activity components.[15]

With the recent introduction of accelerometer-based assessments into cohort studies, an objective approach for measuring distinctive physical activity components at population-scale has emerged.[8,10] We aimed to disentangle the association between physical activity components, including dose and intensity, and detailed brain morphological assessments of adults across a wide age range in a large population-based cohort study. To this end, we systematically investigated whether accelerometer-derived physical activity components were associated with (1) volumes and cortical thickness of predefined temporal, occipital and motor regions, (2) whole-brain voxel-based regional grey matter density, and (3) vertex-based cortical thickness across the entire brain. To identify molecular pathways that potentially underlie the

effects of physical activity on the brain, we linked vertex-based estimates of physical activity to gene expression profiles.

Methods

Study participants

The study was based on cross-sectional baseline data from the first 5,000 participants (age range: 30 – 94 years) of the Rhineland Study, an ongoing community-based prospective cohort study.[16] The data were collected from March 2016 to June 2020. Invitations to participate are send to inhabitants of two municipal districts in Bonn, Germany. To participate in the study, participants are required to have a sufficient command of the German language and be 30 years or older. They are not offered financial rewards for study participation.

We analysed data of in total 2,550 participants out of the first 5,000 participants (Figure e-1). Actimetry data of 1028 participants were not available due to the following reasons: refusal to participate (n = 72), technical/acquisition failure (n = 190) or ineligibility (n = 766). Ineligibility criteria included astasis (inability to stand or walk), unrepresentative physical activity week and/or allergy to medical adhesives. Based on self-reports, it was established whether participants anticipated a typical, representative activity and sleeping pattern during the recording time. Examples of unrepresentative physical activity weeks included vacation, untypical work travel, surgery and hospital stays. To achieve reliable physical activity estimates,[17] we additionally excluded 127 participants with less than 5 valid actimetry recording days. Based on Winkler and colleagues' recommendations, we classified recording days as invalid when meeting one or more of the following criteria: 1. <500 steps/day, 2. ≥95% time spent in one posture, 3. <10 hours estimated waking wear time.[18] Heatmaps of included and excluded data and wear diaries were visually checked to avoid incorrect exclusion. In addition, we excluded 1040 participants who did not undergo an MRI scan and 238 participants with missing covariate data. Lastly, we flagged potential outliers and after visual inspection, excluded 15 participants with erroneous recordings.

Standard protocol approvals, registrations, and patient consents

The study was approved by the Medical Ethics Committee of the University of Bonn and followed the recommendations of the International Council for Harmonization

(ICH) Good Clinical Practice (GCP) standards (ICH-GCP). Participants provided informed consent in accordance with the principles of the Declaration of Helsinki.

Physical Activity

Physical activity was measured using the activPAL3 micro accelerometer (PAL Technologies, Glasgow, UK), a small (55 × 25 × 5 mm) and lightweight (9 g) triaxial accelerometer with a sampling frequency of 20 Hz. Based on acceleration across the vertical, anteroposterior and mediolateral axes, the activPAL can be used to estimate energy expenditure and posture (sitting/lying, standing or stepping) across time. The accelerometer was fixed with a nitrile finger cot and attached on the middle-anterior right thigh with a waterproof transparent dressing (Tegaderm), which allowed the accelerometer to be worn continuously during 7 recording days. Participants were instructed to take off the accelerometer when being exposed to hot environments, strong magnetic fields and before security checks. They were shown how to reattach the accelerometer and instructed to complete a non-wear diary.

Raw data were uploaded using proprietary activPAL software. A customized version of the “activpalProcessing” package was used to extract physical activity dose and intensity.[19] Weighted daily averages were calculated to adjust for accelerometer wear time per day. Physical activity dose was defined as average weighted daily step counts and energy expenditure in average weighted daily metabolic equivalents (METs) per hour. Physical activity intensity was defined based on posture and energy expenditure across time: average weighted daily %time spent in sedentary (sitting/lying posture), light-intensity (standing or step-taking posture and METs<3.0), or moderate-to-vigorous activities (standing or step-taking posture and METS≥3.0).[19,20]

Brain MR Imaging

MRI scans were obtained with a 3T Siemens MAGNETOM Prisma system (Siemens Healthcare, Erlangen, Germany) equipped with an 80 mT/m gradient system and a 64-channel head-neck coil. Using a multi-echo MPRAGE sequence,[21,22] T1-weighted images were acquired at 0.8 mm isotropic spatial resolution (TA=6.5 mins, TR=2560 ms, TI=1100 ms, flip angle 7°, FOV=256x256 mm, 224 sagittal slices). The standard FreeSurfer 6.0 (http://surfer.nmr.mgh.harvard.edu/) preprocessing pipeline was used to extract brain structure volumes and thickness.[23–25] The segmentation quality was visually assessed in 1,872 participants, which were selected based on incidental

findings, examination comments, age-adjusted extreme volumetric values as well as 12% random selection. Cortical thickness was determined at each surface location based on the average closest distance between white and pial surfaces.[23] Cortical structures were parcellated using the “Desikan-Killiany-Tourville” atlas.[24] Subcortical structures were segmented using the automatically segmented brain volume (ASEG) atlas.[25]

Our primary outcome measures were brain volume and cortical thickness of predefined regions of interest, which were selected based on a targeted literature research (Table e-1). In addition, we conducted an exploratory analysis of localized vertex-based thickness estimates across the whole cortex. Individual thickness maps were registered to a group surface and smoothed with a 10mm full-width-half-maximum (FMWH) kernel. Cluster-wise inference and correction for multiple comparisons was done via a permutation simulation.[26]

For the exploratory voxel-based morphological analysis, T1 images were first segmented into grey matter, white matter, and cerebral spinal fluid (CSF) images using Statistical Parametric Mapping (SPM12, Wellcome Department of Cognitive Neurology, UCL) and resampled to 1x1x1mm^3 voxel size.[27] Grey matter images were normalized into MNI space using diffeomorphic anatomical registration through Geodesic shooting and Gauss-Newton optimisation with Geodesic shooting template from CAT12 toolbox (http://www.neuro.uni-jena.de/cat/),[28] and smoothed with an isotropic Gaussian kernel of 8 mm FMWH. Segmented images were checked visually for potential segmentation and registration errors. Estimated total intracranial volume was calculated by combining grey matter, white matter and CSF images generated during segmentation.

Covariates

Participants’ age, sex, and smoking status (current, former, or non-smoker) were determined based on self-reports. Using the International Standard Classification of Education 2011 (ISCED), participants’ highest educational level was classified as low (lower secondary education or below), middle (upper secondary education to undergraduate university level), and high (postgraduate university study). Participants’ medical history of neurological (including dementia, Parkinson’s disease, multiple sclerosis, stroke and TIA) and psychiatric (including depression and anxiety) disorders was obtained based on self-reports and categorized as binary variables (‘yes’ / ’no’).

Mapping Gene Expression to Vertex-Based Estimates of Physical Activity

Normalized microarray-based gene expression data from all six available donors were downloaded from the Allen Human Brain Atlas (AHBA).[29] The donor characteristics and approach used to obtain high-resolution gene expression data from the post-mortem brains have been detailed before (https://help.brain-map.org/display/humanbrain/ Documentation). We linked vertex-based effect estimates of physical activity to gene expression data by first converting FreeSurfer surface-based coordinates (i.e. fsaverage) to MNI volumetric coordinates (i.e. RAS coordinates in MNI152 space) using the registration fusion-advanced normalization approach developed by Wu et al.[30] Next, for each donor, we mapped gene expression data to vertex-based results using rounded MNI coordinates.

Statistical Analysis

Statistical analyses were performed in R (version 3.6.3, The R Foundation). Multivariable regression models were used to assess the association between physical activity (independent variable) and brain structure (outcome). To test for nonlinear effects, initial models also included a quadratic term for physical activity. In addition, we tested for interaction effects between physical activity and age, and between physical activity and sex. All models were adjusted for age, age^2, sex, education, and smoking. The quadratic physical activity and age terms were removed if they failed to reach significance (with $p \leq 0.05$). Volumetric analyses were also adjusted for estimated intracranial volume. Continuous independent variables were z-standardised to allow comparison of effect sizes. Model diagnostics were performed by visual inspection of the distribution of the residuals. We report both multiple-comparisons uncorrected and false discovery rate (FDR)-corrected results, assuming 24 tests for the corresponding number of pre-selected volumetric and cortical regions of interest. To assess the robustness of our findings, we ran a 5-fold cross-validation with 100 iterations. We split up our data set into five equal sized, disjoint folds and examined the physical activity effects in four out of the five folds each time.[31] In a further sensitivity analysis, we tested whether the association between physical activity and brain structure was altered after excluding participants with neurological and psychiatric disorders.

For the voxel-based morphometry analysis, we performed two-sample t-tests (women vs. men) with each actimetry measure as covariate and controlled for age, age^2, education, smoking, and estimated intracranial volume. Resulting maps were

corrected for multiple comparisons using probability threshold-free cluster enhancement method.[32] Statistical inferences were made at *p≤0.01* family-wise error corrected for multiple comparisons across the whole brain and measures. For the vertex-wise analysis, a permutation-based correction for multiple comparisons was performed with a cluster-forming threshold of $p \leq 0.05$ and 1000 iterations.[26] The threshold for statistical significance of cluster sizes was set at a two-sided $p \leq 0.005$.

Generalized additive mixed-effects models were used to assess the association between gene expression (independent variable) and vertex-wise effect estimates of physical activity (outcome) across the brain. In these models, the spatial autocorrelation among the vertices was accounted for by including a smoothed interaction term (i.e. s(x,y,z)) for the MNI coordinates of each vertex. The intra-individual correlation within each donor was modeled through a random intercept for donor. Given the low number of donors, additional adjustments for age or sex were not possible. FDR-correction was applied to adjust for multiple comparisons with $q<0.05$ considered statistically significant.

In silico Functional Analyses

To gain insight into the underlying molecular mediators and functional pathways in the brain that are affected by physical activity, we first selected the set of genes whose expression was significantly associated with vertex-wise effects of physical activity (i.e. FDR $q<0.05$). These genes were used as input for further functional enrichment analyses with the WebGestalt tool,[33,34] using the built-in reference set of the human genome for over-representation analysis. As look-up databases for gene enrichment analyses we queried the KEGG (https://www.genome.jp/kegg/pathway.html), Reactome (https://reactome.org/), WikiPathways (https://www.wikipathways.org/index.php/WikiPathways), and DrugBank (https://go.drugbank.com/) resources.

Data Availability

The Rhineland Study's dataset is not publicly available because of data protection regulations. Access to data can be provided to scientists in accordance with the Rhineland Study's Data Use and Access Policy. Requests for further information or to access the Rhineland Study's dataset should be directed to RS-DUAC@dzne.de.

Results

Characteristics of the sample

Participants were aged 30 to 94 years and had a relatively high education level (Table 1). In comparison to men, women had a lower body mass index, education level and brain volume, were less sedentary and spent more time in light-intensity activities. They also had a higher energy expenditure than men. Age-stratified brain volumetric and physical activity characteristics can be found in Table e-2.

Effects of physical activity on predefined brain regions-of-interest

Predefined regions of interest included temporal, occipital and motor regions (Table e-1). Given our hypothesis-driven approach, here we focus on multiple comparison uncorrected findings. Model statistics and FDR-corrected findings can be found in Table e-3-e-6.

Higher step counts and energy expenditure, as expressed in metabolic equivalent (MET-) hours, were associated with greater total grey matter volume, cerebellar grey matter volume and precentral thickness (Figure 1a&b, Figure 2a). The positive effect of increasing step counts and energy expenditure on brain volumes was most pronounced at the lower end of the physical activity spectrum. Total brain, total grey matter, cerebellar grey matter, hippocampal and precentral volume also increased with a higher proportion of time spent on light- and moderate-to-vigorous intensity activities, whereas the opposite effect was found for sedentary activities (Figure 1c&d, Figure 3c). We found the opposite effect for the cortical thickness of the lateral-occipital cortex (Figure 1d). These results did not change materially after exclusion of participants with neurological or psychiatric disorders (Figure e-2, Figure e-3a&c). Mean and standard deviation of the cross-validation results for the physical activity parameters can be found in Table e-7.

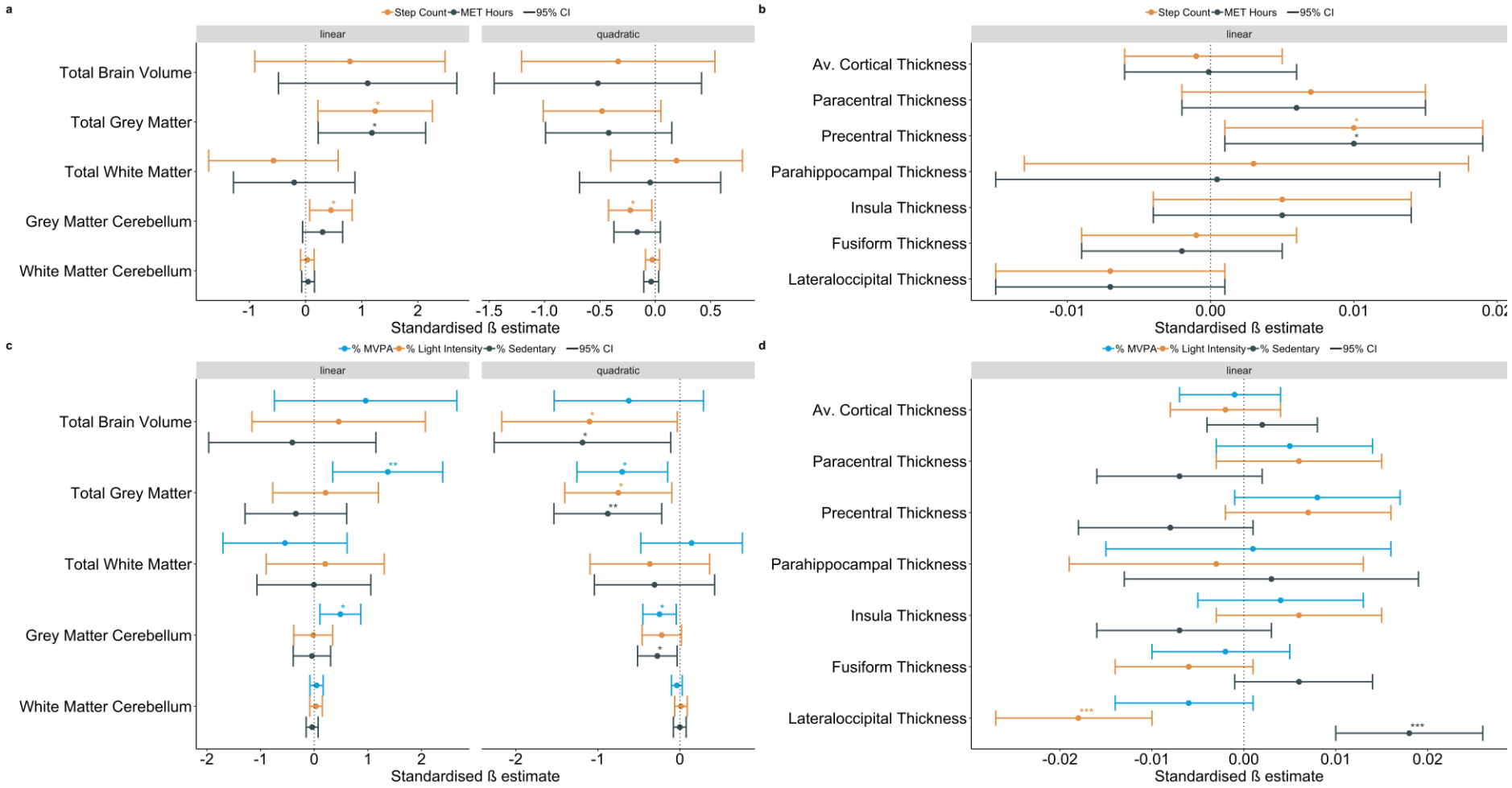
Table 1. Sample Demographics.

	Women (n = 1469)	Men (n = 1081)	p
Age (years), mean (SD)	55.03 (13.38)	54.24 (13.96)	0.151
30-39	238 (16.20)	215 (19.89)	0.194
40-49	258 (17.56)	180 (16.65)	
50-59	409 (27.84)	291 (26.92)	
60-69	343 (23.35)	224 (20.72)	
70-79	182 (12.39)	137 (12.67)	
80-89	38 (2.59)	34 (3.15)	
90+	1 (0.07)	0 (0.00)	
BMI (kg/m^2), mean (SD)	25.29 (4.65)	26.12 (3.50)	<0.001
Smoking, n (%)			0.072
current	182 (12.4)	154 (14.3)	
former	560 (38.1)	440 (40.7)	
never	727 (49.5)	487 (45.1)	
Education ISCED11, n (%)			<0.001
high	689 (46.9)	696 (64.4)	
middle	743 (50.6)	375 (34.7)	
low	37 (2.5)	10 (0.9)	
Neurological Disorders, n yes (%)	34 (2.31)	26 (2.41)	0.986
Psychiatric Disorders, n yes (%)	293 (19.95)	134 (12.40)	<0.001

Daily Sensor Hours Worn (hours), mean (SD)	23.83 (0.47)	23.81 (0.54)	0.440
Daily Energy Expenditure (MET hours), mean (SD)	34.10 (1.29)	33.9 (1.38)	<0.001
Daily Step Count, mean (SD)	8958.25 (3038.77)	8796.33 (3327.51)	0.202
% Daily Light Intensity Physical Activity, mean (SD)	0.23 (0.06)	0.19 (0.06)	<0.001
% Daily Moderate-to-Vigorous Physical Activity, mean (SD)	0.05 (0.02)	0.05 (0.02)	0.964
% Daily Sedentary, mean (SD)	0.72 (0.07)	0.76 (0.06)	<0.001
Actimetry Examination Season, n (%)			0.153
spring	305 (20.76)	219 (20.26)	
summer	343 (23.35)	236 (21.83)	
autumn	431 (29.34)	295 (27.29)	
winter	390 (26.55)	331 (30.62)	
Total Brain Volume (cm^3), mean (SD)	1052.76 (92.51)	1178.09 (107.61)	<0.001
Grey Matter Volume (cm^3), mean (SD)	596.77 (49.00)	661.24 (56.91)	<0.001
White Matter Volume (cm^3), mean (SD)	430.27 (47.20)	489.64 (54.28)	<0.001
Cerebellum Volume (cm^3), mean (SD)	129.50 (12.04)	143.04 (14.46)	<0.001
Hippocampal Volume (cm^3), mean (SD)	7.60 (0.80)	8.23 (0.90)	<0.001

To assess group differences a χ^2 test was used for categorical variables and a two-sample t test for continuous variables.

Figure 1. Association Between Physical Activity and Predefined Brain Regions.



(**a** and **b**) Standardized effect estimates of association between physical activity dose (step counts and MET-hours) and **(a**) larger brain volumes and (b) cortical thickness of predefined regions. (**c** and **d**) Standardized effect estimates of association between physical activity intensity (%moderate-to-vigorous [MVPA],% light-intensity and sedentary activities) and (**c**) larger brain volumes, and (**d**) cortical thickness of predefined regions.

***p≤0.001 **p≤0.01 *p≤0.05 p≤0.06. MET = metabolic equivalents.

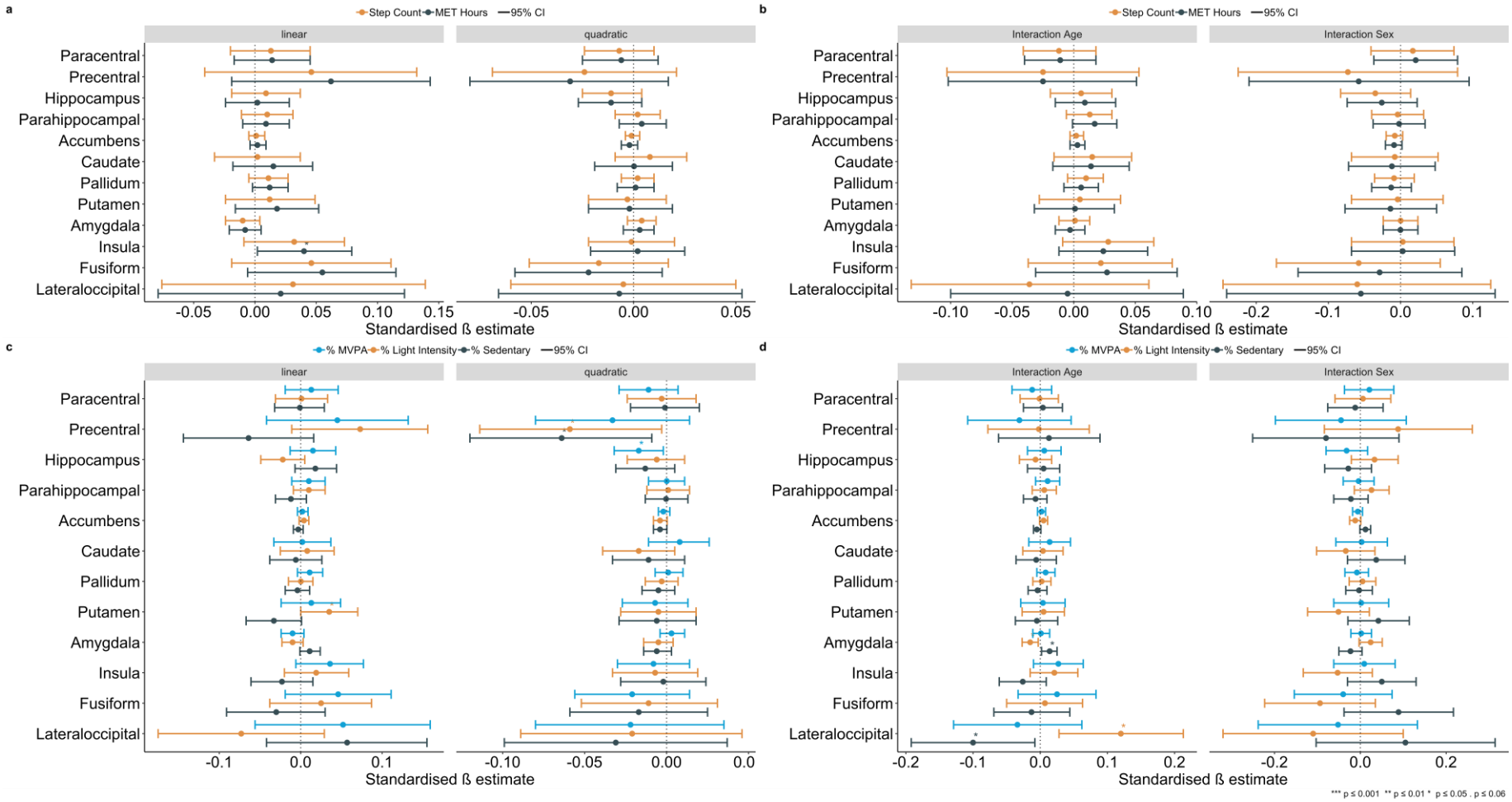
Next, we examined whether the effects of physical activity on these predefined brain regions changed across age and differed between men and women. We observed no differences in the effects of physical activity dose on brain volume and cortical thickness across age and between men and women (Figure 2b, Figure 3a&b). For physical activity intensities, we found that with increasing age the association between more light-intensity activities with higher total brain volume and lower amygdalar volume became more pronounced, whereas the association with lower lateral-occipital volume weakened (Figure 2d, Figure 3c&d). The effect of light-intensity activities on total brain and amygdalar volume was strongest in our oldest age group (70+ years, Figure e-4a&b). Similarly, the effect of sedentary time on greater amygdalar volume strengthened in older age groups (Figure e-4d). However, the opposite was found for the relationship of increasing lateral-occipital volume with more sedentary time (Figure e-4e). Compared to women, men showed a weaker association between more light-intensity activities and greater total cerebellar and cerebellar grey matter volume (Figure 3c). In contrast, the association between more sedentary activities and lower total cerebellar and cerebellar grey matter volume was stronger in men (Figure 3c).

