PHD THESIS

Karina Husted

Proposition of a biological age model and assessment of its value to health promotion

Ph.D. thesis

"Proposition of a biological age model and assessment of its value to health promotion"

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Preface

This thesis concludes the work performed as a Ph.D. student at the University of Copenhagen in the period covering 2017-2022, a period of my life where I also had the pleasure of becoming a mother twice. The thesis is written as a synopsis with four papers as the backbone (study I-III). Together, the four papers elucidate the concept of biological age as a health risk measure from a biomedical perspective.

Before I embarked on this Ph.D. journey, I admit that I did not consider biological age to be of any particular value. My limited knowledge concerning biological age related to television shows using it as a motivational tool under the more populistic term “body age”. Therefore, when my supervisor asked me whether I would be interested in looking into biological age, I was a bit puzzled. However, the more I read, the more intrigued I got. The fact that people at the same age can vary tremendously in physical capacity and disease risk resonated well with my experiences as a physiotherapist before I got into science. The possibility of using biological age in disease prevention raised my curiosity, and so, it began.

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Acknowledgments

First, I would like to thank my supervisor Professor Jørn Wulff Helge for pushing me and believing in me, to jump headfirst into this Ph.D. I do not think I am wrong when I say that some parts of this Ph.D. were new to both of us. However, you convinced me that I could do the job and you have been supportive all the way. Thank you for that! I have had the pleasure of knowing you for seven years, and much has happened during this period in my life. Thus, not only have you been my mentor in science, but I have also learned from you when it comes to combining career and family. Finally, I want to thank you for always keeping your door open. I can see that this is a recurrent appraisal from former students of yours, and with good reason. It means a lot knowing that no question is too big or too small and that you always take your time!

As part of this project, I needed to engage in the somewhat alien mathematical field of health technology and statistical modeling. This has, without question, been my biggest challenge and something I have spent substantial time comprehending. Therefore, I wish to thank Professor Helge Sorensen who gave me an open seat at the department of health technology, and Professor Kaj-Åge Henneberg and Ph.D. student Andreas Brink-Kjær for introducing me to MATLAB language and detangling the concept of principal component analysis. The interdisciplinarity of this project has been an eye-opening experience and it has truly improved the results of the project.

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Professor Flemming Dela, I appreciate your guidance as co-supervisor and your sharp eye when reviewing my work. Especially I would like to thank you for your feedback at conferences. It has improved my communication skills and my confidence on stage. Associate professor Steen Larsen, despite your senior title, I consider you as a fellow colleague and a friend. Dorthe Stensvold, although COVID19 made my stay at NTNU somewhat different than planned, I still learned a lot from seeing firsthand, how you succeed in conducting large-scale population-based interventions.

I am truly grateful for the many great colleagues I have encountered during the years. Sofie, Thomas M., Tine D, Stine, Tine M, Kjestine, Signe, Mimmi, Malene, Marianne, Sune, Ditte, Anja, Magnus, and Ronni thank you for all the laughs, beers, and cakes we consumed during my early years in science. The atmosphere you created was one of the reasons why I stayed in science. Ronni, Jacob, Mikkel, Tue, and Arthur I truly appreciate your company and the trips we have had together. Ronni, I feel lucky that you kept on producing kids (like me) so you could follow me all the way. To my office buddies Stine and Rannva. There is something special about sharing office during the final stages of the Ph.D., that translates colleagues to friends. Sofie Lionett, we had that translation long ago still, I want to thank you for always taking my calls throughout this last period, it means a lot!

To my family and friends. Thank you for showing interest in my project and always being supportive. To Anne Mette and Martin, I appreciate the time you took to proofread the thesis which, makes you part of the small exclusive club of people who have read the whole thesis. Martin, a special thank you for always taking your time for informal research discussions. To my mom and dad and parents-in-law. From the bottom of my heart, thank you for the many times in the past three months you have helped take care of sick children. I think Rasmus still has a job because of that.

And speaking of. Rasmus, how cliché it might sound I feel the urge to say what does not kill us makes us stronger. You have been my solid rock and an amazing temporary single father of two. I hope you did not like it too much because I am coming back full time and then some. Love you forever.

- *Karina Husted*

List of Papers

Paper I:

Karina L.S. Husted*, Sune Dandanell*, Janne Petersen, Flemming Dela & Jørn W. Helge.

The effectiveness of body age-based intervention in workplace health promotion: Results of a cohort study on 9851 Danish employees.

PloS ONE 2020; e0239337

Paper II:

Karina L.S. Husted, Mathilde Fogelstrøm, Pernille Hulst, Andreas Brink-Kjær, Kaj-Åge Henneberg, Helge B. D. Sorensen, Flemming Dela & Jørn W. Helge.
A Biological Age Model Designed for Health Promotion Interventions: Protocol for an Interdisciplinary Study for Model Development.
JMIR Res Protocol 2020; e19209.

Paper III:

Karina L.S. Husted, Andreas Brink-Kjær, Mathilde Fogelstrøm, Pernille Hulst, Akita Bleibach, Kaj-Åge Henneberg, Helge B. D. Sorensen, Flemming Dela, Jens C. B. Jacobsen & Jørn W. Helge.

Proposition of a model estimating biological age from physiological biomarkers of healthy aging: a cross-sectional study.

JMIR Aging, 2022; in Review

Paper IV:

Karina L.S. Husted, Mikkel Hansen, Tue Rømer, Arthur Ingersen, Mathilde Fogelstrøm, Flemming Dela & Jørn. W. Helge.

Proof of concept and change in biological age following 15 weeks of lifestyle intervention.

In Manuscript form

*Shared first authorship

Other contributions

Systematic review

To identify current biological age models and outline the applicability for health promotion a systematic scoping review was conducted. Where a systematic review asks a specific question about the effectiveness of an intervention or treatment, the aim of a scoping review is to explore a particular topic, identify the extent of the available evidence, and highlight gaps in the literature (1). Due to time constrictions, the review has not been finalized into a manuscript, however, it is embedded in the thesis background. The intention is to publish soon.

Study IIII

As part of this Ph.D., a fourth study (as part of a collaborative effort) has been conducted with the aim of investigating the reliability of the new BA model. This study will provide an additional evaluation of the clinical utility of the BA model. Ten women and ten men had their BA estimated three times. The tests were interspersed with a minimum of 48 hours and a maximum of 14 days. The test protocol was performed at the same time of the day (±30 min).

Abbreviations

AFAR: American Federation for Aging Research

AGE: Advanced Glycation End-products

AMP kinase: Adenosine monophosphate-activated protein kinase

BA: Biological Age

BAS: Biological Age Score

BMI: Body Mass Index

BP: Blood Pressure

CA: Chronological Age

CRF: Cardiorespiratory Fitness

CRP: C-Reactive Protein

CVD: Cardiovascular Disease

DBP: Diastolic Blood Pressure

FEV1: Forced Expiratory Volume in one second

FFA: Free Fatty Acids

FVC: Forced Vital Capacity

HbA1c: Glycated Hemoglobin type A1

HDL-C: High-Density Lipoprotein Cholesterol

IPAQ: International Physical Activity Questionnaire

IP3 kinase: Phosphoinositide 3 kinase

IL-6: Interleukin 6

KDM: Klemera and Doubal Method

LDL-C: Low-Density Lipoprotein Cholesterol

MAP: Mean Arterial Pressure

MLR: Multiple Linear Regression

Nm: Newton Meter

PAS: Physical activity scale

PCA: Principal Component Analysis

1^{st}PC: First Principal Component

SBP: Systolic Blood Pressure

SF12: 12 items short-form survey

suPAR: Soluble urokinase Plasminogen Activator Receptor

T2D: Type 2 Diabetes

TG: Triglycerides

TNf-α: tumor necrosis factor-α

VAT: visceral adipose tissue

VO_2max: maximal oxygen uptake

WHO: World Health Organization

Key terminology

Chronological age: quantifies the time (in years) passed since birth

Biological age: quantifies individual physiological aging (in years), in comparison with the average person of that given chronological age within the population from which biological age was generated.

Aging: the general term to describe the process of becoming older. Both biological and chronological age is embedded in this terminology.

Life expectancy: quantifies the average number of years a person can expect to live

Health span: quantifies a period in life (in years) free from major chronic disease and disability

Summary

Background: The prospects of increased life expectancy and prevalence of obesity induce a higher risk of chronic disease. Thus, early, and effective health promotion is increasingly important for a future healthy aging population. Biological age (BA) is employed in general health checks to motivate a healthier lifestyle. BA estimates the risk of future disease and potential life expectancy by comparing individual physiological function to population means with the same sex and age. However, the validity and effectiveness of the BA models applied in public health promotion have not been established. Conversely, the scientific interest in BA has resulted in a large heterogenic pool of BA models, validated against the ability to discriminate between healthy and unhealthy and all-cause mortality in cohort studies. However, without intervention studies to investigate the clinical utility, the evidence for use in health enhancing interventions remains unclear. Therefore, the aim of this Ph.D. was to investigate BA as a motivational- and clinical tool and propose a new BA model to measure healthy aging and estimate the risk of disease.

Methods: A retrospective cohort study investigated the effectiveness of health checks including BA estimation based on their own BA model. Invitations were sent out to employees in 90 different companies. BA was estimated in 9,851 employees at baseline and 3,843 employees at follow-up.

A cross-sectional study was conducted to collect 32 candidate biomarkers of healthy aging for the development of a new BA model. A total of 100 healthy women and men went through an extensive health examination. Correlation analysis was used to select the final biomarkers for BA estimation. Principal component analysis (PCA) was employed to determine the linear combination of the biomarkers and the first principal component was used to form the BA equation. Furthermore, the clinical utility of the BA model was investigated in an intervention study including 27 overweight and obese women and men completing a 15-week lifestyle intervention.

Main results: We found that employment of BA estimation in general health checks leads to an initial high participation rate. An improved BA was observed at follow-up due to small significant improvements in single metabolic risk factors and a high smoking cessation rate.

In the development of a new BA model, we found that nine out of 32 candidate biomarkers were applicable for use in the composite score of BA. The PCA analysis showed that the linear combination of the nine biomarkers differed between women and men, why sex stratified BA models were applied.

Regression analyses indicated that BA and chronological age was highly associated and that the BA model explained a substantial part of the variation in health risk related to chronological age. Furthermore, the BA model was able to distinguish between healthy and high-risk individuals and improved following a lifestyle intervention yielding a clinically significant weight loss.

Conclusion: This thesis advances the knowledge of BA as a concept for use in primary and secondary health promotion and provides a first generation of a new BA model. Our results indicate that the concept of BA has potential for use in health care, both as a motivational tool, but also as a health risk estimator.

Resumé

Baggrund: Den forventede stigning i middellevetid og i antallet af individer med overvægt og fedme øger risikoen for en markant stigning i prævalensen af kroniske sygdomme. Tidlig og effektiv forebyggende indsatser er derfor nødvendige for at fremme sund aldring. Biologisk alder (BA) er et redskab brugt i forbindelse med sundhedstjek for at øge motivationen for sund livsstil. BA estimerer risikoen for sygdom of tidlig død ved statistisk at sammenligne den enkeltes fysiologiske tilstand med den gennemsnitlige i en referencegruppe af samme alder og køn. Indtil nu foreligger der ikke evidens for validiteten eller effekten af anvendelsen af BA i sundhedstjek. Omvendt har der været stor videnskabelig interesse indenfor biologisk alder feltet, hvilket har resulteret i en bred vifte af BA modeller valideret op imod sygelighed og dødelighed i kohorte baseret studier. Indtil nu foreligger der ikke tilstrækkelig med interventionsstudier til at afdække hvorvidt BA som koncept også har klinisk relevans i det forbyggende sundhedsarbejde. Formålet med denne Ph.d. var derfor at undersøge anvendeligheden af BA som motiverende- og klinisk redskab. Som en del heraf, har vi udviklet en ny BA model, der kan måle sund aldring og risiko for sygdom.

Metode: I et retrospektivt kohortestudie undersøgte vi effekten af sundhedstjek, som inkluderer BA estimering baseret på deres egen BA model. Invitationer blev sendt til medarbejdere på 90 forskellige virksomheder. BA blev estimeret i 9.851 medarbejdere i første runde og i 3.843 medarbejdere i den opfølgende test.

I et tværsnitsstudie indsamlede vi 32 potentielle biomarker til at måle sund aldring og indgå i den nye BA model. I alt gennemgik 100 kvinder og mænd, i alderen 18-65 år, en gennemgående sundhedsundersøgelse. Vi anvendte korrelationsanalyser for at udvælge de mest relevante biomarkører til BA modellen. Principal komponentanalyse (PCA) blev brugt til at bestemme den lineære kombination af biomarkørerne, og den første principielle komponent blev brugt til at genere ligningen hvormed BA estimeres. Slutteligt undersøgte vi BA modellens kliniske anvendelighed i et interventionsstudie, hvor 27 kvinder og mænd med overvægt og fedme gennemførte en 15-ugers livsstilsintervention.

Hovedresultater: Data viser at motivation for at deltage i sundhedstjek er høj når BA inddrages. BA var forbedret ved den opfølgende test på grund af små men signifikante forbedringer i enkelte metaboliske risikofaktorer samt et højt fald i antallet af rygere.

Vi fandt at ni ud af de 32 potentielle biomarkører var relevante for at estimere BA. Baseret på PCA fandt vi at den lineære kombination af de ni biomarkører var forskellig for kvinder og mænd, hvorfor vi lavede kønsspecifikke BA modeller. Regressionsanalyserne indikerede at BA og CA var tæt relateret og at BA modellen kan forklare en høj andel af variationen i sundhedsrisiko forbundet med CA. Slutteligt fandt vi at BA modellen kunne diskriminere mellem sunde personer og personer med høj risikoprofil og at BA faldt efter et klinisk relevant vægttab.

Konklusion: Denne afhandling bidrager med ny viden om anvendeligheden af BA som koncept i både primære og sekundære sundhedstilbud samt et bud på en ny BA model. Vores resultater indikerer at BA konceptet er brugbart i det forebyggende sundhedsarbejde som motiverende redskab men også med potentiale for at estimere risiko for sygelighed og dødelighed.

Introduction

We are all living longer in an unhealthy old age

For the last four decades, the aging demographic of the population has changed globally due to, among others, declining fertility and mortality rates (2). From 1980 to 2017, the number of persons ≥ 60 years has more than doubled and by 2050 yet another doubling is expected (**Figure 1A**) (3). This development is especially advanced in Europe where population projections estimate that the percentage of adults > 60 years will increase from 24% in 2017 to 35% in 2060. These demographic changes will challenge the health care systems and affect the labor market as the older working groups (50-64 years) will constitute an increasingly larger proportion of the work force (4). Although aging per se is not a disease, the vulnerability towards disease increases with age (5). Thus, age is considered the most important risk factor for many chronic diseases including cardiovascular disease (CVD), type 2 diabetes (T2D), cancer, and kidney disease (6).

Within that same period, the obesity prevalence doubled from 1980 to 2015 in 73 countries with no indication of levelling off (**Figure 1B**) (7). As the degree of obesity increases, so does the risk of developing chronic diseases such as CVD, T2D, some types of cancer (e.g. breast, kidney and colon cancer), and osteoarthritis (8). Chronic diseases require extensive and expensive treatments for a substantial amount of time following disease manifestation, and they are currently responsible for 71% of all deaths globally (9, 10). Collectively, the increase in life expectancy accompanied by the obesity epidemic will have an additive effect on the susceptibility to chronic diseases and decreased health span. This entails industrialized countries with practical and economic future challenges. To accommodate these challenges, early and effective health promotion is paramount for a future healthy aging population.

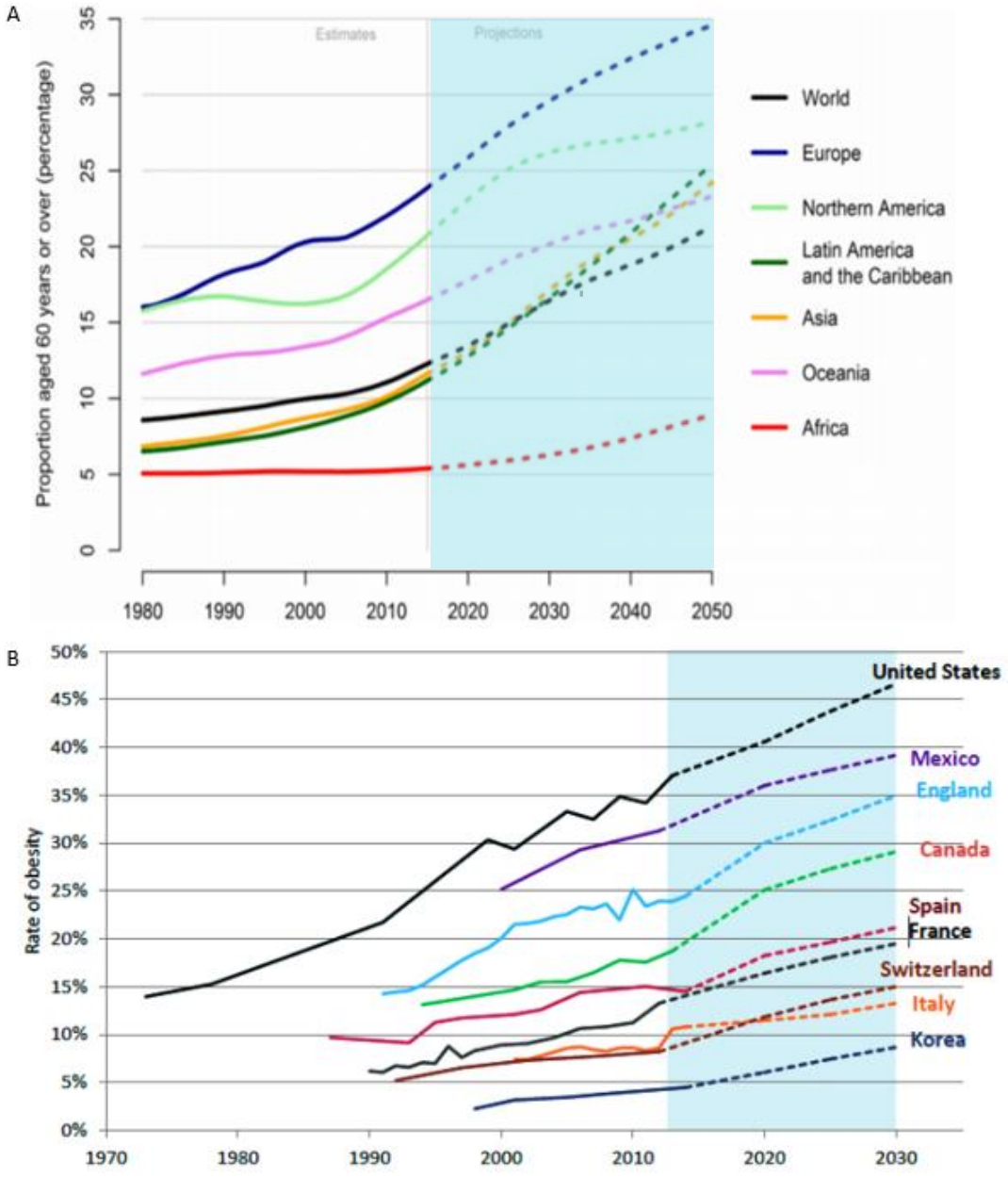

Fig. 1 *Prospects of aging and obesity- solid lines are estimates and broken lines are projections.*

A: Percentage of population aged 60 years or above. Modified from: United Nations (2017). World population Prospects: the 2017 Revision.

B: Percentage of the adult population (age 15-74 years) with a BMI ≥30 kg/m^2. Modified from: OECD analysis of national health survey data (2017)

Health promotion and disease prevention

According to the world health organization (WHO), the aim of health promotion is to target the root cause of poor health instead of focusing solely on treatment and cure, and thereby enable people to increase control over their own health (11). Following the first part of this definition, focus on early detection of disease or risk factors of disease, has been a main strategy to prevent future chronic disease (10, 12).

General health check

A general health check is a model where a person undergoes a screening to identify unrecognized symptoms or risk factors for disease. The theory behind is that providing people with information on individual risks will improve their understanding and motivation for a healthy lifestyle behavior.

The effect of a general health check on all-cause mortality and disease specific mortality was investigated in a Cochrane review and meta-analysis by Krogsbøll et al. (13). The primary analysis included nine randomized studies, investigating general health checks compared with no health check in an adult population performed at the general practitioner (n=3), workplace (n=1), or in the community (n=5). The primary outcome was all-cause mortality, cardiovascular and cancer mortality. The median follow-up time was 9 years. There was no evidence to support that general health checks reduced all-cause mortality (risk ratio 0.99 (95% confidence interval (CI) 0.95-1.03), cardiovascular mortality risk ratio 1.03 (95% CI 0.91-1.17) or cancer mortality risk ratio 1.01 (95% CI 0.92-1.12). In fact, a recent randomized study (Dan-MONICA) demonstrated that the intervention group (who received three health checks) had a higher risk of stroke (Hazard risk: 1.14, 95% CI: 1.04- 1.25, p=0.004) compared to the control group(14).

These surprising and somewhat contra intuitive results do not support the hypothesis that general health checks motivate health behavior change and results in decreased mortality on a population level. However, before rejecting the use of health checks completely as part of health promotion initiatives, some limitations of the analysis should be taken into consideration. Despite a randomized design, participants were primarily allocated based on those who accepted an invitation or answered a questionnaire. Also, in the pre-randomized Dan-MONICA study, a total of 902 individuals in the intervention group (n= 4,789) did not participate in any of the three health checks. These individuals represented an older group with more comorbidities compared to the remaining 3,887 participants. Together, this relates to the volunteer bias effect and difficulty when recruiting equally across social determinants and health. Further, it is worth considering the substantial amount of time (4-22 years)

between the last health check and the follow-up assessment. In the Inter99 study, a substantial improvement in smoking prevalence, physical activity, and dietary habits were observed in high-risk individuals at the end of the five-year intervention, indicating that annual health checks with counselling can be effective. At ten-year follow-up, however, no effect of the five-year intervention was found on stroke incidence or mortality (15). While a long follow-up time is necessary for the hard outcomes to manifest, it is not surprising that the effects of a health check five years ago (or more) do not persist. It is well established that the positive effects of physical activity on cardiovascular function and skeletal muscle health only persists as long as the training stimuli is sufficient (16). This is true for any therapeutic drug as well. The same can be hypothesized to be the case with health checks. Annual health checks are properly necessary throughout the adult life course to maintain adherence to a healthy lifestyle.

Biological age applied in practice

Despite the discouraging results presented above, health checks are still widespread, especially in workplace health promotion (17) and among commercial providers (18). For decades, the workplace has been a prioritized setting for health promotion due to its potential to reach widely within a population and potentially reach individuals not normally engaging in health enhancing interventions (19). In Denmark, and in other countries the health check industry is growing (18). Private actors are the main suppliers of health check interventions performed at the workplaces, and the industry is supported indirectly by the legislated tax exemption for private health insurance (18).

In recent years, biological age (BA) has received public interest and has been included in health checks as an outcome measure, pedagogical aid and motivational tool. BA is a statistical measure of predicted life expectancy and surrogate measure of risk of disease compared to population means with the same sex and age (20). We hypothesize that a reason for the interest in BA when it comes to health promotion is that being older (or younger) than stated on the birth certificate easily translates into disease risk and premature death. Further, it might have an effect by speaking to vanity. When performing health checks, standard protocol is that a health care professional explains how measured risk factors (e.g. blood pressure, lipid profile, and waist circumference) and health behavior (e.g. cardiorespiratory fitness level, diet, and smoking) relates to the risk of future chronic diseases (13). While this might increase the understanding of the relation between health behavior and the underlying pathophysiology of chronic disease, it can be questioned whether this increases the motivation for

reducing high risk behavior. In order to increase motivation for risk reducing behavior, an alternative approach is necessary, and for this purpose, BA could be a useful motivational tool.

The Polar BodyAge system® and the MetabolicAge® by Tanita are two examples of biological age models used in the private health care industry. The Polar model is based on well known risk factors associated with an increased risk of chronic diseases and premature death, affected by aging, and mediated through lifestyle. To estimate BA, the Polar method combines measures of muscular strength, flexibility, cardiovascular fitness level, blood pressure, and cholesterol profile (21). The Tanita model is based on the age-related decrease in basal metabolic rate due to loss of skeletal muscle mass as well as the relation between body composition and risk of disease. The Tanita method uses the basal metabolic rate estimated from the amount of muscle mass, age, height, and sex to estimate a person's Metabolic Age (22).

To the best of my knowledge no studies have confirmed the validity of the above-mentioned BA models in relation to disease incidence or mortality. In addition, the evidence of BA as a motivational tool is inconsistent and sparce. One cluster randomized study (*n*=121) showed no effect of employing BA (the Polar Body Age system) compared to standard feedback on improvements in physical activity level in workplace health checks (23). Another randomized study including patients with coronary heart disease investigated if patients chose to focus on improvement of their lipid profile, blood pressure or other risk factors pending on the specific biological age risk reduction value (intervention group *n*=329) compared to standard recommendations (control group *n*=331) (24). The individual reduction in BA was estimated using a commercially available BA model (RealAge®). A sub analysis showed that if health personal recommended a change in a risk behavior associated with high reductions in BA, patients were more likely to change this health behavior as compared with patients who got the recommendation alone (24). These studies vaguely imply that BA is more useful as motivational tool in tertiary prevention aiming to stop/slow disease progression, compared to secondary prevention targeting unrecognized symptoms or risk factors for disease.

Overall objectives of the thesis

The overall objectives of this thesis was to investigate BA as a concept with implication to health promotion and disease prevention. We aim to answer the following questions: BA is frequently used as a motivational tool, but can we validate the concept as a clinical tool to identify high risk individuals and predict development of chronic diseases? What is the pathophysiology of BA and how does it relate to

lifestyle diseases? How has BA been investigated previously, and can we develop a BA model following scientific standards and still be feasible for use in public health promotion?

Background

Etiology and pathophysiology of (biological) age

Inherent in the aging process is the loss of physiological function and an increase in susceptibility to diseases and disability (25). The mechanisms driving these age-related changes have been the primary focus of interest within basic aging science. Lopez-Otin et al. describes a functional division of the 9 cellular hallmarks of aging as follows (26):

1) Decreased genomic stability, epigenetic alterations, shortening of telomeres and dysregulated protein homeostasis responsible for the cause of cellular damage.

Causing:

2) Deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence.

Leading to:

3) A reduction in stem cell activity and alterations in the neurohormonal communication between cells, leading to chronic low-grade inflammation characterized by elevated levels of tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), C-reactive protein (CRP) among others (26, 27).

Through these complex systems, aging will affect all physiological functions (28). The major physiological clinical features of aging related to the vulnerability to CVD and T2D includes changes in the cardiovascular system (29), changes in cardiovascular response (30), changes in body composition (31) and changes in skeletal muscle (32) (**Figure 2**).

While the aging process is inevitable, the rate of aging is not the same between individuals. Based on twin studies, it is estimated that 25-50% of the variation in life expectancy and susceptibility to disease is driven by genetic factors (33-35).This implies that up to 75% of the variation in susceptibility to disease can be modified by environmental and lifestyle behavior. Hence, aging can be divided into chronological age (CA) measured by years from birth, and biological age driven by the hallmarks of aging combined with environmental, stochastic, and lifestyle factors. The latter makes biological age of particular interest in biomedical science with a focus on healthy aging.

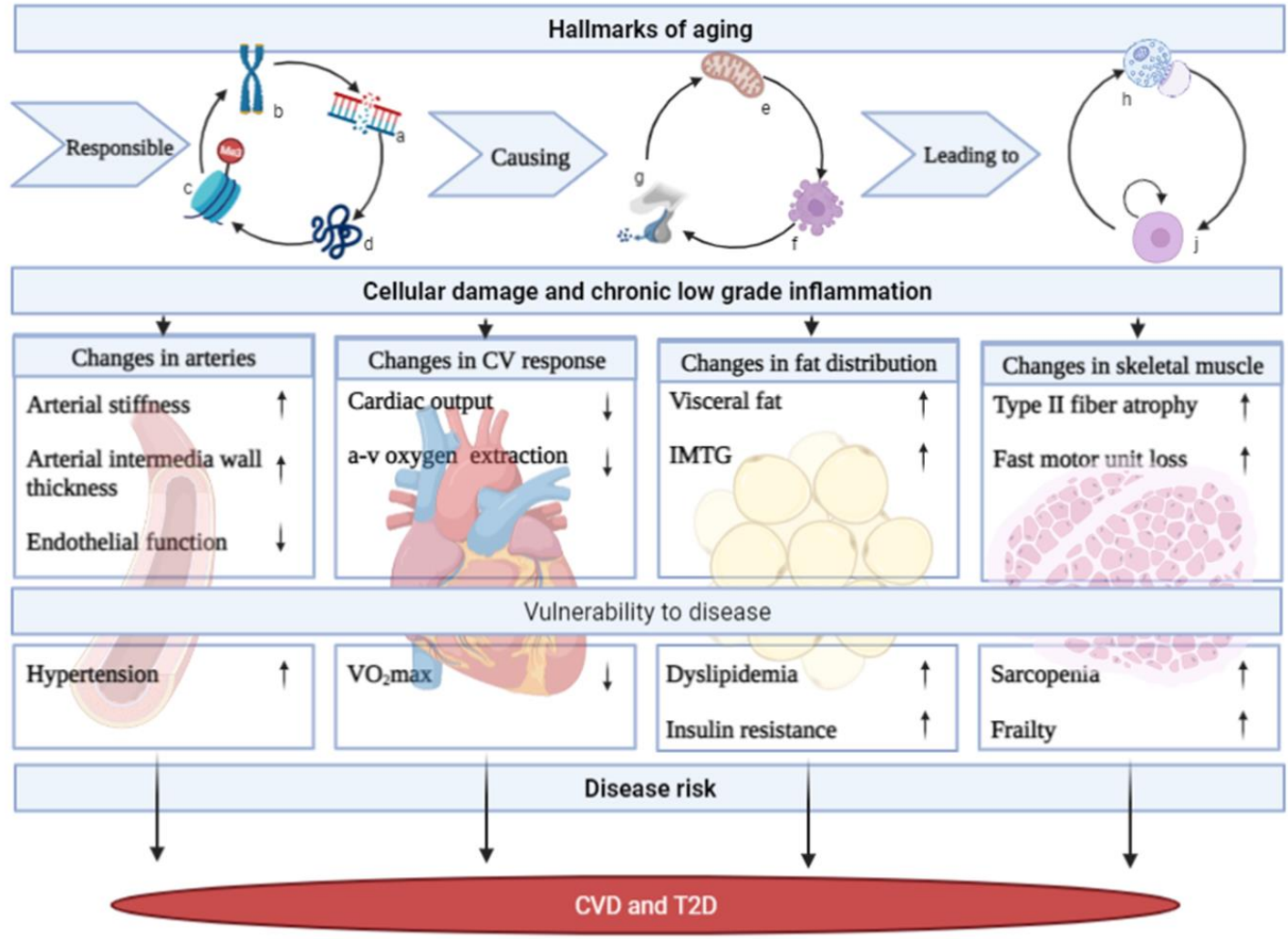

Fig.2. *The nine hallmarks of aging and changes in physiological functions related to risk of CVD and T2D. Aging is caused by progressive cellular damage initiated by genomic instability e.g., DNA damage (a), telomere attrition i.e., shortening (b), epigenetic alterations e.g., histone modifications (c) and disturbances in protein homeostasis e.g. protein metabolism (d). These alteration causes mitochondrial dysfunction e.g., oxygen stress (e), cellular senescence e.g., deficient clearance of senescent cells (f) and deregulated nutrient sensing e.g., reduced growth hormone levels and impaired insulin and Insulin-like growth factor (IGF-1) signalling pathway (g). This leads to altered neurohormonal communication between cells e.g., inflammation (h) reduced stem cell activity e.g. Impaired regeneration (j). The top illustration of the nine hallmarks of aging is modified from Lopez-Otin et al., Cell, 2013, Figure 6, p. 1207.*

Altogether the aging process leads to cell damage and chronic low-grade inflammation responsible for the pathophysiology of age-related changes in arteries, cardiorespiratory fitness (CRF), fat distribution and skeletal muscle. As a result, aging will increase risk of cardiovascular disease (CVD) and type 2 diabetes (T2D). Abbr.: VO_2max: maximal oxygen consumption, IMTG: intramuscular triacylglycerol. Created in BioRender.com

Obesity and biological age

The development of obesity is complex and involves energy imbalance, aging, environmental conditions, genetic factors, and socio-economic status (36, 37). From epidemiology studies, we know that a high BMI is a risk factor for a wide variety of chronic diseases, in particular CVD which is the leading cause of death associated with high BMI (37). Adipose tissue is an important endocrine organ involved in energy metabolism, neuroendocrine and immune function. Obesity and especially excessive visceral adipose tissue (VAT) promotes low grade inflammation by adipose infiltration of macrophages secreting proinflammatory factors to the blood (38, 39). Thus, increased VAT is associated with increased levels of TNF-α leading to hypertriglyceridemia, low concentrations of high-density lipoprotein cholesterol (HDL-C) and increased levels of low-density lipoprotein cholesterol (LDL-C). These changes are associated with impaired endothelial function and development of atherosclerosis which is a risk factor for CVD (40, 41). Adiponectin is an adipokine mediating glucose-metabolism by prompting the uptake and oxidation of free fatty acids (FFA) in skeletal muscles (42). Studies have found that the amount of circulating adiponectin is reduced in individuals with obesity and possibly with increasing age (42-44). The combination of decreased concentrations of adiponectin and a TNF-α stimulated release of FFA entails that plasma levels of FFA increase. Elevated plasma FFA levels are associated with peripheral liver and skeletal muscle insulin resistance, due to increased fat accumulation, and an increased risk of T2D (41, 45).

Altogether, obesity exacerbates the risk of age-related diseases with similar pathophysiological pathways related to chronic low-grade inflammation. In addition, evidence has emerged that obesity accelerates, while physical activity conversely attenuates, the cellular hallmarks of aging *per se* (46, 47). Therefore, a healthy lifestyle is an important biological age mediator and denominator for healthy aging.

Physical activity and biological age

Physical activity is a cornerstone of a healthy lifestyle and involves the activation of skeletal muscles. Like adipose tissue, skeletal muscles is an endocrine organ and are the primary responsible for glucose uptake and therefore act as a regulator of peripheral insulin sensitivity. During physical activity, myokines such as IL-6 are released (41). Physical activity stimulated IL-6 release has anti-inflammatory properties which, among other things, blunt chronic low grade inflammation by reducing plasma TNF-α concentrations (48). Locally, contraction-induced IL-6 release prompts an increase in glucose uptake and

fat oxidation through phosphoinositide 3 (PI3)- and adenosine monophosphate-activated protein (AMP) kinase activation, respectively (41). In addition, the age-related loss of muscle mass (sarcopenia) and reduced peripheral insulin sensitivity is attenuated in individuals with sufficient physical activity levels (49).

VO_2max is the golden standard for cardiorespiratory fitness (CRF) and increases with exercise, however, the size of the response to exercise differ related to genetic variability (≈50%) (50). VO_2max predicts all-cause mortality and particularly the risk of cardiovascular disease within the general population and high-risk individuals (51-54). A reduced maximal cardiac output and loss of muscle mass is the primary cause for the age-related decline in VO_2max (30, 55). This age-related decline in VO_2max was investigated in the large cross-sectional HUNT fitness study. The study measured VO_2max among 4,631 healthy women and men aged 20-90 years (56). For every 10-year increase in CA, VO_2max declined with approximately 6.2% (95% CI 5.9%-6.6%) for both women and men. As expected, across sex and age, VO_2max was consistently lower for inactive individuals compared to individuals with low, medium, and high physical activity level. Furthermore, clustering of risk factors for CVD (obesity, hypertension, and waist circumference) was compared among inactive and regularly active individuals. Noteworthy, for a given VO_2max, inactive individuals in the age category 20-29 years had a cluster of risk factors similar to that observed among physical active individuals in the age category 50-59 years. These sub-analyses were based on self-reported physical activity level and should be interpreted with cation due to a risk of misclassification. Nevertheless, the results indicate that physical activity can attenuate the age-related decline in VO_2max and risk of future disease (56). These results are in line with both longitudinal and cross-sectional studies showing a reduced rate of VO_2max decline among physically active individuals where inactivity is eliminated as a confounding factor (49, 57). The isolated impact of inactivity on all-cause mortality is larger than single conventional risk factors such as obesity, smoking, T2D, and high cholesterol (53, 58, 59). In addition, McGuire et al. showed that inactivity in the form of disuse (three-week bed rest) were more aggravating to VO_2max compared to 30 years of aging (60).

In summary, inactivity accelerates the age-related decline in VO_2max and risk of age-related disease, while physical activity conversely attenuates the decline in skeletal muscle and cardiovascular function observed with age. But what is sufficient physical activity?

A meta-analysis from 2019 concluded that all levels of physical activity, regardless of the intensity, reduces the risk of premature death (59). Exploring the dose relationship between physical activity level and all-cause mortality, the study observed that 24 minutes/day of moderate physical activity

(measured by accelerometry), was sufficient to reach maximal risk reduction of all-cause mortality. This aligns well with Danish national recommendations of physical activity (61). Unfortunately, trends in physical activity are discouraging and displays an inverse relationship between age and level of physical activity (62). In 2017, 28.8 % did not adhere to the physical recommendations in Denmark (63). In 2016, the global prevalence of insufficient physical activity reached 27.5% (95%CI 25.9 - 32.2) and 36.8% (95% CI 34.6 - 38.4) in western countries (64).

Nobel prize winner George Bernard Shaw once said: *"We don't stop playing because we grow old; we grow old because we stop playing"*. Being physically active throughout life and maintaining active in older age is paramount with regards to risk of disease and premature death. However, the overarching question remains unanswered: How do we motivate people to be physically active?