These effects were also observed after excluding individuals with neurological or psychiatric disorders (Figure e-3b&d, Figure e-5). Cross-validation of these interaction models revealed relatively robust interaction effects. For cerebellar volume the interaction between physical activity and sex remained statistically significant across all iterations (Table e-8).

Effects of physical activity on regional grey matter density

Higher step counts and energy expenditure were associated with greater grey matter density in the right temporal pole and bilateral cerebellum (Figure 4a&b). More time spent on light- and moderate-to-vigorous intensity activities was predominantly associated with higher temporal and cerebellar but lower occipital grey matter density (Figure 4c&d). This was reversed for sedentary activities (Figure 4e). Overall, we observed the strongest effects of all the physical activity components on the right cerebellum (Figure e-6). Results corrected for multiple comparisons can be found in Table e-9.

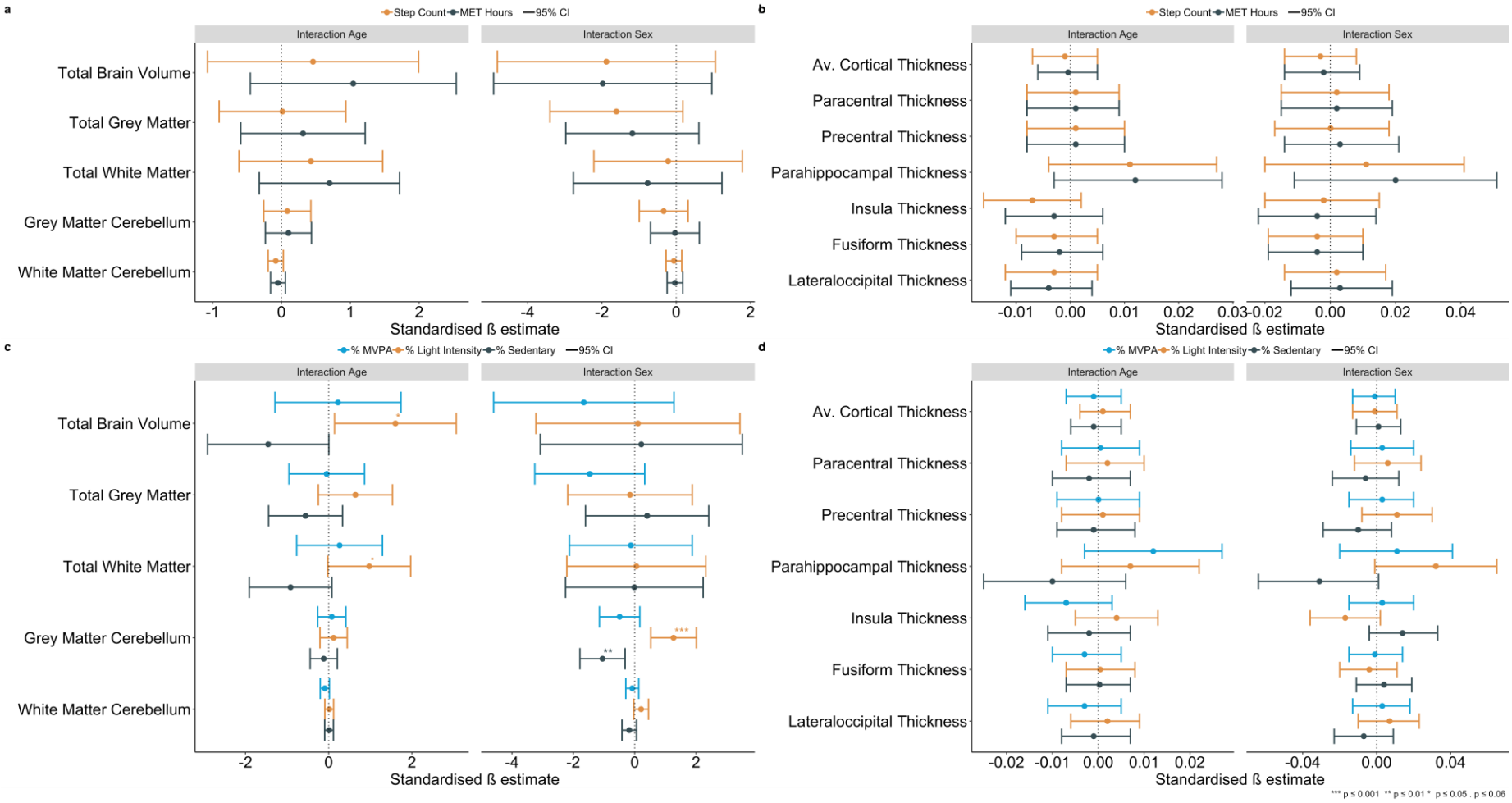
Figure 2. Physical Activity Effects and Interaction Effects With Age and Sex for Additional Smaller Predefined Brain Regions.



(**a**) Standardized effect estimates of association between physical activity dose (step counts and MET-hours) and additional smaller predefined brain regions. (**b**) Standardized effect estimates of interaction between physical activity dose, age, and sex for smaller predefined brain regions. (**c**) larger brain volumes, and (**d**) cortical thickness of predefined regions.

*** p≤0.001 **p≤0.01 *p≤0.05 p≤0.06. MET = metabolic equivalents.

Figure 3. Interaction Between Physical Activity, Age, and Sex for Predefined Brain Regions.

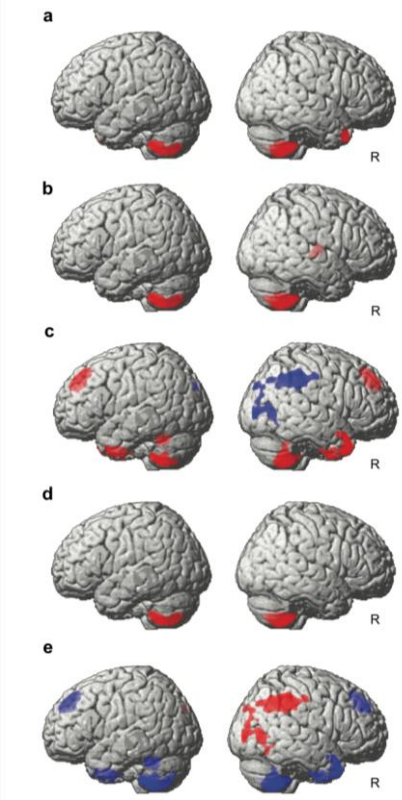


(**a** and **b**) Standardized effect estimates of interaction effects of physical activity dose (step counts and MET-hours) with age and sex for (**a**) larger brain volumes and (**b**) cortical thickness of predefined regions. (**c** and **d**) Standardized effect estimates of interaction effects between physical activity intensity (%moderate-to-vigorous [MVPA], % light-intensity and sedentary activities) with age and sex for (**c**) larger brain volumes, and (**d**) cortical thickness of predefined regions. ***p≤0.001 **p≤0.01 *p≤0.05 p≤0.06. MET = metabolic equivalents.

Effects of physical activity on regional cortical thickness

With higher physical activity intensity and dose, precentral and entorhinal thickness increased whereas lateral-occipital and frontal thickness decreased (Figure 5a). The opposite was found for sedentary activities. After multiple comparison correction, only one cluster in the lateral-occipital region retained significance, showing a negative association with light-intensity and a positive association with sedentary activities (Figure 5b).

Figure 4. Physical Activity Effects on Grey Matter Density.



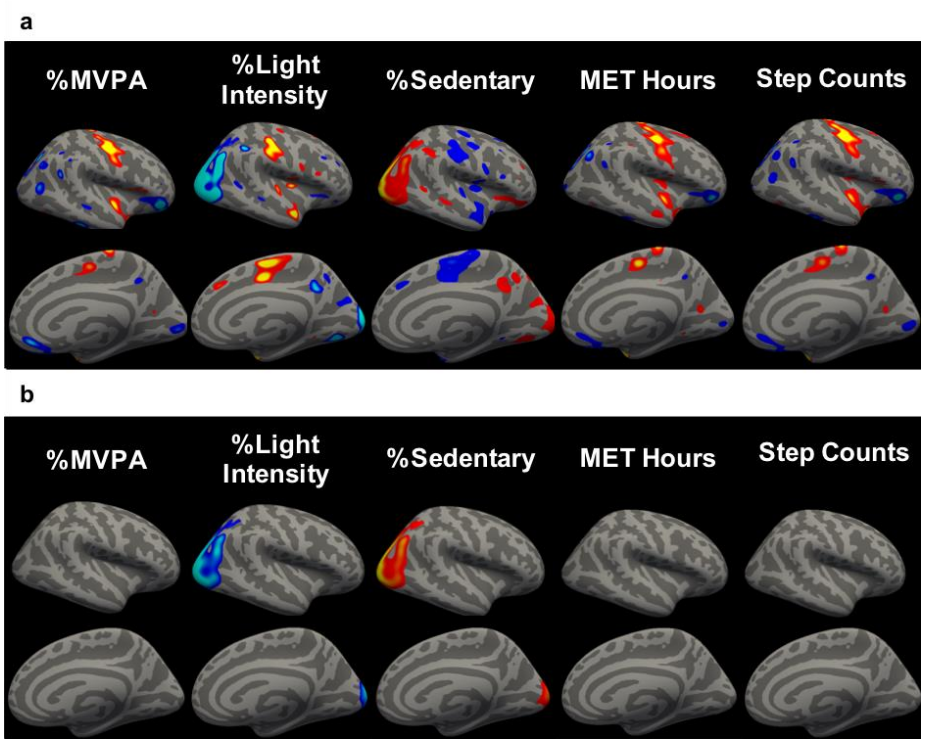
(**a**-**e**) Effects of physical activity on grey matter density for (**a**) MET-hours, (**b**) step count, (**c**) % light-intensity physical activity, (**d**) % moderate-to-vigorous physical activity, (**e**) % sedentary activity are rendered on a template brain using a height threshold of p<0.001 uncorrected and extent threshold of >200 voxels. Positive correlations are depicted in red and negative correlations in blue. MET = metabolic equivalents.

Gene expression profiles associated with the effects of physical activity

Using MET-hours as outcome, the spatial expression patterns of 4504 gene probes were significantly related to the vertex-wise effects of physical activity (FDR q<0.05). Of these 2887 could be unambiguously mapped to unique Entrez gene IDs (Figure e-7). Over-representation analysis with GO terms related to cellular processes, functions and components, demonstrated that these genes were predominantly enriched in

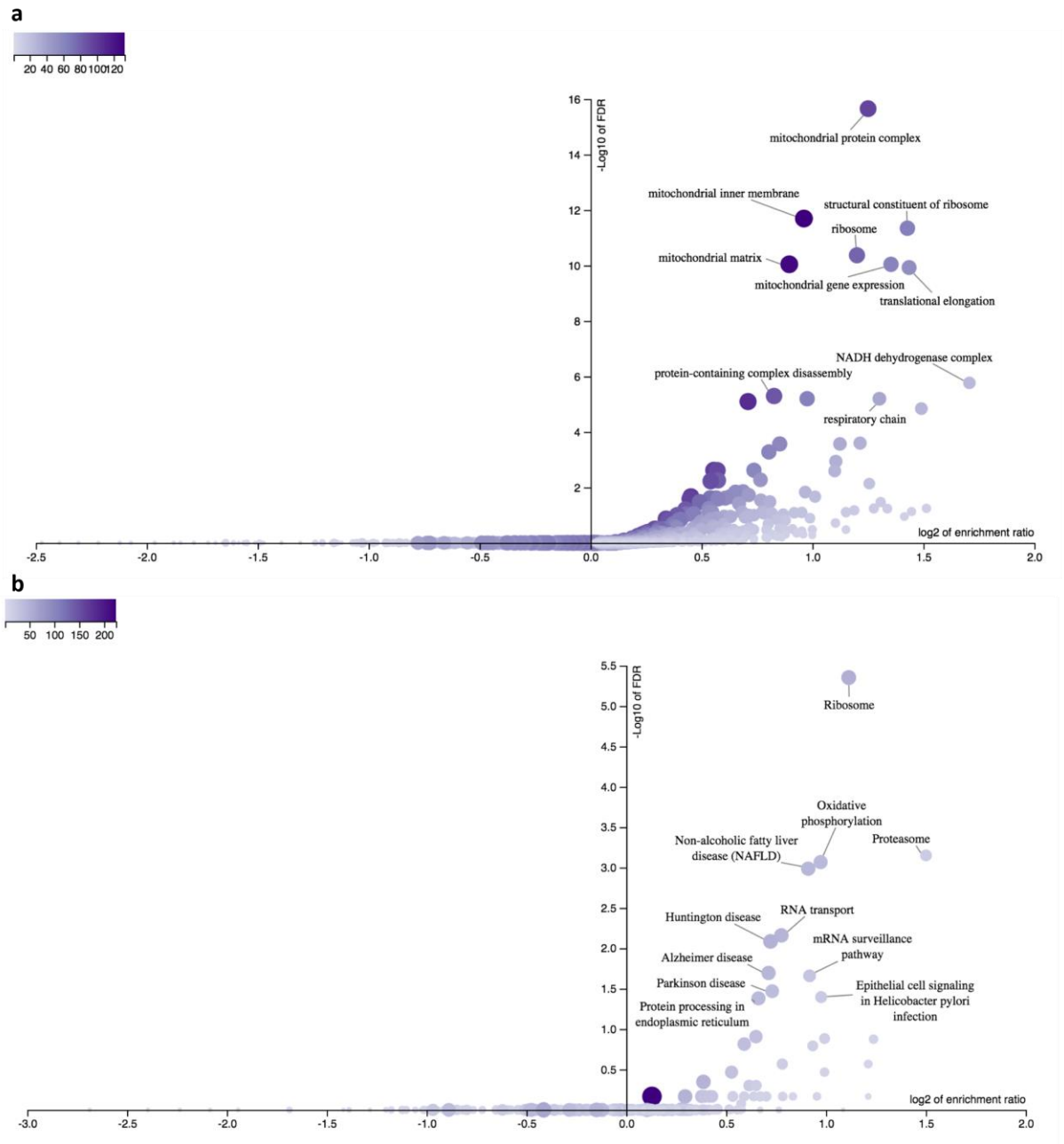
pathways related to mitochondrial constituents and function (Figure 6a, Figure e-8a). Other pathways in which these genes were enriched included (mitochondrial) ribosomal subunits as well as protein-containing complex disassembly (Figure 6a, Figure e-8a). Using the KEGG database resource as a reference also highlighted mitochondrial, ribosomal as well as proteosomal pathways as significantly enriched in genes related to physical activity levels (Figure 6b, Figure e-8b). Interestingly, KEGG pathway analysis also demonstrated an over-enrichment of these genes in pathways related to neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and Huntington's disease (Figure 6b, Figure e-8b). Using Reactome and WikiPathways as reference databases highlighted the involvement of mitochondrial and proteostasis pathways (data not shown). In addition, using the 'DrugBank' resource as reference, we identified two compounds (i.e. phenethyl isothiocyanate and nicotinamide adenine dinucleotide + hydrogen (NADH)), which induced transcriptional changes significantly enriched for genes whose cortical expression patterns coincided with those of physical activity (Figure e-8c).

Figure 5. Physical Activity Effects on Vertex-Based Cortical Thickness.



(**a**-**e**) Effects of physical activity on grey matter density for (**a**) MET-hours, (**b**) step count, (**c**) % light-intensity physical activity, (**d**) % moderate-to-vigorous physical activity, (**e**) % sedentary activity are rendered on a template brain using a height threshold of $p<0.001$ uncorrected and extent threshold of >200 voxels. Positive correlations are depicted in red and negative correlations in blue. MET = metabolic equivalents.

Figure 6. Functional Enrichment Analyses of Genes, Whose Expression Was Significantly Associated With Vertex-wise Effects of Physical Activity.



(**a**) Volcano plot of -log(FDR) vs enrichment ratio of gene ontology terms related to cellular processes, functions, and components with the size and color of the dots proportional to the number of overlapping genes of that category; (**b**) Volcano plot of -log(FDR) vs enrichment ratio of KEGG pathways enriched in genes whose expression levels are associated with physical activity levels, with the size and color of the dots proportional to the number of overlapping genes of that category. FDR = false discovery rate.

Discussion

Physical activity plays an important role in the prevention of age-associated neurodegeneration and promotes health and well-being.[1,2] By combining high-

resolution structural brain imaging with continuous accelerometer-based quantification of physical activity patterns in a large community-based sample of adults across a wide age range, we could disentangle the relation of distinct physical activity components and brain health to an unprecedented level of detail. Importantly, we observed that the effects of physical activity on brain volume are most pronounced at low physical activity quantities. This indicates that relative health gains from additional physical activity are greatest for people leading sedentary lifestyles as compared to those who already engage in at least moderate amounts of physical activity.

Findings from previous population studies were inconsistent with regard to which brain structures are most affected by physical activity. In line with some previous studies, we observed a rise in total grey, but not white, matter volume with increasing physical activity.[6,7,10] Specifically, we observed an increase in grey matter volume and density of motor regions including the precentral cortex and cerebellum with higher physical activity dose and across intensities. Animal studies also found angiogenesis, synaptogenesis and neuronal growth in these motor regions in response to regular exercise.[3] Motor and cognitive skills have been proposed to be anatomically and functionally tightly linked.[35] However, whereas physical activity has been proposed to promote hippocampal neurogenesis,[4] studies examining the association between physical activity and hippocampal volume reported inconsistent findings.[8,10] Discrepancies may be attributable to examining dissimilar physical activity components and utilizing different subjective and objective measurement instruments. In our study, we examined the relation between hippocampal volume and accelerometer-derived physical activity dose and intensity. We observed only a small increment in hippocampal volume with increasing relative time spent on moderate-to-vigorous intensity physical activities but not with increased light-intensity or reduced sedentary time. This parallels findings from exercise studies, which also did not detect major changes in hippocampal volume following exercise interventions.[36,37] Thus, although physical activity may particularly benefit both motor and cognitive regions, its effects on the latter are likely to be comparatively modest.

In contrast to previous population studies, we did not observe an effect of physical activity on total white matter volume.[5,8] A systematic review identified tentative evidence in favor of a small, positive association between physical activity and white matter.[13] Particularly white matter microstructure has been suggested to be affected

by physical activity.[13] A recent population-based study observed motor cortex and basal ganglia structural connectivity to be positively associated with physical activity.[38] Thus, the effects of physical activity on white matter microstructure may be largely confined to structural connectivity between regions involved in motor functions.

Thus far, region-specific effects of distinct physical activity components on cortical thickness had also received little scrutiny. In line with findings of Raffin et al. (2021), we found physical activity levels to be associated with a modestly thicker entorhinal cortex.[12] Similarly, medial temporal lobe thickness has been found to be inversely associated with self-reported sedentary time.[39] It has been suggested that physical activity may protect against ß-amyloid associated thinning of regions, which are particularly affected in Alzheimer's diseases.[40] Interestingly, we observed lateral-occipital thickness to decrease with more light-intensity activity and to increase with reduced sedentary time. As the lateral-occipital cortex is one of the major hubs for visual processing,[41] it could be speculated that longer sedentary time may be associated with relatively more engagement in visual activities, and concomitantly, more stimulation of visual brain areas. Taken together, our findings suggest that the effects of physical activity are not uniformly distributed across the brain, and by inference, may cause remodeling rather than an increase of overall brain tissue.

Both age and sex were found to influence the association of light-intensity physical activity and sedentary time with brain structure. To the best of our knowledge, no study to date had assessed the moderating effects of sex on the relation of physical activity and brain structure, while some studies have examined the moderating effects of age.[8,10] In line with findings from the UK biobank and the Framingham Study, we observed the association between light-intensity physical activity and total brain volume to be strongest in our oldest participants (70+ years).[8,10] Similarly, the association of longer sedentary time with increasing amygdala volume and more light-intensity physical activities with lower amygdala volume became more pronounced with age. The amygdala is thought to play a key role in emotional processing and regulation and its volume has been linked to anxiety levels. However, findings on whether the amygdala volume increases or decreases with higher anxiety levels have been inconsistent.[42,43] Physical activity has been proposed to have an anxiolytic effect by acting upon the 5-HT_{2C}R receptor in the amygdala.[44] Accordingly, our findings suggest that the anxiolytic effect of physical activity may vary across age.

We also observed differences in the effects of light-intensity activity and sedentary time on cerebellar volume between men and women. Compared to women, in men we observed a weaker association between additional light-intensity activities and greater cerebellar volume, but a stronger association between longer sedentary time and lower cerebellar volume. The cerebellum has been found to present a sexually dimorphic anatomy and functional asymmetry.[45] Little is known about the causal link between physical activity and cerebellar structure and function and further research is warranted to establish whether the effect of physical activity on the cerebellum may differ between men and women.

The molecular changes induced in the human brain by physical activity have long eluded detection due to difficulties to extract molecular data from the brain in living subjects. We addressed this challenge by linking our vertex-wise estimates of the effects of physical activity to detailed gene expression data from the Allen Brain Atlas, which enabled identification of a large number of genes whose spatial expression patterns correlated with those of physical activity. Importantly, further *in silico* functional pathway analysis yielded several key insights: First, our findings indicate that the effects of physical activity are strongest in cortical regions with the highest expression levels of genes involved in mitochondrial structure and function. This finding closely parallels the central role of exercise-induced enhancement of mitochondrial function in skeletal muscles,[46] and implies that brain regions with a comparatively high oxidative demand are likely to benefit most from physical activity. Indeed, blood flow to specific brain regions has been shown to increase by almost twofold in response to exercise.[3] Notably, these regions include the motor cortex and the cerebellum,[47] largely coinciding with regions that exhibited the strongest associations with physical activity in our study. Second, pathways previously associated with Alzheimer's and Parkinson's disease, the two most common neurodegenerative diseases, as well as those associated with Huntington's disease, one of the most common genetically determined neurodegenerative diseases, were enriched for genes whose expression patterns coincided with regional effects of physical activity on the brain. These findings further support the beneficial effects of physical exercise in the prevention and treatment of neurodegenerative diseases.[1,2] Lastly, we identified two potentially interesting compounds, phenethyl isothiocyanate and NADH, whose application may partly mimic the cortical gene expression changes induced by physical activity. Of

these, it is noteworthy that supplementation with NAD(+) precursors has been proposed as a potential therapy for neurodegenerative diseases,[48] and was shown to be highly effective in an Alzheimer's disease mouse model.[49] Therefore, further experimental studies assessing the potential efficacy of these two compounds for maintaining or restoring brain health are warranted.

Several limitations of our study should be acknowledged. Our study was based on cross-sectional baseline data of a large population-based study, which did not allow assessment of longitudinal associations. We did not assess the association between physical activity and cognitive and motor function, which could have provided more insights into the effects of physical activity on brain health. Due to lack of more detailed information, we could not account for leisure-time exercise, or the time of day of the MRI assessments. We also excluded participants who were ineligible for MRI scanning due to extreme obesity, as well as those who did not complete an accelerometer recording of at least five days. Recordings were made during regular activity weeks to ensure representativeness of overall activity patterns. Nonetheless, participants may have consciously or unconsciously adapted their physical activities. It also cannot be excluded that our findings may partly have been influenced by selection bias. Our participants were highly educated and comparatively physically active across all age groups. However, one could argue this may have led to an underestimation of the effects of physical activity on brain structure. Finally, for our *in silico* functional pathway analysis we used data from the Allen Human Brain Atlas, which is based on genetic expression data of a limited number of donors.

In summary, we provide a detailed characterization of the effects of physical activity on brain morphology, indicating that physical activity is particularly beneficial to brain regions involved in motor functions. Importantly, we found increases across all physical activity modalities to be associated with larger brain volumes, grey matter density and cortical thickness. The strongest associations between physical activity and brain volumes were observed for additional time spent in moderate-to-vigorous activities and reduced sedentary time. However, in older adults, the association between light-intensity activities and brain volumes was comparatively stronger. Our findings thus indicate that whereas in young and middle-aged adults brain health may particularly profit from additional high-intensity activities, additional light-intensity activities may be sufficient to maintain brain health in older adults. Overall, sedentary behavior was

associated with worse structural markers of brain health and should be reduced across all age groups. Therefore, in line with the new World Health Organization guideline slogan “every move counts”,[50] our findings suggest that incorporating even small increments of additional movement into everyday life is likely to benefit brain health and aid in the prevention of neurodegenerative diseases.

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3.2 Physical activity is associated with slower epigenetic ageing – Findings from the Rhineland Study

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Abstract

Epigenetic ageing, i.e. age-associated changes in DNA methylation patterns, is a sensitive marker of biological ageing, a major determinant of morbidity and functional decline. We examined the association of physical activity with epigenetic ageing and the role of immune function and cardiovascular risk factors in mediating this relation. Moreover, we aimed to identify novel molecular processes underlying the association between physical activity and epigenetic ageing. We analysed cross-sectional data from 3,567 eligible participants (mean age: 55.5 years, range: 30–94 years, 54.8% women) of the Rhineland Study, a community-based cohort study in Bonn, Germany. Physical activity components (metabolic equivalent (MET)-Hours, step counts, sedentary, light-intensity and moderate-to-vigorous intensity activities) were recorded with accelerometers. DNA methylation was measured with the Illumina HumanMethylationEPIC BeadChip. Epigenetic age acceleration (Hannum's age, Horvath's age, PhenoAge and GrimAge) was calculated based on published algorithms. The relation between physical activity and epigenetic ageing was examined with multivariable regression, while structural equation modeling was used for mediation analysis. Moreover, we conducted an epigenome-wide association study of physical activity across 850,000 CpG sites. After adjustment for age, sex, season, education, smoking, cell proportions and batch effects, physical activity (step counts, MET-Hours and %time spend in moderate-to-vigorous activities) was non-linearly associated with slower epigenetic ageing, in part through its beneficial effects on immune function and cardiovascular health. Additionally, we identified 12 and 7 CpGs associated with MET-Hours and %time spend in moderate-to-vigorous activities, respectively ($p<1x10^{-5}$). Our findings suggest that regular physical activity slows epigenetic ageing by counteracting immunosenescence and lowering cardiovascular risk.

Key Words: Epidemiology, Public Health, Cohort Studies, DNA methylation, Epigenetics, Exercise, Cardiovascular Diseases, Cardiovascular Risk Factors

Introduction

Physical activity has been associated with a decreased risk of age-associated diseases, an increased life expectancy and a higher quality of life (World Health Organization, 2020). Several physiological, biochemical and transcriptional changes have been observed in response to acute exercise as well as regular physical activity, which may underlie these benefits (Neufer et al., 2015). Specifically, exercise has been observed to affect the methylation status of genes, with some genes showing hypomethylation and others hypermethylation in response to exercise (Jacques et al., 2019; Voisin, Eynon, Yan, & Bishop, 2015).

Dynamic DNA methylation regulates gene expression and is responsive to environmental and lifestyle changes (Moore, Le, & Fan, 2013). DNA methylation at specific Cytosine-phosphate-Guanine (CpG) sites has been linked to age-associated functional decline and has been suggested as a signature of biological ageing as estimated through *epigenetic clocks* (Hannum et al., 2013). By now, several epigenetic clocks have been developed (Hannum et al., 2013; Horvath, 2013; Levine et al., 2018; A. T. Lu et al., 2019). Whereas first-generation clocks (i.e. Hannum and Horvath's clocks) focus on predicting chronological age, second-generation clocks (i.e. GrimAge and PhenoAge) were developed using clinically relevant biomarkers and a range of different proteins to reflect mortality risk (Topart, Werner, & Arimondo, 2020). The second-generation clocks are therefore also referred to as lifespan estimators. The difference between an individual's estimated biological age and chronological age is referred to as epigenetic ageing, including HorvathAge acceleration, HannumAge acceleration, PhenoAge acceleration, and GrimAge acceleration (Jain et al., 2022; Kim et al., 2021; McCrory et al., 2021; O'Shea, Maynard, & Tremont, 2022).

Physical activity may protect against age-associated functional decline and may slow epigenetic ageing (Oblak, van der Zaag, Higgins-Chen, Levine, & Boks, 2021). Specifically, higher levels of self-reported physical activity have been associated with a lower GrimAge (Kresovich et al., 2021) and Hannum age (Quach et al., 2017). However, large-scale studies assessing the relation between physical activity and epigenetic ageing are sparse. A systematic review reported physical activity to be associated with slower biological ageing as reflected by second-generation clocks, including PhenoAge and GrimAge, but not when using first-generation clocks (Oblak et al., 2021). Importantly, these previous studies predominantly used questionnaire-

based physical activity assessments, which are susceptible to overreporting and cannot discriminate among the different physical activity components (Chastin et al., 2018). To the best of our knowledge, only one study to date has examined the association of objective accelerometer-based physical activity and epigenetic ageing, though only in older adults (Gale et al. 2018). This study found that higher step counts were related to a lower Hannum's age, whereas more sit-to-stand transitions were related to a higher Horvath's age in 79-year-old adults.

Thus far, the mechanisms through which physical activity affects age-associated functional decline are poorly understood. A recent study observed a link between the proportion of naïve and activated T and NK cells and DNA methylation, suggesting that epigenetic ageing may be driven by immunosenescence (Jonkman et al., 2022). Physical activity has been found to directly affect lymphocyte ß2-adrenergic receptor sensitivity, leading to an increased mobilization of T and NK cells, immune surveillance and progenitor cell mobilization, which in turn causes less viral burden on the T cell compartment and reduces the accumulation of senescent T cells (Duggal, Niemiro, Harridge, Simpson, & Lord, 2019). Similarly, regular physical activity leads to a reduced cardiovascular disease risk (World Health Organization, 2020). Poor cardiovascular health increases the risk of age-associated functional decline and has been related to a faster GrimAge acceleration (Joyce et al., 2021). It is unclear, however, whether the advantageous effects of physical activity on epigenetic ageing are solely mediated through their effects on immune function and cardiovascular health or through other (partially) independent mechanisms.

We therefore aimed to examine whether distinct, objectively assessed physical activity components are associated with slower biological ageing in adults over a wide age range. To this end, we leveraged cross-sectional data of a large community-based cohort-study, and assessed whether accelerometer-derived physical activity is associated with epigenetic age acceleration. We focused on the effects of physical activity components on GrimAge acceleration, because 1) second-generation epigenetic clocks have been demonstrated to more closely reflect the high inter-individual variability in the underlying biological ageing processes as compared to first-generation epigenetic clocks, 2) GrimAge acceleration has been demonstrated to be the strongest predictor of age-associated functional decline (A. T. Lu et al., 2019; McCrory et al., 2021), and 3) GrimAge acceleration has been found to outperform the

other epigenetic clocks, both in predicting mortality risk (A. T. Lu et al., 2019; McCrory et al., 2021) and in capturing multisystem dysregulation (Liu, Aziz, Pehlivan, & Breteler, 2023). Nevertheless, for comparison, we also explored the association between physical activity and Hannum's age, Horvath's age and PhenoAge acceleration in additional sensitivity analyses. Furthermore, we investigated to what extent the effect of physical activity on GrimAge acceleration is mediated through its effects on immune function and cardiovascular risk factors, while also examining potential reversed mediation effects - whether GrimAge acceleration mediates the association between physical activity and markers of cardiovascular health. Lastly, we performed an epigenome-wide association study of physical activity and conducted a gene enrichment analysis to gain biological insights into the mechanisms underlying the effects of physical activity on epigenetic ageing.

Results

Sample characteristics

The sample characteristics are presented in Table 1 and Table S1. In our main analysis, 3,567 eligible participants were included, of whom 1,955 were women (54.8%). Participants' mean age was 55.5 years (SD: 14.1, age range: 30 – 94 years). Participants had on average high education and physical activity levels. Physical activity levels were lower in older adults compared to younger adults (Table S1).

Table 1. Sample demographics.

	Eligible participants (n = 3567)	Individuals with cardiovascular data (n = 3357)	Excluded participants (n = 1429)	p[a]
Age (years), mean (SD)	55.5 (14.1)	55.4 (14.0)	54.2 (13.7)	0.002
30-39	594 (16.7)	563 (16.8)	238 (16.7)	[ref]
40-49	610 (17.1)	575 (17.1)	315 (22.0)	0.019
50-59	966 (27.1)	914 (27.2)	392 (27.4)	0.986
60-69	747 (20.9)	707 (21.1)	261 (18.3)	0.172
70-79	492 (13.8)	463 (13.8)	173 (12.1)	0.273
80-89	154 (4.3)	132 (3.9)	45 (3.2)	0.096
90+	4 (0.1)	3 (0.1)	5 (0.4)	0.093
Sex (women), n (%)	1955 (54.8)	1821 (54.2)	865 (60.5)	<0.001
Body-mass index (kg/m^2), mean (SD)	25.86 (4.38)	25.86 (4.33)	26.12 (4.95)	0.008
Waist-to-hip ratio, mean (SD)	0.87 (0.10)	0.87 (0.10)	0.86 (0.10)	0.205
Cardiovascular Event, n (% Yes)	319 (8.9)	289 (8.6)	102 (7.1)	0.338
Smoking, n (% Yes)	448 (12.6)	420 (12.5)	173 (12.2)	0.658
Diabetes, n (% Yes)	192 (5.4)	179 (5.3)	69 (4.8)	0.993
Hypertension, n (%)				
No	2171 (61.5)	2086 (62.1)	893 (63.4)	[ref]
Yes, controlled	613 (17.4)	575 (17.1)	245 (17.4)	0.393
Yes, uncontrolled	699 (19.8)	664 (19.8)	268 (19.0)	0.582
Yes, unknown	46 (1.3)	32 (1.0)	3 (0.2)	0.557

Education ISCED11, n (%)				
high	1867 (52.3)	1751 (52.2)	751 (54.3)	[ref]
middle	1629 (45.7)	1542 (45.9)	602 (43.5)	0.249
low	71 (2.0)	64 (1.9)	30 (2.2)	0.709
Daily Sensor Hours Worn (hours), mean (SD)	23.81 (0.51)	23.81 (0.52)	23.74 (0.76)	0.039
Daily Energy Expenditure (MET hours), mean (SD)	33.98 (1.37)	33.99 (1.37)	33.25 (2.05)	<0.001
Daily Step Count, mean (SD)	8804.97 (3235.32)	8834.85 (3235.77)	7826.58 (3960.50)	<0.001
% Daily Light Intensity Physical Activity, mean (SD)	21.26 (6.08)	21.27 (6.08)	20.86 (9.21)	0.029
% Daily Moderate-to-Vigorous Physical Activity, mean (SD)	4.70 (1.79)	4.72 (1.79)	4.58 (1.97)	0.455
% Daily Sedentary, mean (SD)	74.03 (6.72)	74.01 (6.71)	76.66 (9.43)	<0.001
Hannum's Age acceleration, mean (SD)	0.31 (5.67)	0.11 (5.60)	0.20 (5.58)	0.840
Horvath's Age acceleration, mean (SD)	0.22 (5.28)	0.09 (5.24)	0.40 (5.23)	0.208
PhenoAge acceleration, mean (SD)	0.17 (6.61)	0.04 (6.60)	0.18 (6.57)	0.837
GrimAge acceleration, mean (SD)	0.01 (7.41)	-0.11 (7.42)	-0.34 (7.48)	0.533
% Basophils, mean (SD)[b]	0.77 (0.81)	0.78 (0.81)	0.78 (0.78)	0.674
% Eosophils, mean (SD) [b]	2.44 (1.82)	2.46 (1.81)	2.48 (1.90)	0.668
% Neutrophils, mean (SD)[b]	50.14 (11.66)	49.96 (11.67)	50.34 (11.51)	0.186
% Monophils, mean (SD)[b]	8.95 (2.20)	8.97 (2.20)	8.79 (2.20)	0.076
% Naïve B cells, mean (SD)[b]	3.02 (1.74)	3.06 (1.74)	2.99 (1.58)	0.204
% Memory B cells, mean (SD)[b]	2.05 (3.16)	2.07 (3.24)	1.92 (1.45)	0.219
% Naïve CD4 T cells, mean (SD)[b]	6.76 (4.32)	6.83 (4.32)	6.92 (4.15)	0.576
% Memory CD4 T cells, mean (SD)[b]	11.15 (4.00)	11.14 (3.98)	11.16 (4.01)	0.913
% Regulatory T cells, mean (SD)[b]	0.08 (0.32)	0.08 (0.32)	0.09 (0.33)	0.571
% Naïve CD8 T cells, mean (SD)[b]	2.25 (2.08)	2.29 (2.08)	2.36 (2.15)	0.608

% Memory CD8 T cells, mean (SD)[b]	6.84 (5.69)	6.78 (5.66)	6.70 (5.28)	0.540
% Natural killer cells, mean (SD)[b]	5.67 (2.58)	5.70 (2.59)	5.67 (2.61)	0.811
Systolic blood pressure (mm Hg), mean (SD)	126.60 (16.04)	126.68 (16.00)	125.99 (16.06)	0.358
Diastolic blood pressure (mm Hg), mean (SD)	75.34 (9.47)	75.40 (9.44)	75.40 (9.24)	0.231
Cholesterol (mg/dL), mean (SD)	198.28 (39.74)	198.33 (39.68)	199.10 (37.39)	0.297
High-density lipoprotein (mg/dL), mean (SD)	62.32 (17.74)	62.29 (17.73)	63.00 (17.64)	0.951
Low-density lipoprotein (mg/dL), mean (SD)	126.49 (36.17)	126.58 (36.15)	126.35 (34.05)	0.625
Triglycerides (mg/dL), mean (SD)	111.13 (69.35)	111.07 (69.46)	111.68 (67.25)	0.188
Insulin (mU/L), mean(SD)	10.43 (7.51)	10.40 (7.50)	10.06 (8.11)	0.370

[a]) Group differences (included vs. excluded participants) were assessed using binomial logistic regression, adjusted for age and sex (group differences for the variables age and sex were only adjusted for the other respectively).

[b]) Leukocyte subtypes were derived based on DNA methylation levels (as described by Salas et al., 2022).

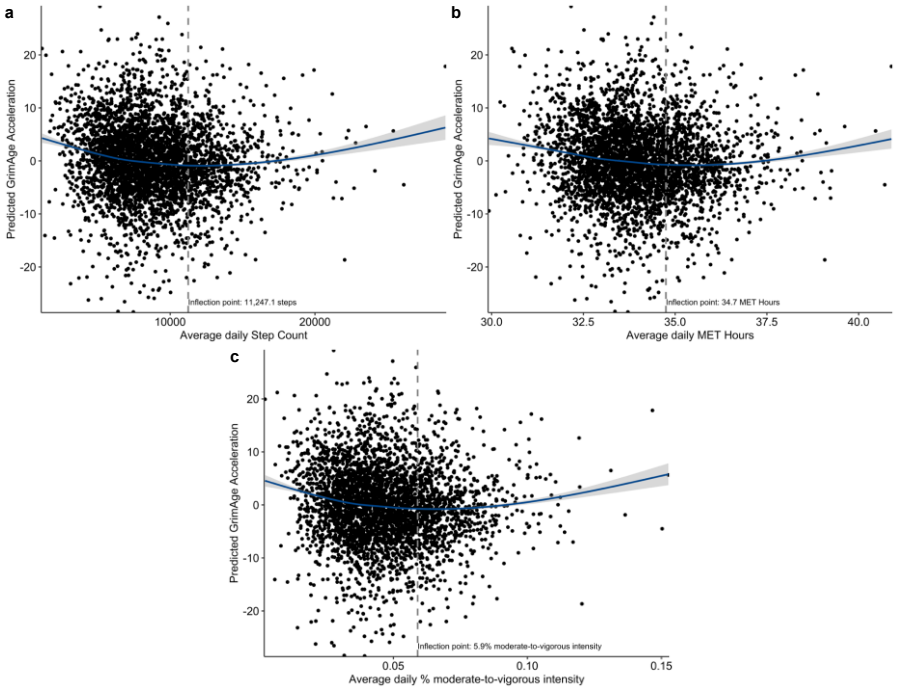
Abbreviations: Metabolic-Equivalent Hours (MET Hours), Standard Deviation (SD), 95% Confidence Interval (95% CI), reference group (ref)

Effects of physical activity on epigenetic age acceleration

Using polynomial regression models, we examined the effects of physical activity on epigenetic ageing. Higher average daily step counts and energy expenditure, as measured in metabolic equivalent (MET-) Hours, were non-linearly associated with lower GrimAge acceleration (Table 2a). For example, for step counts, the difference in GrimAge acceleration for an individual with -2 standard deviations below the mean compared to an individual with an average daily step count (corresponding to ~2300 and ~8800 steps a day, respectively) was around 21 months. Similarly, for MET Hours, the difference in GrimAge acceleration for an individual with -2 standard deviations below the mean compared to one with an average amount of MET Hours (corresponding to ~31 and ~34 MET Hours per day, respectively) was around 18 months. The maximum effect was reached at an average of 11,247 steps and 34.7 daily MET-Hours (Figure 1). The effects of physical activity dose on GrimAge acceleration did not differ between men and women (Figure S1; $\beta_{Steps \times Sex}$ = 0.068, 95%CI = [-0.389; 0.525], p = 0.769; $\beta_{METs \times Sex}$ = -0.052, 95% CI = [-0.509; 0.404], p = 0.823). Additional proportion of time spent in moderate-to-vigorous physical activities (MVPA) was also associated with lower GrimAge acceleration (Table 2a). The effect of MVPA on GrimAge acceleration was also non-linear and was strongest at an average of 5.9% or 1.5 hours of daily MVPA (Figure 1). The effect of MVPA on GrimAge acceleration did not differ between men and women (Figure S1; $\beta_{MVPA \times Sex}$ = 0.088, 95%CI = [-0.370; 0.545], p = 0.708). To examine whether the non-linear shape of the association was driven by a few participants with extreme values, we additionally ran a sensitivity analysis excluding participants with high leverage points. After excluding 74 participants with high leverage points, we observed similar effect estimates (Figure S2).

In exploratory analyses examining the association between physical activity and Hannum's age, Horvath's age and PhenoAge acceleration, we also found greater daily step counts, energy expenditure and time spent in MVPA associated with lower PhenoAge acceleration. The beneficial effects of physical activity dose and MVPA leveled off at higher levels (Table 2b). We did not observe an association between any of the physical activity components and Horvath's and Hannum's age acceleration (Table 2c&d).

Figure 1. Scatterplot of association between physical activity and GrimAge acceleration.



Regression lines were adjusted for age, age2, sex, education, batch effect, cell proportions, season and smoking status.

Abbreviations: Metabolic-Equivalent Hours (MET-Hours).

Table 2. Effect estimates of the association between physical activity and epigenetic ageing.

a Main effect estimates for GrimAge acceleration

Term	ß [95%CI]	p-value	n	Sample
Step Count (linear)	-0.38 [-0.65; -0.11]	0.005	3567	Total Sample
Step Count (quadratic)	0.25 [0.12; 0.39]	<0.001	3567	Total Sample
MET Hours (linear)	-0.28 [-0.53; -0.03]	0.029	3567	Total Sample
MET Hours (quadratic)	0.25 [0.10; 0.39]	<0.001	3567	Total Sample
% Light Intensity (linear)	-0.16 [-0.41; 0.09]	0.214	3567	Total Sample
% Light Intensity (quadratic)	0.12 [-0.05; 0.28]	0.156	3567	Total Sample
% Moderate-to-Vigorous Intensity (linear)	-0.31 [-0.57; -0.04]	0.023	3567	Total Sample
% Moderate-to-Vigorous Intensity (quadratic)	0.23 [0.1; 0.37]	<0.001	3567	Total Sample
% Sedentary (linear)	0.15 [-0.09; 0.4]	0.217	3567	Total Sample
% Sedentary (quadratic)	0.11 [-0.05; 0.27]	0.185	3567	Total Sample

b Effect estimates of the exploratory analysis for PhenoAge acceleration

Term	ß [95%CI]	p-value	n	Sample
Step Count (linear)	-0.29 [-0.50; -0.07]	0.010	3567	Total Sample
Step Count (quadratic)	0.16 [0.05; 0.27]	0.004	3567	Total Sample
MET Hours (linear)	-0.15 [-0.36; 0.05]	0.149	3567	Total Sample
MET Hours (quadratic)	0.12 [0.01; 0.24]	0.037	3567	Total Sample

% Light Intensity (linear)	-0.10 [-0.31; 0.11]	0.341	3567	Total Sample
% Light Intensity (quadratic)	-0.13 [-0.27; -0.01]	0.047	3567	Total Sample
% Moderate-to-Vigorous Intensity (linear)	-0.26 [-0.48; -0.05]	0.018	3567	Total Sample
% Moderate-to-Vigorous Intensity (quadratic)	0.14 [0.03; 0.25]	0.013	3567	Total Sample
% Sedentary (linear)	0.16 [-0.04; 0.35]	0.124	3567	Total Sample
% Sedentary (quadratic)	-0.10 [-0.23; 0.04]	0.153	3567	Total Sample

c Effect estimates of the exploratory analysis for Horvath's Age acceleration

Term	**ß [95%CI]**	**p-value**	**n**	**Sample**
Step Count (linear)	0.04 [-0.15; 0.24]	0.651	3567	Total Sample
Step Count (quadratic)	0.05 [-0.04; 0.15]	0.284	3567	Total Sample
MET Hours (linear)	0.18 [0.01; 0.36]	0.047	3567	Total Sample
MET Hours (quadratic)	-0.01 [-0.11; 0.09]	0.861	3567	Total Sample
% Light Intensity (linear)	-0.09 [-0.27; 0.09]	0.329	3567	Total Sample
% Light Intensity (quadratic)	-0.07 [-0.19; 0.05]	0.232	3567	Total Sample
% Moderate-to-Vigorous Intensity (linear)	0.09 [-0.10; 0.28]	0.333	3567	Total Sample
% Moderate-to-Vigorous Intensity (quadratic)	0.04 [-0.06; 0.13]	0.460	3567	Total Sample
% Sedentary (linear)	0.05 [-0.12; 0.23]	0.566	3567	Total Sample
% Sedentary (quadratic)	-0.09 [-0.20; 0.03]	0.152	3567	Total Sample

d Effect estimates of the exploratory analysis for Hannum's Age acceleration

Term	ß [95%CI]	p-value	n	Sample
Step Count (linear)	-0.06 [-0.24; 0.12]	0.504	3567	Total Sample
Step Count (quadratic)	0.05 [-0.04; 0.14]	0.248	3567	Total Sample
MET Hours (linear)	0.11 [-0.06; 0.27]	0.206	3567	Total Sample
MET Hours (quadratic)	-0.01 [-0.10; 0.09]	0.851	3567	Total Sample
% Light Intensity (linear)	-0.10 [-0.26; 0.07]	0.263	3567	Total Sample
% Light Intensity (quadratic)	0.01 [-0.10; 0.12]	0.874	3567	Total Sample
% Moderate-to-Vigorous Intensity (linear)	-0.03 [-0.20; 0.15]	0.776	3567	Total Sample
% Moderate-to-Vigorous Intensity (quadratic)	0.04 [-0.05; 0.13]	0.351	3567	Total Sample
% Sedentary (linear)	0.07 [-0.09; 0.24]	0.364	3567	Total Sample
% Sedentary (quadratic)	-0.01 [-0.12; 0.10]	0.832	3567	Total Sample

e Effect estimates for GrimAge acceleration of individuals without a cardiovascular event

Term	ß [95%CI]	p-value	n	Sample
Step Count (linear)	-0.39 [-0.67; -0.11]	0.007	3239	Individuals without a cardiovascular event
Step Count (quadratic)	0.26 [0.12; 0.40]	<0.001	3239	Individuals without a cardiovascular event

MET Hours (linear)	-0.27 [-0.53; -0.01]	0.047	3239	Individuals without a cardiovascular event
MET Hours (quadratic)	0.24 [0.09; 0.40]	0.002	3239	Individuals without a cardiovascular event
% Light Intensity (linear)	-0.23 [-0.49; 0.03]	0.088	3239	Individuals without a cardiovascular event
% Light Intensity (quadratic)	0.19 [0.02; 0.37]	0.030	3239	Individuals without a cardiovascular event
% Moderate-to-Vigorous Intensity (linear)	-0.32 [-0.60; -0.04]	0.027	3239	Individuals without a cardiovascular event
% Moderate-to-Vigorous Intensity (quadratic)	0.23 [0.09; 0.37]	0.001	3239	Individuals without a cardiovascular event
% Sedentary (linear)	0.21 [-0.04; 0.47]	0.104	3239	Individuals without a cardiovascular event
% Sedentary (quadratic)	0.19 [0.02; 0.37]	0.033	3239	Individuals without a cardiovascular event

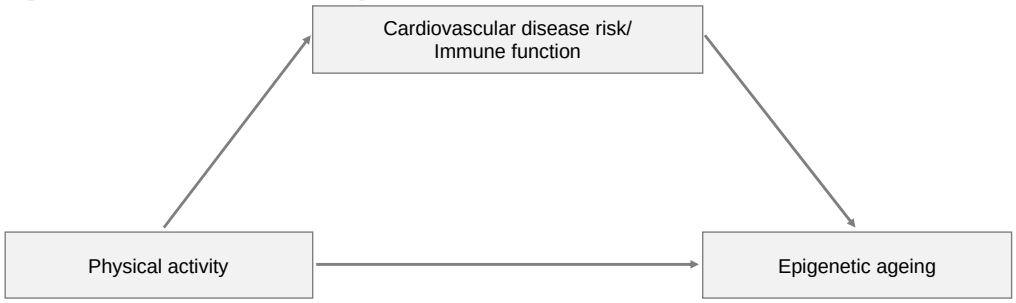
Abbreviations: Metabolic-Equivalent Hours (MET Hours), 95% Confidence Interval (95% CI).