The concept of biological age

The concept of BA was first described in *the Lancet* by Alex Comfort in 1969 (65). He proposed that the combination of multiple biomarkers into a single latent variable of BA could be used to measure individual aging trajectory on a physiological level and thereby the risk of disease (65). As an example, if

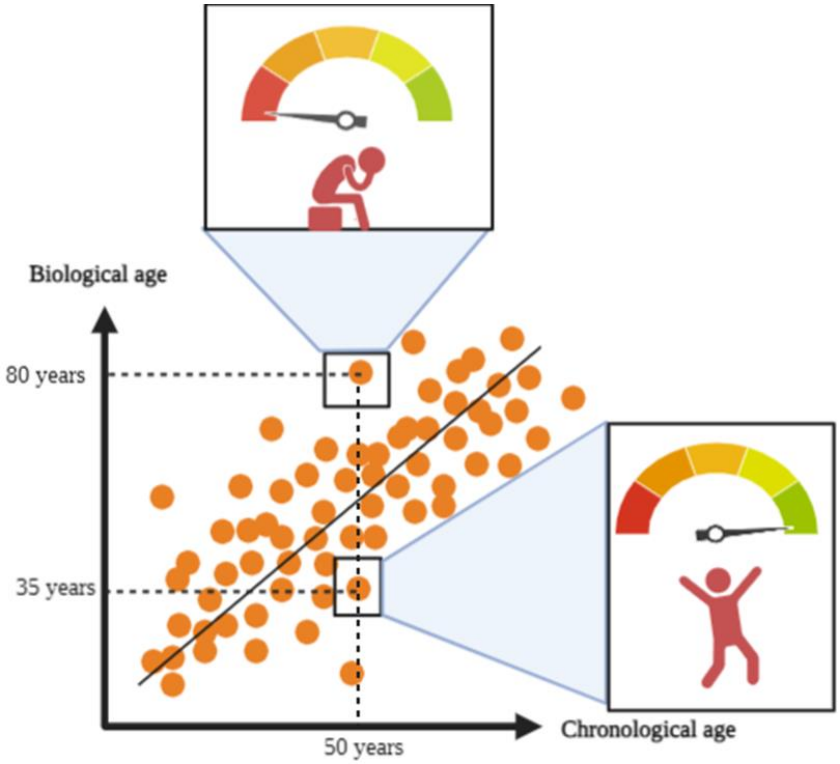

Fig. 3. *A graphical depiction of the concept of biological age. Two persons, born the same year, have very different biological age and thus different vulnerability to age-related disease. Created in BioRender.com*

an individual is 50 years, but 80 years biologically, that person will have a physiological profile (and consequently susceptibility for disease) similar to an individual 30 years older statistically (66) (**Figure 3**).

Specifically, the concept of BA involves the selection of biomarkers representing the integrity of various physiological functions known to decline with aging. The biomarkers are collected in a reference group of individuals free from disease and used to model the healthy aging trajectory (67). However, because it is difficult to distinguish subclinical conditions from age related decrements in older age, some studies allow hypertension and dyslipidemia (68, 69). The reference group should span the entire adult spectrum and ages should be evenly distributed (67). It has been argued that including adults below 40 or 50 years of age is negligible as physiological decline before this age is not expected (5, 70). Conversely, the Dunedin birth cohort study (71) showed that being biologically older, even in a chronologically young age (38 years, n =954), were associated with lower physical performance, increased cognitive decline and lower self-reported health. In addition, being biologically older was associated with a decline in multiple physiological functions over the 12-year follow-up period from age 26-38 years. Collectively, this study refute the premise that biological age estimation is restricted to middle aged populations, and in addition, indicate that BA can discriminate between healthy and unhealthy young adults. These findings are essential when considering BA as a tool in health promotion, as early interventions is key to reduce the risk of future age-related diseases.

Biomarkers of healthy aging

Biomarkers of aging is a loosely defined term. The most cited definition was given by Baker and Sprott in 1988 stating that a biomarker of aging can be any *"biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capacity at some later age than will chronological age" (72).* Based on the last 50 years of research, the idea that a single aging biomarker can predict life expectancy has been abandoned. Instead, a combination of biomarkers is recommended in order to capture the complexity of aging trajectories which supports the applicability of the BA concept proposed by Alex Comfort (66, 73). To increase reliability and validity, the following criteria for biomarkers of aging have been suggested by Ingram et al:

1. Non-lethal

2. Highly reproducible

3. Displays significant alterations during relatively short time periods

4. Critical to effective maintenance of health and prevention of disease

5. Reflects a measurable parameter that can be predicted at a later age

6. Reflects some basic process of aging and metabolism

7. Should have high reproducibility in cross-species comparison (74)

The seven criteria have been modified several times since the first edition, but together with the definition from Baker and Sprott, the main message remains unchanged. Biomarkers must be simple and inexpensive to use and at the same time describe underlying mechanisms of aging. Biomarkers should be investigated in a population free of disease, should correlate with CA, and predict the future incidence of age-related diseases (28, 75). The criteria of cross-species comparison is questionable when validating biomarkers of human aging. Recognizing animal studies as important pre-clinical human trials, animal studies of aging are, however, conducted in well-controlled environments making it difficult to translate the observed mechanisms to that of the free- and long-living human species compared to e.g., rodents.

Focusing on healthy aging, the concept of biological age is appealing as a tool to identify individuals with a future risk of disease and premature death. Further, it is appealing to use when investigating health-span extending interventions (76). An optimal BA model should, therefore, include biomarkers measuring key features of healthy aging. To enable this a clear definition of healthy aging is needed. Healthy aging has been the focus in more than ≈3000 scientific articles in 2016 (77). Still, the terminology is not clear varying between healthy aging (78), successful aging (79), resilient aging (80), and active aging (81). In 2015, WHO changed their terminology from active ageing to healthy ageing (82). Their definition of healthy aging is a process in which functional ability and well-being is retained and emphasize that healthy aging is not only a matter of being disease free (82). This holistic definition opens for a variety of ways to measure healthy aging and the operationalization of the term depends on the context and research question. Our focus is on minimizing risk for age-related disease, why we seek to establish physiological quantifiable biomarkers of healthy aging.

To accommodate this issue, Lara et al. proposed a panel of biomarkers of healthy aging (83). The included biomarkers represented 5 domains: physiological function, endocrine function, physical capacity, cognitive function and immune function (**Figure 4** top row) (83). Their conceptual framework provides an overview of possible biomarkers within each domain, their feasibility of use outside a clinical or laboratory setting, relevance to prediction outcomes (e.g., mortality, CRF, CVD), and cost. The authors

do not provide a discussion of the relative importance of the proposed biomarkers nor the approach to combine the biomarkers into a single score (83). Inspired by this work, we decided to compose a biological age model including biomarkers of healthy aging. We chose to limit the model to the domains of physiological function, physical capacity, and immune function with the aim of developing a BA model useful for the assessment of physiological health and risk of chronic diseases (**Figure 4** bottom row). Our model comprise 32 candidate biomarkers. Following the standards for biomarkers of aging, these 32 candidate biomarkers are valid markers of the central mechanisms of age-related decline in physiological function, essential in the maintenance of health and prevention of chronic disease, technically simple, easy to reproduce and minimally invasive including a blood sample at the most. The relevance of the candidate biomarkers are provided for in depth in paper II. However, it is unlikely that all 32 biomarkers are necessary to model BA and a weighting of biomarkers is therefore appropriate.

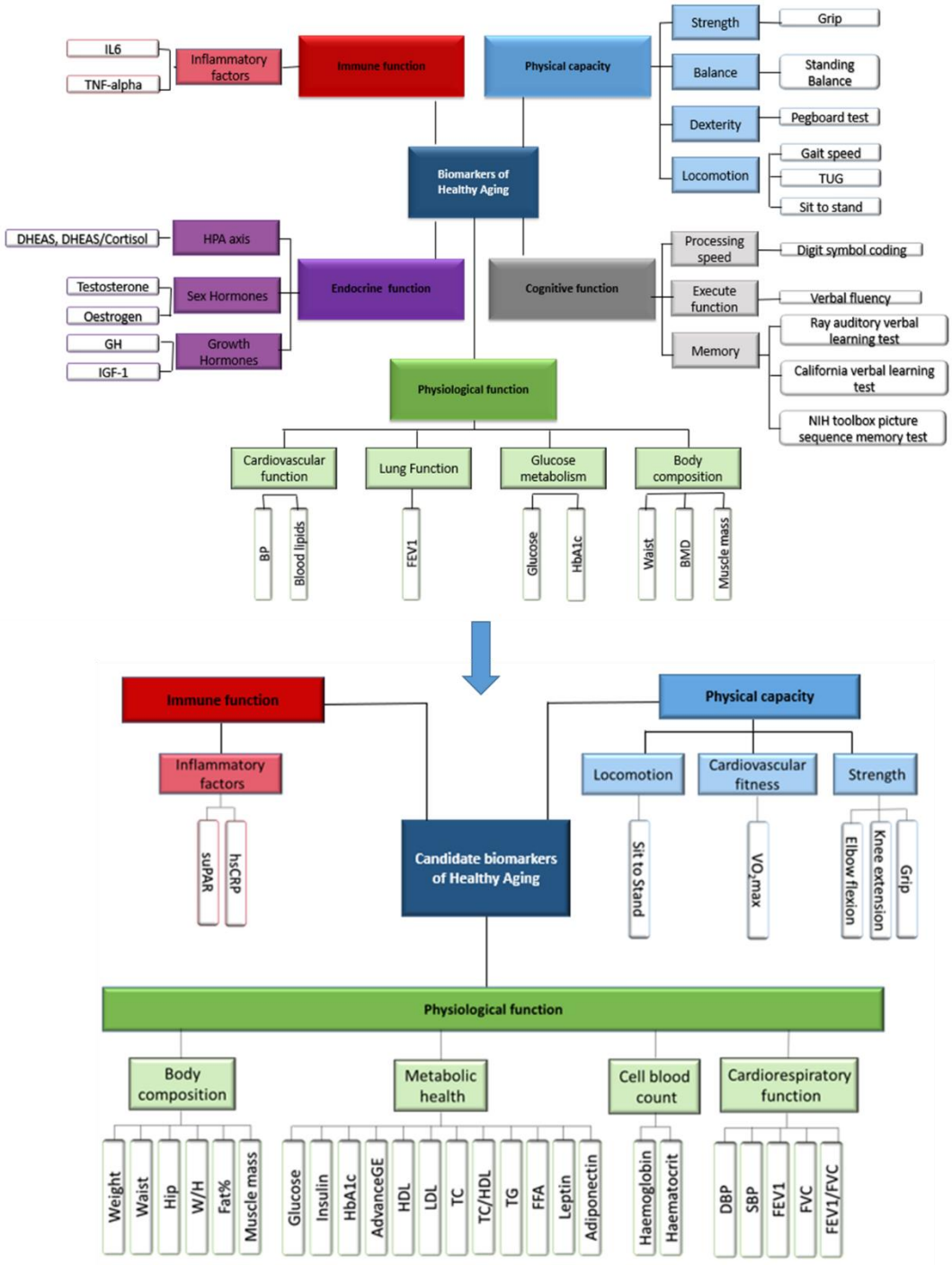

Fig. 4 *(Top) Original model adapted with permission from Laura et al., BMC Med., 2015, Figure 2 page 4. proposing a panel of biomarkers of healthy aging within five domains, and (Bottom) the modified model proposing candidate biomarkers within three domains to measure healthy aging with a focus on chronic disease risk prediction (modified from paper II, Figure 1 page 3). Abbreviations: BMD: bone mass density; DEHAS: dehydroepiandrosterone-sulphate; W/H: waist to hip circumference; HbA1c: glycated hemoglobin; AdvancedGE: advanced glycation end products; HDL: high density lipoprotein; LDL: low density lipoprotein; TC: total cholesterol; TG: triglycerides; FFA: free fatty acids; DPB: diastolic blood pressure; SBP: systolic blood pressure; FEV1: forced expiratory volume within 1 second; FVC: forced vital capacity; suPAR: soluble urokinase plasminogen activator receptor; hsCRP: high-sensitive C-reactive protein; VO_2max: maximal oxygen consumption; TUG: timed up and go.*

Biological age estimation

Different statistical approaches can be employed to determine how biomarkers of healthy aging are combined into the latent score of BA. The following section provides an overview of the most common approaches applied for BA modelling.

Multiple linear regression

We are used to think of a variable, for example mean blood pressure (BP), as a function of CA:

$$(1)\ BP = f(CA)$$

Assuming a linear relationship, the least sum of squared distances are used to find the best fitted line to the data given by:

$$(2)\ BP = w_0 + w * CA$$

Where w_0 is the intercept and w is the slope of the line. However, this relationship can be inverted so that mean CA is a function of BP:

$$(3)\ CA = f(BP)$$

Importantly, this does not mean that BP determines age, but that with the knowledge of BP, an estimate of age can be made (84, 85). This is the basic approach when using multiple linear regression (MLR) to estimate BA. By employing MLR, the individual BA is estimated on the basis of multiple biomarkers:

$$(4)\ BA_i = w_0 + \left(w_1 x_{1(i)}\right) + \left(w_2 x_{2(i)}\right) + \ldots \left(w_k x_{j(i)}\right)$$

Where BA_i is the estimated biological age of the *i*-th individual; $x_{1(i)}$, $x_{2(i)}$….$x_{j(i)}$ are the values of the biomarkers 1, 2, ….*j* of the *i*-th individual with *j* being the number of biomarkers (independent variables); w_k is the slope from the correlation between CA and each biomarker ($x_{j(i)}$) using the least sum squares. This last notion means that BA is estimated as a linearly best fitted value of CA (dependent variable), thereby assuming that CA depend on the included biomarkers which is obviously not the case – CA is defined by the time from birth (86). Moreover, when the MLR equation is used to estimate BA from a healthy population, a perfect equation, which predicts the dependent variable correctly, always predicts the identical CA. Consequently, this approach does not support the concept of BA in that a difference in aging rate may be assessed based on the difference between predicted (BA) and actual ages (CA) (87). Finally, the MLR method produces prediction that tend to be close to the mean of the reference set

values. This means that individuals who are younger than the reference set mean will have a BA that is too high, while the BA for individuals who are older will, conversely, be too low (88).

Principal component analysis

Principal component analysis (PCA) was introduced to avoid some of the limitations mentioned above. PCA is a factor analysis able to reduce the dimensions of a dataset with the aim of retaining as much of the original information (variance) as possible (88). More specifically, PCA transforms the original independent variables into new vectors i.e. principal components (PCs) of a coordinate system where the variance of the data is maximized along the axes (89). In other words, PCA can take four or more variables (dimensions) and make a two-dimensional PCA score plot. Traditionally, the data is plotted in one or more two-dimensional PCA score plots, depending on the number of PCs included in the model. Employing PCA in prediction modelling, usually, all PCs with an eigenvalue above one are included, or alternatively the number of PCs that together contains 80% of the variation in the dataset (90). Thus, it can be used to elucidate the minimum number of the candidate biomarkers necessary to estimate BA (88). However, in 1988, Nakamura et al. proposed that the first principal component (1stPC) alone could be used as an equation for the estimation of BA (87). The loading scores (the proportion of each biomarker and their contribution to the principal component (89)) from the 1stPC was used to generate an individual BA score (BAS_i):

$$(5)\ BAS_i = w_0 + (w_1\, x_1) + (w_2 x_2) + \cdots + \left(w_k x_{j(i)}\right)\ (modified\ from\ paper\ III, eq.1, p.8),$$

where BAS_i is the estimated BAS of the *i*-th individual; $x_{1(i)}$, $x_{2(i)}$….$x_{j(i)}$ represents the original value the biomarkers 1, 2, ….*j* of the *i*-th individual with j being the number of biomarkers (independent variables). Different from MLR, w_k, is the loading score for each of the biomarkers ($x_{j(i)}$) on the 1stPC divided by the standard deviation of each biomarker. This way, CA is not included as the dependent variable.

Furthermore, Nakamura et al. (87) suggested to transform the BAS_i to a T-score (BA_i) using the mean and standard deviation of the CA of the study sample. With this calculation, the BA is presented in units of years. This is valuable in the case of interpretation, however, introducing this relationship between BA and CA can reintroduce the issue of predictions towards the mean which was otherwise eliminated. To correct for this distortion at the regression edges, the correction model proposed by Dubina et al. is frequently used in BA studies employing PCA (91):

$$(6)\ BAc = BA_i + (y_i - \hat{y}) \cdot (1 - b)\ (\textit{Modified from paper III, eq. 5, p. 9}),$$

Where BAc is the corrected BA estimation, $BA_i$is the estimated BA of the *i*-th individual in unit of years, y_i represent individual CA, $\hat{y}$ the mean CA of the study sample and b representing the slope in the linear regression assessing the relationship between BA_i and CA.

Novel methods

There is no consensus on how to estimate BA, hence, a number of seldom applied statistical methods can be found in the BA literature. (76) In the same way that PCA was an extension and improvement of MLR, the method described by Klemera and Doubal (92) was proposed to improve the validity of the estimation of BA compared with both MLR and PCA (93). The model combines the mathematical relations between the biomarkers, CA, and BA allowing nonlinearity of biomarkers (68, 88). The nonlinearity of certain biomarkers is a limitation of both MLR and PCA. Even though the majority of biomarkers are assumed to decline with a slope of 1% per year (94), some biomarkers deviate from this linearity, especially within the last decades of life. In addition, the difference in the clinical consequence of e.g., midlife and late life trajectories of hypertension is not accounted for in a linear model (95, 96).

In 2017, Jee et al. compared BA models estimated by MLR, PCA, and KDM by assessing the fit or closeness to CA values in 912 healthy women (68). The linear relationship between BA and CA expressed by coefficients of determination (R^2) was 0.56 (MLR), 0.67 (PCA), and 0.83 (KDM) with corresponding slopes of the regression lines: 0.56 (MLR), 1.00 (PCA), and 1.00 (KDM) (**Figure 5**, top row). The agreement between BA and CA was further investigated by Bland Altman plots (**Figure 5**, bottom row). The relatively lower coefficient of determination observed with MLR was caused by the over- and underestimation of BA toward both ends of the age spectrum. Despite the good linear fit between BA and CA with both PCA and KDM, the Bland Altman plot reveals that a greater dispersion was found for the PCA model (95% CI: 15.30; -14.09) compared with KDM (95% CI: 9.38; -9.38). Finally, the greatest difference in CA and BA was found with PCA. This indicates that the PCA model overestimates BA values. Jee et al. concluded that KDM is more accurate for predicting BA compared to MLR and PCA which is supported by the results of other comparative studies (76, 88). However, in the comparison study by Levine et al., they found that BA-predicted mortality was better using any of the three algorithms compared with CA-predicted mortality (76).This is important because, as I will show in the next section, employment of KDM in BA research is limited, possible due to low reproducibility of the method as a results of the mathematical complexity of the method (93).

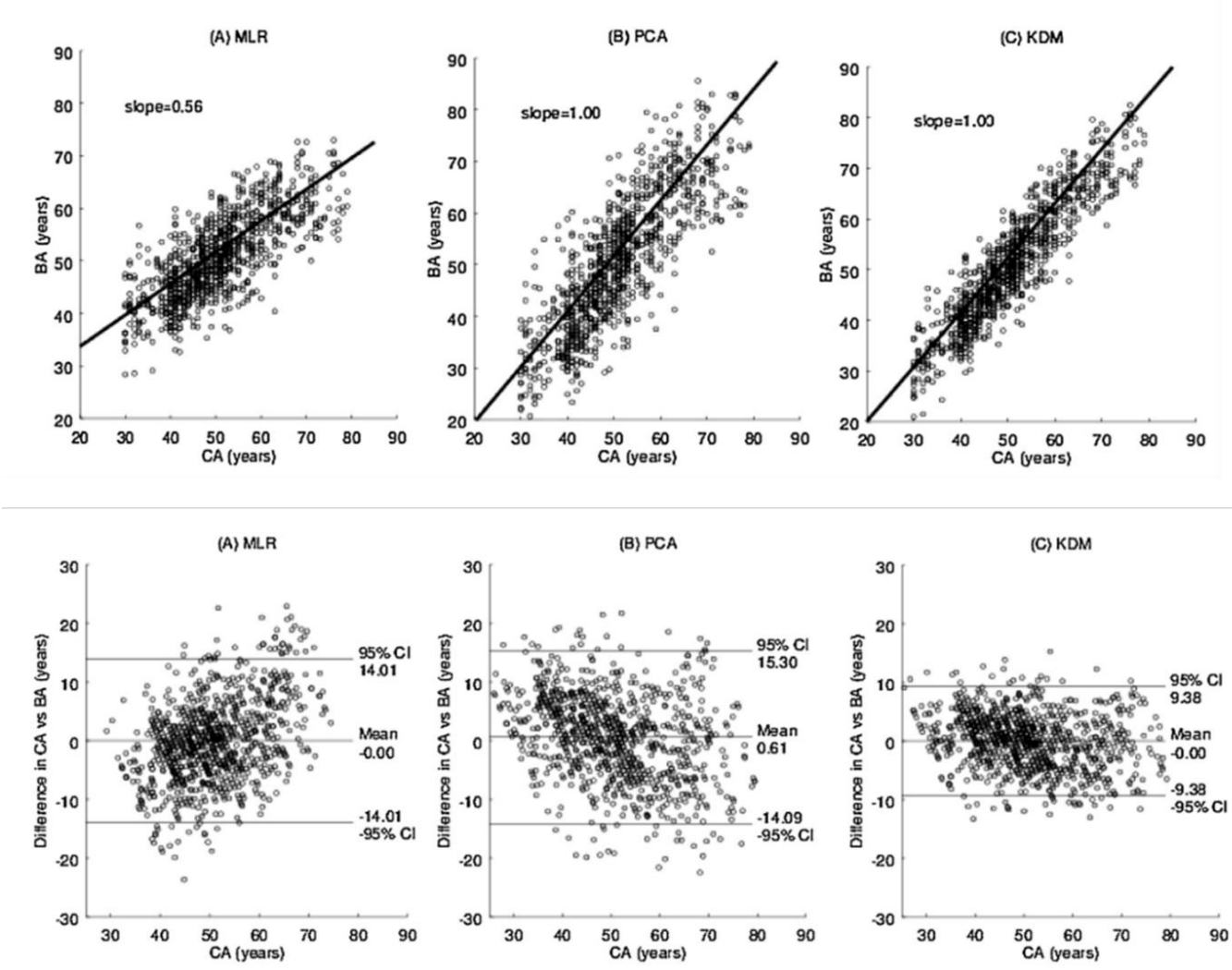

Fig. 5. *Biological age (BA) estimated using A) the multiple linear regression (MLR), B) principal component analysis (PCA), and C) Klemera and Doubal method (KDM) algorithms. Top row: BA in function of chronological age (CA). Bottom row: Bland-Altman plots between CA and BA. Adapted with permission from Jee et al., 2017. Figure 1, p. 87 and Figure 4, p.89.*

How is biological age studied?

We conducted a systematic literature search and review providing an up-to date overview of current BA models. In addition, we used this review to decide on methodology when estimating BA in our study. The search was limited to studies proposing BA models for use in adult human populations *in vivo* with the implication of general health promotion and prevention of chronic diseases. Thus, studies on BA

models including DNA, telomeres, and epigenetics as biomarkers were excluded. Further single organ specific models and studies comparing different algorithms were excluded (**Appendix 1**-search strategy). The systematic literature search was performed in PubMed, Embase, and Web of Science (**Appendix 1** Table 1). The search yielded 2,911 records after removing 1,319 duplicates. Another reviewer (PH) and I independently screened the 2,911 records by title and abstract excluding those articles that did not meet the in- and exclusion criteria. To ensure consistent application of the eligibility criteria we compared a sample (i.e. 10% ≈ 300 records) of the records independently screened. When we had finalized the screening, we compared our choice of eligible records. We discussed any disagreement concerning record eligibility until reaching consensus. The second stage of the eligibility decision constituted full-text reading of 77 records — once again independently assessed. Again, disagreement was resolved through discussion. Finally, 29 articles was included (**Appendix 1** Figure 1). The results from these studies are summarized in **Table 1**. In the case a BA-model and a physical age model was proposed in the same study, only the BA model was included for the sake of comparison.

Study design

BA models have primarily been developed using cross-sectional designs (n=19) and cohort studies (n=9). The investigated populations were primarily Asian (n=19) followed by European (n=6). Of the 29 studies, 15 studies use national registry-based data as the source of candidate biomarkers. A strength of using national databases is the large number of participants enabling longitudinal analyses and the possibility of validating the BA model against hard endpoints such as disease specific mortality and all-cause mortality. A limitation of using national registry-based data is that data is not necessarily statistical representative e.g., due to skewed distributions of sociodemographic variables. In a total of 17 studies, both women and men were included, and in 6 out of the 17 studies, the equation for estimating BA was stratified by sex. In eight studies, BA was derived from male subjects only.

Biomarkers

Selection of candidate biomarkers from national databases could be limited by availability and some of the data rely on self-reported questionnaires. Another consideration is that data from the registers presented in this review were collected 10-40 years ago. Thus, registry-based data reflect a certain era and population behavior which might have changed since then (e.g., smoking behavior and environmental factors). The review identified a large variation in which biomarkers were applied to estimate BA. Some studies cover a narrow area of physiological function e.g. cardiovascular function (97), body composition (98), or metabolic function (99) to assess BA and risk of disease. Other studies focus on biomarkers of physical function (e.g., grip strength, hearing abilities as well as walking and

balance tests) (100-105). However, the majority of the studies combine biomarkers measuring inflammation, cardiovascular, endocrine, and metabolic function in combination with biomarkers measuring bone integrity to estimate BA (87, 98, 106-123).

BA-estimation

Concerning the estimation of BA, more than half of the studies used PCA (n=15), eight studies used MLR, one study used KDM and the remaining five studies applied various other methods. In all but one (122) of the studies employing PCA, the 1stPC is used to estimate BA. When this method is employed correlation analysis was performed *a priory* to select between candidate biomarkers and reduce possible redundancy. Regardless of method, BA estimation relied on a combination of multiple biomarkers.

Validation of BA model

Because BA is estimated based on biomarkers from a healthy reference group, the hypothesis is that BA and CA are closely related within the reference group. Thus, in most of the studies (n=23), a high correlation with CA is used to validate the BA model. To validate the clinical utility of the BA model, a comparison of BA against CA in high-risk groups is a frequently applied method (n=21). High risk groups are defined by body mass index (BMI) ≥25, hypertension, T2D, smoking, alcohol consumption, and fitness level. Only one study validated the BA model by intervention (100). In this study, Shigematsu et al. (2019) validated their BA model through a 3-month training intervention in a group of older women (n= 14, mean age: 79.5 ±3.9 SD years). The model combined four biomarkers measuring physical function (arm-curls, moving beans with chopsticks, sitting and walking around two cones, and reaching forward with the arms while in a standing position). They found a significant decrease in BA together with an improvement in the standardized senior fitness tests with no change in the control group.

In summary, overreaching comparisons of BA-models seem difficult especially due to the heterogeneity of biomarker combinations. The transferability of study results to other non-Asians populations with different genetic and behavioral characteristics is limited. It seems, that despite previous research indicating that KMD is superior for estimation of BA (68, 76) PCA is the most applied method.

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Bae, C. Y. et al. (*2008*) (*106*)	National health screening database Collected 2001-2005	Cross-sectional	*n*=3575 Mean age: 58.0 (8.1 SD) Sex: women and men Ethnicity: Asian	women: *n=14*: W/H ratio, BMI, SBP, DBP, FEV1, LBM, TAS, CR, DOP, ESR, OTC, DHEA-S, IGF-1, FSH men**:** *n=15*: Fat%, W/H ratio, BMI, SBP, DBP, FEV1, ALC, TAS, CR, ESR, PSA, DHEA-S, IGF1, SHBG, TTS	MLR	women: r-sq.=0.66 men: r-sq.=0.62				
Bae, C. Y. et al. (*2013*) (*98*)	National health screening database Collected 2004-2010	Cross-sectional	*n*= 243,778 Mean age: 47 years (11 SD) Sex: women and men Ethnicity: Asian	women: *n=5:* W/H ratio, height, HC, WC LBM% men: *n= 5:* W/H ratio, HC, height, LBM%, and weight	MLR	women: r-sq.=0.76 men: r-sq.=0.71				

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						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Facchini, F., P. et al. (*1992*)(*107*)	Clinical trial	Cross-sectional	*n*= 571 Age range: 25-63 years Sex: men Ethnicity: European	*n= 10*: SBP, DBP, FVC, FEV1, Gunther index, visual reaction time, auditory reaction time, digit-symbol test, glucose, TC	MLR		Alcohol use Tobacco use Level of physical activity	Rural vs. urban Level of education Marital status		
Fedintsev, A. et al. (*2017*)(*97*)	Clinical trial	Cross-sectional	*n*= 303 Age range: 23-91 years Sex: women and men Ethnicity: Russian	*n=5*: minimal thickness of the intima media complex, augmentation index, pulse wave velocity, and maximal of two stenosis values	Machine learning	r sq. =0.69 r-sq.= 0.55	Hypertensive T2D			
Furukawa, T. et al. (*1975*)(*108*)	Clinical trial	Cross-sectional	*n*= 111 Age range: 21-83 years, Sex: women and men	*n=10*: height, weight, SBP, DBP, PSP, FVC, right and left ocular accommodation and threshold of vibratory	MLR	r=0.96	Hypertension Parameters of physical fitness			

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Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
			Ethnicity: Asian	sensation on the right and left hands						
Guéguen, R. et al. (2002)(109)	National preventive health examination database Collected 1995	Cross-sectional	*n*= 24,510 Age range: 25-79 years Sex: women and men Ethnicity: European	women *n= 8*: hearing loss, W/H ratio, SBP, healthy teeth, glucose, TC, MCP men *n= 8*: hearing loss, W/H ratio, SBP, healthy teeth, glucose, TC, Hb, MCV	MLR	women: r=0.67 men: r=0.65	Tobacco use Alcohol use	Occupation		
Heikkinen, E. et al. *(1975)(124)*	Clinical trial	Cross-sectional	*n*= 460 Age range 25-57 years Sex: men Ethnicity: European	*n=3:* VC, thresholds of vibratory, and auditory stimuli	Weighted sum of biomarkers	r=0.79	BP BMI Tobacco use	Rural/urban living Occupation Length of education		

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Jee, H. et al. (*2012*)(*110*)	Clinical trial Recruitment through routine clinical health examinations from 2004-2007	Cross-sectional study	*n*= 4,345 Age range: 30-85 years Sex: women and men Ethnicity: Asian	women *n*= *8*: FEV1, Vertical jump, VO_2max, unilateral stance, SBP, WC, grip strength, whole body reaction time men: *n*= *8*: vertical jump, FEV1, grip strength, lean mass, whole body reaction time, sit and reach test, unilateral stance, and VO_2max	PCA	women: r=0.82 men: r = 0.80	BMI Sarcopenia			
Kang, Y.G., et al. (*2017*) (99)	Routine health screening database	Cross-sectional study	*n*= 263,828 Mean age 44.2 (10.6 SD) Sex: women and men	n= 5: WC, MAP, glucose, TG, HDL-C	PCA	women: r = 0.74 men: r= 0.71	Metabolic syndrome			

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
			Ethnicity: Asian							
Kang, Y.G. et al. *(2018)(112)*	National health insurance database Data collection 2009-2013	Cohort study Follow-up time: 11 years	*n*= 484,724 Mean age: 50.75 (14.11 SD) Sex: women and men Ethnicity: Asian	**women**: *n=10*: height, WC, SBP, glucose, TC, TG, HDL-C, eGFR, AST, and r-GTP **men:** *n=7:* height, WC, SBP, glucose, Hb, eGFR, and AST	PCA	women: r = 0.79 men: r = 0.73				*Hazard Ratios:* Mortality: 1.6% T2D: 4.2% Hypertension: 2.5% Heart disease: 1.3% Stroke: 1.6% Cancer: 0.4%
Kimura, M. et al. *(2012)(104)*	Clinical trail Recruited through routine physical fitness tests	Cohort study Follow-up time: 7 years	*n*= 122 Age range: 60-83 years Sex: women and men Ethnicity: Asian	*n= 5*: open-eyes one leg stand, vertical jump, grip strength, functional reach, and 10 m walk time	PCA	women r=0.67 men r=0.59	Age groups			

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Latorre-Rojas, E. et al. *(2019)(103)*	Clinical trial Recruited from community funded supervised exercise programs	Cross-sectional study	*n*= 459 Mean age: 70.3 Sex: women Ethnicity: European	*n= 6:* 30 second chair stand, arm curl test, 6-minute walk test or 2 min step test, sit and reach test, back scratch test and 8 foot up and go test	MLR	r-sq.=0.81				
Lee, M. et al. *(1996)(102)*	Clinical trial	Cross-sectional study	*n*= 322 Age range: 20-79 years Sex: men Ethnicity: Asian	*n= 4:* VO_2peak, Fat%, trunk flexion, grip strength	PCA	r= 0.81	Ischemic heart disease Hypertension BMI T2D			
Liu, Z. et al. *(2018)(113)*	National health examination database Data collection	Cohort study Follow-up time: 12.6 y.	*n*= 9,926 Age range: 20-84 years Sex: women and men	*n=9*: albumin, CR, glucose, CRP, lymphocyte percent, MCV, RDW, ALP, and WBC	Gompertz proportional hazard model		BMI Chronic disease count Age groups			*Hazard ratios:* Mortality= 9% Heart disease=11% Cancer= 7%

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
	1988-1994		Ethnicity: USA							T2D=19% Chronic lower respiratory disease: 7%
Nakagaichi, M. et al. (*2018)(105)*	Clinical trail	Cross-sectional study	*n*= 688 Age range: 60-94 years, Sex: women Ethnicity: Asian	*n= 4:* grip strength, balancing on one leg with eyes open, sit to stand test, and figure of 8 walking test	PCA	r= 0.76	Frailty Training status			
Nakamura, E. et al. (*1996 (114)*)	Clinical trail	Cross-sectional study	*n*= 221 Age range: 20-85 years Sex: men Ethnicity: Asian	*n=8*: Hb, SBP, GPT, TC, LDH, BUN, FVC, and glucose	PCA	r=0.87	Training status			
Nakamura, E. et al.	Annual health	Cohort study	*n*= 86	*n= 5*: SBP, FEV1,/height^2, Hc,	PCA	r=0.72	Tobacco use	Occupation		

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
(2007) **(115)**	check database Data collection 1992-1998	Follow-up time: 7 y.	Age range 31-77 y. Sex: men Ethnicity: Asian	Albumin, and BUN			Alcohol use Training status			
Nakamura, E. et al. (*1988*) (87)	Annual health check database Data collection 1979-1982	Cross-sectional study	*n*= 462 Age range: 30-80 years Sex: men Ethnicity: Asian	*n= 11*: Hb, albumin, A/G ratio, TC, BUN, GOT, OGTT(1h), vision, pulse, FVC, and SBP	PCA	r=0.75	Hypertension T2D			
Nakamura, E. et al. (*1989*) (116)	Clinical trial	Cross-sectional study	*n*=69 Mean age: 42.6 (9.4) Sex: men Ethnicity: Asian	*n=7:* FVC, pulse, SBP, GOT, AI, BUN, and Hb	PCA	r=0.72	Training status			

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Park, J. et al. (*2009)* (117)	Routine health check database Data collection 2003-2004	Cross-sectional study	*n*= 1,588 Age range: 30-77 years Sex: women and men Ethnicity: Asian	*n= 13*: Fat%, WC, hearing threshold, SBP, VO_2max, FEV1, RBC, HbA1c, LDL-C, HDL-C, albumin, BUN, and ESR	PCA	r=0.76	Glycemic levels			
Rahman, S.A. et al. (2019)(101)	National health and nutritional Survey database Data collection 2003-2006	Cohort study Followed for 12 years	*n*= 4,268 Age range: 18-84 years Sex: women and men Ethnicity: USA	*n=1*: intensity of physical activity for one week: 24 hours in each day with 60 minutes an hour for a time period of 7 days (7 x 24 x 60 minutes of data)	LSTM	r-sq.=0.85	BMI Waist/hip-ratio Surface based body shape index T2D Kidney disease CVD			*Hazard ratio:* Mortality: 7%

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Shigematsu, R., et al. (2001)(100)	Clinical trial	Cross-sectional study	*n*= 373 Age range: +60 years Sex: women Ethnicity: Asian	*n=4*: arm-curls, moving beans with chopsticks, sitting and walking around two cones, and reaching forward with the arms while in a standing position	PCA	r=0.85	Training status		3-month training intervention	
Sternang, O. et al. (2015) (118)	The Swedish Twin Registry database Data collection 1984-1990	Cohort-study Follow-up time: 19 y.	*n*= 740 Age range: 45-85 years Sex: women and men Ethnicity: Europe	*n= 5*: self-perceived vision and hearing abilities, grip strength, time to walk 3 meters (walking speed), and FEV1	LGCM			Culture Shared rearing		
Takeda, H. et al. (*1982)* (119)	Annual physical health check registry	Cross-sectional study	*n*= 200 Age range: 20-69 years Sex: men	*n= 5*: hearing acuity, FEV1, TC, LDH, visual impairment	MLR	BA r=0.61				

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
			Ethnicity: Asian							
Ueno, L. et al. (*2003*) (125)	Medical health check registry database Data collection 1998	Cohort study Follow-up time: 4-7 years	*n*= 110 Age distribution: 28-80 years Sex: women Ethnicity: Asian	*n= 5:* FEV1, SBP, glucose, A/G ratio, MVC	PCA	r=0.77	Age groups			
Uttley, M. et al. (*1994*) (120)	National Institute of Aging registry database Data collection 1980-1981	Cohort study Follow-up time: 10 years	*n*= 543 Age range: 50-90 years Sex: women and men Ethnicity: Russian (Mennonites)	women**:** *n=15*: BUN, albumin, DBP, SBP, LDL-C, globulin, phosphorus, uric acid, Fat%, TC, CR, Hb, total iron, BMI, and triceps strength men**:** *n= 14*: Albumin, DBP, SBP, chloride, potassium, phosphorus, sodium, A/G	Stepwise MR					*Relative risk of death ratios:* women: rr = 1.7 men: rr =2.7

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
				ratio, GOT, glucose, uric acid, LDL-C, HDL-C, TC/HDL-C ratio						
Waziry, R. et al. (*2019*) (121)	Rotterdam study database Data collection 1990-1993 and in 2000	Randomized cohort study Followed-up time: 11 years	*n*= 1,699 Median age: 70 years (IQR= 65-76) Sex: women and men Ethnicity: European	*n= 9:* TC, SBP, FEV1, CR, BUN, ALP, albumin, CRP, cytomegalovirus	KDM		Tobacco use BMI			*Hazard ratios:* Mortality: 15% Stroke: 17% Cancer: 7% T2D: 12%
Yoo, J. et al. (*2017*) (122)	Routine health screenings database Data collection 1994-2004	Cohort study follow-up time: 17 years	*n*= 469,754 Age range: 20-93 years Sex: women and men Ethnicity: Asian	n= 15: WC, SBP, DBP, FEV1, GTP, BUN, HDL-C, LDL-C, TG, glucose, ESR, BMI, Fat%, Muscle%, A/G ratio	PCA					The higher the baseline AgeDiff (Ba-CA) the lower 17-year survival rate.