Models were adjusted for age, age^2, sex, education, batch effect, and smoking status.

Cardiovascular disease risk as mediator

In our mediation analysis, 3,357 eligible participants with available cardiovascular data were included (54.2% women, mean age: 55.4 years, range: 30 – 94 years) (Table 1). We examined whether cardiovascular disease risk could mediate the association between physical activity (i.e. energy expenditure, step counts and % MVPA) and GrimAge acceleration (Figure 2). We assessed the indirect effect of physical activity on GrimAge acceleration mediated through either the Framingham Risk Score (D'Agostino et al., 2008), the ESC SCORE2 (Hageman et al., 2021), the Assessment of Cardiovascular Disease (ASCVD) Risk Score (Goff et al., 2014) or the sample-based (factor analysis for mixed data (FAMD)-derived) cardiovascular disease risk components (Lê, Josse, & Husson, 2008). For the sample-based score, we specifically assessed the mediation effect of the first and second FAMD cardiovascular component, which were predominantly influenced by (1) blood pressure, triglycerides and adiposity measures, and (2) lipoprotein levels, respectively (Figure S3).

Figure 2. Mediation analysis model.



We observed that the Framingham Risk Score, the ESC SCORE2, the ASCVD Score and the first FAMD cardiovascular component partially mediated the association between physical activity and GrimAge acceleration (Table 3). The effects of physical activity on GrimAge acceleration depended on physical activity levels. To illustrate the non-linear association between physical activity and epigenetic age, we report the effects at -1 SD, average (0 SD) and +1 SD levels of physical activity: keeping the covariates at a constant, we found that the direct and indirect effects of physical activity on GrimAge acceleration were strongest at low physical activitiy quanties (e.g. -1 SD) and became progressively weaker at higher levels (i.e. average level and +1 SD). Overall, we observed the strongest mediating effects of cardiovascular risk scores for average daily MET-Hours and step counts. For instance, at one standard deviation

below the mean (i.e. 32.6 MET-Hours and 5,570 steps, respectively), 50.1% and 22.2% of the effects of MET-Hours and step counts on GrimAge acceleration were mediated by the Framingham Risk Score. At the mean (i.e. 34.0 MET-Hours and 8,850 steps, respectively), 40.2% and 27.3% of the effects were mediated by the Framingham Risk Score, with an indirect effect of -0.020 and -0.019 on GrimAge acceleration, respectively. The second FAMD component did not mediate the association between physical activity and GrimAge acceleration (Table 3).

Table 3. Direct and indirect effects of physical activity on epigenetic ageing mediated by cardiovascular disease risk factors and immune function, while keeping the covariates at a constant.

Mediator	Predictor	Indirect effect			Direct effect			Total effect			Percentage mediated		
		-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD	Average	+1 SD
FAMD cardiovascular disease component 1	MET-Hours	NA[a]	-0.0082** [-0.0146; -0.0023]	NA[a]	-0.1283*** [-0.2064; -0.0541]	-0.0550** [-0.0923; -0.0145]	0.0183 [-0.0262; 0.0616]	-0.1365*** [-0.2126; -0.0619]	-0.0632*** [-0.1000; -0.0245]	0.0101 [-0.0356; 0.0547]	6.04%	13.04%	NA[b]
	Step Count	NA[a]	-0.0077* [-0.0140; -0.0020]	NA[a]	-0.1505*** [-0.2199; -0.0743]	-0.0722*** [-0.1112; -0.0307]	0.0060 [-0.0345; 0.0441]	-0.1582*** [-0.2269; -0.0833]	-0.0799*** [-0.1172; -0.0388]	-0.0016 [-0.0437; 0.0366]	4.86%	9.61%	NA[b]
	% MVPA	NA[a]	-0.0071** [-0.0129; -0.0021]	NA[a]	-0.1367*** [-0.2048; -0.0597]	-0.0633** [-0.1013; -0.0216]	0.0102 [-0.0315; 0.0490]	-0.1438*** [-0.2124; -0.0694]	-0.0704*** [-0.1072; -0.0286]	0.0031 [-0.0389; 0.0418]	4.95%	10.11%	NA[b]
FAMD cardiovascular disease component 2	MET-Hours	-0.0044 [-0.0117; 0.0011]	-0.0029 [-0.0075; 0.0008]	-0.0014 [-0.0049; 0.0002]	-0.1336*** [-0.2099; -0.0602]	-0.0607** [-0.0975; -0.0222]	0.0122 [-0.0321; 0.0570]	-0.1380*** [-0.2138; -0.0632]	-0.0636*** [-0.1003; -0.0249]	0.0108 [-0.0333; 0.0557]	3.18%	4.51%	NA[b]
	Step Count	-0.0042 [-0.0113; 0.0011]	-0.0029 [-0.0077; 0.0008]	-0.0017 [-0.0053; 0.0003]	-0.1568*** [-0.2254; -0.0842]	-0.0779*** [-0.1153; -0.0368]	0.0010 [-0.0405; 0.0381]	-0.1610*** [-0.2285; -0.0889]	-0.0808*** [-0.1185; -0.0398]	-0.0007 [-0.0421; 0.0378]	2.60%	3.62%	NA[b]
	% MVPA	-0.0039 [-0.0104; 0.0004]	-0.0028 [-0.0075; 0.0005]	-0.0018 [-0.0054; 0.0002]	-0.1429*** [-0.2132; -0.0699]	-0.0685*** [-0.1060; -0.0278]	0.0059 [-0.0360; 0.0437]	-0.1468*** [-0.2145; -0.0732]	-0.0713*** [-0.1083; -0.0302]	0.0041 [-0.0378; 0.0428]	2.63%	3.97%	NA[b]
Framingham Heart Study cardiovascular score	MET-Hours	-0.0292*** [-0.0471; -0.0155]	-0.0195*** [-0.0292; -0.0114]	-0.0098** [-0.0168; -0.0052]	NA[a]	-0.0291 [-0.0622; 0.0044]	NA[a]	-0.0583*** [-0.0910; -0.0215]	-0.0486** [-0.0806; -0.0164]	-0.0389* [-0.0716; -0.0062]	50.10%	40.15%	25.25%
	Step Count	-0.0268*** [-0.0431; -0.0141]	-0.0186*** [-0.0281; -0.0104]	-0.0104*** [-0.0168; -0.0056]	-0.0942** [-0.1667; -0.0237]	-0.0497* [-0.0927; -0.0118]	-0.0052 [-0.0344; 0.0279]	-0.1210*** [-0.1924; -0.0513]	-0.0683*** [-0.1098; -0.0294]	-0.0156 [-0.0449; 0.0158]	22.15%	27.26%	66.88%
	% MVPA	-0.0249*** [-0.0412; -0.0125]	-0.0176*** [-0.0270; -0.0098]	-0.0104*** [-0.0171; -0.0055]	-0.0873* [-0.1580; -0.0183]	-0.0450* [-0.0868; -0.0065]	-0.0028 [-0.0339; 0.0318]	-0.1122** [-0.1823; -0.0409]	-0.0626** [-0.1041; -0.0225]	-0.0131 [-0.0446; 0.0206]	22.19%	28.13%	79.02%
ESC SCORE2	MET-Hours	-0.0283*** [-0.0414; -0.0177]	-0.0201*** [-0.0280; -0.0141]	-0.0119*** [-0.0175; -0.0071]	-0.1090** [-0.1816; -0.0425]	-0.0430* [-0.0806; -0.0080]	0.0230 [-0.0214; 0.0723]	-0.1373*** [-0.2111; -0.0708]	-0.0631*** [-0.1010; -0.0281]	0.0112 [-0.0331; 0.0594]	20.60%	31.84%	NA[b]
	Step Count	-0.0308*** [-0.0445; -0.0197]	-0.0212*** [-0.0294; -0.0145]	-0.0116*** [-0.0177; -0.0071]	-0.1287*** [-0.2071; -0.0608]	-0.0586** [-0.0976; -0.0208]	0.0115 [-0.0274; 0.0553]	-0.1594*** [-0.2357; -0.0904]	-0.0798*** [-0.1203; -0.0419]	-0.0001 [-0.0395; 0.0423]	19.31%	26.59%	NA[b]
	% MVPA	-0.0287*** [-0.0420; -0.0184]	-0.0197*** [-0.0276; -0.0130]	-0.0106*** [-0.0156; -0.0056]	-0.1174*** [-0.1923; -0.0486]	-0.0509** [-0.0892; -0.0120]	0.0156 [-0.0229; 0.0598]	-0.1462*** [-0.2223; -0.0763]	-0.0706*** [-0.1095; -0.0306]	0.0050 [-0.0336; 0.0501]	19.67%	27.85%	NA[b]

Table 3. [continued]

Mediator	Predictor	Indirect effect			Direct effect			Total effect			Percentage mediated		
		-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD	Average	+1 SD
ASCVD SCORE	MET-Hours	NA[a]	-0.0038* [-0.0080; -0.0009]	NA[a]	-0.1301*** [-0.2069; -0.0500]	-0.0566** [-0.0941; -0.0142]	0.0170 [-0.0321; 0.0645]	-0.1339*** [-0.2105; -0.0560]	-0.0604** [-0.0975; -0.0176]	0.0132 [-0.0370; 0.0604]	2.84%	6.30%	NA[b]
	Step Count	-0.0076* [-0.0176; -0.0017]	-0.0052* [-0.0113; -0.0011]	-0.0027* [-0.0061; -0.0006]	-0.1486*** [-0.2186; -0.0717]	-0.0720*** [-0.1118; -0.0282]	0.0047 [-0.0393; 0.0464]	-0.1563*** [-0.2260; -0.0802]	-0.0771*** [-0.1165; -0.0360]	0.0020 [-0.0425; 0.0432]	4.88%	6.72%	NA[b]
	% MVPA	-0.0074* [-0.0175; -0.0017]	-0.0050* [-0.0108; -0.0012]	-0.0026* [-0.0058; -0.0006]	-0.1362*** [-0.2104; -0.0613]	-0.0635** [-0.1014; -0.0247]	0.0091 [-0.0357; 0.0523]	-0.1435*** [-0.2161; -0.0667]	-0.0685*** [-0.1053; -0.0281]	0.0065 [-0.0367; 0.0516]	5.13%	7.26%	NA[b]
PCA immune function component 1	MET-Hours	NA[a]	-0.0045 [-0.0106; 0.0001]	NA[a]	-0.1306*** [-0.2121; -0.0684]	-0.0633*** [-0.1012; -0.0289]	0.0040 [-0.0392; 0.0475]	-0.1352*** [-0.2183; -0.0711]	-0.0678*** [-0.1065; -0.0341]	0.0005 [-0.0455; 0.0421]	3.36%	6.69%	NA[b]
	Step Count	NA[a]	-0.0059* [-0.0120; -0.0015]	NA[a]	-0.1585*** [-0.2328; -0.0951]	-0.0820*** [-0.1223; -0.0467]	-0.0055 [-0.0464; 0.0322]	-0.1645*** [-0.2414; -0.1013]	-0.0880*** [-0.1304; -0.0545]	-0.0114 [-0.0531; 0.0251]	3.61%	6.75%	51.87%
	% MVPA	NA[a]	-0.0057* [-0.0119; -0.0014]	NA[a]	-0.1447*** [-0.2119; -0.0773]	-0.0724*** [-0.1114; -0.0354]	0.0001 [-0.0411; 0.0407]	-0.1504*** [-0.2293; -0.0848]	-0.0781*** [-0.1193; -0.0433]	-0.0058 [-0.0491; 0.0311]	3.81%	7.35%	NA[b]
PCA immune function component 2	MET-Hours	NA[a]	-0.0027* [-0.0060; -0.0009]	NA[a]	-0.1287*** [-0.2101; -0.0627]	-0.0643*** [-0.1031; -0.0304]	0.0002 [-0.0443; 0.0426]	-0.1314*** [-0.2122; -0.0646]	-0.0676*** [-0.1048; -0.0326]	-0.0025 [-0.0479; 0.0401]	2.05%	4.02%	NA[b]
	Step Count	NA[a]	-0.0025* [-0.0058; -0.0008]	NA[a]	-0.1612*** [-0.2374; -0.0990]	-0.0852*** [-0.1276; -0.0506]	-0.0092 [-0.0499; 0.0271]	-0.1637*** [-0.2403; -0.1022]	-0.0877*** [-0.1290; -0.0537]	-0.0117 [-0.0530; 0.0237]	1.54%	2.87%	21.44%
	% MVPA	NA[a]	-0.0024* [-0.0056; -0.0007]	NA[a]	-0.1462*** [-0.2232; -0.0801]	-0.0751*** [-0.1170; -0.0393]	-0.0041 [-0.0458; 0.0327]	-0.1486*** [-0.2252; -0.0825]	-0.0775*** [-0.1185; -0.0418]	-0.0065 [-0.0473; 0.0307]	1.61%	3.09%	36.90%

Significance levels: *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.

[a]) Models with only linear effect.

[b]) Models with opposite direct and indirect effects.

Abbreviations: Factor Analysis for Mixed Data (FAMD), Metabolic-Equivalent Hours (MET-Hours), %Moderate-to-Vigorous Physical Activity (MVPA), Standard Deviation (SD), 95% Confidence Interval (95% CI)

Immune function as mediator

In addition, we investigated whether changes in immune function could mediate the association between physical activity and epigenetic ageing (Figure 2). Using principle component analysis, and based on DNA methylation levels, we extracted the sample-based first and second immune function composite components based on 12 leukocyte subtypes (Salas et al, 2022). Whereas the first component heavily weighted the proportion of neutrophils, as well as (though to a lesser extent) naïve B cells and CD4T+ T cells (naïve CD4T cells and memory CD4T cells), the second component was largely influenced by CD8T+ T cells (memory CD8T cells, naïve CD8T cells, and natural killer cells) (Figure S4). The immune function composite components were only weakly correlated with the cardiovascular disease risk scores and composite compontents (e.g. $r_{Immune1\ Framingham} = -0.22$; $r_{Immune2\ Framingham} = 0.11$; $r_{Immune1\ ESC\ Score} = -0.19$; $r_{Immune2\ ESC\ Score} = 0.11$; $r_{Immune1\ ASCVD} = -0.15$; $r_{Immune2\ ASCVD} = 0.06$).

We observed that the first and second immune function composite components partially mediated the association between physical activity and GrimAge acceleration. However, the mediation effects were generally smaller compared to those of cardiovascular risk factors (Table 3). Whereas the Framingham Risk Score mediated up to ~50% of the effects of physical activity on GrimAge acceleration, both the first and second immune function composite components mediated only up to ~7% of the effects of physical activity on GrimAge acceleration. We found that the direct effect of physical activity on GrimAge acceleration was strongest at low physical activitiy quanties (e.g. -1 SD) and lessened at the higher end of the physical activity spectrum (i.e. average level and +1 SD).

Reversed mediation: GrimAge acceleration as mediator

In exploratory analyses, we examined whether GrimAge acceleration could mediate some of the effects of physical activity on cardiovascular disease risk. We observed relatively weak mediating effects of GrimAge acceleration (Table S2). Overall, GrimAge acceleration mediated up to ~10% of the effect of physical activity on cardiovascular disease risk.

Sensitivity analysis in individuals without a cardiovascular event

We repeated the polynomial regression and mediation analysis of cardiovascular disease risk in a subset of individuals without a prior cardiovascular event. In these

individuals, the effects of all physical activity components (step counts, energy expenditure, %light intensity activities, %moderate-to-vigorous intensity activities and %sedentary activities) were significantly associated with GrimAge acceleration (Table 2e). Higher physical activity dose and intensity were associated with slower epigenetic ageing. The effects of physical activity were again most pronounced at the lower end of the physical activity spectrum, with the exception of the effects of %sedentary activities. A higher proportion of %sedentary activities was associated with faster epigenetic ageing and the effect was stronger at higher %sedentary levels (Figure S5). Compared to other intensities, the effect of %moderate-to-vigorous intensity activities was strongest.

Mediation analysis in individuals without a prior cardiovascular event largely replicated the results based on the entire sample (Table 4). The ESC score, Framingham Risk Score and ASCVD Score partially mediated the association between average daily energy expenditure, step counts, %MVPA and GrimAge acceleration. For %light-intensity activities and %sedentary activities, we found that the ESC score and Framingham Risk Score fully mediated the association with GrimAge acceleration, whereas we did not observe a mediation effect for the ASCVD Score. Moreover, the first FAMD component only fully mediated the association between %light-intensity activities and GrimAge acceleration. Also here, we did not observe a mediation effect through the second FAMD component.

Mediation analysis using a negative control variable

To test the robustness of the mediation results, we also ran mediation analyses with olfactory performance as a negative control variable. We found that olfactory performance did not mediate the association between physical activity and GrimAge acceleration (Table S3).

Table 4. Direct and indirect effects of physical activity on epigenetic ageing mediated by cardiovascular disease risk in individuals without a prior cardiovascular event, while keeping the covariates at a constant.

Mediator	Predictor	Indirect effect			Direct effect			Total effect			Percentage mediated		
		-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD	Average	+1 SD
FAMD cardiovascular disease component 1	MET-Hours	NA[a]	-0.0062 [-0.0129; 0.0006]	NA[a]	-0.1296** [-0.2061; -0.0514]	-0.0561** [-0.0957; -0.0167]	0.0173 [-0.0310; 0.0691]	-0.1358*** [-0.2106; -0.0586]	-0.0623** [-0.1030; -0.0249]	0.0112 [-0.0356; 0.0630]	4.54%	9.90%	NA[b]
	Step Count	NA[a]	-0.0056 [-0.0122; 0.0006]	NA[a]	-0.1554*** [-0.2363; -0.0839]	-0.0755*** [-0.1181; -0.0351]	0.0045 [-0.0370; 0.0515]	-0.1610*** [-0.2399; -0.0920]	-0.0810*** [-0.1248; -0.0437]	-0.0011 [-0.0430; 0.0449]	3.45%	6.86%	NA[b]
	% MVPA	NA[a]	-0.0051 [-0.0113; 0.0002]	NA[a]	-0.1407*** [-0.2252; -0.0726]	-0.0668** [-0.1097; -0.0260]	0.0071 [-0.0339; 0.0559]	-0.1458*** [-0.2291; -0.0789]	-0.0719*** [-0.1157; -0.0336]	0.0019 [-0.0396; 0.0506]	3.53%	7.15%	NA[b]
	% Light Intensity	NA[a]	-0.0060* [-0.0122; -0.0003]	NA[a]	NA[a]	-0.0165 [-0.0525; 0.0253]	NA[a]	NA[a]	-0.0226 [-0.0566; 0.0187]	NA[a]	NA[a]	26.55%	NA[a]
	% Sedentary	NA[a]	0.0066 [-0.0002; 0.0133]	NA[a]	-0.0251 [-0.0770; 0.0309]	0.0331 [-0.0111; 0.0714]	0.0913* [0.0154; 0.1690]	-0.0186 [-0.0727; 0.0388]	0.0397* [-0.0031; 0.0757]	0.0979* [0.0239; 0.1738]	NA[b]	16.52%	6.69%
FAMD cardiovascular disease component 2	MET-Hours	NA[a]	-0.0020 [-0.0052; 0.0003]	NA[a]	-0.1339*** [-0.2070; -0.0558]	-0.0604** [-0.1015; -0.0239]	0.0132 [-0.0339; 0.0655]	-0.1359*** [-0.2101; -0.0582]	-0.0623** [-0.1030; -0.0247]	0.0112 [-0.0353; 0.0637]	1.46%	3.18%	NA[b]
	Step Count	NA[a]	-0.0020 [-0.0054; 0.0003]	NA[a]	-0.1602*** [-0.2371; -0.0920]	-0.0794*** [-0.1215; -0.0418]	0.0013 [-0.0407; 0.0464]	-0.1622*** [-0.2409; -0.0935]	-0.0814*** [-0.1242; -0.0441]	-0.0006 [-0.0426; 0.0454]	1.23%	2.44%	NA[b]
	% MVPA	NA[a]	-0.0020 [-0.0054; 0.0001]	NA[a]	-0.1456*** [-0.2245; -0.0763]	-0.0706 [-0.1130; -0.0327]	0.0045 [-0.0368; 0.0525]	-0.1476*** [-0.2272; -0.0789]	-0.0725*** [-0.1160; -0.0340]	0.0025 [-0.0392; 0.0516]	1.33%	2.71%	NA[b]
	% Light Intensity	-0.0048 [-0.0128; 0.0002]	-0.0022 [-0.0056; 0.0001]	0.0005 [-0.0008; 0.0039]	NA[a]	-0.0210 [-0.0550; 0.0195]	NA[a]	-0.0258 [-0.0610; 0.0150]	-0.0232 [-0.0578; 0.0172]	-0.0205 [-0.0552; 0.0198]	18.56%	9.27%	NA[b]
	% Sedentary	-0.0006 [-0.0039; 0.0007]	0.0020 [-0.0004; 0.0055]	0.0049 [-0.0014; 0.0130]	-0.0188 [-0.0725; 0.0388]	0.0377 [-0.0045; 0.0742]	0.0942* [0.0178; 0.1719]	-0.0194 [-0.0730; 0.0379]	0.0398* [-0.0032; 0.0755]	0.0991* [0.0248; 0.1749]	2.97%	5.44%	4.95%

Table 4. [continued]

Mediator	Predictor	Indirect effect			Direct effect			Total effect			Percentage mediated		
		-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD	Average	+1 SD
Framingham Heart Study cardiovascular score	MET-Hours	-0.0297*** [-0.0501; -0.0159]	-0.0200*** [-0.0317; -0.0114]	-0.0102** [-0.0182; -0.0046]	NA[a]	-0.0250 [-0.0600; 0.0071]	NA[a]	-0.0548** [-0.0896; -0.0189]	-0.0450** [-0.0795; -0.0122]	-0.0353* [-0.0700; -0.0027]	54.28%	44.38%	29.01%
	Step Count	-0.0234** [-0.0427; -0.0117]	-0.0172*** [-0.0286; -0.0096]	-0.0109*** [-0.0181; -0.0051]	-0.0873* [-0.1535; -0.0104]	-0.0464* [-0.0865; -0.0054]	-0.0056 [-0.0378; 0.0273]	-0.1108** [-0.1753; -0.0317]	-0.0636** [-0.1001; -0.0197]	-0.0165 [-0.0492; 0.0147]	21.17%	27.00%	66.22%
	% MVPA	-0.0217** [-0.0396; -0.0102]	-0.0160*** [-0.0267; -0.0084]	-0.0103** [-0.0174; -0.0047]	-0.0796* [-0.1439; -0.0037]	-0.0423 [-0.0813; 0.0028]	-0.0050 [-0.0360; 0.0290]	-0.1013** [-0.1616; -0.0264]	-0.0583** [-0.0936; -0.0106]	-0.0152 [-0.0454; 0.0186]	21.42%	27.44%	67.76%
	% Light Intensity	-0.0249** [-0.0461; -0.0120]	-0.0146*** [-0.0249; -0.0078]	-0.0043 [-0.0122; 0.0024]	NA[a]	-0.0140 [-0.0492; 0.0208]	NA[a]	-0.0389* [-0.0729; -0.0009]	-0.0286 [-0.0627; 0.0062]	-0.0183 [-0.0535; 0.0166]	64.06%	51.10%	23.51%
	% Sedentary	0.0046 [-0.0024; 0.0125]	0.0165*** [0.0092; 0.0269]	0.0284** [0.0153; 0.0508]	NA[a]	0.0194 [-0.0160; 0.0558]	NA[a]	0.0240 [-0.0112; 0.0620]	0.0359* [0.0016; 0.0720]	0.0478* [0.0121; 0.0835]	19.00%	45.94%	59.43%
ESC SCORE2	MET-Hours	-0.0235*** [-0.0380; -0.0132]	-0.0182*** [-0.0270; -0.0118]	-0.0130*** [-0.0190; -0.0078]	-0.1105** [-0.1836; -0.0299]	-0.0429* [-0.0841; -0.0018]	0.0247 [-0.0258; 0.0768]	-0.1340*** [-0.2087; -0.0529]	-0.0611** [-0.1019; -0.0192]	0.0117 [-0.0393; 0.0605]	17.55%	29.84%	NA[b]
	Step Count	-0.0235*** [-0.0384; -0.0132]	-0.0179*** [-0.0270; -0.0112]	-0.0123*** [-0.018; -0.0074]	-0.1359*** [-0.2050; -0.0568]	-0.0617** [-0.0994; -0.0173]	0.0124 [-0.0338; 0.0553]	-0.1594*** [-0.2288; -0.0805]	-0.0797*** [-0.1195; -0.0356]	0.0004 [-0.0450; 0.0418]	14.77%	22.51%	NA[b]
	% MVPA	-0.0215*** [-0.0359; -0.0118]	-0.0162*** [-0.0248; -0.0098]	-0.0109*** [-0.0165; -0.0061]	-0.1237** [-0.1931; -0.0458]	-0.0547* [-0.0934; -0.0123]	0.0144 [-0.0322; 0.0579]	-0.1452*** [-0.2133; -0.0657]	-0.0709*** [-0.1098; -0.0276]	0.0035 [-0.0421; 0.0478]	14.83%	22.87%	NA[b]
	% Light Intensity	-0.0252*** [-0.0398; -0.0146]	-0.0173*** [-0.0261; -0.0112]	-0.0094** [-0.0164; -0.0039]	NA[a]	-0.0092 [-0.0424; 0.0263]	NA[a]	-0.0344 [-0.0715; 0.0020]	-0.0265 [-0.0606; 0.0091]	-0.0186 [-0.0527; 0.0172]	73.17%	65.20%	50.48%
	% Sedentary	0.0095** [0.0040; 0.0165]	0.0192*** [0.0126; 0.0279]	0.0288*** [0.0167; 0.0455]	NA[a]	0.0140 [-0.0234; 0.0480]	NA[a]	-0.0235 [-0.0133; 0.0578]	0.0332 [-0.0024; 0.0673]	0.0428* [0.0074; 0.0798]	40.45%	57.77%	67.28%

Table 4. [continued]

Mediator	Predictor	Indirect effect			Direct effect			Total effect			Percentage mediated		
		-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD [95% CI]	Average [95% CI]	+1 SD [95% CI]	-1 SD	Average	+1 SD
ASCVD Score	MET-Hours	NA[a]	-0.0035* [-0.0082; -0.0010]	NA	-0.1296*** [-0.2036; -0.0510]	-0.0561** [-0.0968; -0.0116]	0.0175 [-0.0272; 0.0686]	-0.1331*** [-0.2072; -0.0550]	-0.0596** [-0.0998; -0.0155]	0.0140 [-0.0307; 0.0650]	2.64%	5.89%	NA[b]
	Step Count	-0.0066 [-0.0157; -0.0016]	-0.0047* [-0.0105; -0.0012]	-0.0028 [-0.0068; -0.0008]	-0.1518*** [-0.2206; -0.0765]	-0.0735*** [-0.1124; -0.0292]	0.0048 [-0.0373; 0.0497]	-0.1585*** [-0.2279; -0.0848]	-0.0783*** [-0.1165; -0.0339]	0.0019 [-0.0402; 0.0472]	4.19%	6.04%	NA[b]
	% MVPA	NA[a]	-0.0035* [-0.0080; -0.0009]	NA	-0.1386*** [-0.2135; -0.0673]	-0.0656** [-0.1039; -0.0214]	0.0073 [-0.0344; 0.0561]	-0.1421*** [-0.2180; -0.0725]	-0.0691*** [-0.1082; -0.0249]	0.0038 [-0.0393; 0.0508]	2.47%	5.07%	NA[b]
	% Light Intensity	NA[a]	-0.0015 [-0.0047; 0.0003]	NA	NA	-0.0204 [-0.0545; 0.0130]	NA	NA	-0.0219 [-0.0561; 0.0120]	NA	NA[a]	6.85%	NA[a]
	% Sedentary	NA[a]	0.0024 [0.0004; 0.0063]	NA	-0.0216 [-0.0747; 0.0309]	0.0360 [-0.0018; 0.0737]	0.0936* [0.0183; 0.1701]	-0.0193 [-0.0732; 0.0331]	0.0384* [0.0007; 0.0763]	0.0960* [0.0213; 0.1746]	NA[b]	6.14%	2.50%

Significance levels: *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.

[a]) Models with only linear effect.

[b]) Models with opposite direct and indirect effects.

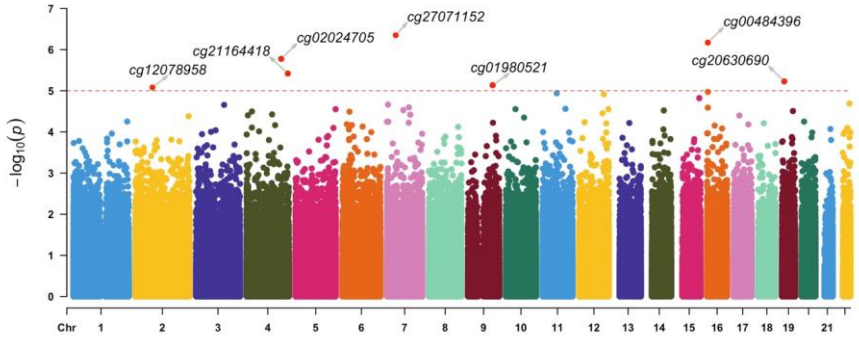
Abbreviations: Factor Analysis for Mixed Data (FAMD), Metabolic-Equivalent Hours (MET-Hours), %Moderate-to-Vigorous Physical Activity (MVPA), Standard Deviation (SD), 95% Confidence Interval (95% CI)

EWAS of physical activity and functional analyses

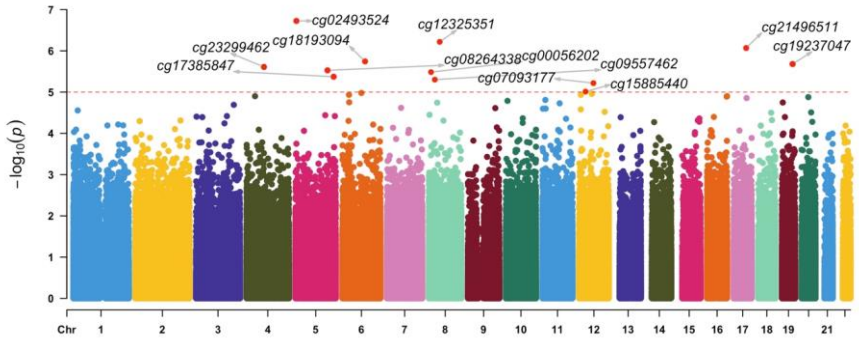
At the nominally significant threshold (defined as $p < 1x10^{-5}$), we identified 7 CpGs associated with %time spent in MVPA (Figure 3a & Table S4a), and 12 CpGs associated with MET-Hours (Figure 3b & Table S4b). However, we did not discover any epigenome-wide significant CpGs (all FDR > 0.05). We did not identify any genomic inflation of the test statistics (Figure S7).

Figure 3. EWAS results of physical activity components.

a.



b.



Manhatten plots of the epigenome-wide association study (EWAS) results for (a) % average daily time spent in moderate-to-vigorous activities and (b) average daily energy expenditure in MET-Hours. The x-axis depicts sites ordered by chromosomal position with the respective -log10 p-value on the y-axis. The horizontal lines represent the level of significance, with the red horizontal dashed line at the norminal significant level (p-value p < 1E-05).

We performed lookup analyses of all the CpG sites and the mapped genes for each of the CpGs, showing an association with %MVPA and MET-Hours at the nominally significant level using the EWAS Catalog (http://ewascatalog.org/), EWAS Atlas (https://ngdc.cncb.ac.cn/ewas/atlas) and GWAS catalog (https://www.ebi.ac.uk/gwas/)

(Table S4a). %MVPA and MET-Hours associated CpGs have been previously linked to immune function (i.e. CD24 on IgD+ CD24+ B cell, CD24 on memory B cell, blood cell counts), cardiometabolic traits (i.e. waist circumference, body mass index (BMI), blood pressure, QT interval, ischemic stroke, myocardial infarction) and other ageing-related traits (i.e. cognitive function, neuritic plaques, white matter hyperintensities, type 2 diabetes). Of note, cg18193094, positionally mapped to the glutamate ionotropic recepter kainite type subunit 2 (*GRIK2*) gene, which has previously been linked to inter-individual differences in heart rate increase and recovery during and after exercise (Verweij, Van De Vegte, & Van Der Harst, 2018).

Over-representation analysis using Webgestalt GWAS catalog (https://www.ebi.ac.uk/gwas/) based on the nearest genes of the CpG sites showing an association with energy expenditure and %MVPA at a nominally significant level did not yield significant Gene Ontology (GO) terms after adjustment of multiple comparisons (data not shown). However, gene set enrichment analysis using clusterProfiler (T. Wu et al., 2021) based on a significance-ranked list of all genes (i.e. from the lowest to the highest p-value of the corresponding CpG site), identified a large number of GO terms related to biological processes, molecular function and cellular components (FDR < 0.05). We observed that the identified genes were particularly enriched in pathways related to regulation of vascular endothelial function, cell proliferation, interaction and signalling and sensory development and perception (Table S5 & Table S6).

To assess potential genetic confounding, we used mQTLdb to examine whether there were previously identified methylation quantitative trait loci (mQTLs) for the CpGs that were found to be associated with MVPA and MET-Hours (Gaunt et al., 2016). There were no mQTLs for 5 CpGs that were associated with MVPA, but several mQTLs were previously identified for cg27071152 and cg00484396. We also found several mQTLs for cg19237047, cg17385847 and cg09557462 that were associated with MET-Hours, but not for the other 9 CpGs (Table S4). These mQTLs might have an impact on the methylation levels of these CpGs.

We performed additional sensitivity analyses to assess whether BMI modifies the association between physical activity and methylation levels of the nominally significant CpGs. We found that although the mediation estimates became smaller after BMI adjustment, they remained directionally consistent (Table S7). We did not find any

interaction effects between cell types and methylation levels of the nominally significant CpGs on physical activity levels (all p values for the interaction terms > 0.05).

Discussion

We found that higher levels of accelerometer-assessed physical activity were associated with slower epigenetic ageing in the general population, with similar effects in both men and women. Health benefits from engaging in physical activity are well-documented (World Health Organization, 2020). Lifestyle interventions incorporating physical activity may thus be cost-effective and easily implementable measures to slow down age-associated functional decline as reflected in decelerated epigenetic ageing. Importantly, both the absolute and relative effects of physical activity dose and intensity on epigenetic ageing were most pronounced at the lower end of the activity spectrum. Our findings therefore indicate that health benefits of additional physical activity are likely to be greatest in adults leading a sedentary lifestyle.

While previous studies predominantly relied on self-reported physical activity levels, to the best of our knowledge, our study is the first to assess the effects of detailed accelerometer-derived physical activity components on epigenetic ageing in adults over a wide age range. In line with previous studies (Kresovich et al., 2021; A. T. Lu et al., 2019), we observed that higher physical activity levels are associated with slower GrimAge acceleration. Specifically, we found slower epigenetic ageing to be related to higher energy expenditure, step counts and more time spent in moderate-to-vigorous intensity activities. In individuals without a history of cardiovascular disease, also more time spent in light intensity activities was associated with slower GrimAge acceleration, whereas longer sedentary time was associated with faster epigenetic ageing. Nonetheless, when considering different levels of physical activity intensity, also in this subset of individuals moderate-to-vigorous intensity physical activity had the strongest effect on epigenetic ageing. Our findings imply that all levels of physical activity may be effective for the prevention of cardiovascular disease, although moderate-to-vigorous intensity activities may yield the strongest benefits.

In further exploratory analyses, we examined whether various physical activity components are also associated with other epigenetic clocks such as PhenoAge, Hannum’s and Horvath’s age. We only observed an association between physical

activity and second-generation (GrimAge and PhenoAge acceleration), but not first-generation clocks (Hannum's and Horvath's age acceleration), which has also been reported in a recent systematic review (Oblak et al., 2021). Slower PhenoAge acceleration was associated with higher energy expenditure, step counts and more time spent in moderate-to-vigorous activites. We found the strongest association between physical activity and GrimAge acceleration, which has been hypothesized to be the prime indicator of age-associated functional decline across all epigenetic clocks (A. T. Lu et al., 2019; McCrory et al., 2021).

The molecular pathways through which physical activity affects epigenetic ageing are thus far poorly understood. To explore the effects of physical activity on methylation status directly, we conducted an epigenome-wide association study across 850,000 CpG sites. We found methylation status of several CpG sites to show a nominal significant association with time spent in moderate-to-vigorous physical activity (n= 7 CpGs) and MET-Hours (n = 12 CpGs). Interestingly, the nearest gene to one of these CpGs, *GRIK2*, was also associated with heart rate response and recovery after exercise in a previous study (Verweij et al., 2018), while mutations in *GRIK2* are known to cause several autosomal recessive forms of intellectual disability (https://omim.org/entry/138244). Gene enrichment analysis of the nearest genes across the CpG sites demonstrated that genes, whose methylation levels were most strongly associated with physical activity, were enriched in pathways related to regulation of vascular endothelial function as well as pathways related to nervous system function and health (including synaptic signalling, sensory development and perception, as well as cell proliferation and interaction). These results thus suggest that physical activity may particularly affect neuronal signalling, thereby pointing to a novel molecular basis for previous findings suggesting that physical activity is beneficial for brain function (Silverman & Deuster, 2014; World Health Organization, 2020), an effect that is particularly evident in brain regions with a high oxidative demand (Fox et al., 2022). Larger EWAS of physical activity are required for the identification of additional molecular mechanisms through which physical activity exerts its beneficial health effects.

Several potential pathways have been suggested through which physical activity could exert its positive effects on health. Exercise has been found to minimize inflammation symptoms and oxidative stress and to promote neuroplasticity and growth factor

expression (Silverman & Deuster, 2014). It is noteworthy that the negative effects of exercise on inflammatory biomarkers such as adipokines (e.g. interleukin-6, tumor necrosis factor alpha) and the stimulating effects of exercise on cortisol and adrenaline secretion are most pronounced for prolonged and high intensity exercise bouts (Gleeson et al., 2011; Peake et al., 2005). It has been suggested that the anti-inflammatory effects of exercise could alter the epigenome and a few small-scale studies have indeed linked exercise to the methylation status of genes linked to inflammation, tumor growth and neuroplasticity (Ferioli et al., 2019). In this study we found that sample-based immune function composites, which were mainly influenced by naïve B cells, CD4T+ T cells and CD8T+ T cells, partially mediated the effect of physical activity on epigenetic ageing. However, it should be noted that the mediated proportion was small. The reduction of naïve cells and accumulation of activated immune T cells is a well-established feature of ageing and immunosenescence. Previous studies suggest that the activation of T and NK cells could be major contributors to epigenetic ageing (Jonkman et al., 2022), whereas physical activity could lead to an increased mobilization of T and NK cells and a decreased accumulation of senescent T cells (Duggal et al., 2019). Our findings thus indicate that particularly regular exercise with a moderate-to-high intensity level may lead to long lasting changes of the epigenome and reduce epigenetic ageing. The beneficial effects of physical activity on epigenetic ageing could be, in part, ascribed to its positive effects on immunosenenscence. However, further experimental studies are needed to study the immunological mechanisms through which physical activity could act upon epigenetic ageing.

Here we also present the first study assessing whether well-established cardiovascular risk factors could mediate the relation between physical activity and epigenetic ageing. Accelerated epigenetic ageing has been associated with a higher cardiovascular disease risk (Joyce et al., 2021). Several studies have found that the effects of physical activity on cardiovascular disease risk is closely linked to DNA methylation changes (Ferrari et al., 2019; Sellami, Bragazzi, Prince, Denham, & Elrayess, 2021). Indeed, we found that the Framingham Risk Score, which is based on the most important cardiovascular risk factors, the Assessment of Cardiovascular Disease (ASCVD) Risk Score, the most recently published guideline to assess cardiovascular risk by the American College of Cardiology, and the Euopean Society of Cardiology Score (ESC

SCORE2), which is scaled to reflect country-specific cardiovascular risk, partially mediated the association between physical activity and epigenetic ageing. To dissect the relative mediation effects of different cardiovascular risk factors, we also created data-driven sample-specific cardiovascular summary measures. Our first sample-based cardiovascular risk component, which primarily captured the combined effects of blood pressure, adiposity markers and triglyceride levels, partially mediated the relation between physical activity and epigenetic ageing. In contrast, the second sample-based cardiovascular component, which primarily captured the effects of lipoprotein levels, did not show a mediation effect. Therefore, our findings indicate that the effects of physical activity on health are preferentially mediated through specific cardiovascular risk factors, especially blood pressure, adiposity and triglyceride levels.

Our findings substantially extend those of previous studies reporting beneficial effects of physical activity in the prevention and treatment of cardiovascular diseases, and suggest that targeting hypertension, hypertriglyceridemia and adiposity may be particularly effective in counteracting cardiovascular ageing (Eriksson, Taimela, & Koivisto, 1997). Blood pressure, triglycerides and adiposity markers have all been found to causally affect methylation status (Mendelson et al., 2017; Richard et al., 2017; Wahl et al., 2017), and several adiposity-related traits have been associated with faster GrimAge acceleration (McCartney et al., 2021). Compared to the Framingham Heart Study sample (D'Agostino et al., 2008), our participants were on average slightly older, were more often treated for hypertension and had lower total cholesterol, but higher HDL levels. Our sample also included more non-smokers. Given that the Framingham Risk Score weighs hypertension markers and age more than cholesterol levels, this might offer an explanation why we observed a mediation effect of the Framingham Risk Score and the first, but not the second, FAMD cardiovascular component. Nonetheless, further studies are warranted to elucidate the molecular underpinnings of the effects of physical activity on hypertension and adiposity markers, as well as their influence on epigenetic ageing.

Limitations

Several limitations of this study should be noted. First, given the cross-sectional nature of this study, we could not assess the causality of the association between physical activity and epigenetic ageing. Second, actimetry recordings were conducted outside of the laboratory and in the participants' normal environment. Even though participants'

daily activities were recorded during regular activity weeks, participants may have consciously or unconsciously changed their activity pattern during this recording period. Third, while the activPAL accelerometer is highly accurate in identifying changes in postures and intensity categories, distinguishing been different postures, such as car driving versus taking steps, might sometimes be unreliable since the device is attached to the thigh; however, through rigorous quality control of the data, we were able to exclude unreliable measurements. Furthermore, classification of moderate versus high intensity activities with the activPAL proprietary software can be imprecise as the algorithm linearly classifies energy expenditure based on cadence. To reach the classification threshold of vigorous intensity (typically defined as ≥ 6 METs), a minimum cadence of 240 steps/min would be required. However, even athletes rarely reach a cadence of more than 200 steps/min for prolonged periods of time. Fourth, it cannot be ruled out that our findings were partly influenced by selection bias as participants of our study had relatively high education and physical activity levels; however, if anything, this is likely to have resulted in an underestimation of the effects of physical activity on epigenetic ageing.

Conclusion

In conclusion, we demonstrated that higher accelerometer-assed physical activity levels are associated with slower epigenetic ageing in adults across a wide age range. We observed that the effect of physical activity on epigenetic ageing can largely be attributed to its beneficial effects on cardiovascular health and immune function. Given the expected rapid rise in the prevalence of cardiovascular diseases, exercise regimens focusing on moderate-to-high intensity activities could serve as inexpensive, easily actionable and effective preventive lifestyle interventions. Particularly adults leading a sedentary lifestyle may profit from engaging in additional exercise.

Experimental Procedures

Study Population

Our analysis was based on cross-sectional baseline data from the first 5,000 participants of the Rhineland Study (age range = 30–94 years), an ongoing population-based prospective cohort study (Fox et al., 2022). Invitations to participate in the Rhineland Study are send to inhabitants of two distinct municipal districts in Bonn,

Germany, who are 30 years or older. To participate, invitees are required to have a sufficient command of the German language to provide informed consent. Participants complete multiple assessments including questionnaires, blood collection, anthropometric and cardiovascular measurements, and accelerometer attachment. The study was approved by the ethics committee of the University of Bonn, Medical Faculty, and is carried out according to the principles of the Declaration of Helsinki.