Table 1. Summary of current BA models

Study	Data source	Study Design	Healthy Reference group[a]	Biomarkers	BA-estimation	Validating the implications for use in health promotion by comparing:				
						The linear relationship between BA and CA[b]	BA in high risk individuals[c]	BA in dif. Socioeconomic groups	BA pre and post intervention	AgeDiff (Ba-CA) and associated risk of mortality and disease
Zhang, W. et al. *(2017)* (123)	Clinical trial	Cross-sectional study	*n*= 1,373 Age range: 19-93 years Sex: women and men Ethnicity: Asian	*n= 5:* pulse pressure, trail making test, minimum intima-media thickness, mitral valve E/A peak, and Cystatin C	PCA	r-sq.=0.77	A group of hospitalized patients (CVD, T2D, disease in the nervous system, kidney, cancer and pulmonary diseases).			

a: The reference group is the population from which the BA-model is based upon. b: The linear relationship are assessed by correlation coefficients (r) or coefficient of determination (R^2). c: High risk groups represent individuals who are in higher risk of chronic disease either by proximal risk factors (e.g. hypertension) or distal risk factors (e.g. smoking, inactivity) or are diagnosed with a disease (e.g. type 2 diabetes mellitus), or are frail.
Abbreviations: ***BA estimation****: MLR: multiple linear regression; PCA: principal component analysis; KDM: Klemera and Doubal method; LGCM: latent growth curve model; LSTM: long short term memory.* ***Anthropometric measures****: W/H ratio: waist to hip ratio; BMI: body mass index; LBM: Lean body mass; HC: hip circumference; WC: waist circumference.* ***Cardiorespiratory system****: SBP: systolic blood pressure; DBP: diastolic blood pressure; FEV1: Forced expiratory volume within the 1. second; FVC: forced vital capacity; VC: vital capacity; VO_2max: maximal oxygen consumption; VO_2peak: peak oxygen consumption; MAP: mean arterial blood pressure.* ***Liver function****: CR: creatinine; AST: aspartate aminotransferase; r-GTP: gamma glutamyl transpeptidase; ALP: alkaline phosphatase; GPT: glutamic pyruvic transmitate concentration; A/G ratio: albumin/ globulin ratio; GOT: glutamate oxaloacetate transmitase.* ***Kidney function****: PSP: phenolsulphonphtalein; eGFR: estimated glomerular filtration rate; BUN: blood urea nitrogen.* ***Inflammation****: CRP: C-reactive protein.* ***Metabolism****: TC: total cholesterol; TG: triglycerides; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; LDH: lactic acid dehydrogenase; OGTT: Oral glucose tolerance test; AI: atherogenic index.* ***Bone turnover markers****: DOP: deoxypyridinoline; OTC: osteocalcin.* ***Complete blood count****: ESR: erythrocyte sedimentation rate; ALC: absolute lymphocyte count; MCV: mean corpsular volume; Hb: hemoglobin; WBC: white blood cell count; Hc: hematocrit; HbA1c: glycated hemoglobin; ESR: erythrocyte sedimentation rate.* ***Endocrine function****: DHEA-S: dehydroepiandrosterone sulfate; IGF-1: insulin like growth factor-1; FSH: follicle-stimulating hormone; TTS: total testosterone; SHBG: sex hormone-binding globulin; RDW: red cell distribution width; RBC: red blood cell count.* ***Tumor markers****: PSA: prostate specific antigen*

Gaps in the literature

A discrepancy exists between the BA models currently used in health checks as motivational tool and health risk estimator and the BA models described in the scientific literature. We know very little about the validity as predictors of disease and mortality as well as the effectiveness of the BA models already employed in health checks. Conversely, the feasibility and clinical utility have not been investigated in the BA models derived from the gerontology field. So far, studies on BA-models have focused on their ability to discriminate between healthy and high-risk individuals (through cross-sectional studies) and their predictive abilities in terms of life expectancy (longitudinal studies). Intervention studies are, nevertheless, important to evaluate if BA is more than a useful concept and whether it has clinical utility to measure the effects of health enhancing interventions. The study by Belsky et al. (126) is, to my knowledge, the only study investigating a change in BA through a randomized intervention. The study was initially excluded from the systematic search as it compares BA estimation methods (search strategy appendix 1). The study included 220 non-obese adults (21-50 years) randomized to either 25% caloric restriction or ad libitum diet (control group) for two years. The BA model included the following ten biomarkers: serum albumin, alkaline phosphatase, CRP, TC, creatinine, HbA1c (estimated from serum glucose), SBP, urea nitrogen, uric acid, and white blood cell count. All biomarkers were from the CALERIE biobank. Adjusted for weight loss, the rate of aging (measured by BA) was reduced in the caloric restriction group compared with the usual diet group at 12- and 24-month follow-up. Despite a low dropout rate (18%), considering the intensive intervention, I question caloric restriction to be a feasible intervention for use in general health promotion. Sustainable caloric restriction requires high individual motivation and comprehensive support (127). Thus, BA should be further investigated in lifestyle interventions preferable applicable outside research settings in order to evaluate the clinical utility of the BA concept.

Aims and hypothesis

As prior indicated the overall objective was to investigate the BA concept as a motivational – and clinical tool with implications to health promotion and disease prevention. This was elucidated through the following four papers.

Paper I

The aim of this paper was to investigate the use and effectiveness of BA as a tool in workplace health promotion performed outside the clinical/research setting. We hypothesized that BA assessment motivates to participate in health risk assessment and that BA assessment has a positive influence on health behavior.

Paper II

The aim of this paper was two-fold. One aim was to provide details of the study protocol employed when developing a new BA model. Another aim was to describe the rationale for selecting the candidate biomarkers, as well as providing subject characteristics of the healthy aging reference group, from which the candidate biomarkers were collected.

Paper III

The primary aim of the third paper was to select final biomarkers for BA estimation, employ PCA to the selected biomarkers and propose a new BA model. The hypothesis is that the BA-model can be used as the base of comparison when estimating individual BA and risk of disease. Thus, on average no difference between BA and CA was expected.

Paper IV

The aim of this paper was to provide initial proof of principle that BA is a relevant health risk measure and is sensitive to a 15-week lifestyle intervention. The hypothesis was that the clinical features of the individuals participating in the lifestyle intervention (obese and inactive) would represent a group of adults deviating from the healthy aging trajectory. We therefore hypothesized that 1) baseline BA is higher in the intervention group compared to the reference population, and 2) we would observe a decrease in BA following the lifestyle intervention.

Methods and methodological considerations

This thesis is based on three studies:

- *Study I:* a field-based population cohort study including women and men participating in a BA-based intervention at their workplace yielding paper I
- *Study II:* a cross-sectional study including healthy women and men for the collection of candidate biomarkers yielding paper II and III
- *Study III:* an intervention study performed in overweight or obese women and men yielding paper IV.

The study design, experimental protocol, and methodical considerations are presented below. A comprehensive description of the measurements is provided for in the articles/manuscripts at the end of the thesis.

Some discrepancies in terminology exists between the thesis and paper I. In paper I, body age and not biological age was used as terminology following the original wording used when the intervention was carried out in practice. In addition, the terminology *biomarker* was not applied in paper I. Instead, *variables* were used to describe components in the body age model. In this thesis I will, however, consistently use *biological age* and *biomarker* when referring to variables included in the BA model.

Study design

Study I

Data originates from the database of a private health care company who offered health checks including to Danish companies. The study was approved by the Copenhagen Research Ethics Committee for Science and Health (504-0056/19-5000) and by the Data Protection Agency (SUND-2018-17). The database includes data from individuals employed at 90 different Danish companies (**Figure 6**). Private companies represent 97% of the 90 companies. The intervention consisted of a health check including BA estimation and a motivational interview. Participation was voluntary and free of charge. The protocol for the BA estimation was designed by a physiotherapist and two human physiologists employed in the Danish health care company. From January 2011

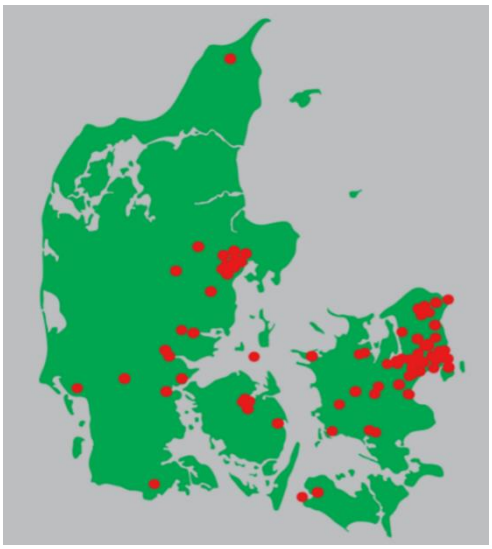


Fig 6*. Map of Denmark showing the locations of the 90 companies.*

to February 2017, the health care company invited 14,071 individuals by email to have a body age health test.

Study II

Women and men were recruited by online advertisement, advertisement at universities and by word of mouth. Our aim was to recruit 100 healthy individuals evenly distributed in sex and the age range of 18-65 years. The study was registered at ClinicalTrials.gov (Identifier: NCT03680768) and approved by the Regional Ethics committee, Copenhagen, Denmark (H-18031350). Exclusion criteria were pregnancy, previous or current CVD, use of medication to reduce blood pressure, cholesterol or glucose levels, and joint pain prohibiting strength and cycle testing. The experimental day was conducted at the facilities of Xlab.

Study III

We recruited participants among the 80 women and men participating in the 15-week lifestyle intervention. The study was registered at ClinicalTrials.gov (Identifier: NCT04279366) and approved by the Regional Ethics committee, Copenhagen, Denmark (H-19073643). Exclusion criteria where use of beta-blockers, pregnancy, and age <18 years or > 65 years. The intervention was carried out at a folk high school constituting supervised daily physical activity, healthy meals, and cognitive therapy. The experimental days was conducted at the facilities of the folk high school.

Measurements and procedures

In study I, participants were encouraged to fast at least three hours prior to the test. To standardize hydration level, participants were asked to drink 0.5 L of water 2 hours prior to the test. In study II and III, participants had fasted over-night (at least 10 hours) and had not exercised 12 hours up to the test.

Body composition

Fat and muscle mass

In study I and III, measurements of body composition was performed with a 2-point and 4-point bioelectrical impedance, respectively (Tanita-SC330 S, Tokyo Japan and Tanita-MC-780MA Illinois USA). In study II, body composition was measured using Dual X-ray Absorptiometry Scan (DXA) (Lunar Prodigy Advance, Lunar, Madison, WI, USA).

Waist and hip circumference

In study II and III, waist and hip circumference were measured with a measuring tape. After an exhalation, waist circumference was measured at the narrowest place between the 12^{th} costae and the crista iliac. Hip

circumference (centimeter) was measured as the widest place around the hip, using trochanter major as reference.

Blood pressure

Blood pressure was measured in all three studies using an automatic monitor (BoSo Medicus Control, BOSCH + SOHN GmbH). Blood pressure was measured three times with one-minute intervals and the average systolic and diastolic blood pressure (SBP and DBP) was used.

Strength measurements

For every strength measure, three attempts were used to find the highest value. Each attempt was separated by a one-minute break. We encouraged maximal performance by cheering on participants.

Handgrip strength

Grip strength was measured by a handheld dynamometer (Jamar J00105, Lafayette, USA (study I), Takei Grip-D TKK5401, Japan (study II + III)) adjusted to individual hand size (**Figure 7A**). Participants stood up, arms by the side and a bit away from the body. Maximal compression was applied, and the highest value (kg) was recorded.

Upper body strength

In study I, arm strength was assessed by number of pushups — women on their knees and men on their toes. In study II, biceps strength was measured using a back strength dynamometer (Takei Scientific Instruments Co, Ltd, Tokyo). Participants stood op straight, with both arms by the side and 90° flexion of the elbows. Maximal isometric flexion of the elbows was applied, and the highest value (kg) was recorded (**Figure 7B**).

Lower body strength

In study I, leg endurance strength was assessed by wall-sit hold. Participants back was against a wall with 90° flexion in the hips. In study II, isometric knee extension strength was measured using a handheld dynamometer (microFET2, Hoggan Health Industries). Participants sat on a table with the knee in 90°flexion and the dynamometer positioned following a standardized belt configuration. This procedure is validated against an isokinetic dynamometer measurement (128). With a straight back and arms crossing the chest and without lifting the thigh, a maximal extension was performed, and the highest value (Nm) was recorded (**Figure 7C**). The lever arm was measured as the distance 5 cm proximal from the distal part of the lateral malleoli to the lateral part of the knee joint (meter). Considering the feasibility of use outside the

laboratory, we choose this method above isokinetic dynamometer. For a better inter individual comparison of strength, the unit Nm was used.

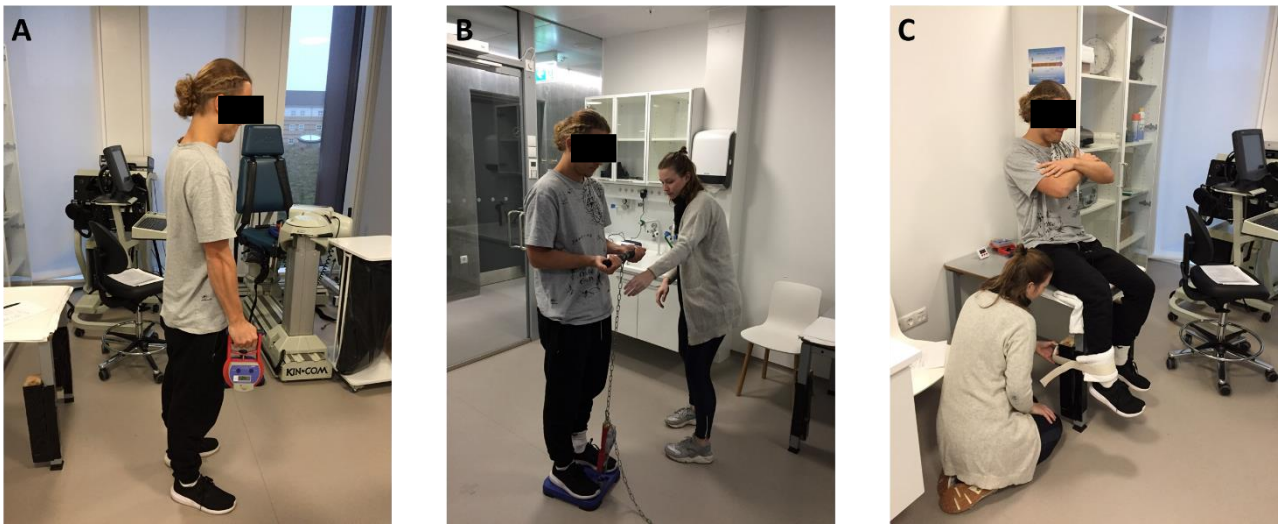


Fig. 7. *Participant in study II performing A) Hand grip strength, B) Biceps strength and C) Knee extensor strength.*

Blood sample and analysis

Whole blood

In study I, blood from a finger prick test was used to measure total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TG) (Alere Cholesterol LDX analyzer, Hayward, USA), and glucose concentrations (Accu-Check Aviva meter, Indianapolis, Indina, USA). The analyzers are calibrated to report values in plasma concentrations.

In study II and III, venous whole blood was used to analyze hemoglobin and hematocrit (Hemo Control Hemoglobin Analyzer, EKF Diagnostics, Madgeburg, Germany) and glycosylated hemoglobin (Hba1c) (DCA 2000+, Bayer Healthcare, Elkhart, IN, USA).

Plasma

In study II and III, plasma from a venous blood sample was used to measure fasting glucose concentration, insulin, adiponectin, leptin, total cholesterol, HDL, LDL, TG, free fatty acids (FFA), soluble urokinase plasminogen activator receptor (suPAR), and C-reactive protein (CRP). Plasma glucose, insulin, FFA, TG, HDL, LDL, and total cholesterol concentrations were analyzed on COBAS (COBAS 6000, C 501, Roche Diagnostics, Mannheim, Germany). Adiponectin concentrations were analyzed by RIA kit (Millipore, MA, USA) and leptin concentrations by ELISA kits (R&D systems, Abingdon, UK). Plasma concentrations of suPAR were analyzed using the commercially available suPARnostic® kit (ViroGates, Copenhagen, Denmark).

Advanced Glycation End-products (AGE)

In study II, participants sat in a chair, with the forearm resting on the AGE Reader (DiagnOptics BV). AGE accumulation was measured by the amount of skin auto fluorescence.

Lung function

In study II and III, forced vital capacity (FVC) and forced expiratory volume in the first second (FEV_1) was measured using a handhold spirometer (Vyntus SPIRO spirometer, Vyaire Medical). Participants sat down in a chair with strait back and both feet on the ground (**Figure 8**). Wearing a nose clip, a maximal inspiration was immediately followed by an expiration with maximal effort. The test was repeated three times and a maximum of seven times to find the highest value.

Fig. 8*. Lung function test of male participant.*

Exercise protocols

Submaximal test

In study I, VO_2max was estimated based on a two-point cycle test performed on an electromagnetically braked ergometer cycle (Monark 828E, Vansbro, Sweden). Workload was based on gender, weight and training status and adjusted to reach a steady state heart rate of 120-130 beats/min at the first rate and 140-150 beat/min at the second rate. Heart rate and workload was recorded at steady state after 6 and 10 minutes respectively. Maximal workload (Wmax) was extrapolated based on maximal heart rate of 220-age and VO_2max calculated based on a cycling efficiency of 23%, an energy-oxygen equivalent of 21.1 kJ/LO_2 and a basal metabolic rate of 0.25 L O_2/min:

$$\mathrm{V}O_2\mathrm{max} = (\frac{Wmax}{0.23} * \frac{60\ sec}{21.1kJ/LO_2}) + 0.25\ LO_2/min$$

Maximal test

In study II and III VO_2max was measured directly using continuously breath by breath measurements sampled into 10 seconds intervals by an automated online system (Quark PFT, Cosmed, Italy). Participants performed a graded exercise cycle test until voluntary exhaustion (**Figure 9**). Heart rate was continuously measured, and rate of perceived exhaustion (RPE) obtained in the end of each workload. Each VO_2max test was evaluated by three criteria. The primary criteria was a plateau of oxygen consumption (VO_2plateu ≤ 150 ml O_2/min increase) between the final two workloads. Secondary criteria was respiratory exchange ratio (RER) ≥ 1.15 (CO_2 expired/ O_2consumed) and maximal heart rate (HR ±10 beats from the estimated maximal HR given by 220-age). The highest average value measured over 30 consecutive seconds determined VO_2max. Duration of the test is important to obtain the highest VO_2max, why we used two different protocols for study II and III aiming to reach exhaustion within 8-12 minutes (129). Thus, in study II warm up was performed at 50W and 100W for women and men, respectively and increased with 25 W/min until voluntary exhaustion. In Study III the warm-up was performed at 30W and 50W for women and men respectively and increased with 20 W/min and 25 W/min, respectively until voluntary exhaustion.

Fig. 9. *VO_2max test performed by males included in study II (left side) and study III (right side).*

Covid19 and study III

During the lifestyle intervention the covid19 pandemic expanded. As a result, we induced several precautions at the follow-up measurements to minimize risk of infection. These included a filter between the flowmeter and mouthpiece, rigorous cleansing of the flowmeter in Rodalon between each test which required a fast-drying procedure where we used a hairdresser. Unfortunately, the follow-up measurements have been negatively affected by these precautions. Going through the data, I found that in 18 out of 28 individuals their VO_2max (ml/min) decreased substantially, despite no change (*n*=7) or an increase in maximal work capacity (*n*=11) (**Figure 10**). With no indication of an error of the watt calibration of the Monarch cycle, or unstable fractions of oxygen in inspired air, a higher maximal work load should result in a higher VO_2max (130). As a consequence, we calculated their follow-up VO_2max (ml/min) based on the VO_2-work rate relation of 9 ml/O2/W/min corresponding to a work efficiency of ~25%. (131) Specifically we added the VO_2-work related oxygen consumption to the VO_2max measured at baseline.

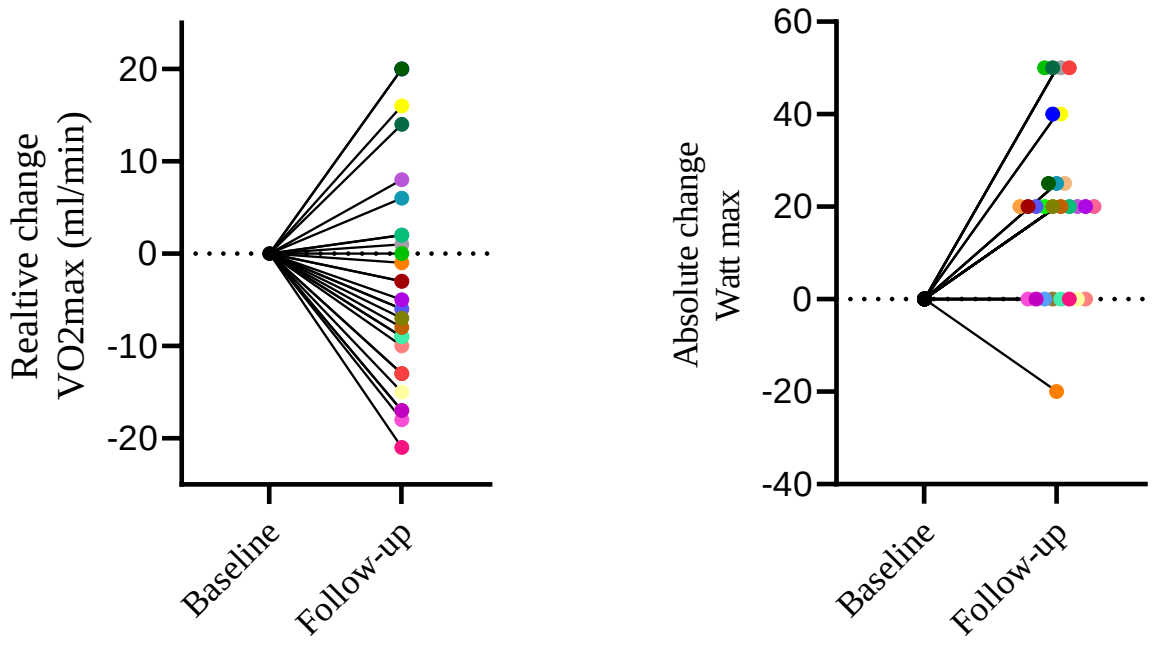


Fig. 10. *Individual plot of relative change (%) in absolute maximal oxygen uptake (VO2max) left hand side with corresponding changes in maximal work capacity (watt max) right hand side. Adapted from paper IV, Suppl. Figure 1, p. 27.*

Functional measures

Sit to stand

In study II and III a 30 second chair stand test was used to assess lower extremity function(132)

Sit and reach

In study I flexibility was assessed by measuring the reach length (cm) using a sit-and-reach bench (ACUFLEX I, Rockton, USA).

Questionnaires

PAS

In study II total physical activity level including sleep, work and leisure time on an average weekday was assessed using the Physical Activity Scale questionnaire, developed to estimate activity behavior in the adult Danish population(133).

SF12

In study II and III quality of life was assessed by the Short Form Health survey (SF-12v2) including 12 generic items covering self-reported health, health related physical and emotional limitations.

Motivational interview

In study I, based on individual BA, a motivational interview was performed as the final part of the health check. Approximately 20 minutes was appointed to the interview and the aim was to clarify potential ambivalence towards health behavior change (134).

Biological age estimation

Study I

BA was estimated through 10 biomarkers: CRF, body fat percentage, TC, FBG, mean blood pressure, WC, handgrip strength, number of push-ups, wall sit endurance, sit and reach flexibility test. Smoking habits also influenced the body age estimate.

BA was calculated as the sum of CA and the BA score. This score was estimated by the following stepwise method:

1: Each test result (e.g., WC) are compared to the mean value in statistical data of age and sex-related peers (*n*=10,000). Depending on the relative variation an age value is given in units of years.

2: This age value is weighted in accordance with its relation and importance to risk of disease and mortality (**Table 2**).

3: For two of the biomarkers (glucose and tobacco use) the age value is not weighted, instead cut off criteria was used to define the age value (**Table 3**).

4: Summing the age values produces the final BA score.

This is expressed in the following equation:

$$BAscore = \sum_{i=1}^{N} \Delta_i^V \times W_i^V + \Delta^{BG} + \Delta^{SH}$$

Where Δ_i^V is the age value given for each biomarker, W_i^V is the corresponding weight and $\Delta^{BG} + \Delta^{SH}$ are the age value given based on blood glucose concentrations and smoking habits. *N* is the total number of biomarkers included in the equation and *i* indicate the specific biomarker (e.g., waist circumference).

Table 2. Weighting of biomarkers adapted from paper I, Table 1 p.6.

Variable	W_i^{Va}
Fitness level	31.1%
Fat percent	17.8%
Total cholesterol	13.4%
Mean blood pressure	13.4%
Waist circumference	6.7%
Handgrip strength	4.4%
Push up	4.4%
Wall sit	4.4%
Sit and reach	4.4%
TOTAL	100%

W_i^V *= the weight in percent assigned to each variable (V), i being the number of variables.*

Table 3. Smoking and blood glucose BA score

	BA score
Cigarettes a day	
1-10	4 years
> 10	8 years
> 15	10 years
Blood glucose concentration	
> 6.1 mmol/L	4 years

Study II

Based on the literature review, we followed the method first proposed by Nakamura et al. in 1988 using the 1stPC alone as a general aging factor (as explained in the background) and used by other since (99, 110, 112, 115, 117). The stepwise method was as follows (**Figure 11**):

1. Collection of candidate biomarkers from a healthy reference population
2. Selection between candidate biomarkers including correlation with CA and exclusion of redundancy
3. Applying the selected biomarkers to PCA
4. Use factor loadings from PC1 to make BA equation
5. Transforming BA score to BA in unit of years
6. Correction of regression towards the mean

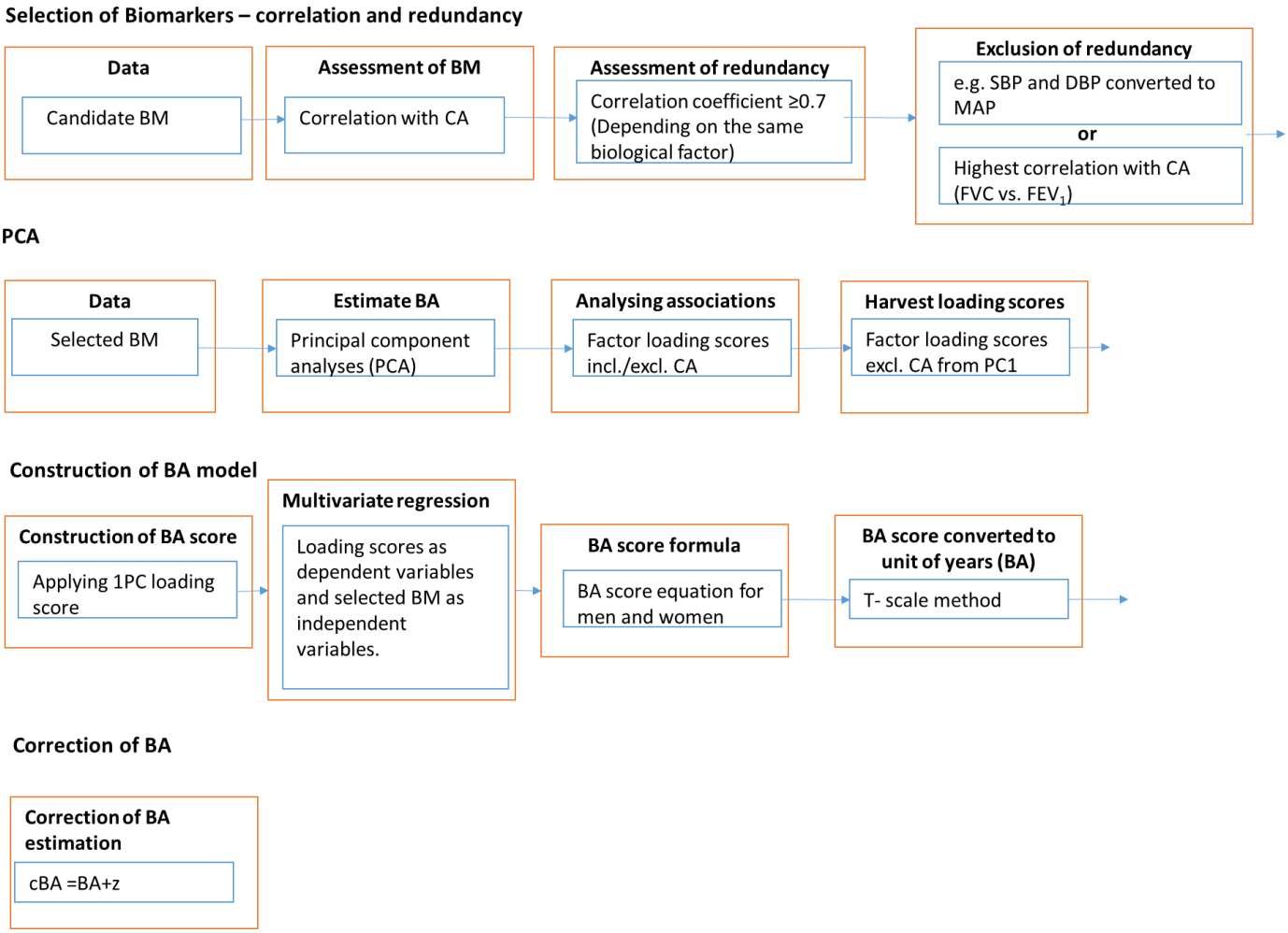


Fig 11. *Boxplot of the conceptual stepwise method to estimate biological age (BA) using the first principal component from the principal component analysis (PCA). BM: biomarkers, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial blood pressure, CA: chronological age, 1PC: first principal component*

Additional measurements

The following section describes the method for measuring facial age and telomere length in study II. As these measures were used to conduct sub analysis only included in this thesis, I describe the method in detail below.

Facial age

Individual portrait pictures were taken with a digital camera (Sony Alpha a3000 ILCE-3000K). We followed the procedure described by Christensen et al. (135). The camera was positioned on a tripod 0.6 meter from the seated participant and adjusted in height. Participants were asked to have a neutral expression and not wear makeup or glasses. Two portrait pictures were taken in case of eyes closed.

Ten independent raters were asked to guess CA of the participants based on the portrait pictures. The assessors are divided in two categories based on profession. Five assessors where academic staff employed at the University of Copenhagen (not expected to have special qualifications to evaluate appearance), and five assessors were employed at a medical center: four general practitioners, and one medical secretary (expected to be used to evaluate appearance, thus be better assessors). They had no information on the CA range of the participants

Relative Telomere length
DNA was isolated from buccal swaps and purified using the Gentra Puregene Buccal Cell kit (Qiagen, California, USA) as recommended by the manufacturer. The purified DNA (5 ul of the 100 ul in hydration solution) was amplified in a 25 µL SYBR Green polymerase chain reaction (PCR) containing 1 × Quantitect SYBR Green Master Mix (Qiagen) and 340 nM of each primer, using telomere and genomic DNA primer sets as given in https://pubmed.ncbi.nlm.nih.gov/32260112. The amplification was monitored real time using the MX3005P Real-time PCR machine (Stratagene, California, USA). The Ct values were related to a standard curve made from serial dilution of human genomic DNA. The specificity of the PCR products was confirmed by melting curve analysis after amplification (broad but reproducible curve for telomeres). Samples were measured as mean of triplicate PCR reactions and the Telomere measures were divided by the genomic DNA measures to obtain a relative telomere length.

Unresolved data

Some data was collected but not included in this thesis for different reasons:

International Physical Activity Questionnaire (IPAQ) *short form*.
In study III participants filled out IPAQ at baseline and follow-up. The questionnaires were intended to support the VO_2max measurements as a measure of physical activity level. The questionnaire unmask the time spent on weekly vigorous, moderate or walking physical activity, with a recall period of seven days and provides a cumulative score of low, moderate or high physical activity level (136). Similar VO_2max can be categorized in very low, low, moderate, high or very high (137). At baseline we found that all but three participants had very low or low VO_2max but a corresponding MET category of moderate to high, mainly due to a high self-reported weekly vigorous activity. In hindsight we should have provided better instructions before handing out the questionnaire. The reliability of the result from the questionnaire is therefore doubtful, why the analysis is dismissed from this thesis.

Adiponectin

In study III plasma was stored for measuring adiponectin concentration. However due to COVID19, delivery of RIA kits from the USA to the EU was to long considering the iodine half time of the kits. This is very unfortunate as adiponectin is part of the BA-model. To accommodate this, I inserted the same adiponectin concentration both at baseline and follow-up for women and men, respectively. This way adiponectin would not influence on the BA change score at follow-up.

Statistical considerations

Individual statistical analyses are provided for in detail in paper I-IV. The following section describes some statistical considerations and general descriptive statistics used in the thesis and included papers.

The statistical analysis conducted in study I was performed in close collaboration with a statistician co-authoring the paper (JP). A priori, data cleaning of the raw dataset extracted from the health care company was necessary. This included detection of outliers (abnormal values), understanding missing values, retrieving correct variable units, and any exclusion criteria for participation.

No sample size calculation was performed for study II. A power calculation rely on the expected change in the primary outcome based on the minimal relevant difference (mirediff) and related standard deviation observed in previous studies, together with the alpha (0.05) and beta value (0.20) of interest. In this study, BA was the primary outcome. The mirediff was, however, not available as we were developing a new model not previously investigated. With the substantial heterogeneity in other BA-models, especially concerning biomarker combinations (Table 1), we found it irrelevant to condition a sample size calculation on the effect sizes found in these studies. Collectively, the explorative nature of the study makes the power calculation impossible.

In study II, the rationale behind the number of individuals in the reference group (n=100) has to do with the explorative nature of the study and is based on the central limit theory stating that sample distribution will normalize with sufficiently large sample size (theoretically above n=30) (138).

All data were checked for normal distribution and log-transformed if necessary to obtain a normal distribution. Baseline data were described as mean ± standard deviation (SD), medians with interquartile range (IQR) in case of skewed distributions or as absolute or relative frequencies when describing categorical variables. Comparisons within and between groups were described as estimated means with 95% confidence interval (95% CI) or absolute and relative frequencies. Statistical significance was

considered at $p<0.05$ in all comparisons. Statistical analysis was performed using SAS Enterprise Guide 7.1 and GraphPad Prism 9. PCA was performed in MATLAB R2018b.

Summary of Results

The results from study I, II and III are provided for in paper I-IV. The following section provides a summary of the main results in paper I-IV together with additional analyses and data from study II and III. The results in paper I is provided for separately followed by a summary of the results from paper II-IV.

Paper I

Study participants

A total of 14,073 employees were invited to a BA health check, and 9,851 (70%) accepted the invitation (baseline test). At follow-up 1.3 years later (IQR 1.0 - 2.1 years), 3,843 participated (40%) and 5,878 were lost to follow-up (60%) (**Figure 12**).

Individuals with both baseline and follow-up tests are referred to as 2 test participants (2TP) and individuals with baseline test only, are referred to as 1 test participants (1TP). Individuals with a BMI ≤18.5 (*n*=130 (1%)) were excluded from the dataset as this study investigate health behavior changes in relation to obesity and inactivity related lifestyle diseases.

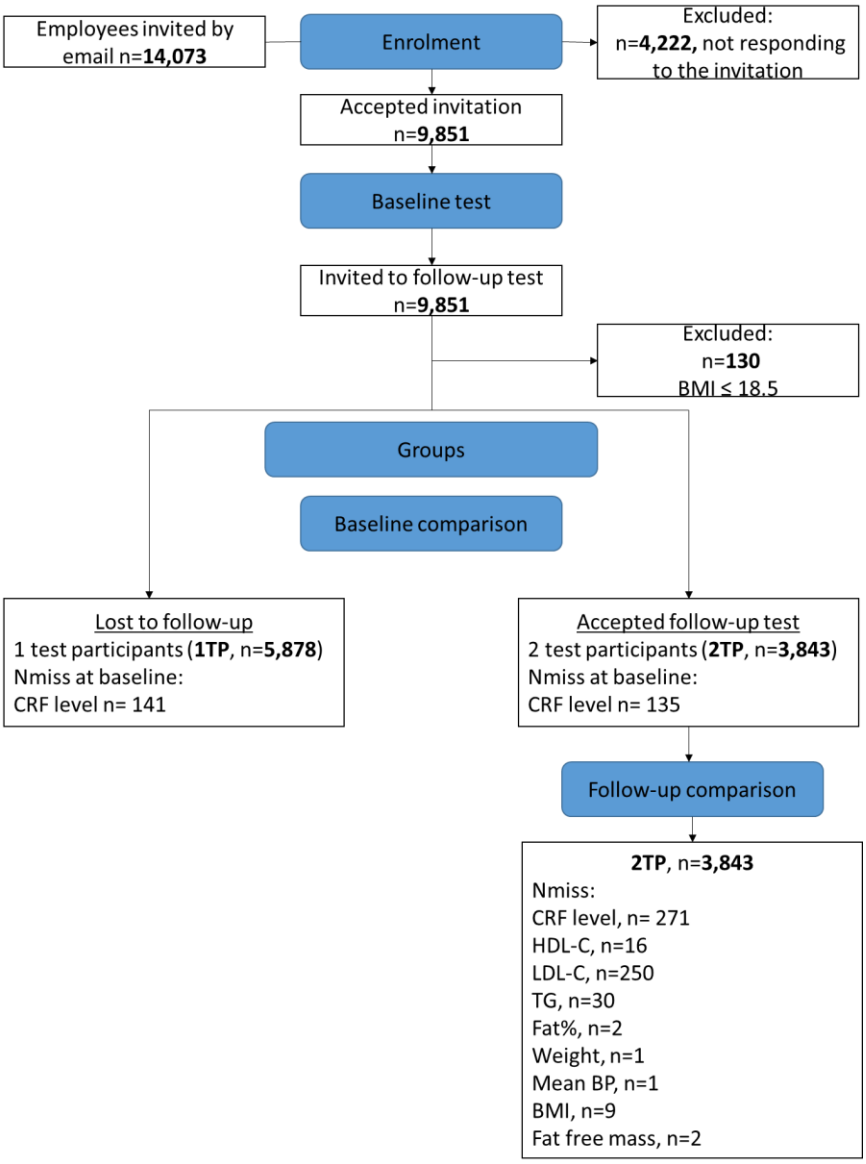


Fig. 12. *Flow chart of individuals participating in the first (baseline) and second (follow-up) body age test. Abbr.: 1TP: 1 test participants; 2TP: 2 test participants; Nmiss: number of missing variables, CRF: cardiorespiratory fitness, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, TG: triglycerides, mean BP: mean blood pressure, BMI: body mass index. Modified from paper I, Figure 1, p. 4.*

Baseline characteristics

1TP were slightly younger compared to 2TP at baseline (p= 0.0006) (**Table 4**). Conversely, 1TP were biologically older compared to 2TP (p <0.0001). **Figure 13** visualize the BA-score as a function of CA and shows how a positive slope is associated with 1TP compared to 2TP associated with a negative slope. This association increases with increasing CA (p<0.0001) and is interpreted as 1TP being less healthy and in higher risk of future lifestyle disease compared to 2TP. In addition, the number of individuals who smoked and the proportion of individuals with obesity (BMI ≥ 30) were higher among 1TP (**Table 4**).