In this study, we analysed data of 3,567 eligible individuals out of the first 5,000 participants of the Rhineland Study (Figure S6). Actimetry recordings were not available for 1005 participants due to the following reasons: refusal to participate (n = 71), technical/acquisition failure (n = 187) or ineligibility (n = 747). Participants were deemed ineligible if they were unable to stand or walk, had an unrepresentative physical activity week and/or were allergic to medical adhesives. Representativeness of the physical activity week was established based on self-reports. Participants were asked to judge whether they anticipated having a regular, representative activity and rest pattern during the recording time. Reasons for an unrepresentative physical activity week included vacation, untypical work trips, surgery or hospital stays, which could result in shifted and unrepresentative activity and rest patterns. In addition, we also excluded 122 participants with less than 5 valid recording days to achieve reliable physical activity estimates (Aguilar-Farias, Martino-Fuentealba, Salom-Diaz, & Brown, 2019). We classified and excluded recording days as invalid based on Winkler and colleagues' proposed criteria: <500 steps/day, ≥95% time spent in one posture, and <10 hours estimated waking wear time (Winkler et al., 2016). We visually checked heatmaps of included and excluded recordings and wear diaries to avoid incorrect exclusion. Using a modified z-score of 3.5, we identified potential outliers and after visual inspection excluded 5 participants with erroneous actimetry recordings. Furthermore, we excluded 60 participants with missing covariate data and 239 participants with missing epigenetic clock data. For the mediation analysis, we additionally excluded 210 participants with missing cardiovascular data.

Physical Activity

We used the activPAL3 micro (PAL Technologies, Glasgow, UK) to measure physical activity intensity, step counts and energy expenditure continuously across seven consecutive days. We processed raw data using the proprietary activPAL software suite. Based on a customized version of the "activpalProcessing" package in R version

3.6.3 (The R Foundation), we extracted information on physical activity dose (energy expenditure, step counts) and intensity (sedentary, light intensity, moderate-to-vigorous intensity) (Lyden, Keadle, Staudenmayer, & Freedson, 2017). We calculated weighted daily averages for all physical activity variables, which were adjusted for accelerometer wear time per day. Physical activity dose variables included average weighted daily step counts and average weighted daily energy expenditure as reflected in metabolic equivalents (METs) per hour. We defined physical activity intensity based on posture and energy expenditure across time: average daily % time spent in sedentary (sitting/lying posture), light-intensity (standing or step-taking posture and METs < 3.0) or moderate-to-vigorous (standing or step-taking posture and METS ≥ 3.0) activities. Further details on the physical activity assessment have been described previously (Fox et al., 2022).

DNA methylation quantification

Genomic DNA was extracted from buffy coat fractions of anti-coagulated blood samples using Chemagic DNA buffy coat kit (PerkinElmer, Germany), and was subsequently bisulfite converted using the DNA methylation kit according to the manufacturer's instructions. DNA methylation levels were measured using Illumina's Human MethylationEPIC BeadChip. The methylation level for each probe was derived as a beta value representing the fractional level of DNA methylation at that probe. Sample-level and probe-level quality control was performed using the 'minfi' package in R (version 3.5.0) (Fortin, Triche, & Hansen, 2017). Samples with sex mismatch or a missing rate at >1% across all probes were excluded. Probes with a missing rate >1% (at a detection p-value >0.01) were also excluded following previously published recommendation guidelines for analyzing methylation data (M. C. Wu & Kuan, 2018).

Estimation of epigenetic age acceleration

DNAm Hannum age, Horvath age, PhenoAge and GrimAge was calculated as described previously (Hannum et al., 2013; Horvath, 2013; Levine et al., 2018; A. T. Lu et al., 2019). The age acceleration estimators were defined as the residuals (in years) that result from regressing the DNAm age estimates on chronological age.

DNA methylation-based immunue phenotyping

Based on DNA methylation levels, the relative proportion of twelve leukocyte subtypes (basophils, eosinophils, neutrophils, monocytes, naïve B cellls, memory B cells, naïve

CD4T cells, memory CD4T cells, regulatory T cells, naïve CD8T cells, memory CD8T cells and natural killer cells) was derived using the "FlowSorted.BloodExtended.EPIC" package in R (version 3.6.3, The R Foundation), which is based on a reference-based deconvolution method described by Salas and colleagues (2022). We created sample-based immune profile composites based on principle component analysis of all leukocyte variables, using the first two principle components (Figure S3).

Covariates

The International Standard Classification of Education 2011 (ISCED) was used to categorize participants' highest education levels as low (lower secondary education or below), middle (upper secondary education to undergraduate university level) and high (postgraduate university study). We determined participants' age, sex, smoking status (smokers vs. non-smokers) and diabetes status (diabetic vs. non-diabetic) based on self-report. Missing smoking values were imputed based on cotinine metabolite levels. Individuals with a cotinine level exceeding the non-smoker sample-defined 97.5 percentile were classified as smokers. We derived the season of the actimetry recording based on the dates of the recordings. Olfactory performance was assessed with the 12-item Sniffin' Stick odour identification test and defined as the total number of correctly identified pens (R. Lu, Aziz, Reuter, Stöcker, & Breteler, 2021). Anthropometric examinations were performed using a SECA 285 measuring station and SECA 201 measuring tape. Waist-to-hip ratio (WHR) was calculated as a ratio of waist circumference to hip circumference and BMI was calculated as weight [kg]/(height [m])^2. Serum levels of cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides were measured using routine methods at the Clinical Chemistry Laboratory of University Hospital Bonn. Resting blood pressure was measured three times with 10-minute intervals and average systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated using the last two measured values. Hypertension was based on regular use of antihypertensive medication, average SBP (≥ 140mm Hg) and average DBP (≥ 90mm Hg). A cardiovascular event was defined based on self-reported medical history of myocardial infarction, coronary artery disease, transient ischemic attack (TIA), cardiac insufficiency, peripheral arterial disease, pacemaker placement, stroke, aortic surgery, carotid artery surgery and peripheral artery surgery.

Cardiovascular disease risk score

Based on previously published algorithms, 10-year cardiovascular disease risk was calculated based on the Framingham Risk Score, the European Society of Cardiology Score (ESC SCORE2) and the Assessment of Cardiovascular Disease (ASCVD) Risk Score (D'Agostino et al., 2008; Goff et al., 2014; Hageman et al., 2021). In addition, we created a sample-based cardiovascular risk component score based on age, waist-to-hip ratio, cholesterol, HDL, LDL, triglycerides and insulin levels, smoking, hypertension and diabetes status as well as SBP and DBP, using factor analysis for mixed data (FAMD) as implemented in the 'FactoMineR' package in R (version 3.6.3, The R Foundation) (Lê et al., 2008). We extracted the first two components as summary measures for our sample-based cardiovascular risk score (Figure S3).

Statistical Analysis

Statistical analyses were performed in R (version 3.6.3, The R Foundation). In the sample demographics, we present mean and standard deviation (SD) for continuous variables and number and percentage for categorical variables. We assessed differences between included and excluded participants using binomial logistic regression adjusted for age and sex.

To examine the association between physical activity (independent variable) and epigenetic age acceleration (outcome), we used multivariable (polynomial) regression models. We tested for potential non-linear effects of physical activity on epigenetic ageing by including a quadratic term for physical activity. In addition, we also assessed whether the effects of physical activity on epigenetic age acceleration differed between men and women by including interaction terms between physical activity and sex. To account for residual confounding, we adjusted the models for age, age^2 as well as for batch effect, cell proportions (CD4T+ T cells, CD8T+ T cells, neutrophils, monocytes and granulocytes), sex, education and smoking status. To allow comparison of effect sizes, we z-standardised all continuous independent variables. Effect estimates are presented with the corresponding two-sided 95% confidence intervals. The threshold for statistical significance was set at $p \leq 0.05$. Visual inspection of the distribution of residuals was performed to evaluate whether model assumptions were met.

In addition, we performed two sensitivity analyses: 1) We re-ran the polynomial regression models excluding data points with a high leverage (i.e. those observations

with values of the independent variables far from those of other independent variables, defined as a hat value exceeding 3 times the average), and 2) We also assessed whether the association between physical activity and epigenetic ageing changed when excluding participants with a prior cardiovascular event.

In follow-up analysis, we wanted to examine whether the association between physical activity (independent variable) and GrimAge acceleration (outcome) was mediated through immune function and cardiovascular disease risk (mediators). We performed structural equation modelling using the 'lavaan' package in R (version 3.6.3, The R Foundation) (Rosseel, 2012). GrimAge acceleration estimates were adjusted for batch effects and cell proportions, while the mediation models were additionally adjusted for age, age^2, sex, season and education. Given the non-linear effect of physical activity on cardiovascular disease risk and epigenetic ageing, we present estimates at the average, 1 SD below and 1 SD above mean physical activity dose and intensity. Quadratic physical activity terms were only added to the final models if they were statistically significant ($p \leq 0.05$). In a sensitivity analysis, we re-examined the mediation effect through cardiovascular disease risk factors while excluding individuals with a prior cardiovascular event. In addition, to test the specificity of the mediation effects, we performed mediation analyses examining whether the effects of physical activity on epigenetic ageing could also be mediated through olfactory performance (negative control variable).

Epigenome-wide association study (EWAS) of physical activity and functional analyses
We examined the association between physical activity components (independent variables) and DNA methylation level (outcome) using multiple linear regression, while adjusting for age, sex, batch effects, blood cell proportion, the first ten genetic principal components (to account for population stratification), and smoking. As our initial analyses suggested relatively high genomic inflation for the MVPA EWAS, we restricted the EWAS analyses to participants from Caucasian descent (n = 3159) which resolved this issue (Figure S7). FDR-adjustment was applied to account for multiple comparisons: FDR adjusted $q < 0.05$ was considered as epigenome-wide significant, while $p < 1E\text{-}05$ was considered to indicate nominal significance.

We looked up CpGs showing associations with physical activity components at a nominally significant level using the EWAS Catalog (http://ewascatalog.org/, downloaded on 16.02.2023) and EWAS Atlas (https://ngdc.cncb.ac.cn/ewas/atlas,

downloaded on 16.02.2023). We also performed a look-up of known associations of the mapped gene for each CpG in previously published GWAS using the GWAS catalog (https://www.ebi.ac.uk/gwas/, downloaded on 16.02.2023). We conducted further gene set enrichment analysis with the WebGestalt (Liao, Wang, Jaehnig, Shi, & Zhang, 2019) and ClusterProfiler (T. Wu et al., 2021). We summarized the results of the latter using the rrvgo R package (Sayols, 2020). We examined whether there were methylation quantitative trait loci (mQTLs) for the CpGs that were associated with MVPA and MET-Hours using mQTLdb (Gaunt et al., 2016).

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3.3 25-hydroxyvitamin D level is associated with greater grip strength across adult lifespan: a population-based cohort study

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Abstract

Objective

Maintaining muscle function throughout life is critical for healthy ageing. Although *in vitro* studies consistently indicate beneficial effects of 25-hydroxyvitamin D (25-OHD) on muscle function, findings from population-based studies remain inconclusive. We therefore aimed to examine the association between 25-OHD concentration and handgrip strength across a wide age range, and assess potential modifying effects of age, sex and season.

Methods

We analysed cross-sectional baseline data of 2,576 eligible participants out of the first 3,000 participants (recruited from March 2016 to March 2019) of the Rhineland Study, a community-based cohort study in Bonn, Germany. Multivariate linear regression models were used to assess the relation between 25-OHD levels and grip strength, while adjusting for age, sex, education, smoking, season, body mass index and vitamin D supplementation.

Results

Compared to participants with deficient 25-OHD levels (<30 nmol/L), grip strength was higher in those with inadequate (30 to <50 nmol/L) and adequate (≥50 to ≤125 nmol/L) levels ($ß_{inadequate}$=1.222 [95%CI: 0.377; 2.067], p=0.005; $ß_{adequate}$=1.228 [95%CI: 0.437; 2.019], p=0.002). Modeling on a continuous scale revealed grip strength to increase with higher 25-OHD levels up to ~100 nmol/L, after which the direction reversed ($ß_{linear}$=0.505, [95%CI: 0.179; 0.830], p=0.002; $ß_{quadratic}$=-0.153 [95%CI: -0.269; -0.038], p=0.009). Older adults showed weaker effects of 25-OHD levels on grip strength than younger adults ($ß_{25OHDxAge}$=-0.309, [95%CI: -0.594; -0.024], p=0.033).

Conclusions

Our findings highlight the importance of sufficient 25-OHD levels for optimal muscle function across the adult lifespan. However, vitamin D supplementation should be closely monitored to avoid detrimental effects.

Keywords: vitamin D, grip strength, sarcopenia, muscle strength, cohort study, ageing

Introduction

Maintaining muscle function throughout life is critical for healthy ageing (1). Progressive loss of muscle mass and function with age is a feature of primary sarcopenia and negatively affects mobility, functional independence and quality of life (2). It also increases the risk of falls and leads to higher healthcare costs and mortality risk (3, 4, 5). Primary sarcopenia has been estimated to affect between 6% and 22% of older adults, making it a major public health burden (5). Its onset may be as early as young adulthood (6). One of the diagnostic criteria of sarcopenia is decreased handgrip strength, a reliable proxy of overall muscle function, which can be measured easily and objectively (4, 5).

Both human and animal *in vitro* studies have shown that vitamin D can modulate skeletal muscle cell function (7). Muscle tissue expresses vitamin D receptors and two biological pathways have been identified by which 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), the biologically active form of vitamin D, could act on muscle tissue: The first pathway activates gene transcription and subsequent protein synthesis, which improves muscle function and structure and promotes cell differentiation and proliferation of type 2 muscle fibres (7, 8, 9). The second pathway has been hypothesized to rely on second-messenger pathways and membrane receptors, which are activated by $1,25(OH)_2D_3$. This could lead to rapid calcium influx and uptake and affect muscle contraction (7, 9, 10). Given the high prevalence of vitamin D deficiency (11), optimizing vitamin D levels may be an easily actionable and cost-effective preventive and curative approach against sarcopenia and age-associated decline of muscle strength.

Although *in vitro* experiments indicate a causal relationship between low vitamin D levels and decreased muscle function (7, 8, 10), findings from observational studies have been inconsistent (12, 13, 14, 15, 16). While a previous cohort study found higher vitamin D levels to be related to greater upper arm strength, but not grip strength (13), other studies observed positive effects on grip strength, although exclusively in men (15, 17), women (18) and/or older adults (15, 19, 20, 21). Discrepancies in previous findings may be attributed to small sample sizes, usually restricted to a specific age or patient group, ethnicity or sex, and residual confounding due to, for example, lack of information on season of measurement and vitamin D supplementation.

While the association between vitamin D levels and grip strength in adults above 65 years of age and athletes has been extensively studied (12, 22), few studies have assessed this relation in young and middle-aged adults. The aim of this study was therefore to examine the relation between serum 25-hydroxyvitamin D (25-OHD) concentration and handgrip strength in adults across a wide age range in a population-based cohort study. In addition, we aimed to assess whether the association differed between men and women, across age and between seasons.

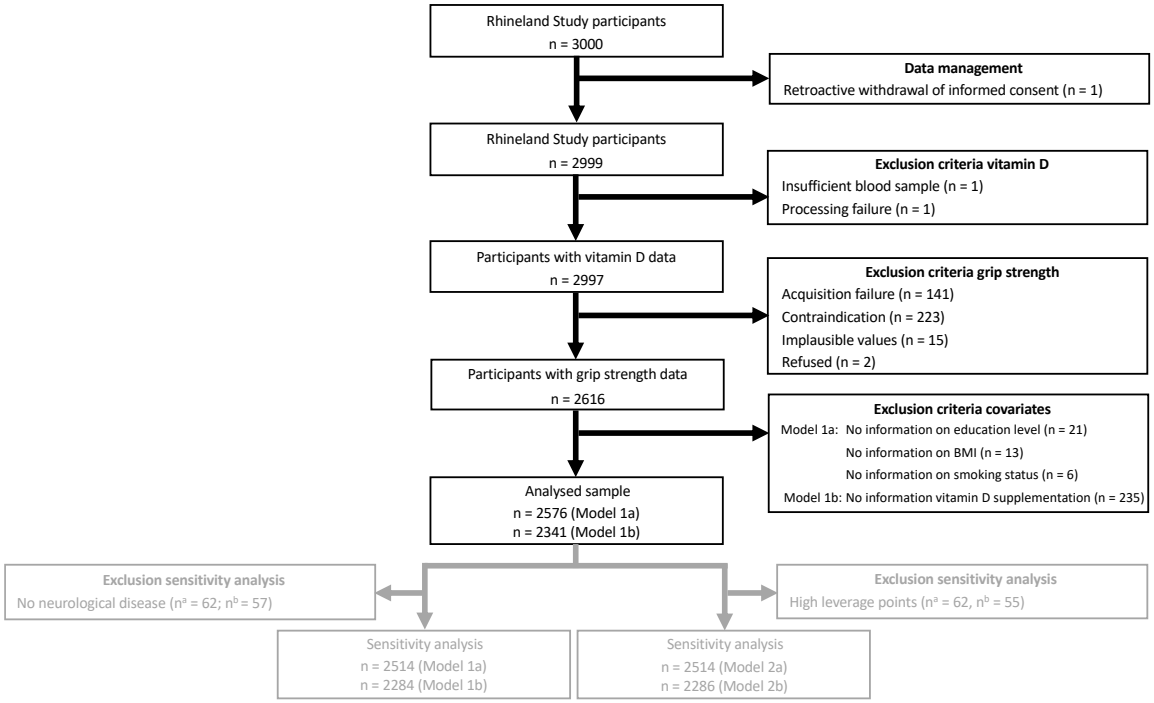
Methods

Study participants

This study used cross-sectional baseline data from the first 3,000 participants (age range: 30 – 95 years) of the Rhineland Study, an ongoing population-based cohort study in Bonn, Germany (23). Participants were recruited from March 2016 to March 2019. They are required to be at least 30 years old and to have a sufficient command of the German language to provide informed consent. Participants complete comprehensive health assessments including anthropometric and cardiovascular measurements, structured interviews as well as physical activity and fitness recordings. No financial incentives were offered for study participation. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Medical Ethics Committee of the University of Bonn.

Our analytical sample consisted of 2,576 participants out of the first 3,000 participants (Figure 1). Serum 25-OHD data of two participants was missing due to acquisition and processing failures, while 381 participants had no grip strength data due to the following reasons: refusal to participate (n = 2), technical/acquisition failure (n = 141) or ineligibility (n = 223). Participants were deemed ineligible if they had an amputation or a fracture of the tested arm within the last month. They were also excluded from participation if they were currently suffering from pain in the tested limb, or if pain could be induced by exerting force. In addition, we excluded 15 participants with implausible, and therefore likely erroneous, grip strength values. Lastly, we excluded participants with missing covariate data: for model 1a, we excluded 40 participants with missing body mass index (BMI), education and smoking data; for model 1b, we additionally excluded 235 participants with missing vitamin D supplementation data.

Figure 1. Recruitment flowchart.



Vitamin D measurements

Venous blood was withdrawn after overnight fasting. Blood samples were collected in tubes (S-Monovetten 7.5ml tubes with coagulation), and kept at room temperature for 30 minutes for clotting to occur, followed by centrifugation for 15 minutes at 2000xg at 4ºC. Samples were automatically divided into aliquots (500 µL each) in FluidX 0.7mL tubes and frozen at -80°C. Serum 25-OHD concentrations were assessed using the non-competitive chemiluminescent enzyme immunoassay (Lumipulse, FujiRebio, Ghent, Belgium) as described previously (24). For this assay, the lower limit of detection was 10 nmol/L. To account for values below the detection threshold (1.7 % of observations), we set those values to a constant of 7, which was computed by dividing the lower limit of detection by the square root of two (25).

Handgrip strength assessment

Handgrip strength was measured using the handheld Jamar Plus Digital Dynamometer (Patterson Medical, USA) according to ASHT and Southampton protocol recommendations (26, 27). Participants were instructed to refrain from intensive exercise for twelve hours prior to the assessment. To determine hand dominance, participants were asked with which hand they cut paper or bread. Previous studies reported a substantially higher maximum grip strength in the dominant hand compared

to the nondominant hand (28). Therefore, for ambidextrous participants (n = 71, 2.8%), we defined the dominant hand based on the maximum measured grip strength across both hands. The maximum grip strength of the dominant hand was based on the measured grip strength of each hand across three trials. Starting with the right hand, the grip strength of each hand was recorded intermittently. The examination was carried out in a neutral sitting position and participants were asked to continuously increase the applied force to the maximum on command.

Assessment of covariates

Participants' highest education level was determined using the International Standard Classification of Education 2011 (ISCED 2011), and was coded as low (lower secondary education or below), middle (upper secondary education to undergraduate university level), or high (postgraduate university study). During a medical interview, a medical history was obtained, including whether the participants had a history of dementia, multiple sclerosis, Parkinson's disease and stroke. We recoded neurological diseases into a binary variable (i.e. 'yes'/'no'). Age, sex and smoking status were determined via self-report. We imputed missing smoking values based on cotinine metabolite levels. Participants were classified as current smokers if they had a cotinine level exceeding the non-smoker sample-defined 97.5 percentile. Vitamin D supplementation status was based on whether participants had taken vitamin D supplements for at least 30 days in the last 12 months or whether they regularly took prescribed cholecalciferol (ATC A11CC05). Height and weight were measured with a wireless measuring station (SECA 285).

Statistical analysis

Sample characteristics are summarized using mean and standard deviation (SD) for continuous variables and number and percentages for categorical variables. Differences between included and excluded participants were examined using binomial logistic regression while adjusting for age and sex.

We used multivariate linear regression models to assess the relation between continuous and categorical circulating 25-OHD levels (independent variable) and maximum handgrip strength of the dominant hand (outcome). We categorized 25-OHD levels using the Endocrine Society (deficient (<50 nmol/L), insufficient (≥50 to <75 nmol/L), sufficient (≥ 75 nmol/L)) and the NAM and NIH guidelines (deficient (<30

nmol/L), inadequate (30 to <50 nmol/L), adequate (≥50 to ≤125 nmol/L), potential adverse (>125 nmol/L)). To test for potential nonlinear effects of vitamin D levels on grip strength, we included a quadratic term for 25-OHD in our models examining the effects of continuous circulating 25-OHD levels. The quadratic 25-OHD term was removed from the model if it failed to reach significance ($p > 0.05$). We computed the saddle point using partial derivatives. We examined interaction effects between 25-OHD levels and age, sex and season of blood withdrawal, respectively. All models were adjusted for age, age^2, sex, education, season, smoking and BMI. In model 1b, we additionally adjusted for vitamin D supplementation. In addition, in sensitivity analyses we examined whether the association between 25-OHD levels and grip strength changed when (1) excluding participants with neurological diseases, and (2) when excluding participants with high leverage observations (i.e. individuals with values of predictor variables far off from other observations, which we defined as a hat value greater than 3 times the average). We z-standardized all continuous variables to enable comparison of effect sizes. Statistical inferences were made at a two tailed $p < 0.05$. The distribution of residuals was visually inspected to check model diagnostics. All statistical analyses were performed using R (version 4.0.3, The R Foundation).

Data Availability

The Rhineland Study's dataset is not publicly available because of data protection regulations. Access to data can be provided to scientists in accordance with the Rhineland Study's Data Use and Access Policy. Requests for further information or to access the Rhineland Study's dataset should be directed to RS-DUAC@dzne.de.

Results

Demographics

The overall and age-stratified characteristics of the analytical sample are presented in Table 1 and Supplementary Table 1. In total 1,427 women (55.4%) were included. Participants' age ranged from 30 to 94 years (54.3 ± 14.2 years). In comparison to excluded participants, those who were included were more often male, younger and had a higher education status. They suffered less often from neurological diseases and were less often prescribed cholecalciferol (Table 1).

Table 1. Sample demographics of included versus excluded participants of the total sample (n = 2999).

	Excluded participants (n = 423)	Included participants (n = 2576)	Individuals with vitamin D supplementation data (n = 2341)	p-value[a]
Age (years), mean (SD)	60.4 (14.6)	54.3 (14.2)	54.4 (14.0)	<0.001
30-39, n (%)	43 (10.2)	477 (18.5)	421 (18.0)	[Ref]
40-49, n (%)	57 (13.5)	520 (20.2)	479 (20.5)	0.396
50-59, n (%)	118 (27.9)	662 (25.7)	596 (25.5)	<0.001
60-69, n (%)	61 (14.4)	495 (19.2)	465 (19.9)	0.150
70+, n (%)	144 (34.0)	422 (16.4)	380 (16.2)	<0.001
Sex (women), n (%)	268 (63.4)	1427 (55.4)	1311 (56.0)	0.001
Body-mass index (kg/m^2), mean (SD)	26.12 (4.68)	25.75 (4.47)	25.71 (4.50)	0.256
Smoking (yes), n (%)	46 (11.1)	355 (13.8)	320 (13.7)	0.578
25-hydroxyvitamin D (nmol/L), mean (SD)	56.76 (27.98)	55.07 (27.51)	55.53 (28.02)	0.880
Vitamin D categories, n (%)				
<30 nmol/L	72 (17.1)	451 (17.5)	408 (17.4)	[ref]
30 - <50 nmol/L	107 (25.4)	658 (25.5)	585 (25.0)	0.976
50 - ≤125 nmol/L	232 (55.1)	1427 (55.4)	1308 (55.9)	0.571
>125 nmol/L	10 (2.4)	40 (1.6)	40 (1.7)	0.601
Vitamin D supplementation status (yes), n (%)	137 (36.1)	678 (26.3)	678 (29.0)	0.186
Regular cholecalciferol intake (yes), n (%)	75 (18.9)	293 (11.9)	293 (12.7)	0.045
Other vitamin D supplementation (yes), n (%)	102 (26.2)	546 (22.8)	546 (23.6)	0.602
Neurological disease (yes), n (%)	23 (5.4)	62 (2.4)	57 (2.4)	0.011
Education ISCED11, n (%)				
low	20 (5.1)	41 (1.6)	35 (1.5)	[Ref]
middle	195 (49.6)	1121 (43.5)	1024 (43.7)	0.006
high	178 (45.3)	1414 (54.9)	1282 (54.8)	0.001
Season of blood withdrawal, n (%)				
spring	89 (21.0)	537 (20.8)	503 (21.5)	[Ref]
summer	95 (22.5)	746 (29.0)	679 (29.0)	0.093
autumn	158 (37.4)	764 (29.7)	661 (28.2)	0.133

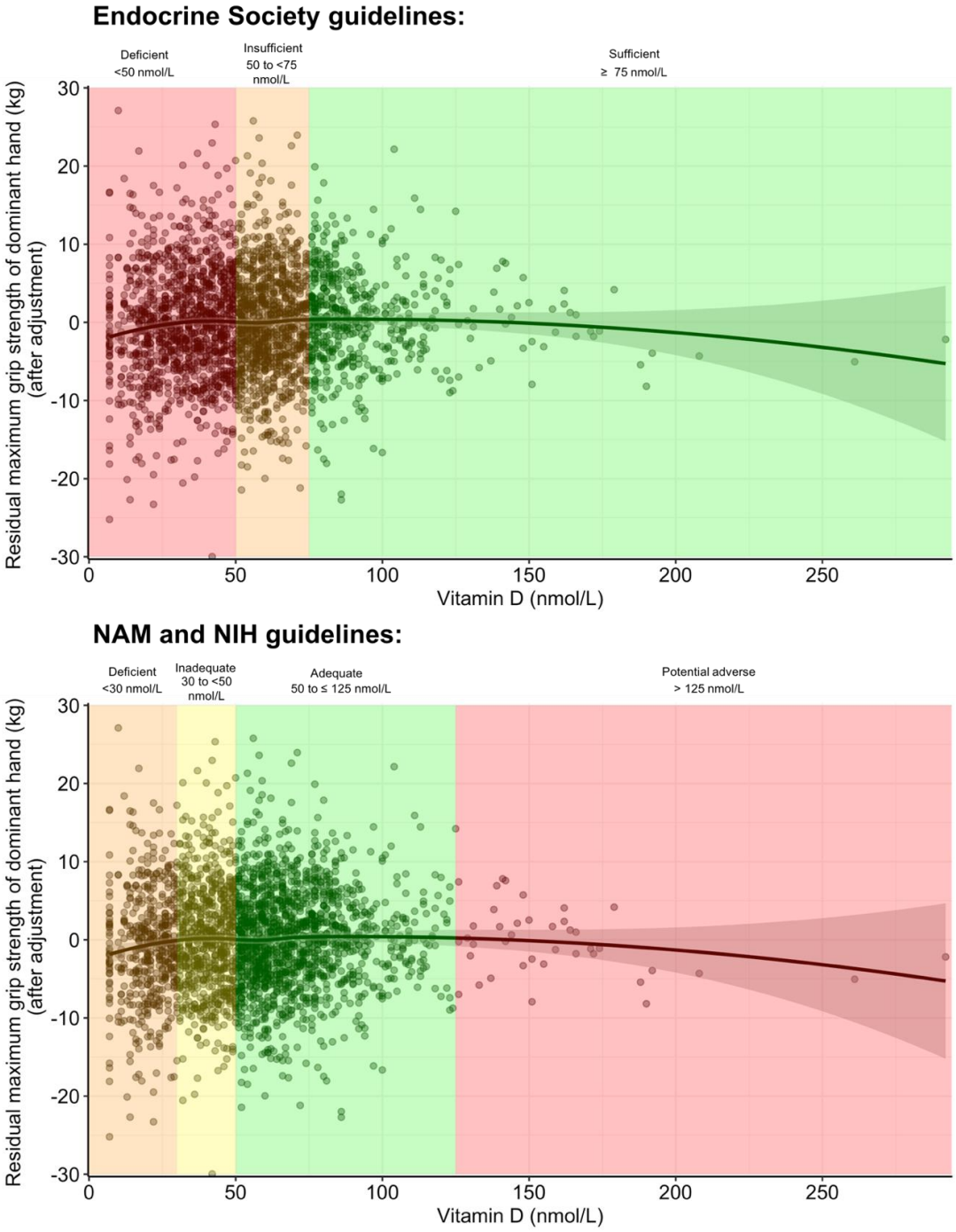
winter	81 (19.1)	529 (20.5)	498 (21.3)	0.688
Handedness, n (%)				
right	212 (92.2)	2368 (91.9)	2153 (92.0)	[Ref]
left	15 (6.5)	137 (5.3)	126 (5.4)	0.475
ambidextrous	3 (1.3)	71 (2.8)	62 (2.6)	0.259
Max. grip strength dominant hand (kg), mean (SD)	33.77 (10.80)	36.68 (11.54)	36.56 (11.53)	0.943

[a] Differences between included and excluded participants were assessed with logistic regression adjusted for age and sex (group differences for the variables age and sex were only adjusted for the other respectively)

Association between circulating 25-OHD levels and maximum grip strength of the dominant hand

First, we examined the effects of 25-OHD levels on maximum grip strength of the dominant hand across vitamin D categories (Figure 2). We used multivariate linear regression models with categorical 25-OHD levels as an independent variable, and adjusting for age, sex, education, smoking, season and BMI (Model 1a). Across the Endocrine Society categories, compared to deficient 25-OHD levels (<50 nmol/L, reference group), maximum grip strength was significantly higher at sufficient (≥ 75 nmol), but not at insufficient 25-OHD levels (≥50 to <75 nmol/L) (Figure 3A). Similarly, across the NAM and NIH categories, maximum grip strength was higher at inadequate (30 to <50 nmol/L) and adequate levels (≥50 to ≤125 nmol/L) compared to deficient 25-OHD levels (<30 nmol/L, reference group; Figure 3B). At potential adverse levels (125 nmol/L), we observed vitamin D to be associated with lower grip strength (Figure 2). Effects at potential adverse levels, however, did not significantly differ from the effects at deficient levels (<30 nmol/L; Figure 3B). These associations did not change after additional adjustment for vitamin D supplementation (Model 1b) as well as when excluding participants with neurological diseases (Sensitivity model 1a and Sensitivity model 1b) or high leverage points (Sensitivity model 2a* and Sensitivity model 2b*; Figure 3).

Figure 2. Association between continuous circulating 25-hydroxyvitamin D on maximum grip strength of the dominant hand across vitamin D categories.



Here we depict the association of circulating 25-hydroxyvitamin and the residuals of the maximum grip strength of the dominant hand with a loess function, after adjusting maximum grip strength of the dominant hand for age, age2, sex, education, smoking, BMI and season.

Second, we examined the association between continuous 25-OHD levels and maximum grip strength of the dominant hand. We found that standardised 25-OHD levels were associated with a greater maximum handgrip strength of the dominant

hand across all models (Table 2). The relation between vitamin D and handgrip strength was curvilinear and most pronounced at low levels of vitamin D (Model 1a: $ß_{linear}$ = 0.505, [95%CI: 0.179; 0.830], p = 0.002; $ß_{quadratic}$ = -0.153 [95%CI: -0.269; -0.038], p = 0.009). At higher levels, the effects of vitamin D on handgrip strength became progressively weaker and reached the saddle point at 100.4 nmol/L. At even higher levels, 25-OHD levels were associated with a weaker handgrip strength (Figure 2). After excluding participants with high leverage points (Figure 4), we found that the relation between vitamin D and handgrip strength became linear (Table 2, Sensitivity models 2 and 2*). One standard deviation increase in 25-OHD levels was associated with 0.375 kg greater maximum handgrip strength of the dominant hand.

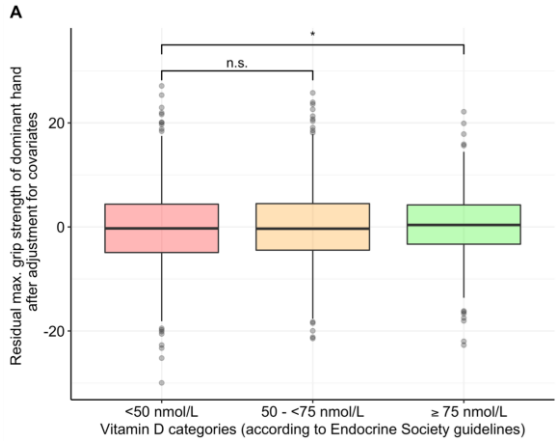
Table 2. Main effects of continuous standardised circulating 25-hydroxyvitamin D levels on maximum grip strength of the dominant hand.

Term	Model[a]	ß [95%CI]	p-value	N
25-OHD (linear)	Model 1a	0.505 [0.179; 0.830]	0.002	2576
25-OHD (quadratic)	Model 1a	-0.153 [-0.269; -0.038]	0.009	2576
25-OHD (linear)	Model 1b	0.634 [0.257; 1.010]	<0.001	2341
25-OHD (quadratic)	Model 1b	-0.162 [-0.283; -0.041]	0.009	2341
25-OHD (linear)	Sensitivity model 1a	0.509 [0.178; 0.840]	0.003	2514
25-OHD (quadratic)	Sensitivity model 1a	-0.156 [-0.273; -0.039]	0.009	2514
25-OHD (linear)	Sensitivity model 1b	0.628 [0.245; 1.011]	0.001	2284
25-OHD (quadratic)	Sensitivity model 1b	-0.165 [-0.288; -0.043]	0.008	2284
25-OHD (linear)	Sensitivity model 2a	0.471 [0.156; 0.785]	0.003	2514
25-OHD (quadratic)	Sensitivity model 2a	-0.146 [-0.332; 0.04]	0.125	2514
25-OHD (linear)	Sensitivity model 2b	0.584 [0.223; 0.946]	0.002	2286
25-OHD (quadratic)	Sensitivity model 2b	-0.131 [-0.32; 0.059]	0.177	2286
25-OHD (linear)	Sensitivity model 2a*	0.375 [0.085; 0.665]	0.011	2514
25-OHD (linear)	Sensitivity model 2b*	0.496 [0.158; 0.833]	0.004	2286

Abbreviations: 25-OHD, serum 25-hydroxyvitamin D; CI, confidence interval.

[a] Model a was adjusted by age, sex, education, season, body mass index, and smoking status.
Model b was additionally adjusted for vitamin D supplementation status.
Sensitivity models 1 excluded participants with neurological diseases (i.e. dementia, Parkinson's disease, multiple sclerosis and stroke). Sensitivity models 2 excluded participants with high leverage points (i.e. hat value > 3 times the average). Sensitivity models 2* excluded participants with high leverage points (i.e. hat value > 3 times the average) and only included a linear 25-OHD predictor term.

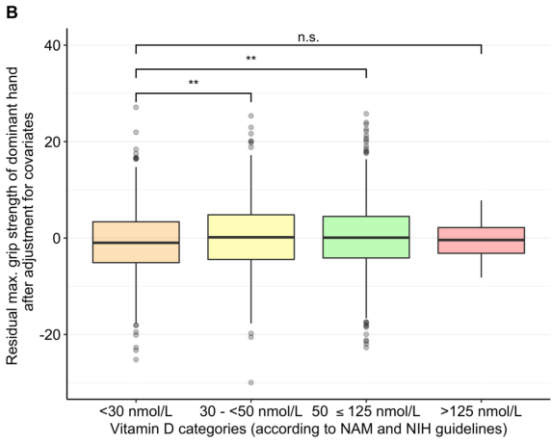
Figure 3. Effects of 25-hydroxyvitamin D on maximum grip strength of the dominant hand across vitamin D categories after adjustment for covariates.



25-OHD Category	Model[a]	ß [95%CI]	p-value	N
Deficient (<50 nmol/L)	Model 1a	[ref]	[ref]	1109
Insufficient (≥50 to 75 nmol/L)	Model 1a	0.232 [-0.394; 0.859]	0.467	937
Sufficient (≥75 nmol/L)	Model 1a	0.795 [0.049; 1.540]	0.037	530
Deficient (<50 nmol/L)	Model 1b	[ref]	[ref]	993
Insufficient (≥50 to 75 nmol/L)	Model 1b	0.391 [-0284; 1.066]	0.256	850
Sufficient (≥75 nmol/L)	Model 1b	1.005 [0.153; 1.858]	0.021	498
Deficient (<50 nmol/L)	Sensitivity model 1a	[ref]	[ref]	1082
Insufficient (≥50 to 75 nmol/L)	Sensitivity model 1a	0.226 [-0.410; 0.862]	0.486	915
Sufficient (≥75 nmol/L)	Sensitivity model 1a	0.775 [0.017; 1.532]	0.045	517
Deficient (<50 nmol/L)	Sensitivity model 1b	[ref]	[ref]	967
Insufficient (≥50 to 75 nmol/L)	Sensitivity model 1b	0.371 [-0.314; 1.057]	0.288	830
Sufficient (≥75 nmol/L)	Sensitivity model 1b	0.949 [0.083; 1.815]	0.032	487
Deficient (<50 nmol/L)	Sensitivity model 2a*	[ref]	[ref]	967
Insufficient (≥50 to 75 nmol/L)	Sensitivity model 2a*	0.183 [-0.452; 0.817]	0.572	838
Sufficient (≥75 nmol/L)	Sensitivity model 2a*	0.867 [0.105; 1.629]	0.026	457
Deficient (<50 nmol/L)	Sensitivity model 2b*	[ref]	[ref]	971
Insufficient (≥50 to 75 nmol/L)	Sensitivity model 2b*	0.356 [-0.327; 1.038]	0.307	839
Sufficient (≥75 nmol/L)	Sensitivity model 2b*	1.078 [0.211; 1.945]	0.015	476

Abbreviations: 25-OHD, serum 25-hydroxyvitamin D; CI, confidence interval.

[a] Model a was adjusted by age, sex, education, season, body mass index, and smoking status. Model b was additionally adjusted for vitamin D supplementation status. Sensitivity models 1 excluded participants with neurological diseases (i.e. dementia, Parkinson's disease, multiple sclerosis and stroke). Sensitivity models 2* excluded participants with high leverage points (i.e. hat value > 3 times the average) and only included a linear 25-OHD predictor term.



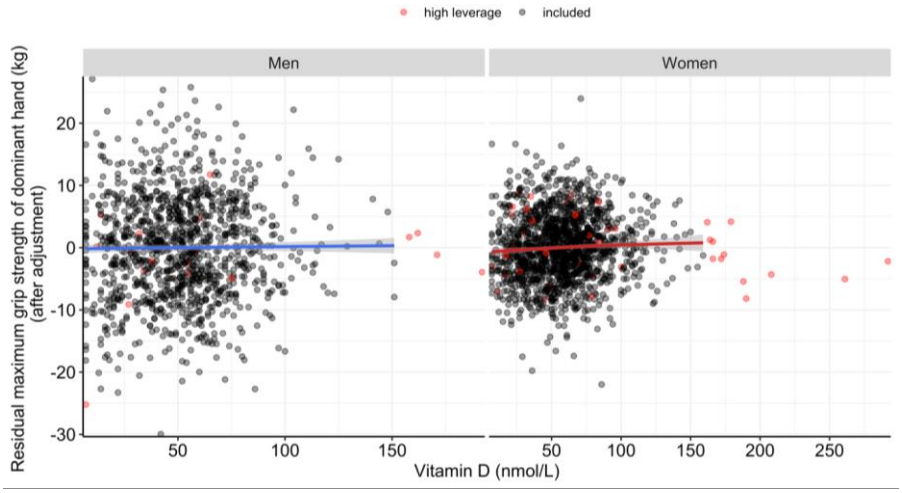
25-OHD Category	Model[a]	ß [95%CI]	p-value	N
Deficient (<30 nmol/L)	Model 1a	[ref]	[ref]	451
Inadequate (≥30 to 50 nmol/L)	Model 1a	1.222 [0.377; 2.067]	0.005	658
Adequate (≥50 nmol/L to ≤125 nmol/L)	Model 1a	1.228 [0.437; 2.019]	0.002	1424
Potential adverse (>125 nmol/L)	Model 1a	0.773 [-1.469; 3.015]	0.449	40
Deficient (<30 nmol/L)	Model 1b	[ref]	[ref]	408
Inadequate (≥30 to 50 nmol/L)	Model 1b	1.467 [0.585; 2.350]	0.001	585
Adequate (≥50 nmol/L to ≤125 nmol/L)	Model 1b	1.525 [0.665; 2.386]	0.001	1308
Potential adverse (>125 nmol/L)	Model 1b	1.378 [-0.925; 3.681]	0.241	40
Deficient (<30 nmol/L)	Sensitivity model 1a	[ref]	[ref]	439
Inadequate (≥30 to 50 nmol/L)	Sensitivity model 1a	1.263 [0.405; 2.121]	0.004	643
Adequate (≥ 50 nmol/L to ≤125 nmol/L)	Sensitivity model 1a	1.242 [0.440; 2.045]	0.002	1392
Potential adverse (>125 nmol/L)	Sensitivity model 1a	0.787 [-1.466; 3.039]	0.494	40
Deficient (<30 nmol/L)	Sensitivity model 1b	[ref]	[ref]	397
Inadequate (≥30 to 50 nmol/L)	Sensitivity model 1b	1.518 [0.621; 2.416]	0.001	570
Adequate (≥50 nmol/L to ≤125 nmol/L)	Sensitivity model 1b	1.530 [0.656; 2.404]	0.001	1277
Potential adverse (>125 nmol/L)	Sensitivity model 1b	1.360 [-0.957; 3.676]	0.250	40
Deficient (<30 nmol/L)	Sensitivity model 2a*	[ref]	[ref]	435
Inadequate (≥30 to 50 nmol/L)	Sensitivity model 2a*	1.188 [0.328; 2.048]	0.007	648
Adequate (≥ 50 nmol/L to ≤125 nmol/L)	Sensitivity model 2a*	1.183 [0.378; 1.989]	0.004	1407
Potential adverse (>125 nmol/L)	Sensitivity model 2a*	1.410 [-1.433; 4.252]	0.331	24
Deficient (<30 nmol/L)	Sensitivity model 2b*	[ref]	[ref]	395
Inadequate (≥30 to 50 nmol/L)	Sensitivity model 2b*	1.395 [0.498; 2.291]	0.002	576
Adequate (≥50 nmol/L to ≤125 nmol/L)	Sensitivity model 2b*	1.466 [0.592; 2.341]	0.001	1289
Potential adverse (>125 nmol/L)	Sensitivity model 2b*	2.130 [-0.645; 4.906]	0.132	26

Abbreviations: 25-OHD, serum 25-hydroxyvitamin D; CI, confidence interval.

[a] Model a was adjusted by age, sex, education, season, body mass index, and smoking status. Model b was additionally adjusted for vitamin D supplementation status. Sensitivity models 1 excluded participants with neurological diseases (i.e. dementia, Parkinson's disease, multiple sclerosis and stroke). Sensitivity models 2* excluded participants with high leverage points (i.e. hat value > 3 times the average) and only included a linear 25-OHD predictor term.

Here we compare the effects of 25-hydroxyvitamin D (25-OHD) categories on the residuals of the maximum grip strength of the dominant hand (reference group: deficient) after adjustment for age, age2, sex, education, smoking, BMI and season across (A): Endocrine Society categories; (B): National Academy of Medicine (NAM, formerly called Institute of Medicine) and National Institute of Health (NIH) categories.

Figure 4. The relation between circulating 25-hydroxyvitamin D and maximum grip strength of the dominant hand stratified by sex after exclusion of participants with high leverage points.

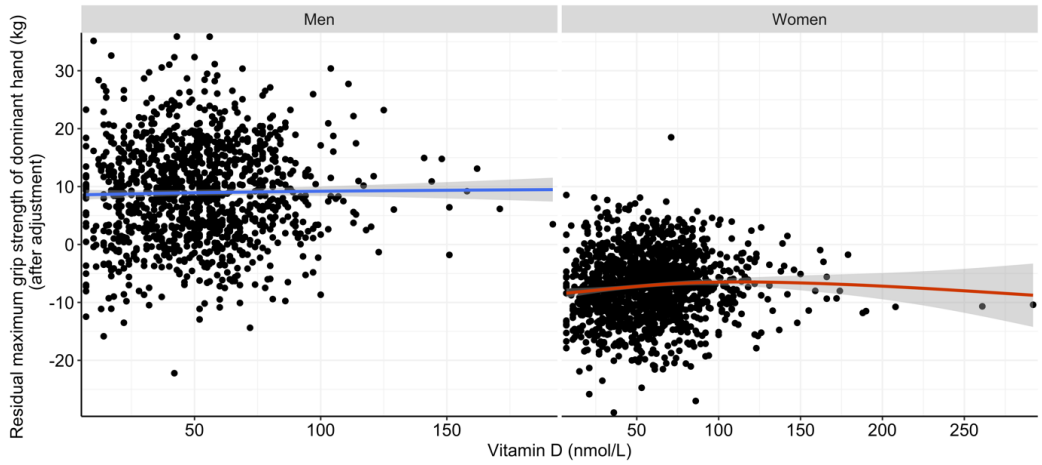


Here we depict the association of circulating 25-hydroxyvitamin and the residuals of the maximum grip strength of the dominant hand, after adjusting maximum grip strength of the dominant hand for age, age2, education, smoking, BMI and season.

Interactions between circulating 25-OHD levels, age, sex and season

Lastly, we assessed whether the effect of vitamin D on maximum grip strength of the dominant hand changed across age, between seasons and differed between men and women. The association between 25-OHD levels and grip strength did not differ between seasons nor between men and women (Table 3, Figure 5).

Figure 5. Effects of circulating 25-hydroxyvitamin D levels on maximum grip strength of the dominant hand stratified by sex.