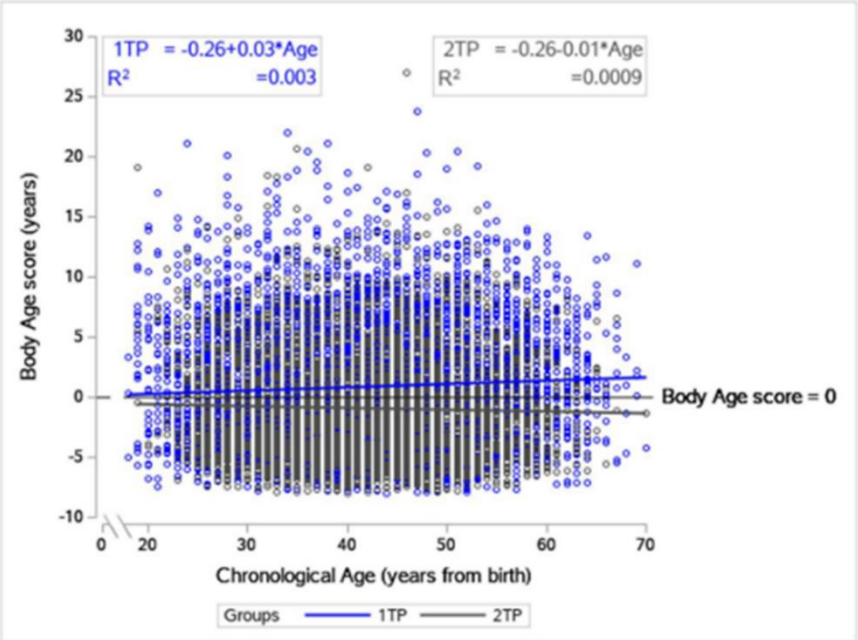


***Fig. 13**. Fitted regression of BA score as a function of chronological age at baseline. The figure shows that there is an association between having a positive body age score, and thereby a higher risk of disease, and not participating at follow-up. The blue line represents body age score for 1TP and the grey line represents body age score for 2TP. The black line is a reference line for body age score of zero, that is no difference between chronological age and body age. Modified from paper I, Figure 2, p. 8.*

Table 4. Baseline characteristics

	Groups		P^a	P^b adjusted
	1TP	2TP		
	Median (IQR)	Median(IQR)		
Women, n *(%)*	2182 (37.1)	1430 (37.2)	0.93	0.89
Chronological age, *years*	41 (33; 49)	42 (35; 48)	0.0006	-
Body age, *years*[c]	41.3 (32.7; 50.4)[*]	40.8 (33.4; 48.2)[*]	<0.001	-
BMI, kg/m^2	24.5 (22.4; 27.1)	24.2 (22.2; 26.3)	<0.001	<0.001
Current Smoker, *(%)*	631 (11%)	245 (6%)	<0.001	<0.001
Mean blood pressure, *mmHg*	105 (98.5; 113)	104 (98; 110)	<0.001	<0.001
Total Cholesterol, *mmol/L*	5.0 (4.3; 5.6)	4.9 (4.4; 5.6)	0.9	0.3
Blood glucose, *mmol/L*	5.0 (4.7; 5.4)	5.0 (4.7; 5.4)	0.4	0.2
Body Fat *%*	22.9 (17.8; 29.4)	21.9 (17.4; 28.2)	<0.001	<0.001
Waist circumference, *cm*	88 (80; 97)	87 (80; 94)	<0.001	<0.001
Fitness level, *ml/min/kg*[c]	37 (31; 44)	40 (34; 47)	<0.001	<0.001
Push Ups, *No. of*	25 (16; 32)	25 (18; 32)	0.02	0.0002
Wall sit, *min*	1.6 (1.1; 2.1)	1.7 (1.3; 2.2)	<0.001	<0.001
Handgrip, *kg*	47 (34; 56)	48 (35; 56)	0.04	0.03
Sit and Reach, *cm*	35 (28; 40)	34 (29; 40)	0.4	0.2

Comparison of baseline characteristics for 1-test participants (1TP, n=5,878) and 2-test participants (2TP, n=3,843). Continuous data are represented as medians with interquartile range (IQR); categorical data as absolute and relative frequencies. Body mass index (BMI), mean blood pressure, total cholesterol, blood glucose, body fat% and waist circumference were log transformed prior to analysis.

P^a: P value using regression analysis and logistic regression.

P^b : P value adjusted for age

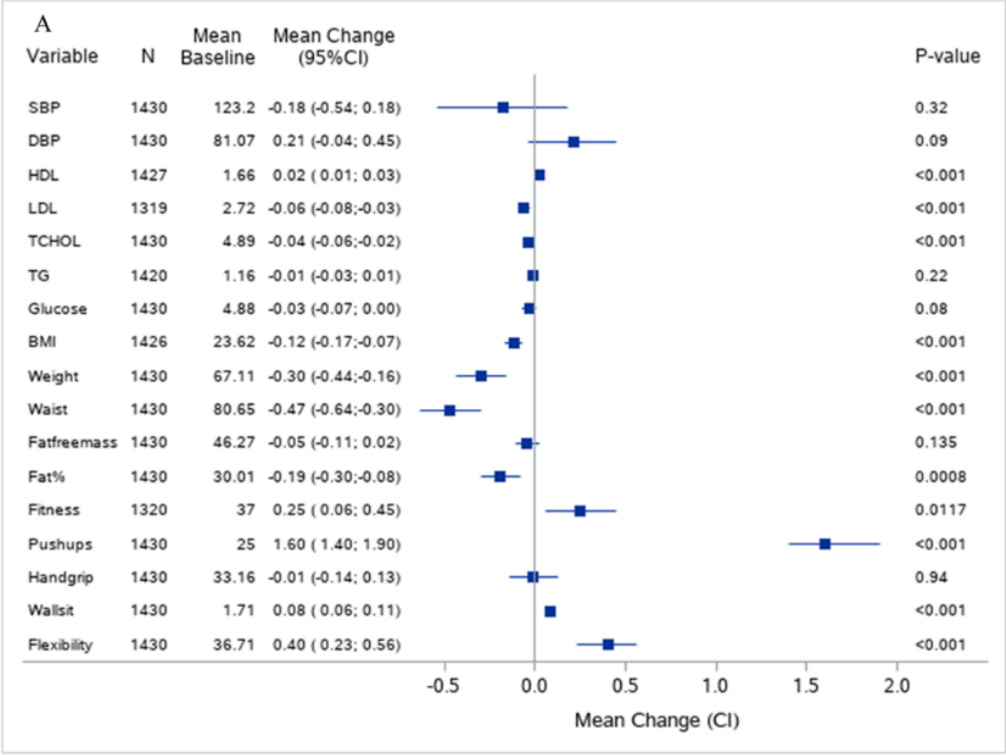
[c] Missing values were observed for fitness level and body age (due to missing fitness level data) why comparison of 1TP and 2TP is between n=5,737 and n=3,708, respectively.

[*] Significant different from chronological age p<0.001 (paired t-test)

Adapted from paper I, Tabel 2, p. 7.

Changes at follow-up

At 1.3-year follow-up (range: 0.02 years - 5.6 years), BA-scores improved among women and men with -0.7 years (95% CI -0.8; -0.5 years) and -0.6 years (95%CI -0.7; -0.5 years), respectively, adjusted for the average age development. The changes in BA-score were partly driven by improvements in cholesterol profile (TC), body composition (fat% and waist circumference), and functional capacity (push up, wall sit hold, and sit and reach test). The changes were observed among both sexes, however, an improvement in CRF was only observed among women (*p*=0.01) (**Figure 14**). These changes resulted in a decrease in number of employees exhibiting metabolic syndrome (n=89 (14%), *p*=0.005). The improved BA-score was also driven by the observed improvement in smoking habits as 42% of employees smoking at baseline no longer smoked at follow-up (n=103).



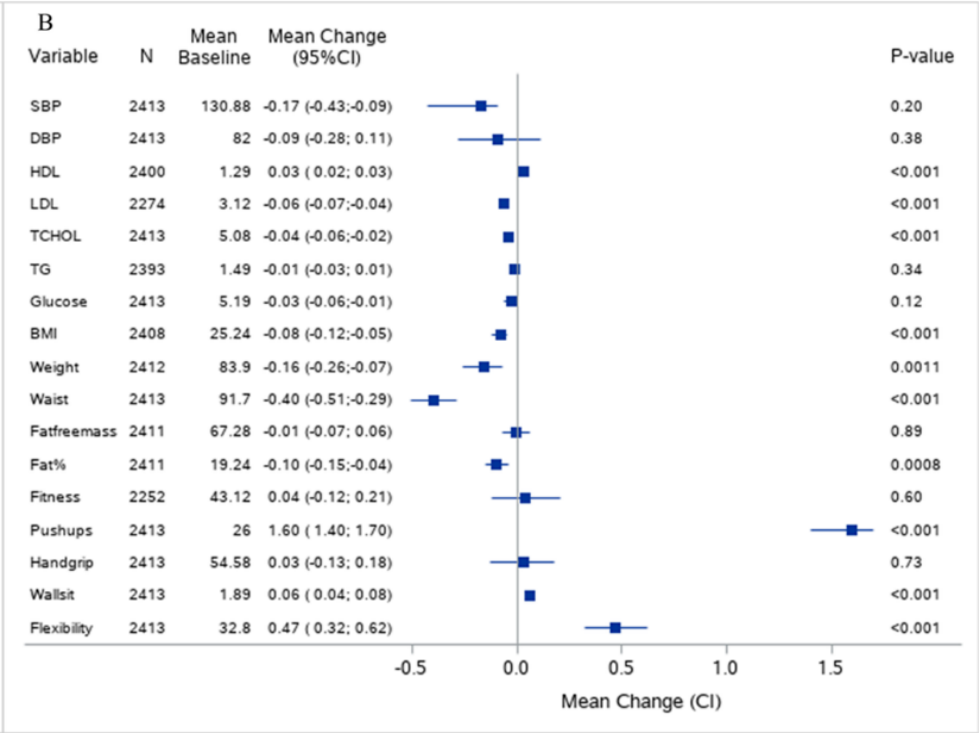


Fig. 14. *Changes in single variables per year beside the average age development. Baseline values and changes (mean, 95% CI) observed at follow-up adjusted for age at baseline and follow-up time by sex:* ***A****= women and* ***B*** *= men. N is the sample size used for calculation of the mean difference. A visualization of the effect size is provided for in the forest plot; squares representing mean change with 95% confidence intervals. P value using a mixed model adjusted for age at baseline and variation in follow-up time. Abbr.: SBP= systolic blood pressure (mmHg), DBP= diastolic blood pressure (mmHg), HDL= high density lipoprotein (mmol/l), LDL= low density lipoprotein (mmol/l), TCHOL= total cholesterol (mmol/l), TG= triglycerides (mmol/l), Glucose = fasting glucose (mmol/l); BMI= body mass index, weight (kg/m^2), weight (kg), waist = circumference (cm), fat free mass (kg), fitness level (ml/min/kg), push up= number of, handgrip strength (kg), wall sit time (min), and flexibility= sit and reach test (cm). Modified from paper I, Figure 4, p. 10.*

Paper II-IV

Reference group

We recruited 100 healthy women (51) and men (49) equally distributed in five age categories (**Figure 15**). They exhibited no signs of insulin resistance (HOMA-IR > 2 (139)) or clinical indications of airway obstruction (FEV_1/FVC ≤ 70% (140)), however, three women (5%) and two men (4%) exhibited metabolic syndrome. Individuals with obesity comprised 6% (BMI≥30). However, across age categories and in both sexes, 80% (women) and 94% (men) adhered to the recommendations of 150 min/week of moderate to vigorous physical activity. CRF level was moderate to high, the latter predominantly in the older age categories (61, 137) (**Table 5**). Besides birth control pills (n=10) and allergy medication (n=3), the study population was free from use of medication. Tobacco use was present among 6% of women (n=3) and 6% of men (n=3).

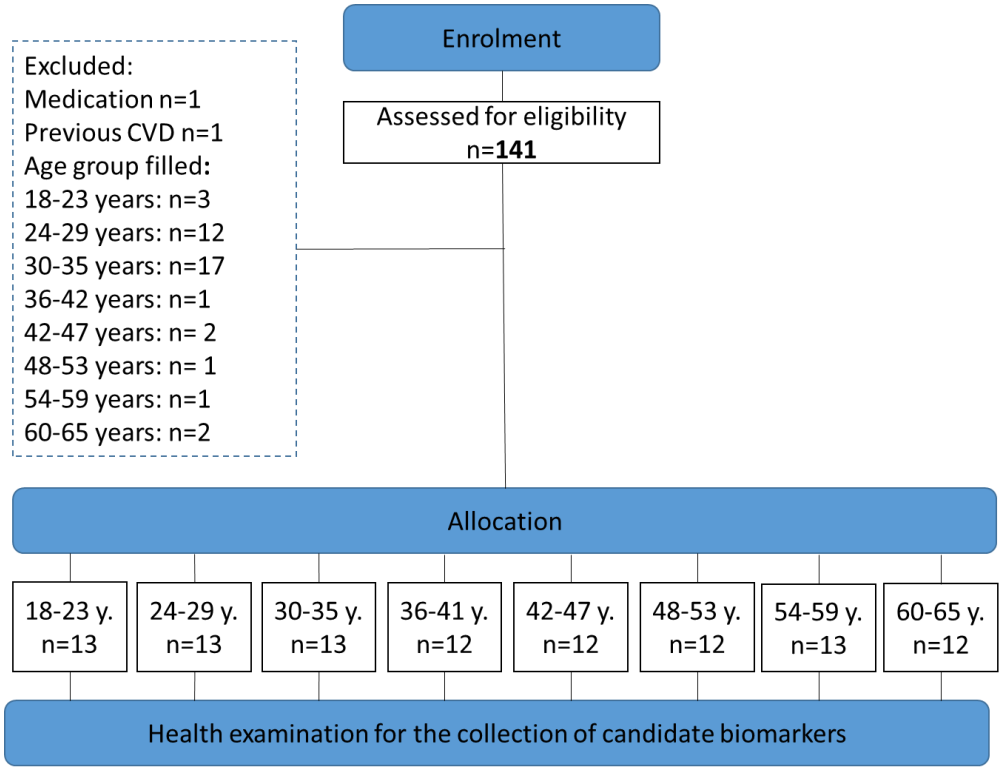


Fig. 15*. Flow chart of the recruitment process in study II. Age group filled, relates to the number of individuals excluded simply due to lack of space within the specific age category.*

Table 5. Cardiorespiratory fitness within the reference group		
Age category (years)	Measured VO_2max (ml/min/kg), mean (SD)	
	Women (n=51)	Men (n=49)
18-23	37 (7)	45 (4)
24-29	37 (3)	45 (8)
30-35	38 (6)	48 (8)
36-41	36 (8)	43 (8)
42-47	40 (7)	47 (7)
48-53	30 (5)	43 (6)
54-59	30 (4)	40 (10)
60-65	32 (5)	40 (4)

Abbr.: VO_2max: maximal oxygen consumption. Modified from paper II, Table 2, p. 7.

Ubberup participants

A total of 43 eligible individuals volunteered to participate at baseline. Unfortunately, only 27 individuals participated at follow-up (**Figure 16**).

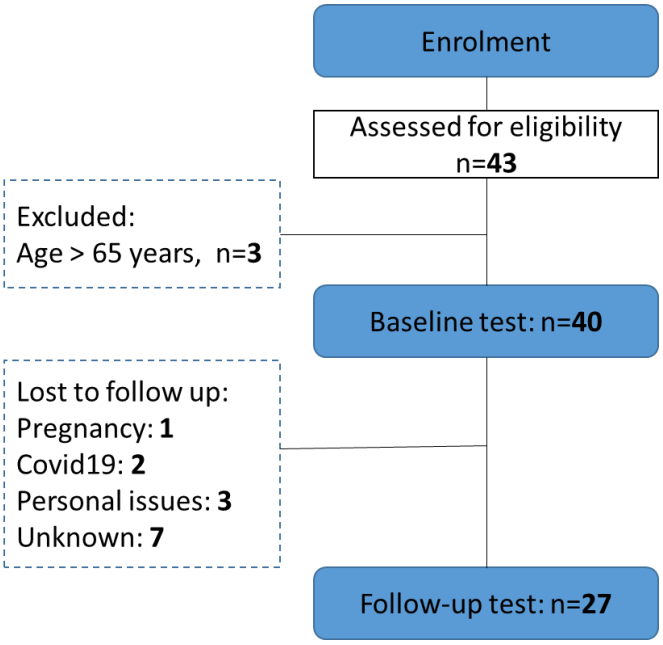


Fig. 16. *Flow chart of the recruitment process in study III. Adapted from paper IV, Figure 1, p.10.*

The women (n=16) and men (n=11) were either overweight (7%, BMI ≥25 and ≤29.9) or obese (93%, BMI≥30). They did not have T2D (HbA1c ≥ 6.5% (141)) or clinical indications of airway obstruction (FEV_1/FVC ≤70% (140)). The CRF level was low within both sexes (82%) (61, 137) and metabolic syndrome was present

in 44% of women (n=7) and 27% of men (n=3). Tobacco use was registered among 44% women (n=7) and 45% men (n=5). The participants used the following medications: birth control pills: n=4, blood pressure lowering medication: n= 3, allergy medication: n=4, obesity medication (liraglutide): n=1, cholesterol lowering medication: n=2, asthma medication: n=7, anti-depressive medication: n=1, and ADHD medication: n=1.

Reference group versus Ubberup group

Table 6 shows characteristics for women and men included in the reference group and the Ubberup group. Ubberup men, but not women, were younger (p=0.02) compared to the reference group. Women and men in the reference group were generally healthier and had higher relative VO_2max, lower weight, BMI, waist circumference, and relative fat mass. The lipid profile was healthier in the reference group based on HDL-C levels. Ubberup women and men had reduced insulin senstivity measured by HOMA- IR. Higher suPAR levels in Ubberup women (p=0.01) and men (p=0.03) indicated higher low grade inflammation compared to reference women and men.

Table 6. *Characteristics of the reference group versus the Ubberup group (baseline measures)*

	Women		***p*-values**	**Men**		***p*-values**
	Reference (n=51)	Ubberup (n=16)		Reference (n=49)	Ubberup (n=11)	
Chronological age (years from birth)	41 ± 13	35 ± 14	0.11[a]	41 ± 14	31 ± 9	**0.02[a]**
Weight (kg)	69 ± 12.8	106 ± 22.6	**<0.001**	82 ± 9.5	133 ± 23.9	**<0.001**
BMI (kg/m^2)	24 ± 4.2	37 ± 7.2	**<0.001**	25 ± 2.8	38 ± 5.4	**<0.001**
Fat mass (%)*	30 ± 6.3	43 ± 5.5	**<0.001**	18 ± 4.8	36 ± 6.2	**<0.001**
Muscle mass (kg)*	45 ± 8.2	57 ± 8	**<0.001**	63 ± 5.9	80 ± 12	**0.0007**
Waist circumference (cm)	79 ± 9.7	110 ± 16.1	**<0.001**	88 ± 1.5	125 ± 17.1	**<0.001**
Systolic blood pressure (mmHg)	118 ± 18.4	122 ± 18	0.53	130 ± 13	131 ± 1	0.89
Diastolic blood pressure (mmHg)	77 ± 11.4	77 ± 9	0.90	78 ± 8.5	80 ± 9	0.58
Total cholesterol (mmol/L)	4.6 ± 0.9	4.5 ± 1.2	0.83	4.3 ± 0.9	4.6 ± 0.7	0.37
HDL-C (mmol/L)	1.7 ± 0.4	1.2 ± 0.3	**0.0001**	1.4 ± 0.3	1.1 ± 0.2	**0.009**
LDL-C (mmol/L)	2.8 ± 0.8	3.2 ± 1.0	0.21	2.8 ± 0.8	3.2 ± 0.5	0.10
suPAR	2.2 ± 0.6	2.9 ± 0.8	**0.01**	1.9 ± 0.3	2.4 ±0.6	**0.03**
HOMA-IR	1.5 (1; 2)	2.5 (1; 5)	**0.0005[a]**	1.5 (1; 2)	2.4 (2; 4)	**0.0005[a]**
VO_2max (ml/min/kg)	35 ± 6.5	26 ± 6	**<0.001**	44 ± 7.1	29 ± 9	**0.0002**

*Data are shown as means ± SD or medians (IQR). Comparison between groups were analyzed by unpaired t-test. Statistically significant differences (p<0.05) are marked in bold. Measurements on Ubberup participants are baseline values. *For the sake of comparison, reference group measurements of fat mass and muscle mass used in the table is estimated with bioelectrical impedance analysis. No adjustments in blood pressure were made in relation to using blood pressure- or cholesterol lowering medication.* [a] *data were log transformed to a normal distribution before the unpaired analyses; Abbr.: BMI: body mass index; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; VO_2max: maximal oxygen uptake.*

BA model development

Selection of biomarkers - correlation and redundancy

Candidate biomarkers collected from the reference group were applied to Pearson's correlation analysis to assess the strength and direction of association between CA and the candidate biomarkers. Low correlation with CA ($|r| < 0.15$) excluded 17 of the 32 candidate biomarkers (**Figure 17**). Thus, 15 biomarkers remained and were assessed for redundancy ($|r| \geq 0.7$) (**Figure 18**). Waist circumference was selected instead of W/H ratio, HbA1c was selected above FBG, TC and HDL was selected instead of LDL, MAP included both SBP and DBP, and FEV1 was selected instead of FVC and the pulmonary ratio (FEV1/FVC) yielding a total of nine biomarkers

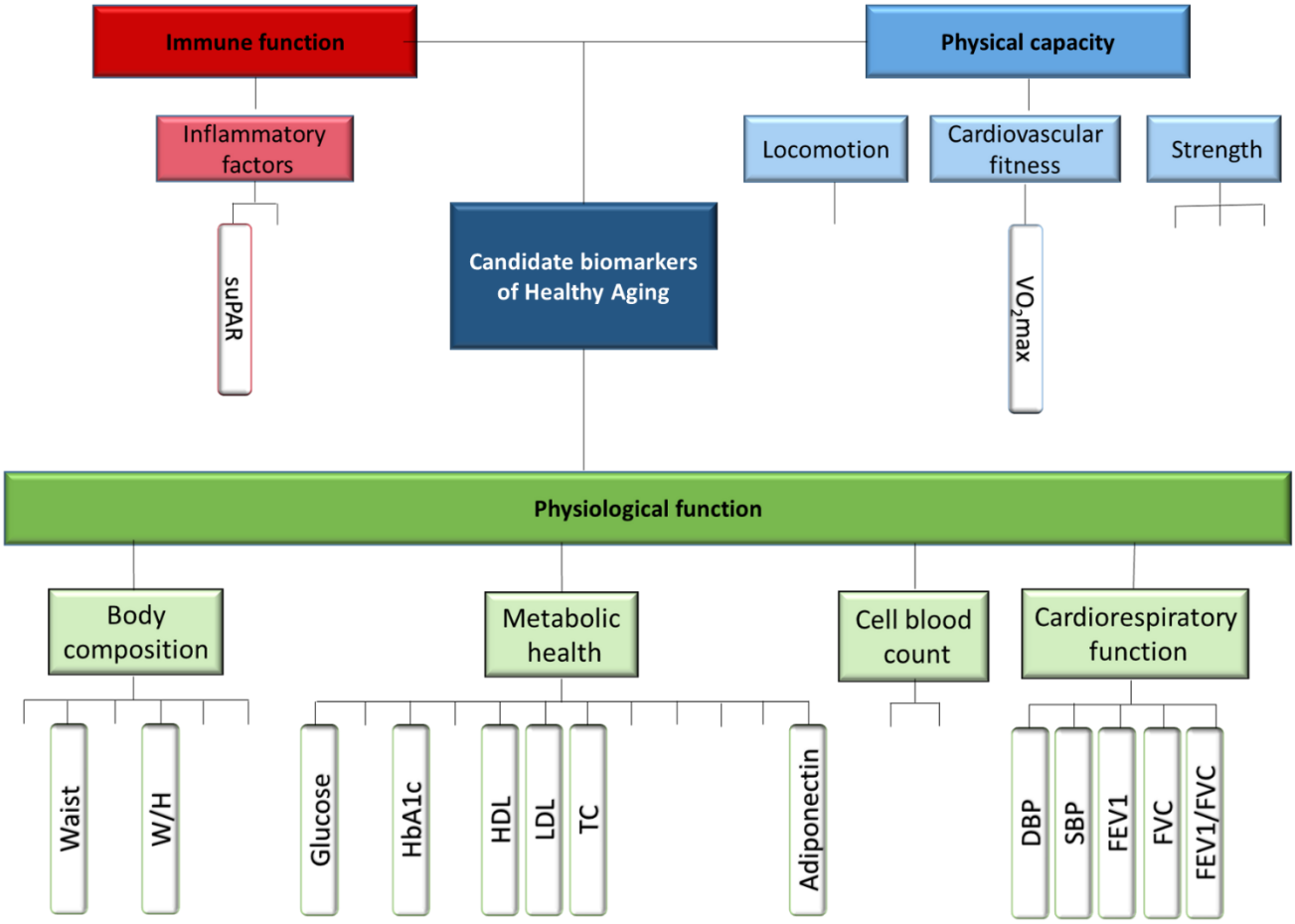
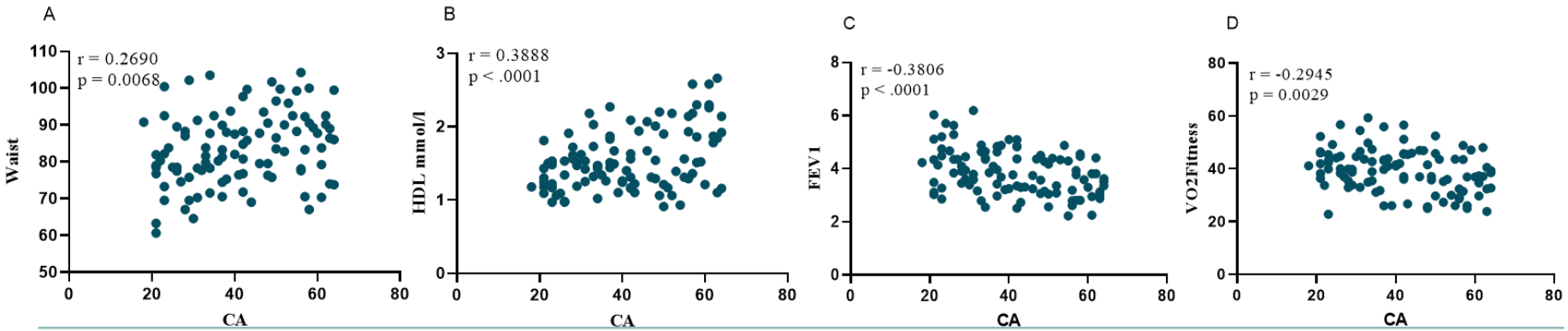


Fig. 17. *Visualizing the exclusion of 17 candidate biomarkers of aging in the modified model of candidate biomarkers (Figure 4). The biomarkers were excluded due to a low correlation with chronological age which is the first step in selecting biomarkers for the BA-model. Abbreviations: W/H: waist to hip circumference; HbA1c: glycated hemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein; TC: total cholesterol; DPB: diastolic blood pressure; SBP: systolic blood pressure; FEV1: forced expiratory volume within 1 second; FVC: forced vital capacity; suPAR: soluble urokinase plasminogen activator receptor; VO_2max: maximal oxygen consumption.*



CA															
0.2690	**Waist**														
0.3654	0.8202	**W/H**													
0.3148	0.5635	0.5333	**FBG**												
0.4922	0.1940	0.3485	0.2945	**HbA1c**											
0.2235	-0.3107	-0.3538	-0.1309	0.0189	**Adiponectin**										
0.3888	-0.3669	-0.3136	-0.1876	0.2088	0.5713	**HDL**									
0.3696	0.3220	0.2946	0.2745	0.2374	-0.0134	0.0361	**LDL**								
0.4952	0.1935	0.1633	0.2176	0.2974	0.2055	0.3937	0.9147	**CHOL**							
0.2828	-0.0829	-0.1137	-0.1832	0.0257	0.2606	0.2663	0.0758	0.1935	**suPAR**						
0.5125	0.2363	0.2315	0.3284	0.2507	0.0936	0.2606	0.2247	0.3333	0.1132	**DBP**					
0.4514	0.3786	0.4283	0.4753	0.2944	-0.0659	0.1272	0.2218	0.2628	-0.0677	0.8135	**SBP**				
-0.3806	0.3256	0.2848	0.2662	-0.0878	-0.3267	-0.3301	-0.0140	-0.1562	-0.2998	-0.1074	0.1730	**FEV1**			
-0.3163	0.3483	0.3400	0.2921	-0.0844	-0.3501	-0.3415	-0.0634	-0.2047	-0.2848	-0.1320	0.1780	0.9510	**FVC**		
-0.3052	-0.0169	-0.1105	-0.0486	-0.0427	-0.0306	-0.0517	0.1258	0.0883	-0.1259	0.0060	0.1780	0.3738	0.0754	**FEV1/FVC**	
-0.2945	-0.1297	0.1140	-0.0116	-0.1248	-0.1305	-0.1404	-0.2644	-0.3435	-0.3063	-0.2438	0.0214	0.4528	0.5093	-0.0455	**VO_2max**

Fig. 18. *Top: Scatterplots and Pearson's correlations of waist circumference (A), high density lipoprotein (B), forced expiratory volume in 1. sec (C), maximal oxygen uptake (D). Bottom: Pearson's correlation coefficients of the 15 biomarkers significantly correlated with age and their inter-correlations. CA: chronological age; W/H: waist to hip ratio; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin type A1c; HDL: High density lipoprotein; LDL: Low density lipoprotein; CHOL: total cholesterol; suPAR: soluble urokinase plasminogen activator receptor; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; FEV1: Forced expiratory volume in 1. sec; VO2max: maximal oxygen uptake. Adapted from paper III, Figure 1, p. 10.*

PCA

Application of PCA revealed the linear combination of the nine biomarkers in the 1stPC, the eigenvalue (sum of squared distances), and how many percent the 1stPC explains of the total variation in the dataset (**Table 7**). We found that the 1stPC had eigenvalues of 2.79 and 2.25 and accounted for 30.96% and 25.04% of the total variance in the BA-model of the nine biomarkers in women and men, respectively. The biomarkers with the highest influence on BA estimation was TC (21.8%) followed by MAP (18.9%) in women and waist circumference (24.1%) and VO_2max (22.6%) in men.

Table 7. The linear combination of normalized variables for the 1stPC by gender and the relative contribution of each biomarker to BA estimation. Modified from paper III, Table 4, p. 14.

	Women		Men	
	Loading scores	Contribution (%)	Loading scores	Contribution (%)
MAP	0.435	18.9	0.349	12.2
Glycated hemoglobin	0.408	16.7	0.324	10.5
Waist circumference	0.173	3.0	0.491	24.1
FEV1.	-0.138	1.9	-0.309	9.5
VO_2max	-0.341	11.6	-0.475	22.6
Adiponectin	0.228	5.2	-0.046	0.2
High density lipoprotein	0.390	15.2	-0.020	0.04
Total cholesterol	0.467	21.8	0.3804	14.5
suPAR	0.238	5.7	0.254	6.4
Eigenvalue	2.79		2.25	
Explained Variance%	30.96		25.04	

Abbr.: BA: Biological age; 1stPC: first principal component comprising the best fit line with the largest sum of squares distances; Eigenvalue: The Sum of Squared distances for PC1; Explained variance %: How many percent does the 1stPC explain of the total variance in the dataset. MAP: Mean arterial blood pressure = ($\frac{1}{3}$SBP + $\frac{2}{3}$DBP); FEV1: forced expiratory volume in 1. sec.; VO_2max: maximal oxygen consumption (ml/min/kg); suPAR: soluble urokinase plasminogen activator receptor.

BA model estimation

The 1stPC loading scores from the PCA analysis (**Table 7**) were used to construct individual standardized BA scores (BAS) as a function of the nine biomarkers (eq. 5, p. 27). These were then scaled into units of years

and corrected for regression towards the mean (eq. 6, p. 28) yielding the following equations for BA estimation in women and men, respectively:

$$BAcfemale = -56.67 + 0.27 \cdot MAP + 1.02 \cdot HbA1c + 0.1453 \cdot Waist - 2.03 \cdot FEV1 - 0.43 \cdot VO2\,\max + 0.0003 \cdot Adiponectin + 7.39 \cdot HDL + 4.06 \cdot TC + 3.24 \cdot suPAR + 0.20 \cdot CA$$

$$BAcmale = -70.37 + 0.34 \cdot MAP + 0.95 \cdot HbA1c + 0.60 \cdot Waist - 3.96 \cdot FEV1 - 0.62 \cdot VO2\,\max - 9.73 \cdot 10^{-5} \cdot Adiponectin - 0.57 \cdot HDL + 4.06 \cdot TC + 7.61 \cdot suPAR + 0.32 \cdot CA$$

Regression analysis on BAc as a function of CA, reveals a symmetrical scatter of BA above and below the regression lines resulting in R^2 values of 0.73 (women) and 0.65 (men) (**Figure 19** top row). Regression lines for both women and men exhibit a slope near one (women: b=0.99 (95%CI 0.83; 1.17) and men: b=1.00 (95%CI 0.79; 1.22)) with an associated variation around the regression lines (standard error of the estimate, SEE) of 8.2 years (women) and 10.2 years (men). The Bland Altmann plot shows high agreement between BAc and CA (BIAS= 0.002 women and BIAS 0.006 men) with no major inconsistency in variability across the graphs (**Figure 19** bottom row).

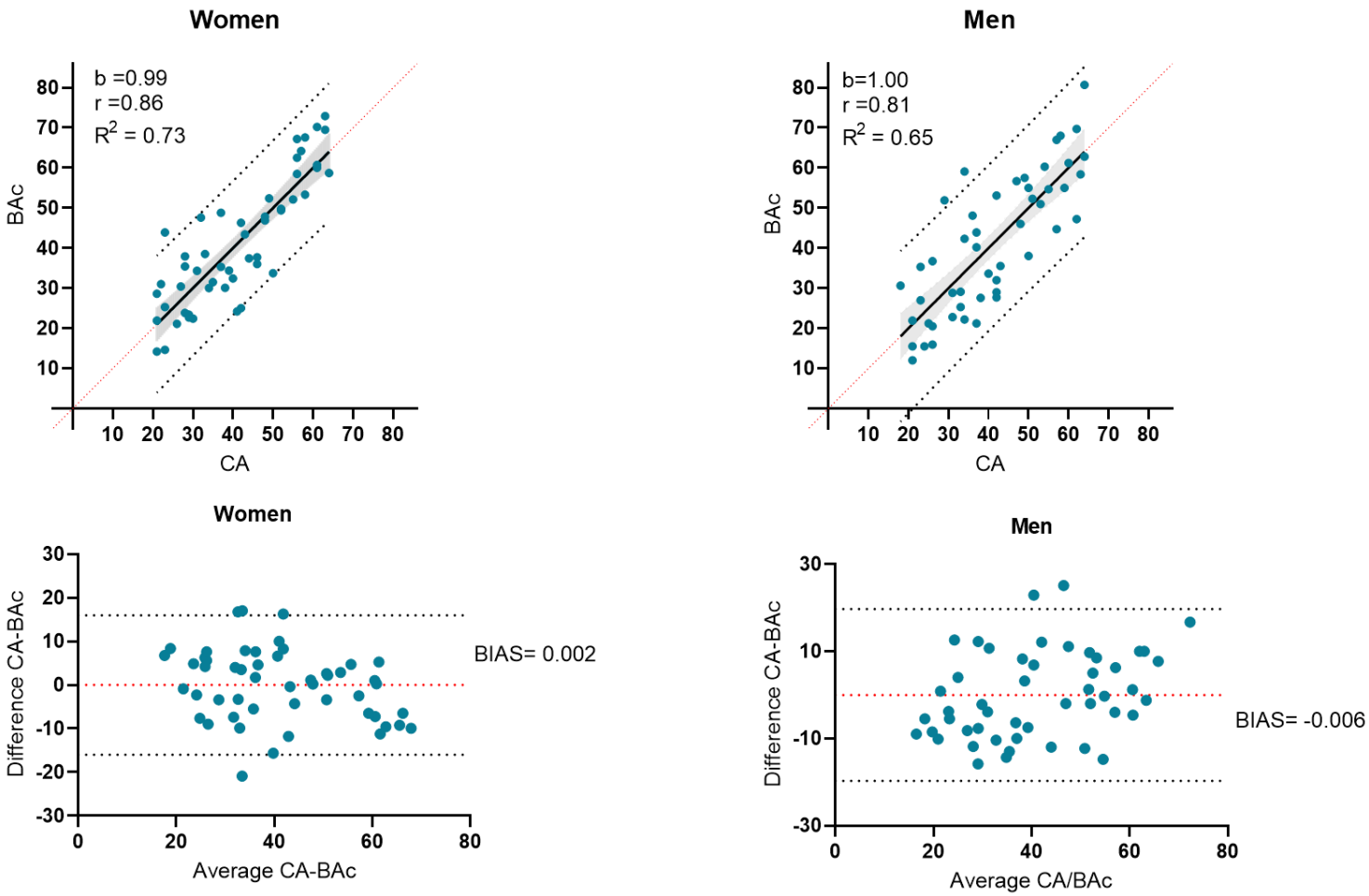


Fig. 19. *Top row: BAc regression lines as a function of chronological age (CA) for women and men, respectively. Shaded area represents 95% confidence interval, black dotted lines represents 95% prediction interval, and red dotted lines represents line of identity. Slope (b), Pearson's correlation coefficient (r) and coefficient of determination (R^2). Bottom row: Bland Altmann plot for women and men, respectively. Red dotted lines represent BIAS, black dotted lines represent upper and lower limits of agreement. The Figure is modified from paper III, Figure 3 and Figure 4.*

Additional analysis

BA distribution

A normal distribution of CA was not present due to the recruitment strategy of including an equal number of women and men within each 5-year age category spanning from 18-65 years (**Figure 15**). In comparison, BA was normally distributed, and the age range of BA was 14-73 years (mean 41 years ± 16 years) in women and 12-81 years (mean 40.2 years ± 17 years) in men (**Figure 20**).

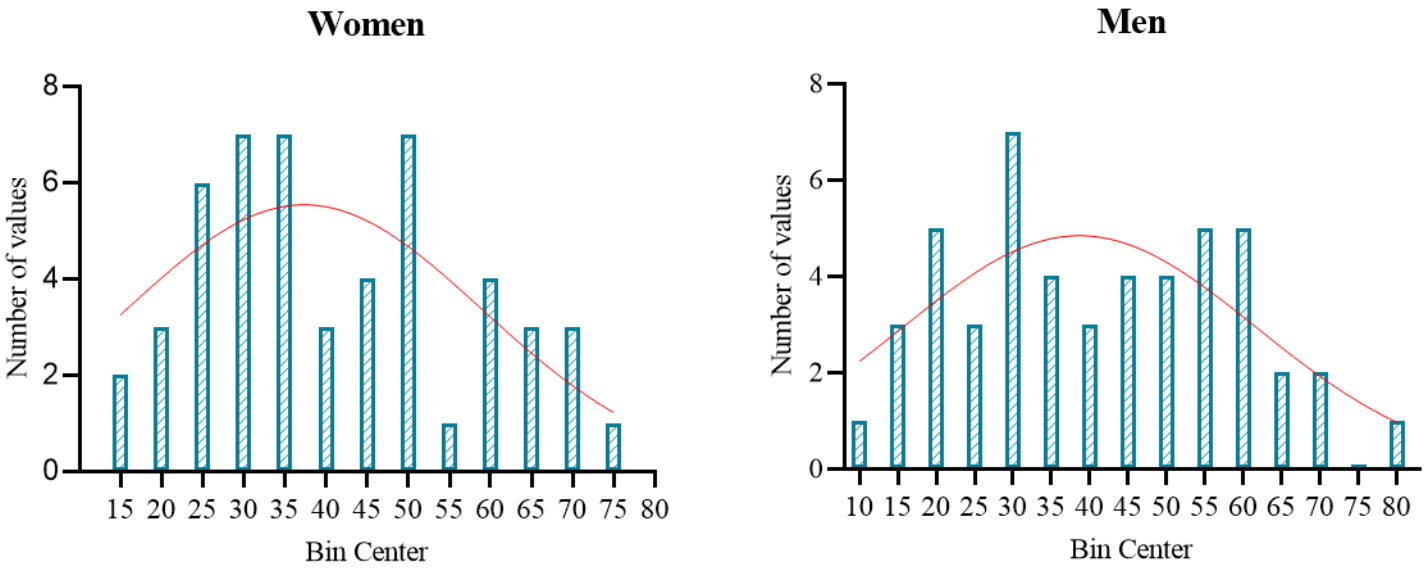


Fig. 20 *Frequency distribution of BA for women (n=51) and men (n=49) in the reference group. Red line shows the Gaussian distribution. The bin width is 5 years. Because the first bin is centred at 15, the bin will contain values between 12.5 and 17.5 years.*

Facial aging

A high correlation (r=0.8, p<0.001 for both AS and MS assessors) was observed between age assessed based on a portrait picture (facial age) and BA (**Figure 21**). No difference in regression slopes (p=0.79) or intercepts (p=0.09) were observed between the two assessor groups.

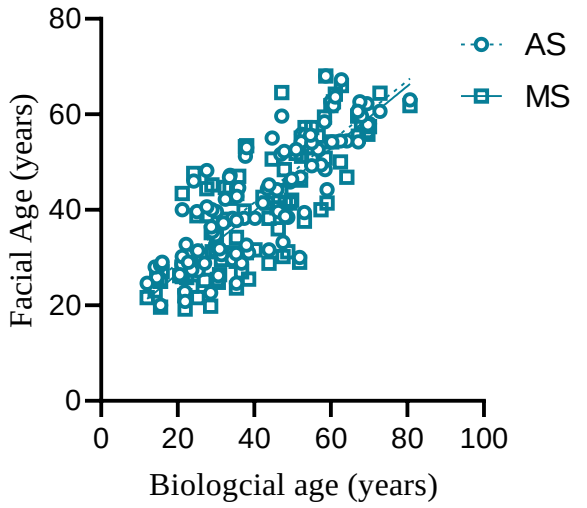


Fig. 21. *Facial age as a function of biological age in the healthy adult reference population (n=100). Assessors were: AS: administrative staff, n=5 (circles and braked line) and MS: medical staff n=5 (squares and solid line). Each point represents mean facial age score from 5 assessors.*

Relative telomere length

We found no association between BA and relative telomere length (r= -0.09, *p*=0.37) (**Figure 22**).

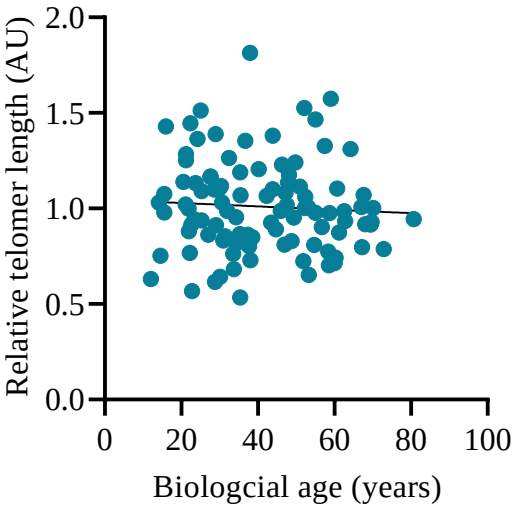


Fig. 22*. Relative telomer length as a function of biological age in the healthy adult reference group (n=100).*

BA and health risk estimation

Reminder to the reader; adiponectin data is missing why BA estimates for Ubberup participants are incomplete. To circumvent this, the same adiponectin concentration was used at baseline and follow-up when estimating BA.

Discrimination between healthy and unhealthy individuals

Figure 23 shows the regression lines of BA as a function of CA in Ubberup and the reference group. Ubberup women had a higher intercept (*p*<0.0001) but a similar slope (*p*=0.87) compared to the reference group. The direction of the regression line did not allow for the same comparison for the Ubberup men.

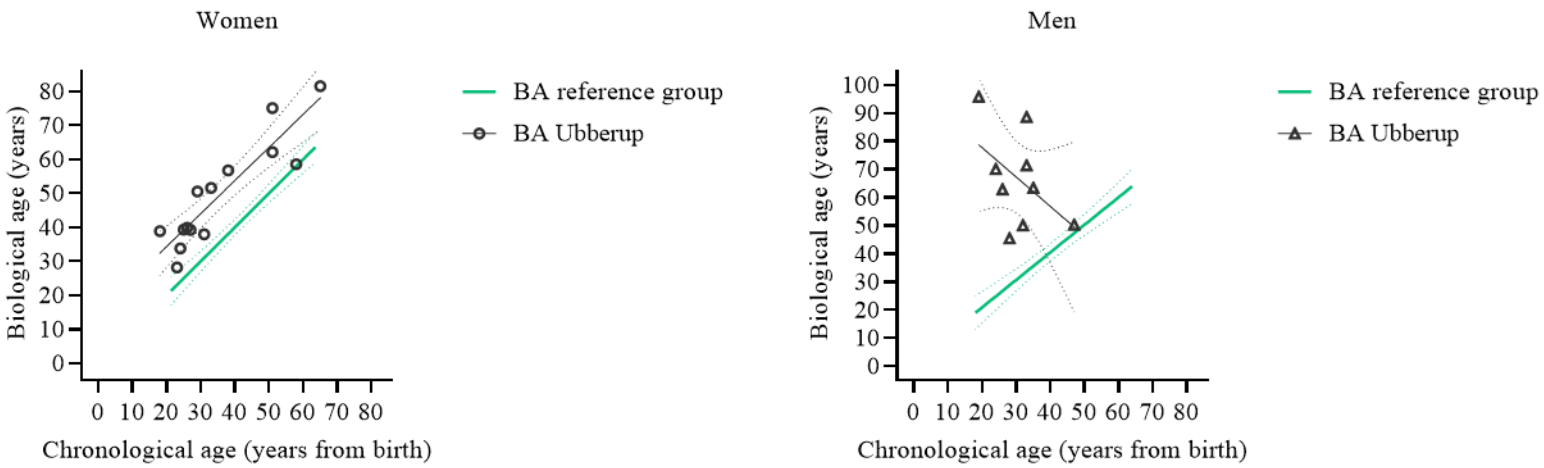


***Fig.23**. Scatterplot of individual biological age (BA) and the relation with chronological age (CA). The green line represents the linear regression of the healthy aging trajectory. The circles and triangles represent the baseline biological age values for women and men, respectively, with related regression lines (black lines). Dashed lines represent 95% confidence intervals. Adapted from paper IV, Figure 2, p. 14.*

The effects of 15-week lifestyle intervention and related change in BA

Participants had a 9% (IQR: 7% to 10%) and 10% (IQR: 5% to 13%) weight loss together with a reduction in waist circumference of -11 cm (95% CI: -8 cm; -14 cm) and -16 cm (95% CI: -9; -23) in women and men, respectively. **Table 8** shows changes for women and men following the 15-week lifestyle intervention. Participants relative CRF level improved with 3.8 ml/min/kg (95% CI: 2.9; 4.8 ml/min/kg) in women and 4.7 ml/min/kg (95% CI: 2.5; 6.7 ml/min/kg) in men. An improvement in blood pressure (MAP) was only observed in male participants (p=0.002). In addition, only men reduced their total cholesterol concentration (p<0.001). The effect of the lifestyle intervention was reflected in BA improvements as BA decreased with -4.1 years (95% CI: -2.1 to -6.1; p=0.0006) and -16.4 years (95% CI: -23.4 to -9.3; p=0.0007) for women and men, respectively.

Table 8 Changes in weight, the 9 biomarkers for biological age estimation and grip strength, divided by sex.

	Women (n=16)			Men (n=11)		
	Baseline	**Follow-up**	*P*	**Baseline**	**Follow-up**	*P*
Weight (kg)	102 (88; 122)	93 (81; 109)	<.0001	130 (115; 152)	116 (109; 132)	0.0001[a]
Muscle mass (kg)	57 ± 9	56 ± 8	0.01	80 ± 12	78 ± 10	0.03
MAP (*mmHg*)	92.0 ± 11.8	92.0 ± 12.5	n.s.	97.0 ± 9.3	92.5 ± 8.4	0.002
HbA1c (*mmol/mol*)	33.9 ± 2.7	33.9 ± 2.7	n.s.	32.5 ± 3.1	32.5 ± 3.1	n.s.
Waist (*cm*)	110 ± 16	99 ± 14	<.0001	125 ± 17	109 ± 13	0.0004
FEV1 (L)	3.1 ± 0.7	3.0 ± 0.6	n.s.	4.4 (3.9 ; 5.2)	4.3 (3.7, 5.1)	n.s.[a]
VO_2max (*ml/kg/min*)	25.5 ± 6.4	29.3 ± 6.7	<.0001	29.1 ± 8.8	33.8 ± 10.7	0.0006
Adiponectin (*mg/mL*)						
HDL-C (*mmol/L*)	1.1 (1.01; 1.32)	1.1 (0.96; 1.31)	n.s. [a]	1.2 (1.05 ; 1.27)	1.1 (0.9; 1.3)	n.s.[a]
TC (*mmol/L*)	4.5 ± 1.2	4.7 ± 1.0	n.s.	4.3 (4.2 - 4.8)	3.8 (3.2 - 4.5)	0.004[a]
suPAR (*ng/ml*)	2.9 ± 0.8	3.1 ± 1.0	n.s.	2.3 (2.1 ; 2.7)	2.3 (1.9 ; 3.6)	n.s.[a]
Grip strength (*kg*)	33 ± 5	33 ± 5	n.s.	49 ± 9	48 ± 9	n.s.
Metabolic syndrome (n)	7	4	n.s.	3	2	n.s.

Abbr.: *MAP*: Mean Arterial Pressure; *HbA1c*: Glycated Hemoglobin*; FEV1*: Forced Expiratory Volume in the 1. Second; *VO_2max*: maximal oxygen uptake; *HDL-C*: High-Density Lipoprotein Cholesterol; *TC:* Total Cholesterol; *suPAR*: soluble urokinase plasminogen activator receptor. Metabolic syndrome was diagnosed using the International Diabetes Federation definition.

Missing values W*omen (W)*: HDL-C n=14, TC n=14, *Men (M)*: suPAR: n=10, TC n=9, HDL n=9, Metabolic syndrome: *W* n=2, *M* n=2; Adiponectin *W* n=16, *M* n=11 [a] log10 transformation was applied. Normal distributed data are represented as Mean ± SD, and log-transformed data as Medians (IQR).

BA and clinical relevance

We observed that BA was positively associated with BMI (r=0.52, *p*=0.01) which is a clinical indicator of overweight and obesity. A suggested by the regression analysis, BA increased 1.5 years (95% CI: 0.4 to 2.7) for every unit increase in BMI. (**Figure 24,** *left side*). Further, BA was positively associated with HOMA-IR which is a clinical indicator of insulin resistance. The positive association (r=0.48, *p*=0.02) was, however, influenced by a single influential observation and should be interpreted with caution (**Figure 24,** *right side*).

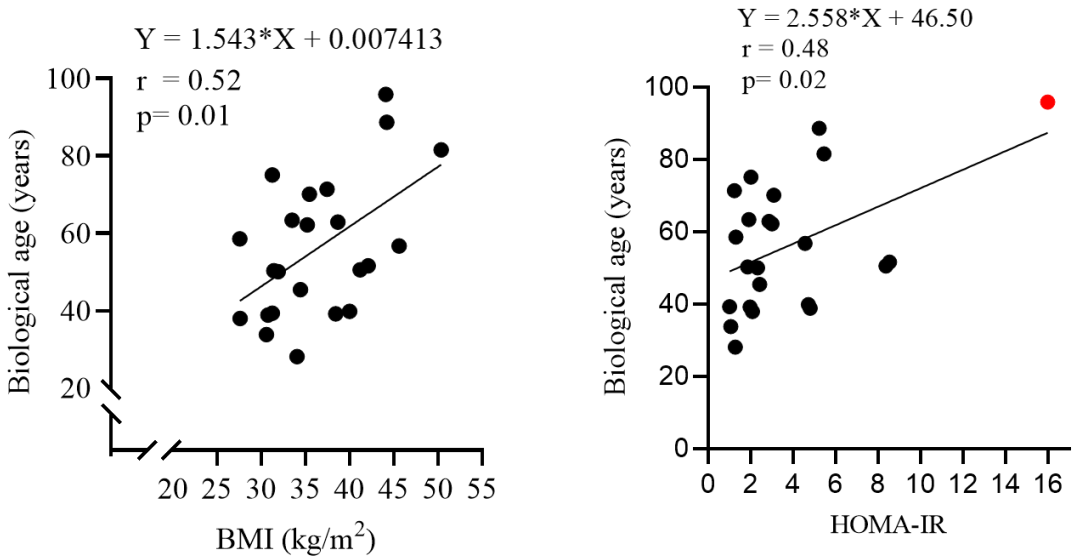


Fig. 24 *Linear regression and Pearson's correlation between biological age (BA) and body mass index (BMI) left side, and the homeostatic model assessment of insulin resistance (HOMA-IR) right side. The red dot represents a highly influential observation to the correlation analysis. Pooled analysis n=22. Modified from paper IV, Figure 4 and 5, p. 17*

Discussion

Our findings provides novel data on the applicability of BA as a concept in general health promotion and its effectiveness in a real-life setting. We developed a new BA model following scientific standards and investigated the strength of the model and clinical utility through a lifestyle intervention commonly used as a treatment against overweight and obesity. The following paragraphs will be a discussion of the main findings and methodological considerations in the development of the BA model. Finally, strengths and limitations of the studies will be elucidated.

BA as motivational tool

Participation

In paper I we found that initial motivation for participation in a body age health check was high (70%). In comparison, results from the fifth round of the Danish Work Environment Cohort study (2010, n=10,605) showed a participation rate of 44.9% in workplace health promotion offering a health check (142). In the specific study, *Social and Health Care* and *Manufacturing* were the dominant industries (19.7% and 10.6%, respectively). In comparison, *Financial* and *Energy* industries dominated in our study (41% and 32%, respectively). Blue-collar workers with physically exhausting work is associated with low participation in workplace health promotion and could partly explain the discrepancy in participation rates (142, 143). In addition, the body age health check was offered during working hours and not in leisure time which is another key factor for participation (142). To my knowledge, our study is the first to investigate workplace health promotion offering health checks whit BA estimation as an outcome measure. Whether or not the prospect of BA estimation has an additional positive effect on the motivation for participation is not possible to evaluate without a proper controlled study design. However, the high participation rate at baseline could be an indication of this.

During follow-up, the participation rate fell from 70% at baseline to 40% as 60% were lost to follow-up (1TP). The comparison of BA and the single metabolic risk factors (**Figure 13 and Table 4**) indicate that 1TP are less healthy compared to 2TP. This confirms a recurrent issue in workplace health promotion involving lower participation rate among the unhealthiest employees (144). BMI, mean blood pressure, waist circumference, and body fat percentage was statistically higher among 1TP compared to 2TP. Whether these differences are clinically relevant remains unknown hence, do the two groups differ from a health risk perspective? We observed higher frequencies of employees who smoked and had lower cardiorespiratory fitness among 1TP. These variables are two important determinants of CVD and risk of premature mortality (51-54, 145). Collectively, these results indicate that 1TP have higher risk of future lifestyle related diseases despite the rather similar medians at baseline between 1TP and 2TP.

Finally, it could be interesting to have data on the 4,222 employees who did not respond to the invitation in the first place (**Figure 10**). Do they also represent a less healthy fraction of the cohort, or do they simply not consider BA a relevant measure? We can only speculate on this matter, however, one consideration could be that not one size fits all in motivation for health behavior change, and BA as a motivational tool is no exception.

Change in health behavior

At follow-up, we observed small to moderate effect sizes in seven (TC, waist circumference, fat%, CRF, pushups, wallsit and flexibility) out of eleven biomarkers included in the BA estimation in women and six

(TC, waist circumference, fat%, pushups, wallsit and flexibility) out of eleven biomarkers included in the BA estimation in men (**Figure 14**). These results partly drive the decrease in BA observed in women and men. A lower BA at follow-up implies lower health risk and thus a change in health behavior since the baseline measurements.

Change in diet and/or the physical activity level are health behaviors that could be responsible for the changes observed in metabolic risk factors and the reduction in weight loss observed in both women and men. As only women improved their CRF, and the improvement on average reached 0.25 ml/min/kg (95% CI 0.06, 0.45), it is unlikely that change in physical activity behavior was the main determinant for the observed improvements in single metabolic risk factors. In addition, an effect on blood pressure would be expected in case of a clinically relevant increase in the level of physical activity which we did not find (146). Small improvements in metabolic risk factors can, however, be influential. The Framingham Offspring Study found that a small increase in HDL-C of 0.06 mmol/L was associated with a 2-3% reduction in CVD risk (147). In our study, the prevalence of employees with metabolic syndrome, a clinical indicator associated with future risk of CVD and T2D (148), had decreased at follow-up (14%), indicating that the effect sizes observed can be considered clinically relevant. Importantly, regression towards the mean effect should be considered when interpreting small effect sizes in a retrospective study without a control group. Measurement errors and random fluctuation in measurements could potentially confound the changes observed at follow-up (149). Thus, a common regression towards the mean phenomenon is that on follow-up the measured risk variables will be lower than the starting value, even in the absence of an effect of the intervention (150).

Based on these data it is difficult to interpret whether BA estimation motivates to a change in health behavior related to physical activity and/or dietary behavior. However, a smoking cessation rate of 42% indicates that knowing the impact smoking has on BA estimation (**Table 3**) motivates positively on smoking behavior. The cessation rate observed in the present study is high compared to cessation rates found in previous workplace health promoting interventions offering health checks (20% and 8%, respectively) (17, 151) and closer to the cessation rates found in anti-smoking interventions per se performed at the workplace (53%) (152). Following the implementation of the Danish law against smoking at public- and workplaces in 2007, 320,000 Danes quit smoking (153). From 2011 to 2016, the prevalence of smokers was stable around 21-23% (153). This comprises most of the sample period (2011-2017), why we believe that our finding on cessation rates is an effect of the intervention rather than secular trends. The result could, however, be inflated by self-reported measurement of smoking habits.

BA model development

Recruitment of healthy agers

Baker and Sprott stated that biomarkers should be investigated in a population free from disease *(72).* While free from known disease, we found that metabolic syndrome was present in five participants. In addition, four women and eight men had a difference between BA and CA of ≥10 years indicating an increased risk of disease. A BMI > 24.9 was overrepresented among those exhibiting metabolic syndrome. In general, high BMI is considered causally related to morbidity and mortality (7, 154). It is therefore worth discussing whether individuals with a high BMI should have been included in the reference group meant to represent healthy aging.

A meta-analysis by Flegal and colleagues found that all-cause mortality was lower among overweight individuals compared to normal weight individuals (HR 0.94; 95% CI 0.91-0.96) and that grade 1 obesity (BMI 30-35kg/m^2) was not significantly associated with increased mortality (HR 0.95; 95%CI 0.88-1.01) (155). Despite that this study did not consider morbidity risk or body composition, it adds to the discussion concerning the obesity paradox stating that a J-shape association exists between mortality rate and BMI which becomes more like a U-shape with increasing age (156). Whether this is an occurrence of reverse causality, i.e. low BMI and mortality could be confounded by underlying disease, is still to be investigated (157). However, neglecting to consider CRF level is a considerable confounding factor in the assessment of BMI and associated mortality. A meta-analysis showed that regardless of BMI unfit individuals had twice the risk of death compared to fit counterparts (158). In addition, and relevant to the present reference group, the risk of disease was attenuated among fit overweight and obese individuals (158).

Similarly, CRF level should be considered in aging studies when comparing decline in physiological function in young versus old individuals (159, 160). It has been argued that without a clear definition of the minimum requirements of physical activity to remove the confounding effects of inactivity, the age-related changes in physiological function should be studied in individuals with a high physical activity level (49, 161). The women and men included in the reference group had moderate to high VO_2max across all age categories (**Table 5**) but where not recruited based on activity level and cannot be characterized as professional athletes or master athletes. However, the reference group represents a sample of the general population overall adhering to the Danish health authorities recommendations of a minimum of 150 min/week of moderate to vigorous physical activity. Thus, as comparison group, the reference group is considered to represent a sample of an adult population where the deleterious effects of inactivity are abolished or at least diminished. Considering socioeconomic status and specifically level of education, the reference group represents a sample of the Danish population with a higher level of education compared to

the general Danish population. Only 7.5% of men and 0% of women reported lower secondary education (i.e. primary school 7-10 grade) as their highest level of education. In comparison, 25% of women and men in the Danish population (age 15-69 years) had lower secondary education as their highest level of education registered in 2020 (162).

Selected biomarkers and age-related pathophysiology

The selection of biomarkers for BA estimation was based on the established correlation with age (criteria 5 p. 17, *biomarkers of aging*). Nine biomarkers were significantly associated with CA and included in the BA estimation representing five subdomains: *body composition:* WC, *metabolic health:* HDL, TC, adiponectin and HbA1c, *cardiorespiratory function:* MAP and FEV1, *inflammation:* suPAR, and *cardiovascular fitness:* VO_2max. Valid biomarkers of aging should describe underlying mechanisms of aging, and be critical in the maintenance of health and prevention of disease (criteria 4 and 6, p. 17, *biomarkers of aging*) (74). This subject will be discussed in the following section.

Body composition

Redistribution and increase of fat mass together with loss of muscle mass are key characteristics of the age-related change in body composition (163). Aging is associated with a reduced storage of fat subcutaneously due to decreasing size of adipose depots (164). Instead, fat is increasingly stored in visceral depots and ectopic depots such as in skeletal muscle or liver (164). Studies have shown that WC is a valid anthropometric measure of VAT accumulation (165) and have been found to predict age-related disease such as T2D (166) and CVD (167). Both cross-sectional and longitudinal studies found that between 20-80 years of age WC increases but the magnitude of the increase differs depending on study design and sex (31). We found that WC was significantly correlated with CA (r=0.3) and waist to hip ratio (r=0.8202). Waist to hip ratio showed a higher correlation with CA (r= 0.4). The inherent limitation that an individual with central obesity can display the same ratio as an individual without central obesity ultimately made us select waist circumference despite a lower correlation with CA (**Figure 18**).

Surprisingly, due to insignificant correlation with age, no strength-related measures (grip strength, biceps strength, or quadriceps strength) were included in the BA model. Aging is associated with sarcopenia including loss of muscle mass and especially a decline in muscle strength (168). Grip strength is an isometric strength measure and a robust and commonly used measure of whole-body strength (169). The missing association between age and strength contrast previous cross-sectional and longitudinal studies consistently showing a linear decline in grip strength (170, 171). This discrepancy could be an indication of the difficulty to identify biomarkers of aging within a healthy sample size of the population compared to within a general population (49). However, an age-related decline in grip strength has been shown in highly

active men (p<0.001) but not in highly active women (p=0.055) (49). This indicates that despite the relation between level of physical activity and grip strength, the age-related decline in strength is only attenuated through sufficient physical activity (172). Strength has been found to decrease in a linear manner from the age of 40 (170), other studies showed an accelerated decline from age 50 and onwards (171). In comparison, the age range of the reference group was 18-65 years, and a total of 32 were above 50 years. Thus, the combination of a physically active reference group, a small absolute proportion of individuals above 50, and the oldest being 65 years can possibly explain the lack of observed age-related decline in strength.

Metabolic health

Aging is associated with metabolic changes such as dyslipidemia and reduced glycemic control (173, 174). The underlying pathophysiology is complex and interrelated. Chronic low-grade inflammation caused by the cellular hallmarks of aging and the derived inflammation from the afore mentioned redistribution of fat, especially VAT, is considered the underlying etiology (25, 27).

Adiponectin is an adipokine with anti-inflammatory properties secreted only from adipose tissue to the blood stream (42). Regulation of plasma adiponectin concentration is suppressed by TNF-a and is negatively correlated with VAT which potentially explains the decreased level of adiponectin observed with ageing (31, 175). Adiponectin is a relevant biomarker of the metabolic disturbances related to glycemic control and hypertension (42). Studies have shown that plasma concentration of adiponectin is lower among individuals with T2D and hypertension (176, 177). In addition, lifestyle interventions focusing on weight loss through exercise and healthy dieting increase plasma concentration of adiponectin (178).

We investigated HbA1c and FBG as biomarkers for glycemic control. HbA1c is a clinical indicator of elevated plasma glucose concentration over the preceding months related to the erythrocytes life span (approximately 120 days) (179). We found that both HbA1c and FBG concentration correlated significantly with CA. The intercorrelation between HbA1c and FBG concentration (r=0.2945, p=0.003), was beneath the cut off set for redundancy (r≥0.7). In theory, this indicates that the biomarkers are not considered as measures of the same physiological function. This contrasts with previous studies showing highly significant correlations between HbA1c and FBG concentrations (179, 180). This relation has been used to validate the use of HbA1c instead of FBG concentrations in the diagnostics, control, and management of T2D within the clinic. The sample size could be a confounding factor in this regard, and we excluded FBG due to a lower correlation with age and a higher clinical feasibility of HbA1c (ref).

The age-related changes in the lipid profile constitutes increases in TC and/or LDL-C cholesterol and a decrease in plasma HDL-C. These changes have been observed both among women and men from age 20

to 60 years (181-183). We identified similar changes and included HDL-C and TC as biomarkers. Despite consistent results showing that lowering LDL-C plasma concentrations decreases the risk of atherosclerosis and concomitant CVD, we did not include LDL-C. The reason for this was methodical entailing that TC and LDL-C were highly intercorrelated (r= 0.9) and TC had a higher correlation with CA. The redundancy observed between TC and LDL-C is related to LDL-C being the main carrier of cholesterol emphasizing that both TC and LDL-C can be used in risk prediction related to CVD as examplified in the Framingham risk score which includes TC and not LDL-C (184). HDL-C has been shown to reduce CVD risk among individuals already achieving a plasma concentration <1.7 mmol/L through statin treatment. This highlights the protective effect of HDL-C (185) and makes a rationale for the inclusion of both TC and HDL-C as biomarkers in the BA model.

Cardiorespiratory function

Blood pressure and especially SBP represents an essential and commonly used biomarker across the BA-literature (67). In the included review SBP and/or DBP were used in 62% of the studies (n=18). This represents a high prevalence considering the great heterogeneity otherwise seen in biomarker combinations (**Table 1**). Hypertension is one of the most important CVD risk factors and predictor of CV mortality (186, 187). Hypertension is related to increasing arterial stiffness and its prevalence increases with CA (29). In general, high SBP is considered more important compared to DBP as a risk factor for CVD (188). This is a possible explanation as to why SBP is employed more often (n=13) compared to DBP in BA studies (**Table 1**). We did not include SBP instead of DBP as both correlated equally with CA. Instead, we used MAP to cover both parameters and estimate the average arterial pressure throughout one cardiac cycle, i.e. both systole and diastole (189). Using MAP as a biomarker provides a functional assessment of the cardiovascular system including cardiac output and peripheral resistance. This is important when estimating risks in patient specific groups or different age ranges. It has been shown that MAP is a predictor of CVD on the same level as SBP and pulse pressure in individuals with T2D and that the risk in younger individuals is better predicted using the combination of MAP and pulse pressure than SBP alone (190, 191).

FEV1 represents one of three lung function measures (FVC and FEV1/FVC ratio), and FEV1 was selected as biomarker of aging as it comprised the highest correlation with age compared to FVC and the FEV1/FVC ratio. Like blood pressure, lung function is another frequent component in BA estimation (n=14, **Table 1**) favoring the use of FEV1 (n=9) above FVC (n=4) or both (n=1) (**Table 1**). FEV1 is the most common clinical measure of lung function and declines from age 35 in women and men (192). A linear decline with age has been found in cross-sectional studies. However, longitudinal analyses show that the decline remains linear up until the age of 70 years after which the decline accelerates (193). FEV1 is a predictor of respiratory as

well as non-respiratory diseases (e.g. CVD) and all-cause mortality which indicates that impaired lung function acts as a component in the pathophysiology of other age- related diseases (194, 195).

Inflammation

Aging is accompanied by a pro-inflammatory status such as elevated TNF-α and IL-6 plasma concentrations (27) due to, among others, cellular senescence (**Figure 2**) (26), decreased physical activity (39, 62), and increased VAT ((196)). We found no association between CRP and CA in the reference group and hence CRP was not included as a biomarker for the BA model. CRP is the gold standard biomarker when measuring low grade inflammation (197). It is synthesized by the liver and stimulated by IL-6 (198). Studies showing an increase in CRP with age is, however, restricted to generally healthy populations >65 years (199-201). In the estimation of BA in young adults, Belsky and colleagues applied a BA model including CRP (71). They found that CRP did not change over the course of 12 years in a young adult population with a baseline age of 26 years. Thus, the age range 18-65 years in the reference group represent the adult lifespan except for the elderly >65 years which potentially explains the lack of association between CRP and CA.

Instead, we found a positive association between suPAR and CA. suPAR is a new inflammatory biomarker. Like CRP, it is thought to reflect low grade inflammation (197). The intracellular pool of uPAR is especially abundant in vascular endothelial cells and neutrophils constituting the majority of the white blood cells (202). A pro-inflammatory status with increased TNF-a and IL-6 levels stimulates the translocation of uPAR to the cell surface and to plasma generating the soluble form of the receptor (203). The age-related increase in plasma suPAR levels has been shown in a Danish cross-sectional study (n=5,538) of 30-60 year old individuals (197) and reconfirmed in a cohort study exhibiting a 6% increase in plasma suPAR levels over a five-year period (204). suPAR has been found to predict the risk of T2D, CVD, and mortality independent of CRP levels (205). In addition, the association between suPAR levels and the risk of CVD and mortality were stronger among young compared to old individuals (205). These findings all advocate for the use of suPAR in the present BA model. We found that plasma concentrations of suPAR was higher in Ubberup participants compared to the reference group, however we found no change in suPAR plasma concentrations after the 15 week-lifestyle intervention. This contrasts with the findings from Haupt et al. showing that lifestyle changes did lower plasma concentrations of suPAR (204). Specifically, they found that the age-related increase in suPAR, during a five-year period, was attenuated by healthy dieting and physical activity which are two core components in the lifestyle intervention. suPAR is, in contrast to CRP, not an acute phase reactant and studies suggest that the two biomarkers of inflammation belongs to different pathways (206). For example, following surgery, plasma concentrations of CRP were increased whereas suPAR remained stable (207). In addition, and in contrast to CRP, no correlation between anthropometric measures (e.g. BMI and waist circumference) and suPAR have been found (208). Instead, suPAR has been

found to correlate with endothelial dysfunction and atherosclerosis (208). Collectively, these results indicate that suPAR is related to subclinical organ damage and disease progression, probably explaining the stable suPAR levels after the 15-week lifestyle intervention.

Cardiovascular fitness

As anticipated, VO_2max was negatively associated with age in the reference population. The decline in VO_2max with age is well established (30) and the inverse relationship with all-cause mortality (52) makes VO_2max an important biomarker of aging. Only three studies included in the review (102, 110, 117) included VO_2max, and Lara et al. did not mention VO_2max in their proposal of biomarkers of healthy aging (83). While non-invasive, direct measures of VO_2max may not be feasible in longitudinal studies of the general population and is typically not part of national health registry databases which is a common source of biomarkers in previous BA studies (**Table 1**). Instead of direct measurements, VO_2max could be estimated through submaximal exercise protocols (209, 210) or by employing technology aids (211, 212). This way VO_2max could be part of a BA estimation which would increase the ability to predict risk of future age-related disease. It has been argued that VO_2max, or at least an assessment of physical activity habits, should be a part of any clinical examination conducted at the general practitioner (53). In addition, considering BA estimation as a tool in health promotion, it is beneficial to include VO_2max with the aim of motivating the general population to be more physically active.

In summary, the nine biomarkers are considered important manifestations of the biological age process that occurs at the physiological functional level as well as clinical important age-related variables. We found that within the reference group, estimation of BA using the nine biomarkers, took on a normal distribution and resulted in a larger age range in both ends of the age spectra compared to CA. Collectively these findings indicate that despite the reference group being largely free from disease and physical inactivity, some are considered biologically older. This confirms the complexity when trying to measure healthy aging and emphasize the integrative nature of BA where healthy lifestyle is only one component that influence the rate of aging and susceptibility to age related diseases.

The initial idea to develop a BA model useful for health enhancing interventions, steered us towards a composite model of phenotypic biomarkers of aging (clinical measures e.g. BP and HDL-C), excluding molecular biomarkers such as telomere length. Relative telomere length is one of the cellular hallmarks of aging (**Figure 2**). Aging is associated with a decreased telomere length and relative telomere length is related to risk of CVD and mortality (213). Telomeres are positioned at the end of chromosomes and protects the chromosome but shortens during each cell division. When telomeres reach a critical short length, the stability of the chromosome is reduced, and cellular senescence is induced (26). Thus, relative

telomere length has been recognized as an important biological age predictor (213). Since we had the capacity, it was interesting to examine the relationship between relative telomere length and BA as a kind of control measure. We found, however, no association between BA and relative telomere length (**Figure 22**). This indicates that biologically older individuals in the active healthy reference group did not exhibit increased molecular signs of aging, in line with Werner et al. showing that physical activity protects against telomere shortening from middle age (214).

Our model is limited by the omission of environmental factors and socioeconomic status affecting healthy aging. Facial aging could be considered an indicator of these risk factors as facial aging is influenced by sun exposure, smoking, social status, depression score, and marital status (215). In addition, Christensen et al. have suggested that facial aging should be used as a biomarker of aging due to the correlation between facial age, telomere length and survival in twin pairs ≥70 years (135). In line with Christensen et al. we found that biologically older individuals looked older than their biologically younger counterparts (**Figure 21**). In addition, we found that evaluation of facial aging did not depend on professional background which increases its use and feasibility. These results might even suggest that facial aging, a simple noninvasive measure, should be used instead of a composite measure of BA to identify high risk individuals. However, I question that being told, based on your facial appearance alone, that your risk of disease and potential premature mortality is high, is a beneficial way to communicate health risk. In addition, and unlike facial aging, BA estimation provides a framework to guide risk reduction behavior. Thus, facial aging is not suitable as a biomarker of aging either alone or in a composite BA model. Instead, the results emphasize that health care professionals can trust their "clinical visual view" in the meeting with patients.

Model assessment

PCA was used to detect the structural relationship between the nine biomarkers of aging and the 1stPC was used as a general aging factor to construct the equation for BA estimation. We found that the factor loadings of the nine biomarkers were different for women and men. The factor loadings explain how each biomarker contribute to the linear combination of the nine biomarkers. The largest discrepancies in factor loadings between women and men were found in the contribution from HDL and waist circumference. HDL contributes 15.8% in women but only 0.04% in men. This could be an indicator of the sexual dimorphism in lipid profile and body composition (216). During menopause, estrogen levels decrease causing women to have a more atherogenic risk factor profile. This include, among others, that HDL levels decrease and TC increases (217). Conversely, waist circumference contributes 24.1% in men but only 3.0% in women. Due to

the sex hormones, fat distribution varies between women and men starting in puberty. Thus, men have a relatively higher distribution of fat centrally which increases with increasing age. This also applies in the absence of weight gain (218) and potentially mediates the higher CVD incidence observed among men compared to women (219).

In the assessment of the BA model, the quality of the PCA fit and the quality of the BA regression fit is important. When using the 1stPC as the general aging factor it is relevant to report how much of the original information (variance) from the nine biomarkers is captured in this component alone. We found that the total variance explained by the 1stPC was 31% for women and 25% for men. This is similar to previous studies employing the 1stPC as the aging factor for BA estimation with variance varying from 23-42% in women (99, 110, 112, 125) and 20-37% in men (99, 110, 112, 115) (**Table 1**).

R^2 is a goodness of fit measure used to assess the quality of BA regression models derived from the 1stPC. A high correlation coefficient with age (r) or its squared counterpart, the coefficient of determination (R^2), is the most commonly used method to assess the quality of the BA models included in the review (employed in 79% of studies, **Table 1**). In general, a high R^2 is thought to be better than a low R^2 because a high R^2 indicates that the BA model explain a large proportion of the variation in health risk related to CA and that the data fit the predicted values well. Our BA model produced an unbiased R^2 value of 0.73 in women and 0.65 in men. To evaluate the magnitude of the R^2 values, one need to take into consideration how much of the variability is considered explainable. Explaining the interrelation between BA and CA is a complex matter and BA is hard to predict considering the interplay between genetics, environmental and lifestyle factors, and the considerable stochastic component (220). With that in mind, the R^2 value found in our study is considered good and, in any case, the same or better than previous BA models have exhibited (99, 102, 106, 110, 112). In some cases, a low R^2 value is not necessary a problematic result, and the regression model can still be used to compare groups as in the case of paper I (1TP: R^2=0.0003 and 2TP: R^2=0.0009) (**Figure 13)**. Thus, the statistically significant coefficients allow for assessment of the relationship between BAS and CA despite a low R^2 value. We found that given a one unit increase in CA, 1TP have a positive mean increase in BAS compared to 1TP exhibiting a negative mean increase in BAS.

R^2 cannot stand alone in the quality assessment of the BA model because it does not say anything about the prediction error expected when BA is estimated from the BA model. Thus, absolute error measurements should be included in the assessment of the model (221). Standard error of the estimate (SEE) indicates the size of error in the predicted BA value compared to the actual BA value. We found a SEE of 8.2 years in women and 10.2 years in men. In comparison with other studies exhibiting SEE <1 year our results are high (104, 115, 125). The regression model in these studies are based on repeated measures

why the number of datapoints used to fit the regression is high (>500). Thus, the high SEE observed in our study is explained by the low number of datapoints (n=51 women and n=49 in men). This is a first-generation model, and the quality of the model should be further investigated in a larger sample of healthy active adults to evaluate whether the linear combination of biomarkers represents an age-related mechanism applicable to a new dataset from a similar population (221).

BA and the clinical relevance

The clinical relevance of the BA model relies on its ability to discriminate between healthy and non-healthy individuals, its sensitivity towards health enhancing interventions, and its ability to estimate health risks.