Here we depict the association of circulating 25-hydroxyvitamin and the residuals of the maximum grip strength of the dominant hand, after adjusting maximum grip strength of the dominant hand for age, age2, education, smoking, BMI and season.

However, age had a significant moderating effect on this association, after adjusting for sex, education, smoking, season and BMI (Model 1a: $ß_{25OHDxAge}$ = -0.309, 95%CI: [-0.594; -0.024], p = 0.033), but not when additionally adjusting for vitamin D supplementation (Model 1b: $ß_{25OHDxAge}$ = -0.213, 95%CI: [-0.514; 0.087], p = 0.164). We observed that the effect of vitamin D on grip strength was weaker in older adults compared to younger adults, both in men and women (Figure 6). Particularly in older women, we observed extremely high levels of vitamin D to be associated with lower grip strength. When adjusting for vitamin D supplementation, several older individuals with high leverage points were excluded due to missing data on vitamin D supplementation (Figure 7).

Table 3. Interaction between continuous circulating 25-hydroxyvitamin D, age, sex and season.

Interaction term	**Model[a]**	**ß [95%CI]**	**p-value**	**N**
25-OHD × Age				
	Model 1a	-0.309 [-0.594; -0.024]	0.033	2576
	Model 1b	-0.213 [-0.514; 0.087]	0.164	2341
	Sensitivity model 1a	-0.321 [-0.609; -0.033]	0.029	2514
	Sensitivity model 1b	-0.220 [-0.520; 0.081]	0.152	2284
	Sensitivity model 2a*	-0.329 [-0.602; -0.056]	0.018	2514
	Sensitivity model 2b*	-0.231 [-0.518; 0.057]	0.116	2286
25-OHD × Sex				
	Model 1a	0.035 [-0.535; 0.606]	0.904	2576
	Model 1b	0.200 [-0.397; 0.797]	0.511	2341
	Sensitivity model 1a	0.037 [-0.543; 0.618]	0.900	2514
	Sensitivity model 1b	0.195 [-0.413; 0.802]	0.530	2284
	Sensitivity model 2a*	0.014 [-0.539; 0.567]	0.960	2514
	Sensitivity model 2b*	0.192 [-0.386; 0.771]	0.515	2286
25-OHD ×				

Spring	Model 1a	[ref]	[ref]	2576
Summer	Model 1a	0.132 [-0.740; 1.005]	0.766	2576
Autumn	Model 1a	-0.191 [-0.981; 0.599]	0.636	2576
Winter	Model 1a	-0.586 [-1.442; 0.270]	0.180	2576
Spring	Model 1b	[ref]	[ref]	2341
Summer	Model 1b	0.147 [-0.763; 1.057]	0.751	2341
Autumn	Model 1b	-0.146 [-0.970; 0.677]	0.728	2341
Winter	Model 1b	-0.531 [-1.411; 0.349]	0.237	2341
Spring	Sensitivity model 1a	[ref]	[ref]	2514
Summer	Sensitivity model 1a	0.056 [-0.830; 0.943]	0.901	2514
Autumn	Sensitivity model 1a	-0.221 [-1.022; 0.581]	0.590	2514
Winter	Sensitivity model 1a	-0.662 [-1.531; 0.207]	0.135	2514
Spring	Sensitivity model 1b	[ref]	[ref]	2284
Summer	Sensitivity model 1b	0.089 [-0.835; 1.013]	0.85	2284
Autumn	Sensitivity model 1b	-0.157 [-0.994; 0.679]	0.713	2284
Winter	Sensitivity model 1b	-0.601 [-1.494; 0.292]	0.187	2284
Spring	Sensitivity model 2a*	[ref]	[ref]	2514
Summer	Sensitivity model 2a*	0.077 [-0.788; 0.943]	0.861	2514
Autumn	Sensitivity model 2a*	-0.208 [-0.996; 0.581]	0.606	2514
Winter	Sensitivity model 2a*	-0.558 [-1.386; 0.27]	0.186	2514
Spring	Sensitivity model 2b*	[ref]	[ref]	2286
Summer	Sensitivity model 2b*	0.072 [-0.823; 0.966]	0.875	2286
Autumn	Sensitivity model 2b*	-0.187 [-0.999; 0.626]	0.652	2286
Winter	Sensitivity model 2b*	-0.52 [-1.366; 0.326]	0.228	2286

Abbreviations: 25-OHD, serum 25-hydroxyvitamin D; CI, confidence interval.

[a] Model a was adjusted by age, sex, education, season, body mass index and smoking status.

Model b was additionally adjusted for vitamin D supplementation status.

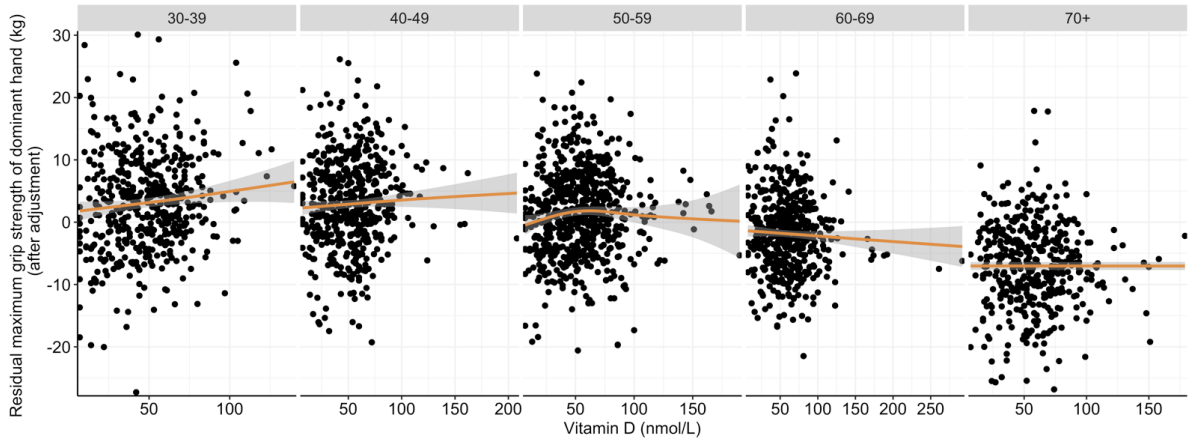
Sensitivity models 1 excluded participants with neurological diseases (i.e. dementia, Parkinson's disease, multiple sclerosis and stroke).

Sensitivity models 2* excluded participants with high leverage points (i.e. hat value > 3 times the average) and only included a linear 25-OHD predictor term.

Discussion

We aimed to examine the relation between circulating 25-OHD levels and handgrip strength across adult lifespan in a large population-based cohort study. In our cross-sectional sample of 2,576 individuals aged 30 to 94 years, we found a robust association between circulating vitamin D levels and maximum grip strength of the dominant hand after adjustment for age, sex, education, season, smoking, BMI as well as for vitamin D supplementation. Our findings highlight the importance of adequate vitamin D levels for the maintenance of muscle function in adults across a wide age range.

Figure 6. Effects of circulating 25-hydroxyvitamin D levels on maximum grip strength of the dominant hand stratified by age.

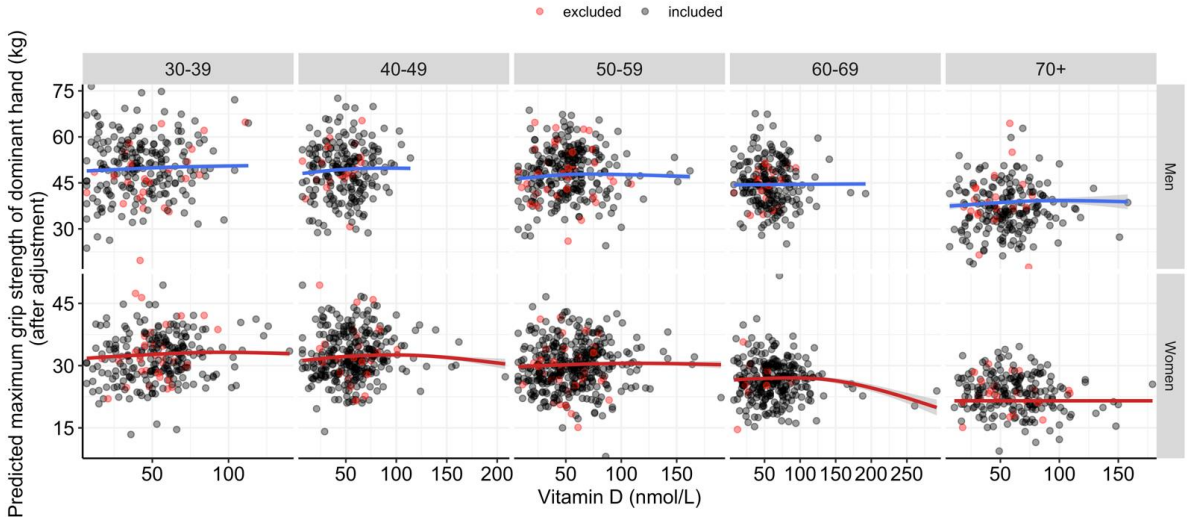


Here we depict the association of circulating 25-hydroxyvitamin and the residuals of the maximum grip strength of the dominant hand, after adjusting maximum grip strength of the dominant hand for sex, education, smoking, BMI and season.

Thus far, findings from observational studies examining the relation between vitamin D levels and grip strength have been contradictory (13, 15, 17, 19, 20). While some studies reported higher vitamin D levels to be associated with greater upper arm and grip strength (13, 15), others could not replicate these findings (29, 30). The heterogeneity of these findings may reflect different study designs and populations: particularly, differences in skin pigmentation, latitude, dietary patterns, lifestyle factors as well as genetic polymorphisms should be taken into account when comparing findings of studies across different regions and ethnicities (31, 32, 33). In addition, varying vitamin D thresholds have been used to cluster participants and to define vitamin D deficiency across studies with an ongoing controversy about recommended

vitamin D targets (34). This hampers comparisons of the findings of different studies and may lead to biased conclusions (35).

Figure 7. The relation between circulating 25-hydroxyvitamin D and maximum grip strength of the dominant hand stratified by age and sex after exclusion of participants with missing data on vitamin D supplementation.



Regression lines were adjusted for age, age2, sex, education, smoking, BMI, season and vitamin D supplementation.

Thus, to allow better comparison to previous findings, here we report results based on the Endocrine Society's, the NAM and the NIH's classification guidelines for circulating 25-OHD levels (36, 37, 38). Across NAM and NIH categories, we found that individuals with deficient 25-OHD levels (<30 nmol/L) show lower grip strength than individuals with inadequate (30 to <50 nmol/L) or adequate levels (≥50 to ≤125 nmol/L). However, no differences in the effects of 25-OHD levels on grip strength between the deficient (<30 nmol/L) and the potential adverse (>125 nmol/L) categories were observed. Across the Endocrine Society's categories, we only found differences in the effects on grip strength between individuals having deficient 25-OHD levels (<50 nmol/L) and individuals having sufficient levels (≥ 75 nmol) but not between individuals having deficient 25-OHD levels and individuals having insufficient levels (≥50 to <75 nmol/L). Next to dividing 25-OHD levels into categories, we also examined the association between vitamin D and grip strength on a continuous scale. We could replicate findings from a previous cohort study, which examined the effects of continuous vitamin D levels in 419 healthy men and women aged 20–76 years and reported a positive effect

on upper arm strength (13). Specifically, we found that the effect of vitamin D on grip strength was strongest at low levels of 25-OHD (<50 nmol/L) and weakened at higher levels. We observed the maximum effect at around 100 nmol/L. At even higher levels, we observed a strong negative effect of 25-OHD levels on grip strength across all ages. Taken together, in line with NAM and NIH recommendations (36, 38), our findings suggest a dose-response relationship between vitamin D levels and grip strength with an optimum around 50–100 nmol/L and an increased risk of adverse effects at excess levels of circulating 25-OHD levels above 125 nmol/L. Moreover, our findings highlight that examining the effects of 25-OHD across narrow categories, such as proposed by the NAM and NIH guidelines, or examining the effects of 25-OHD levels on a continuous scale may provide valuable insights such as the potential adverse effects at excess 25-OHD levels that may be hidden when using broad cut-offs. Nonetheless, it should be noted that we detected potential adverse vitamin D levels only in a few participants. Further studies are warranted to study the association between extreme vitamin D levels and grip strength in greater detail.

While previous studies largely focused on older adults, our study examined the association between vitamin D levels and grip strength in adults across a wide age range. This allowed us to examine whether the effect of vitamin D is modified by age. Surprisingly, we observed a weaker effect of vitamin D on grip strength in older adults compared to younger adults. A systematic review of randomized controlled trials found that overall muscle strength of older adults could profit more from vitamin D supplementation than that of younger adults (39). Compared to other cohort studies in Germany (35, 40), a substantially higher percentage of our participants reported to take vitamin D supplementation (29.0% versus 2.81% men and 3.8% women of the German National Health Interview and Examination Survey (GNHIES) 1998 (40)) and a substantially lower percentage of our participants were showing a vitamin D deficiency (43.0% < 50nmol/L versus 56.8% < 50 nmol/L in the GNHIES 1998 (40) and ~62.1% <50 nmol/L in the Studie zur Gesundheit Erwachsener in Deutschland (DEGS1) (41)). This is in line with a recent report observing a drastic increase in prescribed cholecalciferol in recent years (42). In our sample we observed that compared to younger individuals, a greater proportion of older adults had been regularly taking prescribed cholecalciferol and had higher vitamin D levels. Indeed, we

found that after accounting for vitamin D supplementation, the relation between vitamin D levels and grip strength did not change with age.

In addition, we aimed to examine whether the effects of circulating vitamin D levels on grip strength varied between seasons and differed between men and women. Little is known about seasonal variations of muscle function in relation to vitamin D levels. Bird and colleagues (2013) found seasonal variations in ankle strength and serum vitamin D levels in community-dwelling older adults. Similarly, Milani and colleagues (2021) reported season-dependent effects of vitamin D on physical fitness performance in male adolescents. To the best of our knowledge, no study to date has examined the modifying effects of season or sex on the association between vitamin D and grip strength in adults across a wide age range. In our study, we neither observed a modifying effect of season, nor of sex. Several observational studies noticed an association between vitamin D levels and grip strength exclusively in healthy middle-aged men (15), older men (17) or stronger associations in older men than in older women (20). However, other studies observed effects both in older men and women (19, 43). A recent narrative review points to potential sex differences in the synthesis and metabolism of vitamin D, which may lead to differences in the effects of vitamin D on grip strength between men and women (44). Further research is needed to study factors that could modulate the effects of vitamin D on muscle strength.

A number of limitations of our study should be noted. First, this study examined cross-sectional associations between vitamin D levels and grip strength using baseline data of a large cohort study. Therefore, the longitudinal relationship between circulating vitamin D levels and grip strength could not be explored and a causal relationship cannot be inferred. Second, given the relatively high education levels and the high prevalence of vitamin D supplementation across all age groups in our sample, it cannot be ruled out that our findings may have been partly influenced by selection bias. Third, our findings in predominantly white individuals from European descent may not be generalizable to other ethnicities. Fourth, while grip strength has been found to be a reliable proxy of muscle strength (4, 5), several studies highlight the usefulness of measuring muscle strength of multiple muscle groups to achieve more precise estimates of overall muscle function (45, 46).

To conclude, here we present a detailed characterization of the relation between circulating 25-OHD levels and muscle strength in adults aged 30 to 94 years. We

observed a robust association between vitamin D levels and grip strength, which was most pronounced at deficient levels and dropped at potential adverse levels. Our findings suggest that optimizing vitamin D levels could be an easily actionable and inexpensive strategy for improving muscle function and protecting against sarcopenia in adults across a wide age range. Nonetheless, vitamin D supplementation should be closely monitored to avoid overdosing and potential detrimental effects.

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4. Discussion with references

Due to population ageing, the prevalence of age-associated noncommunicable diseases such as cancer, cardiovascular and neurodegenerative diseases are expected to rise rapidly in the near future (United Nations, 2020). By 2060, it is estimated that almost a third of Germans will be aged 67 years and above and that the working-age population will shrink by about 10 million people (Federal Statistical Office (Destatis), 2019). To maintain the economic stability and social security of an ageing society, it is key to prevent disability, maintain mobility and promote quality of life up to an old age.

Targeting modifiable behavioural risk factors of noncommunicable diseases such as promoting healthy nutrition, encouraging physical activity and reducing sedentary behaviours could be key to mitigating the impact of population ageing. In a recent meta-analysis, 7.2% of all-cause deaths and 7.6% of cardiovascular disease deaths were linked to insufficient physical activity (Katzmarzyk, Friedenreich, Shiroma, & Lee, 2022). On the other hand, regular participation in physical activity and maintaining fitness up to an old age has been implicated to be effective in the prevention, treatment and management of cancer, cardiovascular and neurodegenerative diseases (Ascherio & Schwarzschild, 2016; Hamer & Chida, 2008; World Health Organization, 2020).

During this dissertation project, I examined whether physical activity and fitness are related to markers of healthy ageing. We showed that physical activity is associated with detailed measures of brain morphology across several brain regions. In line with animal studies observing exercise-induced hippocampal neurogenesis, we observed physical activity to be associated with a greater volume of the hippocampus, which is a major hub for cognitive function (Voss, Vivar, Kramer, & van Praag, 2013). Accordingly, physical activity may preferentially benefit a brain region that is affected in early stages of dementia and whose atrophy is associated with one of its most debilitating symptoms - the loss of memory and impaired memory storage (Buckner, 2004). However, while the majority of previous studies focused on the effects of physical activity on brain regions predominantly involved in cognitive function (Bherer, Erickson, & Liu-Ambrose, 2013), we found the most pronounced effects in motor regions and regions with a high oxidative demand. Thereby, our findings suggest that the effects of physical activity may lead to remodelling not only of cognitive regions but also of several other brain regions.

In addition, we demonstrated that physical activity is related to epigenetic ageing, a measure of biological ageing, which is linked to functional impairment and mortality risk. We could replicate and extend findings from previous studies showing that physical activity is associated with slower epigenetic age acceleration (Kresovich et al., 2021; A. T. Lu et al., 2019), whereas sedentary time had the opposite effect. By distinguishing between physical activity components, we obtained novel, in-depth insights into the effects of physical activity on epigenetic ageing as well as on epigenome-wide methylation levels. Overall, we observed the strongest effects for moderate-to-vigorous intensity activities. This is in line with a recent study, which found the strongest association between the highest accelerometer-based physical activity counts and cardiometabolic risk in middle-aged to older adults (Dempsey et al., 2022). Hence, while all forms of physical activity may be beneficial for physiological health, comparatively, increases in moderate-to-high intensity activities may lead to the greatest health benefit.

In the presented articles, my co-authors and I investigated the dose-response relationship between physical activity and several physiological markers of health. We observed that the effects of physical activity are most pronounced at low physical activity levels and weaken at higher levels. Also, previous studies suggested that the relation between physical activity and markers of physiological health follows an inverted U-shape curve: For instance, Armstrong and colleagues (2015) noted that the risk of vascular diseases decreased with more frequent engagements in high-intensity physical activity as well as any kind of physical activity. The lowest risk associated with strenuous activities was reached at a frequency of 2 – 3 times per week, whereas engaging even more often in high-intensity activities was linked to a greater vascular risk. Similarly, Stamatakis and colleagues (2022) found the relation between short bouts of high-intensity physical activity and all-cause mortality risk, cardiovascular disease mortality risk and cancer mortality risk to follow a dose-response curve with the greatest risk reduction for small increments of high-intensity activity bouts at the lower end of the spectrum. Nonetheless, mortality risk decreased even further, although to a lesser extent, with more frequent bouts of high-intensity activities. Altogether these findings suggest that particularly inactive individuals, who predominantly engage in low intensity activity, may profit from increases in physical activity frequency and duration, especially at a high intensity level.

Physical activity is essential to strengthen and maintain muscle function and, by extension, mobility up to an old age. Importantly, vitamin D supplementation has been observed to be effective in the prevention of loss of muscle strength, especially in old age (Beaudart et al., 2014). In this dissertation we show that circulating vitamin D levels are associated with greater handgrip strength, a proxy of muscle mass and function, in adults across a wide age range. However, at very high, potentially adverse vitamin D levels, we observed the opposite effect. Our findings imply that adequate vitamin D levels are essential for muscle maintenance but an oversupply may be linked to detrimental effects. Thus, to preserve mobility and prevent functional impairment and falls, vitamin D levels should be carefully observed across the adult lifespan.

Several limitations of the presented studies should be considered: First, we assessed associations between physical activity, fitness and markers of physiological health using cross-sectional baseline data of the Rhineland Study. Therefore, we were unable to infer directionality of the observed relationships. Second, physical activity recordings were conducted in the participants' free-living (including home, work and leisure) environments and we cannot rule out that some participants' might have adapted their behaviour during the recording. Similarly, while grip strength has been observed to reliably reflect muscle strength, it has been proposed that the assessment of multiple muscle groups could be used to reach an even more precise estimate of muscle mass and function (Guadalupe-Grau et al., 2015; Yeung et al., 2018). Third, education, physical activity and fitness levels of participants across all age groups were comparatively high in our study. It is likely that we have underestimated the strength of the association between physical activity, fitness and markers of physiological health in our analyses as the effect sizes were generally higher at the lower end of the spectrum for both physical activity and grip strength.

4.1 Summary & Outlook

In summary, utilizing accelerometers in a large prospective cohort study of adults across a wide age range allowed us to assess the relation between physical activity, fitness and markers of physiological health in considerable detail. We showed that physical activity is associated with improved markers of physiological health including biological ageing, brain volumes, grey matter density, and cortical thickness. Furthermore, we identified effects of physical activity on the methylation level of several CpGs and studied molecular

mechanisms underlying the effects of physical activity on brain morphology. We also demonstrated that a sufficient vitamin D supply is essential for muscle strength and that vitamin D levels should be monitored for optimal muscle performance and maintenance.

Additional studies are required to further unravel the mechanisms behind the beneficial effects of physical activity. For instance, next to its effect on brain morphology, physical activity has been suggested to be linked with improved structural and functional connectivity of the brain (Maleki et al., 2022; Sexton, Betts, Demnitz, Dawes, & Ebmeier, 2016; Won et al., 2021). However, the majority of previous studies have employed questionnaire-based physical activity measures and focused on the effects of physical activity on global white matter microstructure, thus far. Regional effects have been proposed to be particularly pronounced in tracts connecting cognitive regions such as the corpus callosum, uncinate fasciculus and fornix (Maleki et al., 2022; Sexton et al., 2016; Won et al., 2021). However, the relation of detailed, objectively assessed physical activity components and white matter microstructure remains unclear.

Accelerometers allow activity and rest profiling that go beyond summary measures. In-depth assessments of physical activity dimensions such as the frequency, duration (i.e. bout length) and time-of-day has the potential to elucidate the physiological effects of largely unexplored behavioural patterns. Recently, Stamatakis and colleagues (2022) observed that an accumulation of extremely short bouts (1-2 minutes) of high intensity activities across the day can reduce mortality risk by up to 40%. Thus, individuals may partake in health promoting activities, which require vigorous muscle engagement but are not consciously aimed at engaging in exercise. These activities may not be recalled by individuals in questionnaires but will be reflected in accelerometer recordings.

The recent introduction of medical wearables allows near real time health data collection on a global scale that could revolutionize our healthcare system and clinical research. By now, almost everyone owns a smartphone with an inbuilt accelerometer sensor that could provide accurate activity and rest recordings. Wearables could be utilized for inclusive personalized physical activity interventions while taking into account individuals' capabilities, preferences and medical history. In addition, these technical advances can be used to boost health education, engage with hard-to-reach population groups and facilitate novel insights into risk prediction, diagnosis and treatment effectiveness.

4.2 References

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