Healthy vs. unhealthy

Within Ubberup women and men, we found that BA was scattered above the healthy aging trajectory. The limited sample size and age range (18-47 years) prohibited comparison of regression lines in men. However, the scatter distribution of BA and the parallel upward shift in the regression line for Ubberup women indicate that Ubberup participants are biologically older and in higher risk of future age-related diseases compared to their CA-related counterparts in the healthy aging reference group (**Figure 23**). This is in alignment with the increased risk of future CVD and T2D as indicated by the high HOMA-IR and BMI among Ubberup women and men at baseline (**Table 6**).

Lifestyle intervention and change in BA

With a mean reduction in BA of -4.1 years in women and -16.4 years in men, we demonstrate that the BA measure is sensitive towards lifestyle interventions resulting in a weight-loss of approximately 10% from baseline. However, despite a similar weight-loss in women and men, the impact on BA is 4-fold greater in men. This is partly related to the differences in factor loadings between women and men of the nine biomarkers. As previously discussed, the sexual dimorphism can explain why some biomarkers are more influential to the BA estimate (like HDL and waist circumference). Other discrepancies, however, are harder to explain from a physiological point of view. For example, VO_2max contributes 11.6% in the BA estimation for women and 22.6% in men. As a result, an improvement in VO_2max will have a greater effect on BA in men than women. Generally, low VO_2max is a strong predictor for mortality and CVD in both women and men (52, 53, 56). However, the same absolute mortality risk has been found in women and men despite women exhibiting a relative lower VO_2max (222). These results indicate that women tolerate lower VO_2max better than men, arguing for the difference in the VO_2max loading scores in the BA estimate for women and men. On the other hand, the age-related decline in VO_2max is expected to be similar between sexes (56).

Due to the strong evidence that VO_2max is the strongest predictor of age-related disease and premature death, it would be natural to rank VO_2max as the most important biomarker for both sexes. However, when it comes to BA estimation, researchers have not been able to identify one single biomarker specifically measuring the basic underlying processes of BA, VO_2max included (223). Instead, to capture the complexity of biological age, the composite measure is recommended and PCA is an objective method to combine relevant biomarkers of aging based on their covariance structure (68, 76).

Health risk estimation

We utilized that BMI is a well-established clinical risk predictor of CVD and T2D and applied BMI as a comparator to assess the predictive value of BA. We demonstrate a significant relationship between BA and BMI which can be interpreted as an improvement in BA of -1.5 years with one unit reduction in BA i.e., a weight loss. Because waist circumference is one of the biomarkers in the BA model, one can speculate if the relationship between BA and BMI is inflated by the strong correlation between waist circumference and BMI (224). However, a recent meta-analysis showed that waist circumference, independent of BMI, was strongly associated with the risk of T2D. We therefore argue that the positive correlation between BMI and BA can be interpreted as initial evidence that BA can predict risk of future disease.

Relative vs. absolute risk prediction

The concept of combining risk factors, or biomarkers, to identify individuals in relatively higher risk of CVD and T2D is not new. Metabolic syndrome and Framingham risk score are examples used by researchers and clinicians in health promotion. The BA model resembles the key features of metabolic syndrome including central obesity, insulin resistance, and dyslipidemia (225). Despite their similarities, BA fulfill some of the limitations inherent in the metabolic syndrome as pointed out by the WHO Expert consultation (226). One of the limitations is the dichotomization used when assessing metabolic syndrome (226). While cut-off criteria can increase the ease of use for clinicians, the risk related to e.g. cholesterol is continuous. In addition, no weighting of variables are provided for despite for example, FBG and SBP relates to higher risk of CVD compared to for example HDL (184, 226).

Like metabolic syndrome, BA estimation does not provide an absolute risk directed at a specific outcome as is the case with the Framingham risk score predicting the absolute risk of future CVD (227). An inherent issue with absolute risk prediction is, however, that young adults have low absolute risks despite a high relative risk (228). For example, the risk of future CVD incidence is not increased in the young adults (<40 years) at Ubberup Højskole using Framingham risk score, despite a metabolic high-risk profile (i.e. high relative risk). This is problematic as early prevention and health promotion is important for health behavior change and reduces the absolute risk of chronic disease at an older age. Instead, risk prediction among

younger adults with higher relative risk might better be assessed and guided by their biological age. Conversely, the omission of CRF in the assessment of future CVD increases the risk of overestimating the absolute risk of future CVD in older adults. Again, biological age might be a more useful way to assess health risk. However, validation of the BA model against incident CVD and T2D are important objectives for future studies to assess the predictive ability of BA estimation and hence the clinical utility.

Translational perspectives

Different BA models have been proposed and validated in both cross-sectional and longitudinal cohort studies (**Table 1**). In these studies, the applicability for use in health promotion has been indirectly evaluated focusing on the ability of the BA models to discriminate between people with low and high-risk profiles (e.g. individuals with hypertension, high alcohol consumption, smoking, BMI and metabolic syndrome). In addition, some of these studies assess how BA and socioeconomic status associate (107, 109, 118, 124) to provide evidence that BA also captures the socioeconomic health-related inequality (229).

To the best of my knowledge, we are the first to apply a BA model to a health enhancing intervention that directly benefits health promotion and found that BA can be used to evaluate health improvements related to weight loss. However, the feasibility for health personal to obtain the measurements in lifestyle interventions, health checks or similar health promoting interventions is debatable. Blood lipids and HbA1c can be measured by simple finger prick test on portable analyzers. MAP, FEV1, and the measure of waist circumference are simple measures which are easy to perform in clinical practice. Direct measurement of VO_2max is time demanding and requires technical equipment and knowledge on integrative physiology to obtain reliable measurements. Instead, indirect measures of VO_2max can be employed using submaximal test protocols or future technological aids. Study I clearly shows that a similar test battery including a submaximal test to obtain VO_2max measures is feasible to conduct on a large scale. Measurements of adiponectin and suPAR levels are more difficult to obtain without biochemical laboratory analysis available. With a focus on applicability to health enhancing interventions, future studies should investigate how exclusion of adiponectin and suPAR affects the ability of the BA model to identify high risk individuals and assess the effect of lifestyle change.

Strengths

A major strength of study I was that the database from the private health care company allowed us to examine workplace health promotion, including BA as a motivational tool as it occurs in practice. The large

sample size included employees from several different occupations and industries in a representable sample of the Danish workforce in the private sector.

In comparison with previous BA model studies, a major strength of study II was that the reference group represented a healthy aging group, not only free from known disease, but in addition fulfilling the criteria for daily physical activity. Thus, they represent a group largely free from inactivity as a confounding factor, without being categorized as athletes or highly active individuals. This increase the application as a base of comparison in public health promotion aiming to increase adherence to the minimum requirements of daily physical activity. Another strength was that the reference population included young adults making the BA model applicable for use in health promoting interventions targeting the primary working population. This is important because early prevention is needed to prevent disease from manifesting later on and because a substantial part of health promoting interventions are performed in the workplace.

Lifestyle interventions represents one of the secondary health promoting strategies that are conducted in society. Thus, a strength of study III was that BA was sensitive towards a realistic improvement in health among overweight and obese individuals. Collectively, the study designs advance the current knowledge of the applicability of BA estimation in primary and secondary health care initiatives.

Limitations

Despite the strengths of investigating BA as close as possible to real life scenarios, omission of controlled studies have the limitation that no causal conclusion can be drawn. In study I we have no information on other health enhancing or aggravating line of events between baseline and follow-up. Lost to follow up could be affected by leave of absence or shift in employment. Covariates like alcohol consumption and drug initiation, might confound the interpretation of changes in smoking habits and metabolic syndrome. Finally, the improvement in BA is susceptible to bias due to a learning effect when executing wall sit and push-ups.

A limitation of study II is that we measure the effects of aging in a cross-sectional design. Effects of aging are best examined using longitudinal data, but repeated measures are not as common as cross-sectional studies (**Table 1**). This is probably because of the need to obtain biomarkers of aging over decades to cover the adult human lifespan (74). However, the change in biomarkers observed with increased age in our study is a snapshot in time covering different birth cohorts. By chance, we could have recruited a sample of women during or done with menopause confounding the age-related changes in lipid profile. Also, the study population may represent a genetically sample free from heritable risk factors such as dyslipidemia. Thus, it is difficult to separate the age effect from cohort effects. Longitudinal stability of the biomarkers is

a priority in future assessment of the reliability and validity of the included biomarkers. Also, further testing of the BA model should be conducted in a representative validating sample of the Danish population.

Smoking is a major risk factor for chronic obstructive pulmonary disease, cancer, and CVD (230). Considering that FEV1 and cholesterol profile is part of the BA-model, in hindsight, it would have been appropriate to have smoking as an exclusion criterion. Observational studies have found that individuals who smoke ≥1 package a day had 9% lower HDL-C cholesterol levels compared to individuals who did not smoke (231). The HDL-C concentrations in the reference group were not reduced (<1.29 in women and <1.03 in men), if anything they were high (≥1.55 mmol/L) (225, 232). In addition, no clinical indications of obstructive lung disease was found in the FEV1/FVC ratio in the individuals who smoked comprising four individuals smoking ≤2 cigarette a day and two individuals smoking ≥10 cigarettes a day. The acute effects of smoking (increased plasma LDL-C and insulin concentrations and rise in blood pressure) were excluded as participants refrained from smoking at least 4 hours prior to the examinations (233).

Study III is limited by sample size especially concerning male participants prohibiting meaningful comparison of regression lines between Ubberup males and the reference group. Therefore, we have already planned to repeat the study in the spring and rerun the analysis on a larger sample size and a larger age range. Unfortunately, we were unable to retrieve the kits for analyzing adiponectin. Thus, imputed values of adiponectin was included in the BA estimation limiting the precision of the estimate in study III. We did not account for medication use when estimating BA. In cases of blood pressure and cholesterol lowering medication Levine et al. suggest replacing measured values of SBP and TC with 140 mmHG for SBP and 5 mmol/L for TC if SBP and TC were below these levels (234). Four Ubberup participants used blood pressure and/or cholesterol lowering medication but only one individual fulfilled the criteria for imputation. Recalculating BA with the imputed values increased the BA estimate with four years. This indicate that adjustment for medication use is relevant in future estimation of BA, especially going from controlled research environments to clinical practice.

Ethical consideration

I began this thesis by stating that aging per se is not a disease and that the age-related susceptibility for disease is better assessed by BA determined by the hallmarks of aging, genetic variability, environmental factors, lifestyle factors, and some level of stochasticity. In theory, if aging was a disease, a disease-free state should be achievable. Conversely, aging is an inherent and inevitable ongoing process occurring in all living organism, which is highly unlikely for any disease (5, 70). The association between BA and the risk of disease does, however, give the understanding that aging should be avoided or defeated. Importantly,

while the BA concept presented in this thesis concerns with risks related to lifestyle and adverse age-related changes in physiological function, not all age-related changes are connected to increased susceptibility to disease. Some age-related changes are positive such as acquisition of wisdom and without an adverse association to chronic disease such as greying of hair or baldness (75). Furthermore, mental health and cognitive function is equally important for healthy aging according to WHO (82).The proposition of BA as a tool in health promotion should therefore not be used as a scare campaign against increasing CA. Rather it should be clearly explained that it is the discrepancy between CA and BA, not BA in itself, which potentially influences the risk of early incidence of age-related disease.

The BA model presented in this thesis was developed as a tool to identify high risk individuals and a motivational tool applicable for health promotion interventions with voluntary participation. Introducing BA as a risk predictor could induce ethical dilemmas in different hypothetical scenarios. An important consideration is that BA could induce unequal opportunities and create a more polarized society of healthy versus non-healthy, as we have seen it with individuals who smoke and among individuals with obesity. This is a relevant consideration if BA estimates become available in journal records or used in insurance cases. On the other hand, BA estimation could also improve the opportunities for individuals otherwise discriminated by their CA. This could be the case for cancer treatment and the decision making for surgery or not in elderly patients (235, 236).

Conclusion

The overall objective of this thesis was to investigate the concept of biological age in a context of health promotion and disease prevention. We aimed to bridge the gap between unvalidated BA models applied in health enhancing interventions and BA models derived from the gerontology field. In real-life, third-party health care promoters are one of the main providers of public health promotion and user of the BA concept. We investigated the effectiveness of BA health checks in a large sample of the Danish workforce provided by a private health care company. Our results indicate that including BA estimation in general health checks, positively influence the motivation to participate. In addition, our results indicate that knowing the exact consequence of smoking behavior might increase motivation for smoking cessation. These findings speaks in favor of using BA estimation in health care as BA provides an intuitively meaningful outcome easily translated into risk of disease. Furthermore, we show that waist circumference, HbA1c, VO_2max, FEV1, Adiponectin, suPAR, MAP, HDL-C and TC in combination provides a BA model able to identify high risk individuals among young and older adults. The nine biomarkers of aging represent different important pathways in the pathophysiology of CVD and T2D occurring with age and aggravated by

obesity emphasizing the potential of a BA model with a focus on healthy aging. We demonstrate initial evidence that the BA model is useful as a measure to assess individual effects of change in lifestyle related to increased physical activity, healthy diet, and weight loss. The translation from science to health care is not completed with this first-generation model however, the results of this thesis indicate that BA estimation has potential to be more than a theoretical concept.

Appendix 1

This appendix contains the search strategy and the in- and exclusion criteria used to identify relevant studies. The flow chart of the study selection is also presented.

Search strategy

In August 2019, we performed a pilot search in PubMed. From this search, we retrieved 281 references that were screened for relevance. We used this pilot search to harvest keywords and subject headings from relevant articles. We developed a comprehensive search strategy in collaboration with an information-specialist from the Library at the Faculty of Health, at the University of Copenhagen. She provided advice on choice of databases and helped refine relevant keywords. She devised the initial search strategy and showed how to convert the search string to other databases.

We searched the electronic databases PubMed, Embase and Web of Science on 02.03.2020. We ran the final search strategy in PubMed (**A. Table 1**) and subsequently converted it to fit Embase and Web of Science. The search strategy combined both subject headings and free text terms for "Biological age", "Model" and "Health promotion". To remove studies indexed as animal studies we added the following NOT statement in PubMed and Embase: NOT ((exp animal /or nonhuman) NOT exp human/). In Web of Science NOT (SU=veterinary sciences) was applied.

Finally, searches were limited to English language studies. We checked records retrieved from the pilot search to ensure that relevant articles were included in the review. The number of references generated by PubMed and Embase were similar producing 1609 and 1657 records, respectively. From Web of Science 964 records were retrieved.

Based on the pilot search, the following inclusion criteria were selected:

Type of publication: journal articles

Time frame: any

Language: English

Population: adults, 18 years and above

Type of studies: aiming to improve general health promotion and/or primary and secondary prevention of the four main non-communicable diseases (cardiovascular disease, type 2 diabetes, chronic respiratory disease, and cancer).

Type of model: the biological age model can have a variety of names such as real age, fitness age, body age, or similar.

Based on the pilot search, we agreed to exclude records using the following criteria:

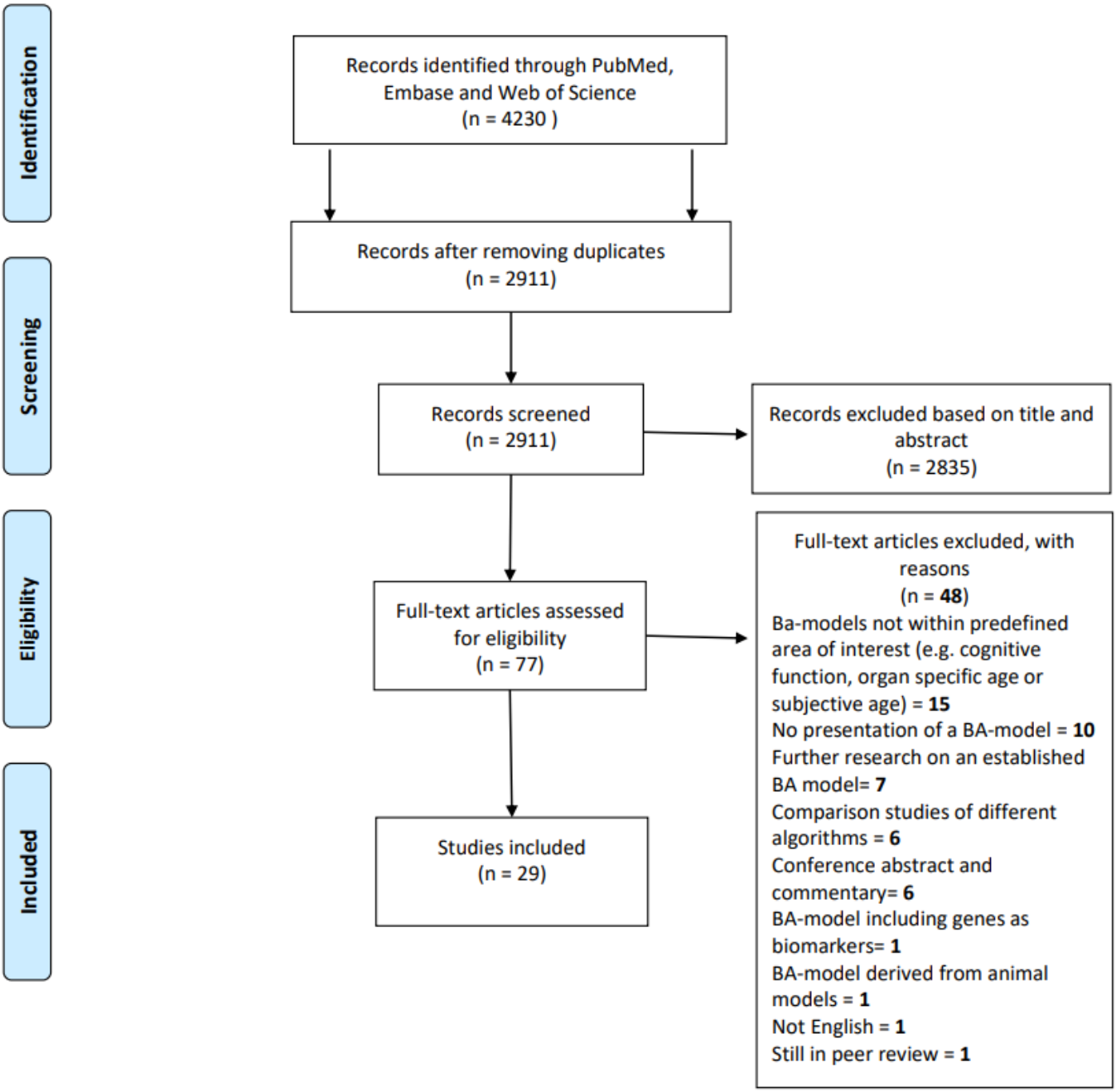
Type of publication: books, conference abstracts, commentaries, or editorials.
Type of studies: Studies aiming to validate variables as biomarkers without integrating them into a biological age model. Comparison studies of different mathematical algorithms.
Type of biomarkers: biological age models including DNA, telomere, or epigenetics.
Type of model: BA model on specific organ and models derived from animals and in vitro models.

Table 1 Search strategy in PubMed

Search date	Limits	Database	Search strategy (items found)
Searched 02/03/20	Limited to English language, and human studies.	MEDLINE/PubMed	1 Biological Variation, Individual [MeSH Terms] (207) 2 “Biological age*” (1303) 3 “Body age*” (14) 4 “Chronological age” (7006) 5 OR/1-4 (8150) 6 Model, Biological [MeSH Terms] (804089) 7 index (1021765) 8 model* (2225235) 9 estimat* (1110354) 10 protocol* (564266) 11 determination (8362529) 12 predict* (1599587) 13 “technology aided”(89) 14 OR/6-13 (11578856) 15 Health Promotion [MeSH Terms] (74890) 16 Preventive health Services [MeSH: noexp] (13231) 17 Primary Prevention [MeSH: noexp] (18055) 18 Health Status [MeSH Terms] (325859) 19 Health Behavior [MeSH Terms] (308913)

	20 health (4641010) 21 Risk reduction*(28919) 22 Lifestyle (163816) 23 OR/15-22 (4914636) 24 5 AND 14 AND 23 (1726) 25 24 NOT ((“animals” [MeSH Terms]/ NOT “humans”[MeSH Terms])) (1695) 26 limit 25 to (English language) (1609)

Figure 1. Flow chart of included studies



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

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Paper I

PLOS ONE

RESEARCH ARTICLE

The effectiveness of body age-based intervention in workplace health promotion: Results of a cohort study on 9851 Danish employees

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Abstract

Introduction

The aging population emphasize the need for effective health promotion interventions. The workplace is a prioritized setting for health promotion to reach widely within a population. Body age can be used as a health-risk estimate and as a motivational tool to change health behavior. In this study we investigate body age-based intervention including motivational interview and its effect on health, when applied to real life workplace health promotion.

Material and methods

Body age-based intervention was performed in 90 companies on 9851 Danish employees from 2011–2017. Metabolic risk factors were assessed, body age score was determined and an individualized motivational interview was conducted at baseline and follow-up. Change in body age score, single risk factors, smoking habits and metabolic syndrome were analyzed. The body age score is a composite score comprising 11 weighted variables. A body age score ≤ 0 is preferred, as this elicit a younger/healthier or equal body age compared to chronological age.

Results

At 1.3 year follow-up the unhealthiest employees were less likely to participate. Within follow-up participants (39%, n = 3843) body age had improved by a decline in mean body age score of -0.6 and -0.7 years for men and women, respectively ($p<0.001$). Number of employees with metabolic syndrome had decreased from 646 at baseline to 557 at follow-up ($p = 0.005$) and 42% of smokers had quit smoking ($p<0.001$).

design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusion

On the basis of this study, we suggest that body age assessment motivates to participate in workplace health promotion, affect high risk behavior such as smoking thus have potential in public health promotion.

Introduction

The macroeconomic implications of the aging workforce depends mainly on how long people continue to work [1]. In western countries legislated statutory retirement age is set to increase in line with the increase in life expectancy [2] thus, a healthy aging workforce is increasingly important. Unfortunately, a healthy aging workforce is challenged by inactive lifestyle and increasing prevalence of obesity and non-communicable diseases (e.g. cardiovascular disease and type 2 diabetes) [3, 4]. The workplace is a favorable setting to reach widely within a population because individuals with similar profiles, (e.g. lifestyle and socio-economic status) tend to cluster at different work-sites [5, 6]. A frequently used tool in workplace health promotion is health risk assessment (HRA) [7]. HRA produce a risk profile for individuals based on demographic, behavioral and biometric information. Motivational interview (MI), a client centered technique focusing on exploring and resolving ambivalence towards behavior change, has proven effective when it comes to change of health behavior [8]. It has been suggested that adding motivational interview (MI), will increase the effectiveness of HRA in workplace health promotion [9].

Recently, HRA used to produce individual biological age (also named body age and fitness age) as a health-risk estimate and motivational tool is increasingly applied [10, 11]. Heterogeneity in functional status and vulnerability to disease can be assessed by biological age. This heterogeneity is due to non-modifiable factors such as genetics and modifiable factors such as lifestyle [12, 13]. Biological age is commonly constructed from a number of modifiable risk factors assessed as reliable biomarkers of aging and proven valid as a health-risk estimate and prediction of mortality compared to chronological age [14–16]. Being older (or younger) than ones chronological age is easily translated into risk of disease and vigor and can be a motivation for healthy lifestyle. To our knowledge, only one study has been identified evaluating the effect of using body age estimation in workplace health promotion [11]. To adequately assess the potential of body age as a tool in workplace health promotion more research is needed.

Therefore, this study aims to evaluate the effectiveness of a commercially developed body age-based intervention (BAI) including MI in a large representative sample of the Danish workforce. The objectives are change in body age score and the associated changes in health behavior and single risk factors. In addition, we will include metabolic syndrome as an effect measure related to risk of future non-communicable diseases.

Material and methods

Study design

This is a retrospective database cohort study carried out by a Danish private health-care company in 90 Danish companies from January 2011 to February 2017. The companies were recruited from the Danish private health-care company's list of previous costumers. The 90 companies were primarily private representing 97% (41% Financial, 32% Energy, 5%

Transport, 5% Consultancy, 2% Food Sector Industry and 12% other), and public companies representing 3%.

The Danish private health-care company is the founder and provider of the BAI. The company had previously used the body age estimate developed by Polar (Polar Electro Inc., Kempele, Finland) [17]. Because the use of risk scores based on foreign population have been shown to produce poor results [18] the Danish company developed their own body age estimate on the basis of data from previous health screenings of 10.000 Danish employees. The BAI is designed to measure changes in variables associated with aging due to behavior and lifestyle.

The intervention

The BAI consisted of an individual 60–70 minutes session including a health screening, body age estimation and MI (comprising ≈20 minutes). The BAI was conducted at the workplace within working hours. The health screening included assessment of metabolic function (fasting blood glucose and cholesterol profile), cardiorespiratory function (blood pressure and maximal oxygen consumption (fitness level)), body composition (weight, height, waist circumference, fat percentage and fat-free mass) and physical performance (handgrip strength, upper arm strength, thigh strength and flexibility of the hamstrings). Immediately after the health screening a standardized report was given to the employees containing their body age. Employees where informed on how the test results had influenced their body age result. This report formed the basis for the MI.

The Danish private healthcare company visited the companies twice, providing the employees with the opportunity of a baseline and follow-up test. No other intervention beside the BAI including MI was provided. The Danish private health care company aimed to do follow-up tests within 1 year, however, this was essentially decided by the employers of the companies. The test was carried out by health care professionals (sports physiologists and physiotherapists) educated, trained and supervised in the body age protocol and in techniques of MI [19]. Participation was voluntary for the employees and financed by the employers, without incentives to participate or criteria's for participation.

Study population

In total 14073 employees received an invitation by email, and 9851 (70%) chose to participate. Of the 9851 employees the study population consist of 5878 (60%) employees who participated at baseline only (from here on referred to as 1 test participants (**1TP**) and 3843 (39%) who also participated at follow up (from here on referred to as 2 test participants (**2TP**). As this study investigated the beneficial effects on obesogenic related lifestyle, employees with a low BMI ($\leq$ 18.4) at baseline were excluded from the study population (n = 130 (1%)) (Fig 1).

Ethical approval

This study was approved by the University of Copenhagen Research Ethics Committee for Science and Health (504–0056/19–5000) and by the Data Protection Agency (SUND–2018–17). Data was retrieved from the database of the Danish private healthcare company fully anonymized, why consent was not collected from participants. Data is reported in accordance to the STROBE statement and follows the checklist included in reports of cohort studies [20].

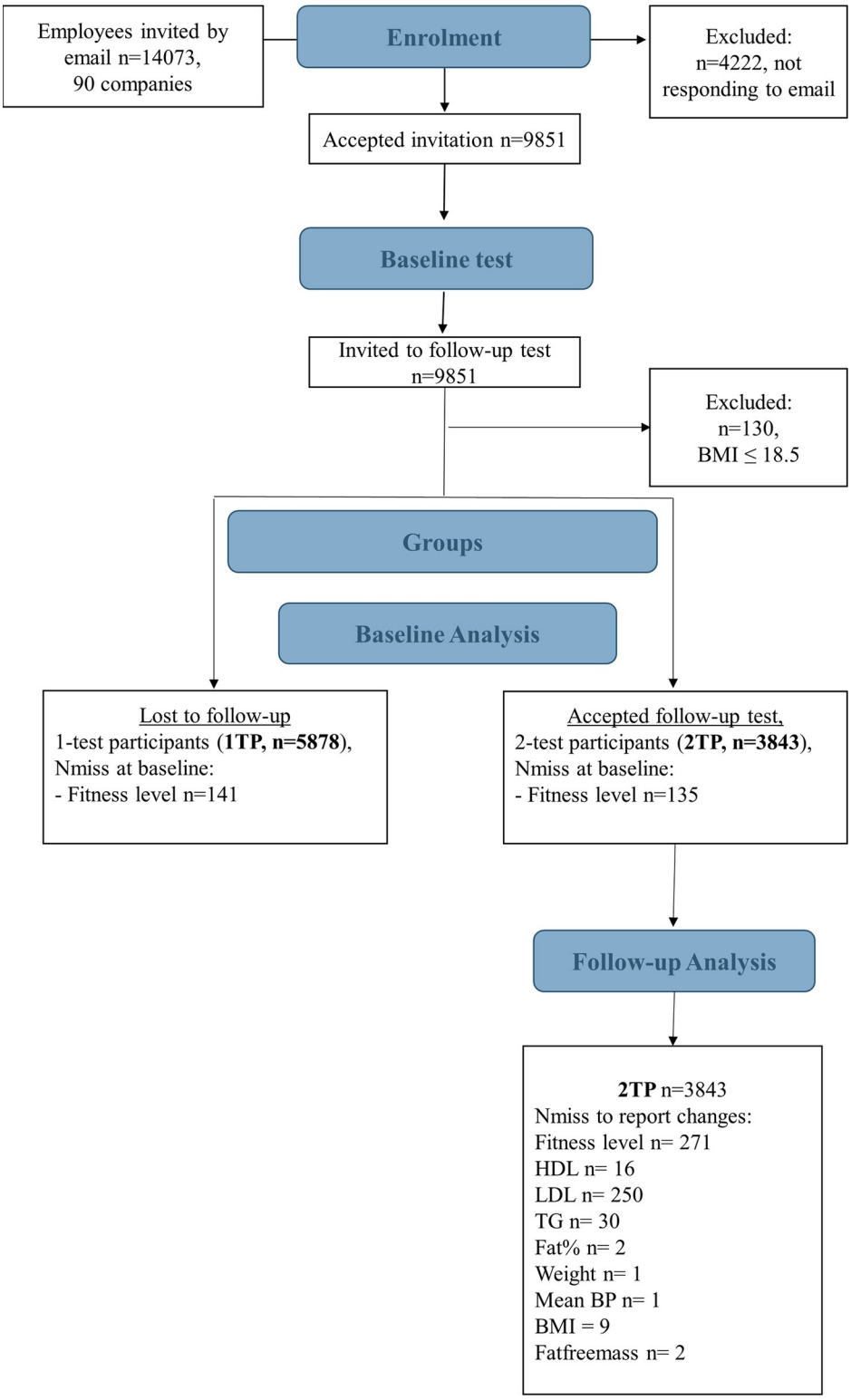

Fig 1. Flow chart of study population. *Nmiss* number of missing variables, *HDL* High density lipoprotein, *LDL* Low density lipoprotein, *TG* Triglycerides, *FAT%* body fat percent, *MeanBP* mean blood pressure, *BMI* Body mass index.

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Measurements and procedures

Employees were tested individually and were requested to fast minimum 3 hours before the test. Additionally, to standardize hydration level, employees where ask to drink 0.5 L of water 2 hours prior to the test. Blood from a finger prick test was used to measure glucose concentration (Accu-Check Aviva meter, Indianapolis, Indiana, USA), total cholesterol (TC), high-density lipoproteins (HDL), low-density lipoprotein (LDL) and triglycerides (TG) (Alere Cholestech LDX analyzer, Hayward, USA). With an alcohol swab the center of a finger was cleaned. When dry, a single-use lancet pricked the selected site. The finger was gently squeezed and the first drop of blood was wiped away as it may contain tissue fluid. Again, the finger is squeezed gently and 30μl is collected in a capillary tube, taking caution that the blood drop does not touch the skin. The capillary tube must be filled within 10 seconds. Secondly, a new blood drop is applied on the blood glucose meter test strip, and inserted in the meter. Blood pressure was measured at rest in the supine position using an automatic monitor (Boso-medicus control, Jungingen, Germany) and body composition was measured by bio impedance (TANITA -SC330 S, Tokyo, Japan). Waist circumference was measured between the 12^{th} costae and crista iliaca. Fitness level was assessed using a two-point submaximal cycle test ad modum Ekblom-Bak [21] on an electromagnetically braked cycle ergometer (Monark 828E, Vansbro, Sweden). Heart rate and workload was recorded at steady state after 6 and 10 min, respectively. Maximal workload (W_{max}) was extrapolated on the basis of theoretical maximal heart rate (220-age) and $\dot{V}O_{2\ peak}$ calculated based on the assumption of a cycling efficiency of 23%, an energy-oxygen equivalent of 21.1 kJ/L O_2 and a basal metabolic rate of 0.25 L O_2/min. Grip strength was measured with handheld dynamometer (Jamar J00105, Lafayette, USA) in a standing position with the arm by the side. Three attempts were allowed and the highest result registered. Leg endurance strength was assessed by wall-sit hold, performed with the back against a wall, and a 90˚ flexion of the hip and knee joint. Upper body strength was assessed via the maximal number of pushups. A valid pushup was defined by the chest touching a foam roller on the floor—men being on their toes, women being on their knees as starting position. A sit-and-reach test was used to assess flexibility. The participants sat on the floor with 90˚ flexion of the hips and with a straight back. The sit-and-reach bench (ACUFLEX I, Rockton, USA) was pushed against the feet and the participant reached forward pushing the reach indicator away, in a fluent movement. Finally, participants were asked about number of cigarettes smoked per day.

Determination of body age

Body age is the sum of chronological age and the body age score:

$$Body\ age = Chronological\ age + BAscore.$$

The body age score is a composite score comprising 11 weighted variables assessed in the health screening. The variables included are: fitness level, fat%, total cholesterol, blood glucose concentration, mean blood pressure, waist circumference, handgrip strength, push up, wall sit, sit and reach, and smoking habits. A body age score ≤ 0 is preferred, as this elicit a younger/healthier or equal body age compared to chronological age. The body age score was determined using the following algorithm:

$$BAscore = \sum_{i=1}^{N} \Delta_i^V \times W_i^V + \Delta^{BG} + \Delta^{SH}$$

Where Δ_i^V is the age value given for each of the 9 variables (not including blood glucose concentration and smoking habits), the W_i^V is the corresponding weight. The age value depends on how the corresponding test results varies in statistical data of age and sex-related peers. National recommendations of variables relationship with health and risk of disease was used to determine the weighting (Table 1).

N is the total number of variables applied in the algorithm (11 variables) and *i* indicate the specific variable (e.g. fitness level). Parameters Δ^{BG} and Δ^{SH} represent the age value given based on blood glucose (BG) concentration and smoking habits (SH). A blood glucose concentration >6.1 mmol/L results in an age value of +4 years, and smoking attributes an age value in accordance to the number of cigarettes smoked per day (CPD): 1–10 CPD attributes +4 years, >10 CPD attributes +8 years and >15 CPD attributes +10 years. Summation of the 11 age values produce the overall body age score.

Statistics and data analysis

A merged dataset from the 6-year data collection was extracted by the IT department of the Danish private healthcare company and cleaned before analysis. Normal distribution was checked using q-q plots and histograms, variance homogeneity was checked by plotting residuals versus predicted values. Descriptive statistics at baseline were presented as medians with IQR. Baseline comparison of 1TP and 2TP was analyzed using linear regression for continuous variables and logistic regression for categorical variables. Changes in outcomes between baseline and follow-up were compared using the *proc mixed* procedure of each outcome on follow-up adjusted for age at baseline and follow-up time. Thus, interpretation of results are how much more the outcome has changed per year beside the average age development. Residuals for the analysis where checked for normal distribution to ensure that the underlying assumptions of the statistical model were met. When normal distribution did not fit the model log transformation was used successfully. Linearity of covariates were checked by visual inspection of residuals plots against covariates, if linearity assumption was not met, covariates was modelled using splines. McNemar's test was used to analyze changes in smoking habits and Chi-square test was used to test for independency of sex. All statistical analyses were done in SAS Enterprise Guide 7.1. Statistical significance was considered at $p<0.05$ in all comparisons. Metabolic syndrome was assessed using the definition set out by the International Diabetes Federation [22].

Table 1. Weighting of variables.

Variable	$W_i^{V\,a}$
Fitness level	31.1%
Fat percent	17.8%
Total cholesterol	13.4%
Mean blood pressure	13.4%
Waist circumference	6.7%
Handgrip strength	4.4%
Push up	4.4%
Wall sit	4.4%
Sit and reach	4.4%
TOTAL	100%

The weight of each variable relative to its estimated importance of health and risk of disease [a] W_i^V = the weight in percent assigned to each variable.

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Results

Based on the email invitation 70% (n = 9851) accepted and participated in the BAI at baseline. The study population was 41.3 years on average (range 18–70 years) and 63% where men. Overall 57% were normal weight (BMI 18.5–24.9) and 9% were smokers.

1TP versus 2TP baseline characteristics

Table 2 shows the baseline characteristics of the 11 variables included in the body age model by groups and Fig 2 shows the related body age scores as a function of age.

Fig 2 visualize that 1TP are associated with positive body age scores and 2TP are associated with negative body age scores, indicative of an unhealthier profile for employees lost to follow-up (1TP). With increasing age this association becomes more pronounced *($p<0.001$)*. This is reflected in a lower body age for 2TP compared to 1TP *($p<0.001$)* (Table 2).

The majority of variables included in the body age model were significantly different between groups, except for total cholesterol and blood glucose concentration (Table 2). BMI, mean blood pressure, body fat% and waist circumference were lower for 2TP and this was concurrent with a lower prevalence of smokers. Baseline fitness level and strength related measures were higher in 2TP, with no difference in flexibility between the groups. These differences remained significant after adjusting for age (Table 2). The proportion of participants with obesity (BMI ≥30) were higher in 1TP compared to 2TP (p< 0.0001) with no

Table 2. Baseline characteristics.

	Groups		P^a	P^b adjusted
	1TP	2TP		
	Median (IQR)	Median (IQR)		
Women, n *(%)*	2182 (37.1)	1430 (37.2)	0.93	0.89
Chronological age, *years*	41 (33; 49)	42 (35; 48)	0.0006	-
Body age, *years*[c]	41.3 (32.7; 50.4)*	40.8 (33.4; 48.2)*	<0.001	-
BMI, kg/m^2	24.5 (22.4; 27.1)	24.2 (22.2; 26.3)	<0.001	<0.001
Current Smoker, *(%)*	631 (11%)	245 (6%)	<0.001	<0.001
Mean blood pressure, *mmHg*	105 (98.5; 113)	104 (98; 110)	<0.001	<0.001
Total Cholesterol, *mmol/L*	5.0 (4.3; 5.6)	4.9 (4.4; 5.6)	0.9	0.3
Blood glucose, *mmol/L*	5.0 (4.7; 5.4)	5.0 (4.7; 5.4)	0.4	0.2
Body Fat %	22.9 (17.8; 29.4)	21.9 (17.4; 28.2)	<0.001	<0.001
Waist circumference, *cm*	88 (80; 97)	87 (80; 94)	<0.001	<0.001
Fitness level, *ml/min/kg*[c]	37 (31; 44)	40 (34; 47)	<0.001	<0.001
Push Ups, *No. of*	25 (16; 32)	25 (18; 32)	0.02	0.0002
Wall sit, *min*	1.6 (1.1; 2.1)	1.7 (1.3; 2.2)	<0.001	<0.001
Handgrip, *kg*	47 (34; 56)	48 (35; 56)	0.04	0.03
Sit and Reach, *cm*	35 (28; 40)	34 (29; 40)	0.4	0.2

Comparison of baseline characteristics for 1-test participants (1TP, *n = 5878*) and 2-test participants (2TP, *n = 3843*). Continuous data are represented as medians with interquartile range (IQR); categorical data as absolute and relative frequencies. Body mass index (BMI), mean blood pressure, total cholesterol, blood glucose, body fat% and waist circumference were log transformed prior to analysis.

[a]P value using regression analysis and logistic regression.

[b]P value adjusted for age.

[c]Missing values were observed for fitness level and body age (due to missing fitness level data) why comparison of 1TP and 2TP is between n = 5737 and n = 3708, respectively.

*Significant different from chronological age p<0.001 (paired t-test).

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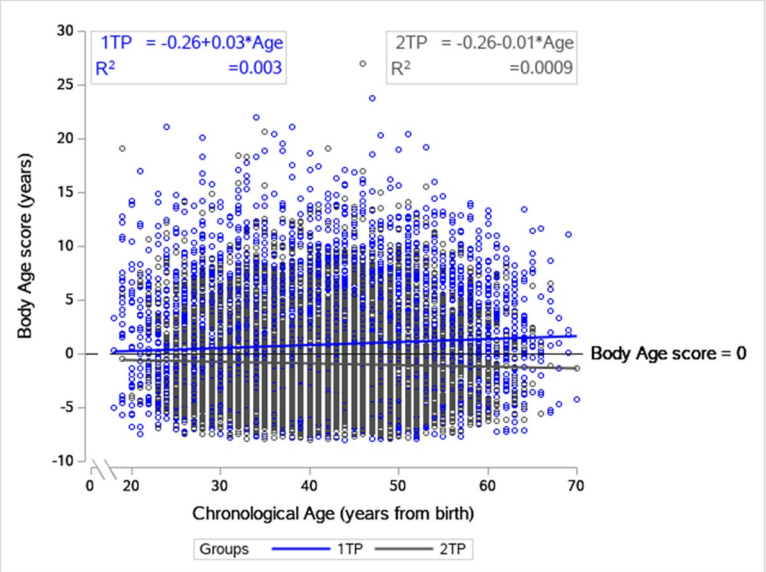

Fig 2. Fitted regression of body age score as a function of chronological age at baseline. The figure show that there is an association between having a positive body age score, and thereby a higher risk, and not participating at follow-up. The blue line represents body age score for 1 test participants (1TP) and the grey line represents body age score for 2 test participants (2TP). The black line is a reference line for body age score of zero that is, no difference between chronological and body age.

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differences in the proportion of normal weight (BMI 18.5–24.9) and overweight (BMI 25–29.9) participants.

Change in body age and metabolic syndrome

The median follow up time for 2TP was 1.3 years (IQR 1.0; 2.1 years; min: 0.02 years, max: 5.6 years). Fig 3 shows the body age score at baseline and follow-up for men and women.

Body age improved for both men and women as the mean body age score decreased with -0.6 years (95% CI -0.7; -0.5) and -0.7 years (95% CI -0.8; -0.5), respectively ($p<0.001$) (Fig 3). Number of employees with metabolic syndrome had decreased from 646 at baseline to 557 at follow-up (p = 0.005). Fig 4 shows the changes per year beside the average age development in single variables included in the health screening.

After adjusting for age at baseline and variation in follow-up time we found a small but significant positive change in cholesterol for women with and increased HDL (0.02 mmol/l) and decreased LDL (-0.06 mmol/l) concentration. BMI was lower at follow up, together with weight, body fat percent and waist circumference. The same pattern was seen in men. Only women increased their fitness level (0.25 ml/min/kg), but both men and women increased number of push-ups, wall sit time and flexibility indicative of higher physical capacity. No changes in blood pressure or glucose concentration was found in male or female employees.

Change in smoking habits

At baseline 245 employees smoked in the 2TP group (Table 2). At follow-up, 61% of these had quit/or reduced the number of cigarettes smoked per day (n = 149, no sex differences ($p = 0.73$)), quitters representing 42% (n = 103, no sex differences ($p = 0.98$)). 21% of smokers (n = 52) increased their cigarette smoking and 18% (n = 43) reported no change. About 0.6%

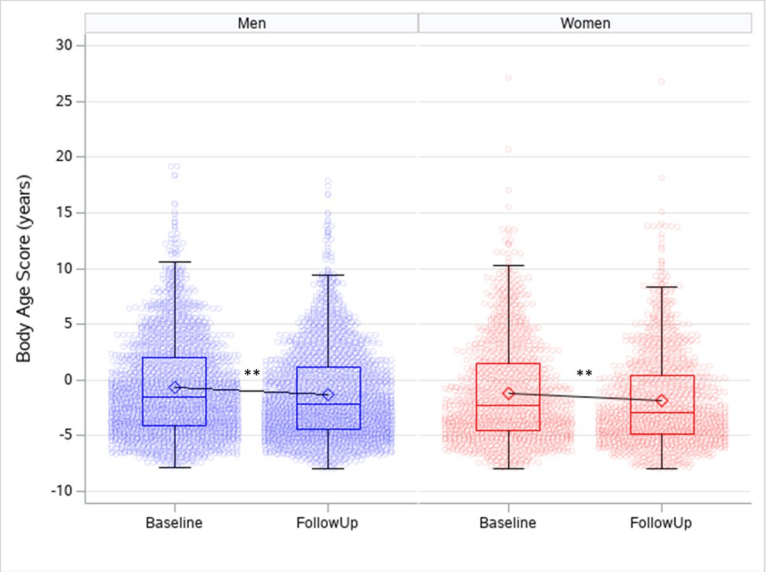

Fig 3. Body age score observed at baseline and follow-up by sex. Men (blue, n = 2251) and women (red, n = 1321). Only those with a body age score comprising all 11 parameters at baseline and follow-up are included in the analysis. P-value using paired t-test. $^{**}p<0.001$.

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of nonsmokers at baseline had initiated smoking at follow-up (n = 20, no sex differences ($p = 0.84$)).

Discussion

The results of this study show an overall positive effect of using BAI in workplace health promotion. Body age as health-risk estimate improved due to an overall improvement in metabolic risk factors (Fig 4) and an impressive smoking cessation rate of 42%. As visualized in the forest plot in Fig 4 the effect sizes in single metabolic risk factors were small to moderate and the effect on risk of future morbidity could be questioned. This is comparable with effect sizes found in a review on the effect of HRA in workplace health promotion [7] and the reason for the skepticism of using HRA in primary prevention in general [23]. Nevertheless, the changes in metabolic risk factors were associated with a decrease in the number of employees with metabolic syndrome, commonly used in the clinic to assess individuals with high risk of future cardiovascular disease and type 2 diabetes [24, 25]. Furthermore, small effect sizes have been found to independently affect the risk of non-communicable diseases. Data from the Framingham Offspring Study suggest that 0.055 mmol/L increase in HDL relates to a 2–3% decrease in risk of CVD [26], which is somewhat similar to the effect sizes seen in our study.

Reducing the proportion of employees who smoke, substantially reduce absenteeism from work as well as the duration of absenteeism [27]. Our finding that 42% had quit smoking is high compared to previous results from workplace health promotion interventions showing cessation rates of 20.3% and 8.1% [7, 28] but lower compared to workplace interventions solely focusing on smoking prevention (53.2%) [29]. The unique combination of MI and the awareness of the influence smoking have on body age might be important determinants when changing smoking behavior. Studies on anti-smoking interventions have shown that MI outperforms traditional advice [30, 31]. On the other hand, the documented effect of body age as a motivational tool is sparse. A cluster randomized study (n = 121) of healthy workers found that body age assessment did not increase encouragement and motivation for changes in

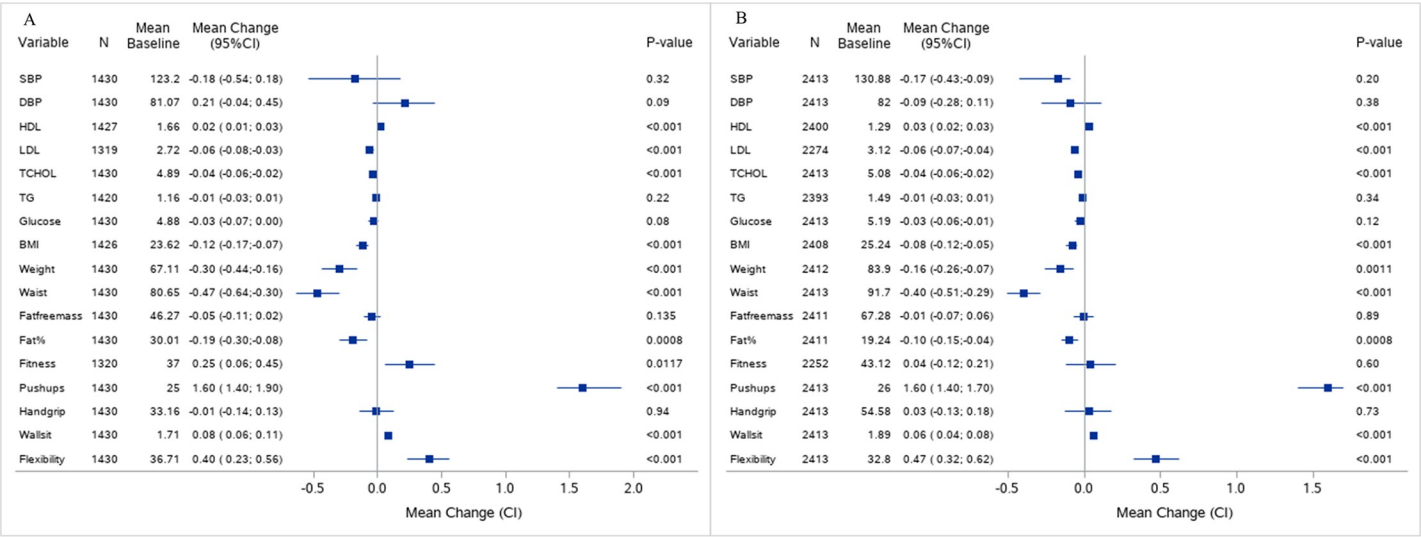

Fig 4. Changes in single variables per year beside the average age development. Baseline values and changes (mean, 95% CI) observed at follow-up adjusted for age at baseline and follow-up time by sex: A = women and B = men. N is the sample size used for calculation of the mean difference. A visualization of the effect size is provided for in the Forest plot; squares representing mean change with 95% confidence intervals. *P value using mixed model adjusted for age at baseline and variation in follow-up time. SBP = systolic blood pressure (mmHg), DBP = diastolic blood pressure (mmHg), HDL = high density lipoprotein (mmol/l), LDL = low density lipoprotein (mmol/l), TCHOL = total cholesterol (mmol/l), TG = triglycerides (mmol/l), Glucose = fasting glucose (mmol/l); BMI = body mass index (kg/m^2), weight (kg), waist = circumference (cm), fat free mass (kg), fitness level (ml/min/kg), push up = number of, handgrip strength (kg), wall sit time (min) and flexibility = sit and reach test (cm).

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health behavior [11]. In contrast a randomized study on heart patients (n = 660), found that patients aware of the net-value (change in body age) were more likely to choose certain health-risk behavior to change if the change resulted in a high reduction in body age [10]. Our finding on smoking cessation could have been affected by secular trends and not the intervention per se. Following the new Danish law against smoking at public- and workplaces in 2007, 320.00 Danes quit smoking. However, from 2011 to 2016 the amount of smokers was stable at 21–23% comprising almost the entire sample period [32].

Comparison of 1- and 2TP at baseline suggest that 1TP are less healthy (Table 2). Lower participation rate among the unhealthiest employees is a known issue in workplace health promotion [33]. Fig 2 also indicate that employees with the highest body age score are less likely to participate at follow-up especially within the oldest part of the study population. However, we acknowledge that the highly significant differences between groups in BMI, mean blood pressure, waist circumference and body fat percentage (Table 2) could be ascribed to the large sample size, and it can be questioned whether the differences between the groups have clinical relevance thus, if the two groups differ from a health risk perspective. On the other hand, smoking increase the risk of premature death mediated through cardiovascular- and lung diseases [34]. Thus, a higher prevalence of smokers in the 1TP group will increase their risk of future lifestyle related diseases. Also, as smoking has great impact on body age we speculate that reluctance to change smoking behavior influenced the motivation to attend at follow-up. Another strong predictor for cardiovascular disease and premature mortality is fitness level [35]. We found a median difference between the groups of 3 ml/kg/min (Table 2). Studies have shown that an increase of 1MET (approximately 3.5 ml/kg/min) in fitness level was associated with 21% lower risk of future CVD [36] and that a 1 ml/kg/min increase in fitness level was equivalent to a CVD risk reduction of 10% and 9% in women and men respectively [37].

Collectively, these results indicate that 1TP have higher risk of future lifestyle related diseases despite the close values of medians at baseline between the groups.

Other limitations include that body age as a health risk-estimate has not been tested for reliability, although it includes separate measurements that have been used and tested. Wall sit-hold and push up are susceptible for bias due to a learning effect, thus the improvement in body age seen at follow-up could partly be due to this. The study design does not allow for causal conclusions and we do not know if other health promotion activities have been conducted by the companies within the follow-up time. No information on drug use was given, and some of the changes we found could be assigned to drug initiation. Smoking habits were self-reported which might inflate the result regarding reduced CPD. Finally, it is worth noting that factors like alcohol intake, education level, socioeconomic status and marital status could confound the interpretation of change in smoking habits and metabolic syndrome. The strengths of this study are the performance in a real-life setting, in a large sample of the Danish workforce using a novel approach to worksite health promotion. This improves the generalizability that formal RCT-like designs struggles with. Our study shows that in a real-life setting 70% of the invited employees wanted to participate in at least 1 health risk assessment. High participation rate is a crucial part of successful health promotion and the result implies that body age assessments attracts employees across work fields and across a wide health profile spectrum ('2). In comparison the Inter99 study, a large Danish population-based randomized longitudinal study, using individual health risk assessment plus individual and group-based counselling as intervention, had a baseline participation rate of 52.4% [38]. The Inter99 study was conducted in the participants' spare time whereas our study was conducted during working hours, which have been shown to be important determinants for participation rate [39]. However, we are aware that the participation rate observed at baseline attenuates through follow-up. Effort to reduce lost to follow-up is a recurring issue in health promotion in general, why future research on this matter should be highly prioritized.

Conclusion

In this study we investigated BAI as workplace health promotion in a large representative sample of the Danish workforce. The effectiveness on single metabolic risk factors were small and due to the study design difficult to translate into effect on risk of future disease. Even so, using BAI including MI was associated with a decrease in the proportion of employees with metabolic syndrome and a surprisingly high smoking cessation rate of 42%. This could indicate that body age as health-risk estimate makes it easy to understand to what extent health behavior affects vigor and risk of disease and thus has potential as motivational tool in health promotion. This study demonstrate that BAI including MI is feasible on a large scale as workplace health promotion, but further research should be aimed towards a) validating body age towards morbidity and b) comparing BAI with standard HRA in workplace health promotion and c) qualitatively assessing body age as motivational tool in order to recommend BAI as a tool in health promotion.

Supporting information

S1 Checklist. STROBE statement—Checklist of items that should be included in reports of observational studies.
(DOC)

Acknowledgments

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Writing – original draft: Karina L. S. Husted.

Writing – review & editing: Sune Dandanell, Janne Petersen, Flemming Dela, Jørn W. Helge.

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Paper II

Protocol

A Biological Age Model Designed for Health Promotion Interventions: Protocol for an Interdisciplinary Study for Model Development

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Abstract

Background: Actions to improve healthy aging and delay morbidity are crucial, given the global aging population. We believe that biological age estimation can help promote the health of the general population. Biological age reflects the heterogeneity in functional status and vulnerability to disease that chronological age cannot. Thus, biological age assessment is a tool that provides an intuitively meaningful outcome for the general population, and as such, facilitates our understanding of the extent to which lifestyle can increase health span.

Objective: This interdisciplinary study intends to develop a biological age model and explore its usefulness.

Methods: The model development comprised three consecutive phases: (1) conducting a cross-sectional study to gather candidate biomarkers from 100 individuals representing normal healthy aging people (the derivation cohort); (2) estimating the biological age using principal component analysis; and (3) testing the clinical use of the model in a validation cohort of overweight adults attending a lifestyle intervention course.

Results: We completed the data collection and analysis of the cross-sectional study, and the initial results of the principal component analysis are ready. Interpretation and refinement of the model is ongoing. Recruitment to the validation cohort is forthcoming. We expect the results to be published by December 2021.

Conclusions: We expect the biological age model to be a useful indicator of disease risk and metabolic risk, and further research should focus on validating the model on a larger scale.

Trial Registration: ClinicalTrials.gov NCT03680768, https://clinicaltrials.gov/ct2/show/NCT03680768 (Phase 1 study); NCT04279366 https://clinicaltrials.gov/ct2/show/NCT04279366 (Phase 3 study).

International Registered Report Identifier (IRRID): DERR1-10.2196/19209

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KEYWORDS

biological age; health promotion; protocol; healthy aging; principal component analysis

Introduction

Healthy aging is of paramount importance when considering the trajectory of future aging populations [1,2]. Healthy aging refers to a healthy aging phenotype constituting a course of aging with high autonomy, no major chronic diseases, high quality of life, and an extended health span [3,4]. Following a healthy lifestyle earlier in life (eg, consuming alcohol moderately, not smoking, maintaining a healthy diet, and conducting regular physical activity) improves the chances of healthy aging [5,6]. Unfortunately, the steady increase in the prevalence of overweight and obesity in parallel with insufficient physical activity threatens healthy aging and emphasizes the need for effective health promotion of the general population [7-9].

Development of health literacy is a key element to promote a healthy lifestyle in the general population [10]. Applying various forms of health screenings, such as health risk assessment and health checks, is one way to track health status and thereby enable people to make qualified health decisions before diseases are manifested or progress. However, while knowledge is an important factor, it may not, by itself, motivate a change in lifestyle behavior. Health screenings often include measurements of well-established risk factors such as blood cholesterol, fasting blood glucose, and waist circumference. Although some people can understand the risk connected with these risk factors, they may be unaware of the extent to which their lifestyle affects their capability of maintaining youthful vigor and delaying morbidity to an older age. Such awareness might be pivotal and motivate changes in health behavior. Biological age plays a key role in this respect. We suggest that being “older” than stated on one’s birth certificate readily translates into disease and mortality risks, and is thus effective as health literacy to improve people’s lifestyles. In addition, we propose that biological age can be used as an outcome measure to quantify the overall placement of an individual on the healthy aging trajectory and their susceptibility to disease, which are useful in the context of primary and secondary health promotion interventions.

Unlike chronological age, biological age assesses the heterogeneity in functional metabolic status and vulnerability to disease. The increase in chronological age is uniform, whereas biological age can increase more rapidly for some and slower for others. This is due to nonmodifiable factors, such as genetics, and modifiable factors, such as lifestyle (smoking, diet, physical activity, etc) [11,12]. Biological age has been studied since the 1960s [13,14]. Much research has been directed toward finding the best biomarkers of aging [15,16] as well as the optimal method to estimate biological age [17,18]. Studies have shown that biological age can predict mortality better than chronological age and incidence of age-related diseases such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) [18,19]. These results were obtained from large cross-sectional data and were derived statistically. Moreover, these studies rarely investigated the clinical use of the model in health promotion interventions.

This study aims to develop a biological age model that can distinguish between healthy and unhealthy aging among individuals with the same chronological age and sex, and investigate its clinical applicability. We apply acknowledged mathematical methodology to estimate biological age; use a combination of commonly used biological age modelling biomarkers that are minimally invasive, represent healthy aging [20], and denote the processes that change with age [15]; and explore their usefulness.

Methods

Overview

When developing a biological age model, it is optimal to combine knowledge of integrative physiology and health technology. Thus, our approach is interdisciplinary and involves expertise in human physiology, healthy aging, prediction modeling, and human data science.

This study protocol comprises three consecutive phases: (1) conducting a cross-sectional study to gather indicators from 100 individuals representing normal healthy aging (the derivation cohort); (2) defining a novel biological age model and estimating biological age using principal component analysis; and (3) investigating the clinical use of the model in a validation cohort of overweight adults attending a lifestyle intervention course.

Phase 1: Derivation Cohort

Study Design

We recruited 100 healthy individuals equally distributed in sex and evenly spread out within the age range of 18-65 years. It is difficult to distinguish normal aging from pathological aging because physiological and functional decrements (or pathological changes) at the outset of a disease occurs as part of the normal aging process. Considering this, we excluded individuals with a history of previous or current CVD, and using medicine to reduce blood pressure, cholesterol, or glucose levels. Pregnancy is marked by physiological dynamics and is very different from the nonpregnant state (eg, the blood volume increases in the former) [21]. Thus, pregnant women or women who breastfeed were excluded from participation. In addition, people with conditions that would prevent them from enduring the cycle exercise and strength tests (eg, knee osteoarthritis) were also excluded. The study was approved by the Regional Ethics Committee, Copenhagen, Denmark (H-18031350) and was performed in accordance with the Helsinki Declaration. The study was recorded as a clinical trial (NCT03680768).

Candidate Biological Age Model Biomarkers

Eligible women and men arrived at the laboratory for a 2-hour examination after fasting overnight and abstaining from vigorous exercise in the prior 24 hours. The examination involved measuring 50 parameters to assess the health of the participants and collecting candidate biomarkers for the biological age model. Thus, the examination included measures of anthropometrics, physiological and metabolic health, and physical capacity as well as answering quality of life and daily physical activity questionnaires.

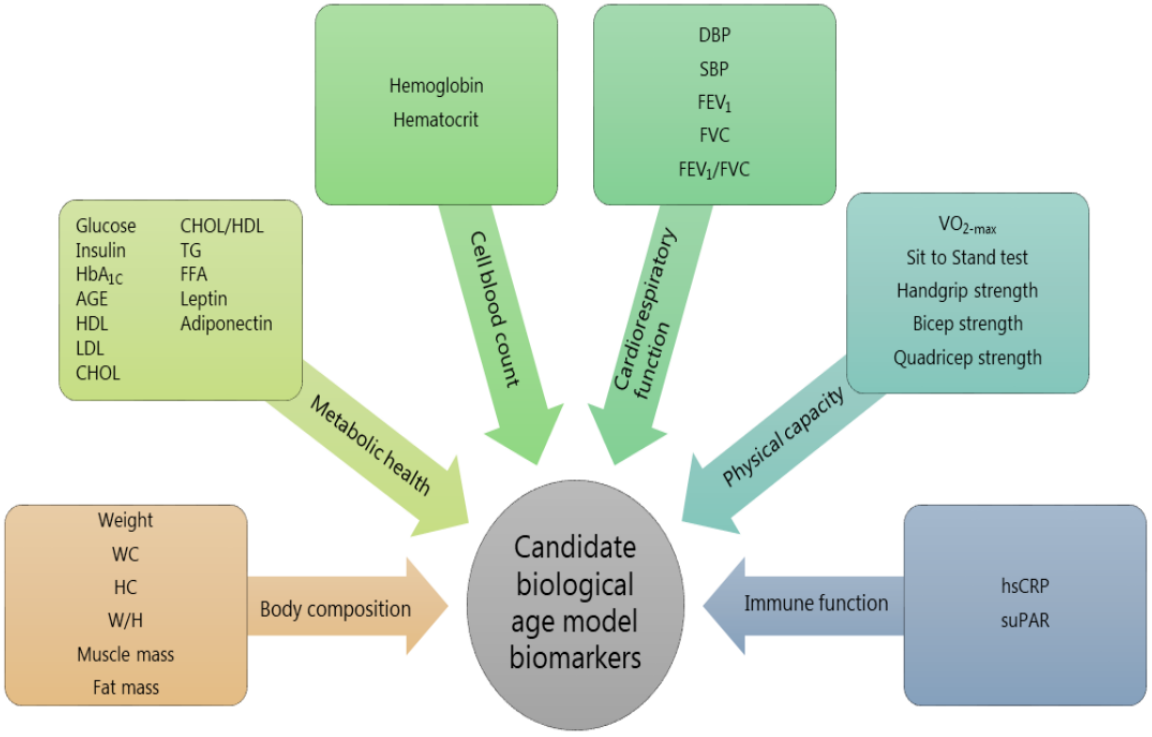
When selecting candidate biomarkers for the biological age model, we focused on variables that (1) characterize features

of the healthy aging phenotype [20], (2) are associated with aging and age-related diseases, (3) are affected by lifestyle, and (4) are possible to obtain in a variety of settings (ie, that are not limited to use in a research setting).

Due to the mathematical approach used to estimate biological age, binary/discrete variables (eg, quality of life and education level) were not considered for the biological age model although we recognize that some of these variables are important for assessing social and mental wellbeing in the healthy aging phenotype [3].

In total, 32 variables were selected as candidate biomarkers and categorized in the following 6 domains: (1) body composition, (2) metabolic health, (3) cell blood count, (4) cardiorespiratory function, (5) physical capacity, and (6) immune function (Figure 1).

Figure 1. Candidate biomarkers proposed for the BA model. Each square represents 1 of the 6 following domains: (1) Body composition, (2) metabolic health, (3) cell blood count, (4) cardiorespiratory function, (5) physical capacity, and (6) immune function. The candidate biomarkers for the BA model are listed within each domain. AGE: advanced glycation end products; CHOL: Total cholesterol; CHOL/HDL: HDL to CHOL ratio; DBP: Diastolic blood pressure; FEV_1: Forced expiratory volume within 1 second; FEV_1/FVC: FEV_1-FVC ratio; FFA: Free fatty acids; FVC: Forced vital capacity; Hb_{A1c}: Glycated hemoglobin; HC: Hip circumference; HDL: High-density lipoprotein; hsCRP: High-sensitive C-reactive protein; LDL: Low-density lipoprotein; SBP: Systolic blood pressure; TG: Triglycerides; suPAR: soluble urokinase Plasminogen Activator Receptor; VO_{2max}: Maximal oxygen uptake; WC: Waist circumference; W/H: waist to hip ratio.



Relevance of Domains

In this section, we outline the variables included as possible biomarkers for the biological age model. We describe the variables and explain their relevance in a model that assesses healthy aging.

Body Composition

Aging is associated with loss of muscle mass and strength (sarcopenia) and an increase in fat mass and central adiposity. Muscle mass has been reported to begin showing a negative association with age as early as 27 years [22], with the decline in strength exceeding that in muscle mass [23]. This characteristic is related to loss in muscle quality, gradual muscle denervation, loss of type 2 muscle fibers, reduced muscle capillary density, reduced oxidative capacity, and fat infiltration [24,25].

Excess fat mass, and especially fat distribution, are important risk factors for the development of CVD and T2DM. Waist circumference and hip to waist ratio are used as surrogate measures for central adiposity and visceral adipose tissue [26,27].

Metabolic Health

Aging and unhealthy lifestyle are associated with reduced glucose homeostasis [28]. Fasting blood glucose concentration, HbA_{1c}, and insulin sensitivity are markers of glucose homeostasis and are associated with incidence of CVD, T2DM, and mortality [20]. The prevalence of metabolic syndrome (a cluster of risk factors for T2DM and CVD) increases with age [29,30]. According to the International Diabetes Federation, the risk factors of metabolic syndrome are central obesity and any two of the following: raised triglyceride concentrations, reduced

high-density lipoprotein concentrations, raised blood pressure, and raised fasting plasma glucose concentration [31]. The increase in metabolic syndrome prevalence observed with aging is associated with the age-related redistribution of fat, particularly increased central adiposity. Low levels of adiponectin are induced by visceral fat accumulation, recognized as a risk factor for CVD and T2DM, and associated in an inverse correlation with insulin resistance [32,33]. Leptin regulates the appetite, and high levels of leptin induced by subcutaneous fat accumulation may indicate decreased leptin sensitivity in obese individuals [34]. Finally, high levels of free fatty acids associated with obesity contribute to the development of peripheral insulin resistance [35].

Advanced glycation end products (AGEs) are a result of the nonenzymatic reactions between sugars and amino groups such as proteins and lipids [36]. As some AGEs have typical fluorescence bands [37], skin autofluorescence can be used as a robust noninvasive biomarker of AGE accumulation in tissues [38]. AGEs accumulate with age in healthy individuals and have been observed to accumulate faster in people with diabetes and inflammatory diseases [39]. AGEs can predict the severity of complications in diabetes [40]. The inclusion of skin autofluorescence in the Finnish Diabetes Risk Score improved the ability to detect undiagnosed diabetes and reclassify people in the intermediate risk category [41].

Cell Blood Count

A decrease in blood hemoglobin with age and anemia in older people is associated with functional and cognitive impairment as well as mortality [42,43]. In addition, studies on biological age modelling often include hemoglobin and hematocrit as biomarkers of aging due to their correlation with age [18,44]. Therefore, we included hemoglobin and hematocrit as candidate variables for the biological age model despite the notion that anemia is not a physiological finding related to aging per se but is associated with nutrient-related iron deficiency or unexplained anemia [45].

Cardiorespiratory Function

Blood pressure is a biomarker of cardiovascular (CV) function and is one of the most important modifiable risk factors that strongly predicts CV morbidity and mortality [46]. High blood pressure is a common medical condition, and its prevalence increases with age [47]. As excess fat mass represents the major factor predisposing high blood pressure, lifestyle interventions targeting obesity (and smoking) are highly relevant [48]. Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and the FEV_1-FVC ratio are biomarkers of dynamic lung function [49]. FEV_1 declines in a nonlinear manner with age, with the estimated decline of 25-30 mL/year starting at the age of 35-40 years and increasing up to 60 mL/year after the age of 70 years; however, the interindividual variability can be considerable [49].

Physical Capacity

A main indicator of physical activity and cardiorespiratory fitness is maximal oxygen uptake (VO_{2max}). Functional independence is dependent on VO_{2max} [50], and its association with mortality and morbidity of noncommunicable diseases is well established [5]. Aging is associated with a decline in VO_{2max} (about 6.2%/decade) [51], primarily due to a reduction in maximal cardiac output and, secondly, due to a reduced oxygen extraction capacity at the muscle level (maximal arteriovenous oxygen difference) [52,53]. Importantly, the decline in VO_{2max} is decelerated in trained compared to sedentary subjects [54]. Finally, physical inactivity accelerates secondary aging by reduction in VO_{2max}, skeletal muscle strength, and bone mineral density [55]. A Norwegian study found that sedentary time increased by 4.4 and 3.2 min/day for women and men, respectively from the age of 65 years, concomitant with a decrease in both low and moderate to vigorous physical activity [56]. Handgrip strength is a robust measure of overall strength, which correlates with mortality and declines in a linear manner with age (0.34 and 0.65 kg/year for women and men, respectively) [57,58]. Knee extension and elbow flexion are associated with functional independence and health. They are important for daily activities and have been used in previous epidemiological health investigations [59,60]. The sit to stand test is part of the “Short Physical Performance Battery” [61], which assesses lower extremity function and predicts disability in older age [59,62].

Immune Function

Adipose tissue is an endocrine organ and a major regulator of inflammation [63]. Excess adipose tissue is an important contributor to the elevated C-reactive protein (CRP) concentrations observed in obese people [64] and is related to the production of interleukin 6 (IL-6) and its stimulation of hepatic CRP production [65]. Chronically elevated levels of pro-inflammatory markers such as IL-6 and tumor necrosis factor-α are also key features of the aging phenotype defined as “inflammaging” [66]. Chronic low-grade inflammation (LGI) is thought to be part of the T2DM [67], CVD [68], cancer [69], and Alzheimer disease [70] pathophysiologies. CRP is considered a gold standard biomarker of low-grade inflammation and chronic inflammation. Recently, soluble urokinase plasminogen activator receptor (suPAR) was proposed as a biomarker of inflammation and was shown to predict T2DM, CVD, and cancer independently of CRP [71]. Plasma suPAR concentration increases with aging and unhealthy lifestyles (eg, unhealthy dieting and smoking) [71,72].

Measurements and Procedure

The examination was conducted in the order described below. Arterial blood pressure was measured in triplicate in the supine position using an automatic monitor (BoSo Medicus Control, BOSCH + SOHN GmbH). Venous blood samples were obtained for measuring concentrations of total cholesterol, high- and low-density lipoproteins, triglycerides, glucose, insulin, adiponectin, glycated hemoglobin (HbA_{1c}), hematocrit, hemoglobin, CRP, and suPAR. Body composition was assessed by dual-energy X-ray absorptiometry scanning and visceral fat measurements using the CoreScan software (Lunar Prodigy Advanced, Lunar). Body composition was also assessed by bio-impedance (MC-780MA, Tanita Corporation of America Inc), which is commonly used in clinical settings. Measures of waist and hip circumference were collected. A high-quality

portrait picture was taken for a subanalysis on perceived age. Skin autofluorescence was measured by an AGE Reader (DiagnOptics BV). Lung function was assessed in terms of FEV_1 and FVC (Vyntus SPIRO spirometer, Vyaire Medical). We tested three isometric strength measures. The first test involved measuring knee extension strength. The participant was made to sit on a table. The test was performed with one leg, with the knee in 90° flexion serving as the starting position while the thigh was stabilized against the table with a standard gait belt so that it could not be lifted during the test. A standardized belt stabilization configuration was used to position the dynamometer (microFET2, Hoggan Health Industries) against the back of the table leg using a flat attachment. This method has been validated against the "gold standard" isokinetic dynamometer [73]. The second test involved measuring handgrip strength. Keeping the arm by the side, the participant was asked to squeeze a handgrip dynamometer (Takei Digital Hand Grip Dynamometer, Takei Scientific Instruments Co, Ltd). The third test measured bicep strength. The participants were asked to keep both arms by the side and flex both elbows by 90° using a Takei TKK 5402 Digital Back Strength Dynamometer (Takei Scientific Instruments Co, Ltd, Tokyo). Participants performed a minimum of 3 test trials and continued until no increase in strength occurred. A graded exercise test (Quark PFT Ergo, Cosmed) was conducted to determine VO_{2max} with an electromagnetically braked cycle ergometer (Lode Excalibur, Groeningen). The exercise protocol consisted of 5 minutes of warm-up time at 50 and 100 W for females and males, respectively, followed by a 25 W increase in load every minute until voluntary exhaustion. Finally, the participants filled out the quality of life (SF-12v2 Health Survey) and Physical Activity Score (PAS 2.1) questionnaires [74], and their education level and smoking habits were recorded.

Phase 2: BA Estimation

Mathematical Approach

The three most common approaches to estimate biological age are (1) multiple linear regression (MLR) [14,75-78]; (2) principal component analysis (PCA) [19,44,79-82]; and (3) Klemera and Doubals' method (KDM) [83,84]. Each method has its own benefits and limitations and has been compared substantially in the literature [17,18,85]. The MLR method is considered the basic approach to estimate biological age but is criticized for over- and underestimating biological age at each end of the age spectrum and the risk of biomarker multicollinearity. The PCA method derives from MLR but uses the first principal component from the PCA to form the biological age equation. This reduces the over- and underestimation observed in the MLR method and resolves the risk of multicollinearity [79]. In comparison with the MLR and PCA approaches, the KDM is a comprehensive mathematical approach. The biological age estimation is based on minimizing the distance between *m* regression lines and *m* biomarker points within an *m*-dimensional space of all included biomarkers [83]. Although the biological age estimated by the KDM has been shown to predict mortality better than that estimated by MLR and PCA [18], the majority of the studies on biological age models using minimally invasive biomarkers (essential for the use of a biological age model in health promotion) have been conducted using PCA [86]. Therefore, we will use PCA in our model development. Doing so will also allow a wider comparison of our results against more data and the findings of prior studies that had applied this approach to their models, thus facilitating an evaluation of the external validity of our model.

PCA was originally proposed by Nakamura et al [79] to select the fewest possible physiological variables to estimate biological age. Biological age construction when applying PCA includes (1) selection of the variables using correlation analysis, redundancy assessment, and loss of informative value caused by internal consistency among the variables; (2) use of PCA to obtain the principal components; (3) application of the first principal component to develop the normalized biological age score; and (4) transformation of the normalized biological age score into biological age expressed in years so that it is comparable with the chronological age [79,86]. The mathematical and statistical analysis will be completed using SAS Enterprise Guide 7.1 and MATLAB R2018b.

Phase 3: Validation Cohort

Study Design

We intend to recruit overweight and obese subjects as obesity increases the risk of age-related diseases early in life. Thus, individuals with obesity are expected to deviate from the pathway of a healthy aging phenotype, resulting in a higher biological age compared to chronological age. Recruitment for the study will commence at a Danish folk high school conducting lifestyle interventions. We seek to recruit 80 overweight or obese adults (≥18 years) attending a 15-week lifestyle intervention course. Pregnancy, history of CVD, and using β-blockers are the exclusion criteria for participation in the study. The aim of the lifestyle intervention is an 8%-10% weight loss. Initial moderate weight loss induces improvements in most CV risk factors [87,88]. Therefore, this setting will allow us to explore the clinical relevance of the biological age model in assessing healthy aging. The intensive lifestyle intervention includes key features to achieve healthy aging and compress morbidity. Daily activities from 7 AM to 4 PM include supervised training (1-3 hour/day), class-based theoretical teaching focusing on changes to healthy behavior, and individual cognitive therapy. Participants are served healthy hypocaloric diets, individually prepared in accordance to an energy balance required for a normal BMI of 25 kg/m^2. For more information on the intensive lifestyle intervention, refer to the work of Dandanell et al [89].

Measurements and Procedure

The results from the PCA will determine the measures to be included in the protocol. The procedure will be similar to the one described in the Phase 1 study, with the exception that we will use the short version (4 generic items) of the International Physical Activity Questionnaire and a modified exercise protocol to assess VO_{2max}. To ensure that the exercise protocol elicits a valid VO_{2max}, warm up will be performed at 30 and 50 W for women and men, respectively, and thereafter increased by 20 and 25 W every minute until exhaustion for women and men, respectively. Biological age will be estimated at the beginning

and end of the course based on the results of the PCA. In addition, we will estimate the metabolic syndrome and Framingham risk score in the validation cohort [90,91]. Doing so will allow us to evaluate the response variation in biological age after an expected moderate weight loss and improved aerobic capacity, and we will compare these results with the changes observed in the existing validated health metrics used in health promotion and disease prevention (Framingham risk score and metabolic syndrome) [92,93]. Furthermore, we will (1) compare the biological age results in the healthy study population (the derivation cohort) with the overweight study population (the validation cohort) and (2) evaluate our biological age model against existing models to assess the feasibility of the former for health promotion.

Results

Phase 1

The derivation cohort consists of 51 women and 49 men. The distributions of their demographic and clinical characteristics are presented in Tables 1 and 2.

Table 1. Characteristics of study participants in the derivation cohort.

Variables	Women (n=51)	Men (n=49)
Age groups (years), n (%)		
18-23	7 (13.7)	6 (12.2)
24-29	7 (13.7)	6 (12.2)
30-35	6 (11.7)	7 (14.3)
36-41	6 (11.8)	6 (12.2)
42-47	6 (11.8)	6 (12.2)
48-53	6 (11.8)	6 (12.2)
54-59	7 (13.7)	6 (12.2)
60-65	6 (11.8)	6 (12.2)
BMI (kg/m^2), n (%)		
<25	33 (64.7)	27 (55.1)
≥25	13 (25.5)	21 (42.9)
≥30	5 (9.8)	1 (2.0)
HbA_{1c}[a] (mmol/mol), mean (SD)	32.3 (3.2)	33.4 (3.1)
Lung function - FEV_1/FVC (%), mean (SD)	79.4 (6.0)	79.1 (5.3)
Physical activity[b] (min/week), n (%)		
≥150	41 (80.4)	46 (93.9)
<150	10 (19.6)	3 (6.1)
Education[c] (years), n (%)		
<10[d]	0 (0.0)	3 (7.5)
10-12[e]	32 (69.6)	29 (72.5)
≥13[f]	14 (30.4)	8 (20)
Smoking status, n (%)		
Yes	3 (5.9)	3 (6.1)
No	48 (94.1)	46 (93.9)

[a]HbA_{1c}: Hemoglobin A_{1c}.

[b]Leisure-time spent on moderate (5 metabolic equivalents) and vigorous (6 metabolic equivalents) physical activity.

[c]Level of education was reported by 86.0% (86/100) of the total study population (46/100, 46.0% women; 40/100, 40.0% men).

[d]Lower secondary education.

[e]Upper secondary education.

[f]First- and second-stage tertiary education.

Table 2. Maximal oxygen consumption of study participants in the derivation cohort.

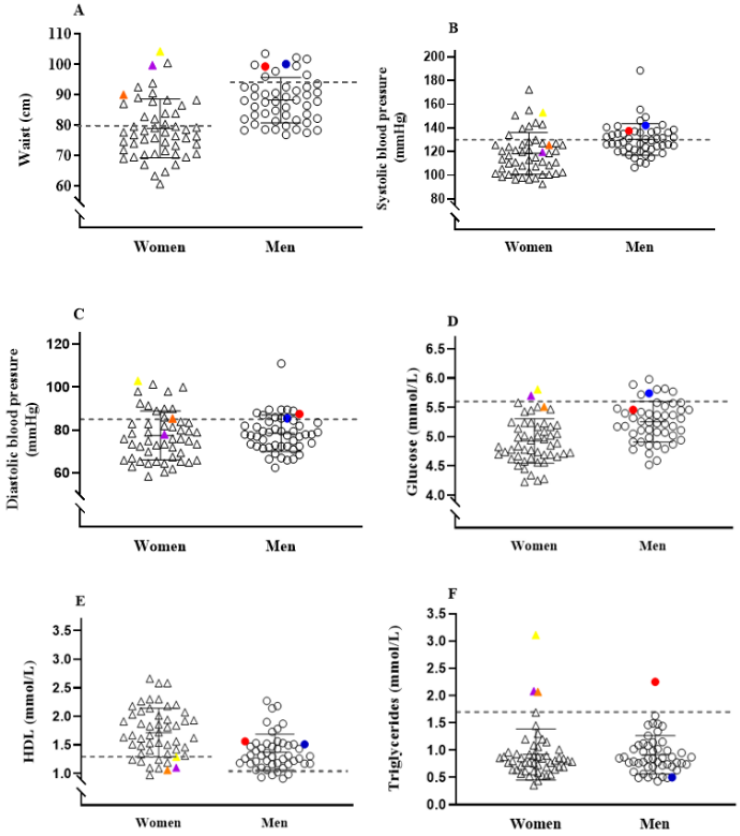
Age group (years)	VO_{2max}[a] (mL/min/kg), mean (SD)	
	Women (n=51)	Men (n=49)
18-23	36.9 (7.3)	45.2 (4.0)
24-29	37.4 (2.5)	44.8 (7.8)
30-35	37.7 (5.6)	47.9 (7.8)
36-41	36.0 (7.8)	43.2 (7.7)
42-47	39.7 (7.4)	47.1 (6.5)
48-53	29.8 (4.7)	42.7 (6.0)
54-59	30.1 (3.9)	39.7 (9.8)
60-65	31.8 (4.6)	40.1 (4.1)

[a]VO_{2max}: Maximal oxygen consumption.

The majority of the participants reported having an upper secondary education (eg, high school diploma; women: 32/46, 69.6%; men: 29/40, 72.5%). Very few within the cohort (6/100, 6% in total) smoked. Cardiorespiratory fitness (VO_{2max}) was moderate to high in women and men throughout the age range, the majority (women, 41/51, 80.4%; men, 46/49, 93.9%) adhering to the national recommendations of a minimum of 150 min/week of moderate to vigorous physical activity [94]. No indications of decreased lung function or T2DM were found.

Although free from diseases, we found variations in metabolic health when assessing the cohort in terms of metabolic syndrome. Metabolic syndrome was present in 3 women and 2 men. The distribution of risk factors related to metabolic syndrome are visualized in Figure 2. We used the definition provided by the International Diabetes Federation to assess metabolic syndrome [31].

Figure 2. Health profile in relation to metabolic syndrome variables. The triangles represent women, and the circles represent men. Three women (yellow, orange, and purple triangles) and two men (red and blue circles) fulfilled the criteria for metabolic syndrome. The solid lines represent the mean (SD) for each group. The dashed lines represent the cut-off criteria (values mentioned in the brackets that follow) for each variable in accordance with the definition provided by the International Diabetes Federation. A: Waist circumference in females (≥80 cm) and males (≥94 cm); B: Systolic blood pressure (≥130 mm Hg); C: Diastolic blood pressure (≥85 mm Hg); D: Fasting plasma glucose (≥5.6 mmol/L); E: High-density lipoprotein (HDL) for females (1.29 mmol/L) and males (1.03 mmol/L); F: Triglycerides (≥1.7 mmol/L).



Phase 2

Correlation analysis and principal component analysis have been performed. Interpretation of the model, including sensitivity analysis, internal consistency reliability, and model refinement, will follow.

Phase 3

This study has been approved by the Local Research Ethics Committee, Copenhagen, Denmark (H-19073643; Clinical Trial Number NCT04279366). We have established collaboration with the staff at the folk high school and recruitment for participation is forthcoming.

Discussion

Findings

The primary objective of this pilot study was to develop a biological age model that could be applied for health promotion of the general adult population, given its ability to distinguish healthy and unhealthy aging trajectories among individuals with the same chronological age and sex. Within this objective lies the practical limitation of including as few and minimally invasive biomarkers in the model as possible despite the complexity of aging. Therefore, to develop a reliable biological age model, it is essential to select biomarkers that accurately show significant change with age, reflect the aging status independent of disease, have high reproducibility, cover essential areas of human function, and are appropriate for in vivo studies of humans [15,79,95]. A limitation of this study is that this biological age model is designed to assess a healthy aging trajectory only on a physical level; the assessment of the cognitive aspects of maintaining functional independence for a socially active life, an important part of the healthy aging phenotype, are not included herein [4]. Another limitation is that while the biomarkers included in the proposed biological age model align with the phenotypic biomarkers of aging (eg, clinical measures such as grip strength and glucose concentration), the model overlooks the molecular-based biomarkers of aging (ie, DNA-related markers). Short telomere length is associated with risk of CVD, age-related decline in physical function, and mortality [96]. Furthermore, DNA methylation, a biomarker for biological age (DNAm age, also referred to as the "epigenetic clock"), predicts all-cause mortality independent of the classic risk factors (age, body mass index, smoking, etc) as well as frailty, self-related health, and chronological age [96]. While such models seem promising, the lack of feasibility regarding use in community-based interventions is the main reason for not including these biomarkers in our biological age model. We do, however, plan to validate the biological age model against telomere length at a later time, when data from the derivation cohort become available. Our secondary objective involves investigating the usefulness of the model. Validating the model against mortality and morbidity is preferable but beyond the scope of this study. Instead, we plan to validate the clinical use of the model in Phase 3 by comparing the change in biological age against that in already validated prediction metrics commonly used in health promotion (eg, the Framingham risk score and metabolic syndrome) in relation to a lifestyle intervention. As the validation cohort is not randomly assigned from the general population, there is a risk that it might represent a selected group whose physiological state is independent of behavioral factors (eg, diet and physical activity) and biased by genetics. Regardless, the change in biological age after an intensive lifestyle intervention can provide initial evidence about the potential of the biological age model for health promoting interventions.

Conclusions

We expect to find that the biological age model is a useful indicator of the risk of metabolic dysfunction and disease. Given future challenges, our expectation calls for further optimization of the model (eg, extending the sample size of the derivation cohort) and validation (by including hard endpoints such as mortality and morbidity).

Acknowledgments

KH and JWH designed and conceptualized the study. KH, MF, and PH collected the data. KH and ABK analyzed the data. KH, ABK, K-ÅH, JWH, and HBDS interpreted the data. KH wrote the first draft, and FD, HBDS, ABK, and JWH revised the manuscript. All authors read and approved the final manuscript. This work was supported by the Copenhagen Center for Health Technology, the Center for Healthy Aging, and University College Copenhagen. The sponsors had no involvement in the study design, writing of the manuscript, and choice of publication.

Conflicts of Interest

None declared.

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Abbreviations

AGEs: advanced glycation end products

CV: cardiovascular
CVD: cardiovascular disease
CRP: C-reactive protein
FEV_1: forced expiratory volume in 1 second
FVC: forced vital capacity
Hb_{A1c}: glycated hemoglobin
IL-6: interleukin 6
KDM: Klemera and Doubals' method
MLR: multiple linear regression
PAS: physical activity score
PCA: principal component analysis
suPAR: soluble urokinase Plasminogen Activator Receptor
T2DM: type 2 diabetes mellitus
VO_{2max}: maximal oxygen uptake

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Paper III

Proposition of a model estimating biological age from physiological biomarkers of healthy aging: a cross-sectional study.

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Abstract

Background: Individual differences in rate of aging and susceptibility to disease are not accounted for by chronological age alone. These individual differences are better explained by biological age, which may be estimated by biomarker prediction models. In the light of the aging demographics of the global population and the increase in lifestyle related morbidities, it is interesting to invent a new biological age model to be used for health promotion.

Objectives: To develop an model that estimate biological age based on physiological biomarkers of healthy aging.

Methods: Carefully selected physiological variables from a healthy study population of 100 women and men were used as biomarkers to establish an estimate of biological age. Principal component analysis was applied to the biomarkers and the first principal component was used to define the algorithm estimating biological age.

Results: The first principal component accounted for 31% in women and 25% in men of the total variance in the biological age model combining: mean arterial pressure, glycated Hemoglobin (HbA1c), waist circumference, Forced Expiratory Volume within the first sec. (FEV_1), maximal oxygen consumption (VO_2max), adiponectin, High Density Lipoprotein (HDL), total cholesterol and Soluble urokinase-type Plasminogen Activator Receptor (suPAR). The correlation between the corrected biological age and chronological age was r=0.86 (p<.0001) and r=0.81 (p<.0001) for women and men, respectively and the

agreement was high and unbiased. No difference was found between mean chronological age and mean biological age, and the slope of the regression line was near one for both sexes.

Conclusion: Estimating biological age from these nine biomarkers of aging can be used to assess general health compared to the healthy aging trajectory. This may be useful to evaluate health interventions and as an aid to enhance awareness of individual health risks and behavior, when deviating from this trajectory.

Introduction

Biological age (BA) is a measure that quantifies where an individual is on the aging trajectory, assessed by the physiological profile, in comparison with the average person of that given chronological age within the population from which the equation was generated (1, 2). The predictive abilities of BA have been investigated in relation to age-related diseases such as cardiovascular disease and type 2 diabetes and some BA models have been found to predict mortality better than chronological age (CA) (3-5). Parallels can be drawn between the changes that occur with aging and the changes that occur with an unhealthy lifestyle (especially related to physical inactivity and obesity) and the risk of developing cardiovascular disease and type 2 diabetes (6, 7). Therefore, the objective assessment of BA is an appealing approach for risk stratification and health literacy within public health promotion. However, to truly measure the current state of aging, and thereby objectively determine BA, would entail studies that follow people until they die and biomarkers representing all bodily functions. This is practically impossible and objectively unfeasible for use in a clinical setting. To circumvent this, BA models conceptualizing some mechanisms of aging are proposed as surrogate measures of BA. Despite a substantial research effort (8-10), there is still no agreement upon which panel of biomarkers to use when defining BA (11). Targeting health promotion and management of lifestyle-related diseases, studies have developed a number of BA models which evaluate the degree of severity of the metabolic syndrome (12), the relation to waist circumference (13), the relation to physical fitness level (14, 15) and the organ-specific health status (16) just to mention a few.

Increasing life expectancy and low fertility rates will have a profound impact on future resources and health care needs (17, 18). Forecasts anticipate that by 2050, people of 65 years or above will constitute more

than 20% of the population worldwide (19, 20). This is the decade in life where chronic diseases (e.g. cardiovascular disease, cancer and type 2 diabetes) frequently manifest (21) making healthy aging a key objective for research (22-24). Healthy aging is defined as an extension of healthspan (25) also characterized by the "healthy aging phenotype" avoiding major chronic diseases as well as cognitive and physical impairments (22). The important work from Lara and colleagues (26) have resulted in a panel of biomarkers of healthy aging. The purpose of our study was, to apply a novel approach, to incorporate biomarkers of healthy aging into a BA-model. For this purpose, we used the first principal component obtained from principal component analysis (PCA) as the method to assess individual BA. The goal was to create a biological age model based on the healthy aging phenotype. In this way, the model can be used to identify those deviating from the healthy aging trajectory. Thus, no difference between average CA and estimated BA was expected in the study population of healthy individuals.

Methods

Subjects

We included 100 healthy Danish subjects, 51 women and 49 men, between 18–65 years of age to participate in an extensive health examination and the data collection of candidate biomarkers for the BA-model. The study was approved by the Regional Ethics Committee, Denmark (H-18031350), recorded as a Clinical Trial (Clinical Trial number: NCT03680768) and performed in accordance with the Helsinki declaration. Participants were informed orally and in writing about the study protocol and the potential risks before written consent was obtained.

Candidate biomarkers

At the day of the health examination, participants came to the laboratory following an overnight fast and having avoided exercise activities and alcohol consumption for 24 hours and restrained from smoking for at least 4 hours. Information on the participants' previous and current health status included weekly alcohol consumption, smoking habits, present medications; past medical history and self-administered questionnaires on physical activity level (PAS 2.1) (27) and quality of life (SF12v2). We gathered data on the candidate biomarkers listed in Table 1. These 32 variables are all physiological components of healthy aging

that are associated with aging, age-related diseases, and are affected by changes in lifestyle. In addition, this panel of biomarkers covers multiple areas of human function and they are suitable to study in humans *in vivo*. For a more comprehensive description of the rationale for including these 32 variables as candidate biomarkers, we refer to our protocol paper (Clinical Trial number: NCT03680768) (28).

Table 1. Candidate biomarkers measured in the study participants (n=100) showing means with standard deviations (SD) and outcome units pr. year increase (regression slope with 95% confidence interval (CI))

	Mean (SD) (SD)	Slope (CI) (CI)
Body composition		
(1) Weight, *kg*	75.7 (13.1)	0.03 (-0.2, 0.2)
(2) Waist circumference, *cm*	83.4 (9.8)	0.2 (0.05, 0.3)
(3) Hip circumference, *cm*	101.4 (7.1)	-0.001 (-0.1, 0.1)
(4) Waist/Hip ratio	0.8 (0.07)	0.002 (0.001, 0.003)
(5) Fat mass, *%*	26.8 (8.3)	0.09 (-0.03, 0.2)
(6) Muscle mass, *kg*	52.8 (10.9)	-0.05 (-0.2. 0.1)
Metabolic health		
(7) Fasting blood glucose, *mmol/l*	5.1 (0.4)	0.01 (0.004, 0.015)
(8) HbA1c, *mmol/mol*	32.8 (3.2)	0.12 (0.08, 0.16)
(9) AGEs, *AU*	1.8 (0.5)	0.027 (0.022, 0.031)
(10) Insulin, *pmol/l*	44.4 (25.3)	0.05 (-0.32, 0.42)
(11) Triglycerides, *mmol/l*	0.9 (0.4)	0.002 (-0.004, 0.008)
(12) Free fatty acids, *µmol/l*	440 (212)	2.36 (-0.72, 5.46)
(13) Leptin, *pg/mL*	8411 (9472)	-60.0 (-199.8, 79.9)
(14) Adiponectin, *mg/mL*	11515 (6490)	106.6 (13.4, 199.8)
(15) HDL, *mmol/l*	1.5 (0.4)	0.01 (0.006, 0.017)
(16) LDL, *mmol/l*	2.8 (0.8)	0.02 (0.01, 0.03)
(17) TC, *mmol/l*	4.5 (0.9)	0.03 (0.02, 0.04)
(18) TC/HDL- ratio	3.1 (0.9)	0.003 (-0.01, 0.02)
Immune function		
(19) CRP, *mg/l*	1.6 (3.4)	-0.04 (-0.09, 0.01)
(20) suPAR, *ng/ml*	2.09 (0.5)	0.01 (0.003, 0.017)
Cell blood count		
(21) Hemoglobin, *mmol/l*	8.7 (0.8)	0.004 (-0.01, 0.02)
(22) Hematocrit, *%*	41.6 (3.8)	0.03 (-0.03, 0.09)
Cardiorespiratory function		
(23) Diastolic BP, *mmHg*	78.0 (10.1)	0.4 (0.3, 0.5)
(24) Systolic BP, *mmHg*	124.2 (16.7)	0.6 (0.3, 0.8)
(25) FEV1, *L*	3.9 (0.9)	-0.02 (-0.04, -0.01)
(26) FVC, *L*	4.9 (1.0)	-0.02 (-0.04, -0.01)
(27) FEV1/FVC ratio, *%*	77.8 (11.6)	-0.13 (-0.20, -0.05)
Physical capacity		
(28) VO_2max, *ml/min/kg*	39.3 (8.11)	-0.18 (-0.28, -0.06)
(29) STS, *stands*	23.4 (5.2)	-0.07 (-0.14, 0.01)
(30) Handgrip strength, *kg*	36.0 (9.4)	-0.8 (-0.2, 0.1)
(31) Biceps strength, *kg*	35.0 (11.5)	-0.1 (-0.3, 0.03)
(32) Quadriceps strength, *Nm*	152.4 (51.3)	-0.7 (-1.4, 0.1)

HbA1c; g*lycosylated hemoglobin type A1c,* AGEs*; Advanced glycation end products,* HDL; *High density lipoprotein*, LDL; *Low density lipoprotein*, TC; *Total cholesterol*, CRP; *C-reactive protein,* suPAR; *soluble urokinase plasminogen activator receptor*, BP; *Blood pressure*, FEV1; *Forced expiratory volume in first second*, FVC; *Forced vital capacity*, STS; *30 sec. sit-to-stand chair rise*, VO_2max; *maximal oxygen consumption*. Missing values were present in Leptin (n=99), CRP (n=87), Hematocrit (n=97) and Hemoglobin (n=99) and Bicep's strength (n=98).

Procedures

Variables of *Body composition* were measured by dual-energy X-ray (DXA) absorptiometry scanning (Lunar Prodigy Advance, Lunar, Madison, WI). Waist and hip circumference were measured twice, using a standard measuring tape. Variables of *Metabolic health* and *Immune function* were measured from venous blood samples. We extracted plasma and stored it at -80 °C before analysis. Plasma concentrations of C-reactive protein (CRP), total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides (TG), free fatty acids (FFA) and glycerol were measured separately by spectrophotometry (Cobas 6000 c501, Roche, Glostrup, Denmark). Plasma fasting blood glucose (FBG) concentration was measured on an automated analyser (Hitachi 912; Roche, Mannheim, Germany). Plasma insulin, adiponectin and leptin concentrations were measured by RIA kits (Millipore Cat., HADP-61HK, MA, USA). Plasma concentrations of soluble urokinase plasminogen activator receptor (suPAR) were measured using the commercially available suPARnostic® ELISA kit, according to the manufacturer's instructions (ViroGates, Copenhagen, Denmark). Advanced glycation end products (AGE) were measured non-invasively using an AGE reader (Diagnoptics Technologies, Groningen, the Netherlands). We measured glycosylated hemoglobin (HbA1c) on whole blood using DCA Vantage Analyser (Siemens Healthcare, NY, USA) for the analysis. Resting arterial blood pressure (BP) was measured in triplicate (with one-minute intervals) using an automatic monitor (Boso-medicus control, Jungingen, Germany). Forced vital capacity (FVC) and forced expiratory volume in the 1 sec. (FEV_1) was assessed by spirometer measurements (VyntusTM SPIRO spirometer, North Riverwoods, USA) where participants were sitting on a chair, wearing a nose clip and mouthpiece. Initially, participants

breathed normally before conducting a rapid maximal inspiration immediately followed by an expiration with a maximal effort that continued until no more air could be expelled while maintaining an upright posture. The procedure was repeated a minimum of three times and a maximum of seven. The trial with the highest reading was used and the VyntusTM SPIRO software (SentrySuite) automatically assessed the repeatability, acceptability and usability criteria defined by the American Thoracic Society and European Respiratory Society (29). The handgrip, biceps and quadriceps strength were measured by a handheld dynamometer (Takei, A5401, Physical company, High Wycombe, UK), a digital back strength dynamometer (Takei TKK 5402, Takei Scientific Instruments Co. Ltd., Tokyo, Japan) and a handheld dynamometer (microFET2, Hoggan Health Industries, Inc., Utah), respectively. At least three attempts were used until no rise in strength occurred. Each test was interspersed with one minute rest. Maximal oxygen consumption (VO_2max) was measured by a graded exercise test, performed on a bicycle ergometer (Lode Excalibur, Groeningen, Netherlands) using breath by breath (Quark PFT Ergo, Cosmed, Rome, Italy) oxygen consumption measurements. After five minutes of warm-up at 50 W and 100 W for women and men, respectively the load increased with 25 W every minute until voluntary exhaustion. VO_2max was determined as the highest 30 s. rolling average of VO_2.

Exclusion and inclusion of candidate biomarkers

To observe the trajectory of normal healthy aging, we excluded participants diagnosed with or having a previous history of, diabetes mellitus, cardiovascular disease, cancer and thyroid dysfunction and free of the use of medication to lower cholesterol levels, glucose concentration, and blood pressure (16, 30-32). In addition, a 99% reference interval (mean ±2.96xSD) was applied to examine any potential outliers (30). To acknowledge age-related decrements within the healthy aging spectrum, however, extreme values below or above the reference interval were individually assessed (33). We excluded the candidate biomarker AGE from the study due to technical problems affecting the reliability of the measurements.

The actual selection between the remaining 31 candidate biomarkers followed a systematic stepwise method in alignment with previous studies (30, 34, 35). To begin with, all candidate biomarkers were

submitted to Pearson's correlation analysis to assess the strength and direction of association between CA and the candidate biomarkers. All biomarkers that were significantly correlated with CA ($|r| > 0.15$, $P \leq .05$) were included. To minimize redundancy arising in the analysis, we assessed inter-correlation between the included biomarkers. If the correlation between biomarkers were high ($|r| \geq 0.7$) and they have a similar clinical function, they are likely to be dependent on the same biological factor and one is excluded depending on the strength of the relationship with CA and the clinical relevance.

Principal component analysis

PCA is a factor analysis that reduces dimensions but preserves most of the information in the original dataset. PCA is a linear transformation that applies orthogonal rotation to find factors/principal components that capture the largest amount of information in the data (36). As the PCA produce uncorrelated principal components disclosing which variables are most valuable for clustering the data, it can be used to elucidate the minimum numbers of candidate biomarkers necessary for estimating BA (37). Traditionally, all principal components with an eigenvalue above one are included, or alternatively the number of principal components that together contain 80% of the variation in the dataset. However, we will follow the approach, first applied by Nakamura et al. in 1988 (38) and applied by others since (39-42), and use the 1. principal component from the PCA to estimate individual BA.

To do so, included biomarkers were normalized to a mean of zero and unit standard deviation, which gives them equal weight in the PCA. The subsequent estimation of BA was performed in three steps. First, based on the PCA loading scores a standardized individual BA score (BAS) was modelled:

$$(1)\ BAS = w_0 + (w_1\ x_1) + (w_2 x_2) + \cdots + (w_N x_N),$$

where x represent the original value of each of the *N* biomarkers (without units) and the coefficients w_n are defined as:

$$(2)\ w_n = loading\ score_n/\sigma_n,$$

and the constant w_0 is defined as:

$$(3)\ w_0 = \sum_{n=1}^{N} loading\ scores_n\ .\ (\overline{x_n}\ /\sigma_n),$$

Where w_n represent each of the *N* biomarkers and $\overline{x}$ and σ representing the original mean and standard deviation for each biomarker. The loading scores represent the contribution of each biomarker to one unit vector of the principal component.

Second, transforming the BA score into BA in units of years by application of the T-scale method (43):

$$(4)\ BA = BAS\ \cdot \sigma_{CA} + \overline{\hat{x}_{CA}},$$

where σ_{CA} and $\overline{\hat{x}_{CA}}$ is the standard deviation and mean of the CA of the sample size. However, this introduces a regression towards the mean effect (overestimation of younger subjects BA and underestimation of older subjects BA) (44), why the correction model proposed by Dubina et al. is applied (45):

$$(5)\ BAc = BA + (y_i - \hat{y}) \cdot (1 - b),$$

where y_i represent individual CA, $\hat{y}$ the mean CA of the study sample and b representing the slope in the linear regression assessing the relationship between BA and CA.

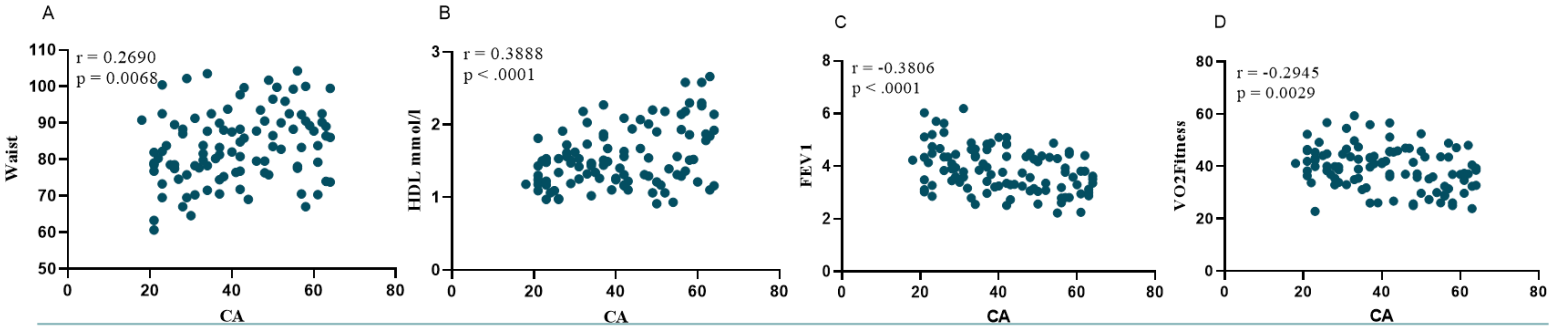
Statistics

We present candidate biomarkers as means with standard deviations and by linear regression to describe the direction and change of the candidate biomarkers per year. We assessed normal distribution using q-q plots, histograms, and checked variance of homogeneity and assessment of linearity by plotting residuals versus predicted values. Paired t-test was used to assess differences within sex and the difference between BAc and CA (Agedif) calculated as CA-BAc. The statistical analyses were done in SAS Enterprise Guide 7.1 and MATLAB R2018b. Statistical significance was considered at $p \leq 0.05$ in all statistical tests.

Results

Correlation analysis

Pearson's correlation coefficient was calculated for each of the 31 candidate biomarkers as a function of CA (supplementary Table 1). Overall, 15 biomarkers significantly correlated with CA covering five domains. *Body composition*: waist circumference and waist/hip ratio; *Metabolic health*: FBG, HbA1c, adiponectin, HDL, LDL, TC; *Immune function*: suPAR; *Cardiorespiratory function*: diastolic and systolic BP, FEV1, FVC, FEV1/FVC ratio and VO_2max. We observed positive correlations in waist circumference, waist/hip ratio, FBG, adiponectin, HbA1c, HDL, LDL, TC, suPAR, diastolic blood pressure (DBP) and systolic blood pressure (SBP) and negative correlations for FEV1, FVC, FEV1/FVC ratio and VO_2max (Figure 1.)



CA	Waist	W/H	FBG	HbA1c	Adiponectin	HDL	LDL	CHOL	suPAR	DBP	SBP	FEV1	FVC	FEV1/FVC	VO_2max
0.2690															
0.3654	0.8202														
0.3148	0.5635	0.5333													
0.4922	0.1940	0.3485	0.2945												
0.2235	-0.3107	-0.3538	-0.1309	0.0189											
0.3888	-0.3669	-0.3136	-0.1876	0.2088	0.5713										
0.3696	0.3220	0.2946	0.2745	0.2374	-0.0134	0.0361									
0.4952	0.1935	0.1633	0.2176	0.2974	0.2055	0.3937	0.9147								
0.2828	-0.0829	-0.1137	-0.1832	0.0257	0.2606	0.2663	0.0758	0.1935							
0.5125	0.2363	0.2315	0.3284	0.2507	0.0936	0.2606	0.2247	0.3333	0.1132						
0.4514	0.3786	0.4283	0.4753	0.2944	-0.0659	0.1272	0.2218	0.2628	-0.0677	0.8135					
-0.3806	0.3256	0.2848	0.2662	-0.0878	-0.3267	-0.3301	-0.0140	-0.1562	-0.2998	-0.1074	0.1730				
-0.3163	0.3483	0.3400	0.2921	-0.0844	-0.3501	-0.3415	-0.0634	-0.2047	-0.2848	-0.1320	0.1780	0.9510			
-0.3052	-0.0169	-0.1105	-0.0486	-0.0427	-0.0306	-0.0517	0.1258	0.0883	-0.1259	0.0060	0.1780	0.3738	0.0754		
-0.2945	-0.1297	0.1140	-0.0116	-0.1248	-0.1305	-0.1404	-0.2644	-0.3435	-0.3063	-0.2438	0.0214	0.4528	0.5093	-0.0455	

Figure. 1 *Top:* Scatterplots and Pearson's correlations between chronological age (CA) and waist circumference (A), high density lipoprotein (B), forced expiratory volume in 1. sec (C), maximal oxygen uptake (D).
Bottom: Pearson's correlation coefficients of the 15 biomarkers significantly correlated with age and their inter-correlations. Abbr.: W/H: waist to hip ratio; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin type A1c; HDL: High density lipoprotein; LDL: Low density lipoprotein; CHOL: total cholesterol; suPAR: soluble urokinase plasminogen activator receptor; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; FEV1: Forced expiratory volume in 1. sec; VO_2max: maximal oxygen uptake.

Assessment of redundancy

We observed high inter-correlations for some of the variables (Figure 1, bottom part) and we selected those with the strongest correlation with age and/or with the highest clinical significance within each cluster. Therefore, as FEV_1, FVC and FEV_1/FVC ratio all represent pulmonary function and FEV_1 has the highest correlation with age (r = - 0.3806, *P*< .001) compared to FVC (r = -0.3163, *P*= .001) and FEV_1/FVC (r= -0.3052, *P*=0.002), FEV_1 was selected. In the same manner we selected total cholesterol (r = 0.4952, *P*< .001) above LDL (r = 0.3696, *P*= .0002). HbA1c and FBG concentration are both markers of glycemic control, and a high correlation between HbA1c and FBG has been shown in people with and without diabetes (46, 47). We suggest that the moderate inter-correlation (r = 0.2945 *P* = .003) found in this present study is due to the sample size. HbA1c, which shows a higher correlation with age, has previously been used in the literature in BA models (48) and is generally preferred over FBG due to its higher applicability in a clinical setting. Thus, to reduce redundancy we only include HbA1c as a marker of glycemic control despite an inter-correlation < 0.7.

We observed a high inter-correlation between waist circumference and waist-to-hip ratio, the latter having the highest correlation with CA. Despite this, waist circumference was selected due to its strong association with visceral adipose tissue (49), its clinical importance as the best single anthropometric measure able to identify individuals at high risk of cardiovascular disease and type 2 diabetes, and its simplicity (50-52). In addition, the inherent problem of the equation that a morbidly obese could have the same W/H ratio as a normal weight individual made us select waist circumference. Finally, DBP and SBP had an inter-correlation of r = 0.8135 (*P*< .001), and a very similar correlation with age (r = 0.5125 (*P*< .001) and r = 0.4514 (*P*< .001), respectively). Instead, we calculated mean arterial pressure (MAP = $\frac{1}{3}$SBP + $\frac{2}{3}$DBP) to capture both parameters. MAP had a correlation with age of r = 0.510 (*P*< .001) and an inter-correlation with SBP and DBP of r = 0.943 (*P*< .001) and r = 0.961 (*P*< .001), respectively. Thus, a total of nine biomarkers were submitted to the PCA: waist circumference, FEV_1, HbA1c, Adiponectin, HDL, TC, suPAR,

MAP and VO_2max (Scatterplots and Pearson's correlation with age for all nine biomarkers are available in Suppl. Fig. 1).

Applying PCA

Following the normalization of the dataset comprising the nine biomarkers, we applied PCA for women and men separately, with and without the inclusion of CA. By including and excluding CA, we could assess if the direction of the 1. principal component (1PC) was similar in both cases, thus assuming that the 1PC can be seen as a general aging factor. The analysis showed high loading scores for CA on the 1PC for both women and men (0.473 and 0.515), respectively confirming the close relationship between age and 1PC (Table 3). In the second PCA we excluded CA and found that the relationship between the 9 biomarkers and the 1PC persisted. The 1PC's had eigenvalues above 1.0 and account for 30.96 % (females) and 25.04 % (males) of the total variance in the battery of nine biomarkers (Table 4). These results indicate that the 9 biomarkers reflect underlying measures of a healthy aging trajectory.

To clarify how the variables contribute to the estimation of the BA model, we calculated the percentage contribution of each variable using the following equation:

$$\frac{a_n^2}{\sum a_n^2} * 100, n = 1,2, \dots, N$$

Where a_n^2 is the given loading score and *N* is the number of variables (Table 4). In women, we see that total cholesterol concentration contributed most (21.8%) followed by MAP (18.9%) and HbA1c (16.7%). For men, waist circumference contributed most (24.1%) closely followed by VO_2max (22.6%) and total cholesterol concentration (14.5%).

Table 3. The linear combination of normalized variables for the PC1 by gender (chronological age included).

	Loading scores for PC1	
	Women	Men
Chronological age	0.473	0.515
Mean arterial blood pressure	0.392	0.294
Glycated hemoglobin	0.348	0.352
Waist circumference	0.144	0.378
Forced expiratory volume in 1. sec.	-0.164	-0.340
Maximal oxygen consumption	-0.287	-0.321
Adiponectin	0.199	0.078
High density lipoprotein	0.346	0.127
Total cholesterol	0.405	0.337
suPAR	0.220	0.167
Eigenvalue	3.50	2.90
Explained variance (%)	35.04	28.96

PC1: first principal component comprising the best fit line with the largest sum of squares distances; Eigenvalue: the Sum of Squared distances for PC1; Explained variance %: How many percent does the PC1 explain of the total variance in the dataset. Mean arterial blood pressure = ($\frac{1}{3}$SBP + $\frac{2}{3}$DBP); suPAR: soluble urokinase plasminogen activator receptor.

Table 4. The linear combination of normalized variables for the PC1 by gender (chronological age excluded) and the relative contribution of each physiological variable to BA estimation

	Women		Men	
	Loading scores	Contribution (%)	Loading scores	Contribution (%)
Mean arterial blood pressure	0.435	18.9	0.349	12.2
Glycated hemoglobin	0.408	16.7	0.324	10.5
Waist circumference	0.173	3.0	0.491	24.1
Forced expiratory volume in 1. sec.	-0.138	1.9	-0.309	9.5
Maximal oxygen consumption	-0.341	11.6	-0.475	22.6
Adiponectin	0.228	5.2	-0.046	0.2
High density lipoprotein	0.390	15.2	-0.020	0.04
Total cholesterol	0.467	21.8	0.3804	14.5
suPAR	0.238	5.7	0.254	6.4
Eigenvalue	2.79		2.25	
Explained Variance%	30.96		25.04	

BA: Biological age; PC1: first principal component comprising the best fit line with the largest sum of squares distances; Eigenvalue: the Sum of Squared distances for PC1; Explained variance %: How many percent does the PC1 explain of the total variance in the dataset.

Mean arterial blood pressure = ($\frac{1}{3}$SBP + $\frac{2}{3}$DBP); suPAR: soluble urokinase plasminogen activator receptor.

Biological age model

By application of equation (1), the loading scores from the PCA were used to construct individual standardized BAS as a function of the nine biomarkers as shown in the following equations:

$$BAS_{female} = -11.04 + 0.03 \cdot MAP + 0.126 \cdot HbA1c + 0.018 \cdot Waist - 0.018 \cdot FEV1 - 0.053 \cdot VO2\,\max + 3.205 \cdot 10^{-5} \cdot Adiponectin + 0.909 \cdot \mathrm{HDL} + 0.500 \cdot \mathrm{TC} + 0.400 \cdot \mathrm{suPAR}$$

$$BAS_{male} = -11.23 + 0.037 \cdot MAP + 0.103 \cdot HbA1c + 0.066 \cdot Waist - 0.431 \cdot FEV1 - 0.067 \cdot VO2\,\max - 1.058 \cdot 10^{-5} \cdot Adiponectin - 0.062 \cdot HDL + 0.442 \cdot TC + 0.828 \cdot suPAR$$

Subsequently, the BAS was scaled by the application of equation (4).

$$BA_{female} = (BAS \times 13.6) + 41.3$$

$$BA_{male} = (BAS \times 13.8) + 41.1$$

Scaling the score into units of years makes it more feasible to use when applying it to health promotion in the general population. Introducing this relationship between CA and BA has been shown to create some bias at the regression ends. Thus, following the previously mentioned correction model of Dubina et al. (45) (equation 5) the final BA models are expressed as:

$$BAcfemale = -56.67 + 0.27 \cdot MAP + 1.02 \cdot HbA1c + 0.1453 \cdot Waist - 2.03 \cdot FEV1 - 0.43 \cdot VO2\,\max + 0.0003 \cdot Adiponectin + 7.39 \cdot HDL + 4.06 \cdot TC + 3.24 \cdot suPAR + 0.20 \cdot CA$$

$$BAcmale = -70.37 + 0.34 \cdot MAP + 0.95 \cdot HbA1c + 0.60 \cdot Waist - 3.96 \cdot FEV1 - 0.62 \cdot VO2\,\max - 9.73 \cdot 10^{-5} \cdot Adiponectin - 0.57 \cdot HDL + 4.06 \cdot TC + 7.61 \cdot suPAR + 0.32 \cdot CA$$

The corrections are visualized in Figure 2, showing how the overestimation of BA in younger adults and underestimation of older adults are attenuated. In addition, Figure 3 visualize the regression of BAc on CA (R^2=0.73, *P* < .001 and R^2=0.65, *P* < .001). BAc is scattered relatively close and symmetrically above and below the regression line with a standard error of the estimate (SEE) of 8.2 years (women) and 10.2 years (men). We found no statistical difference between mean CA and mean BAc in women (*P*= .998) or men (*P*= .996). To assess the agreement between CA and BAc we made a Bland Altman plot and found a mean difference of 0.002 in women and – 0.006 in men, respectively (Figure 4).

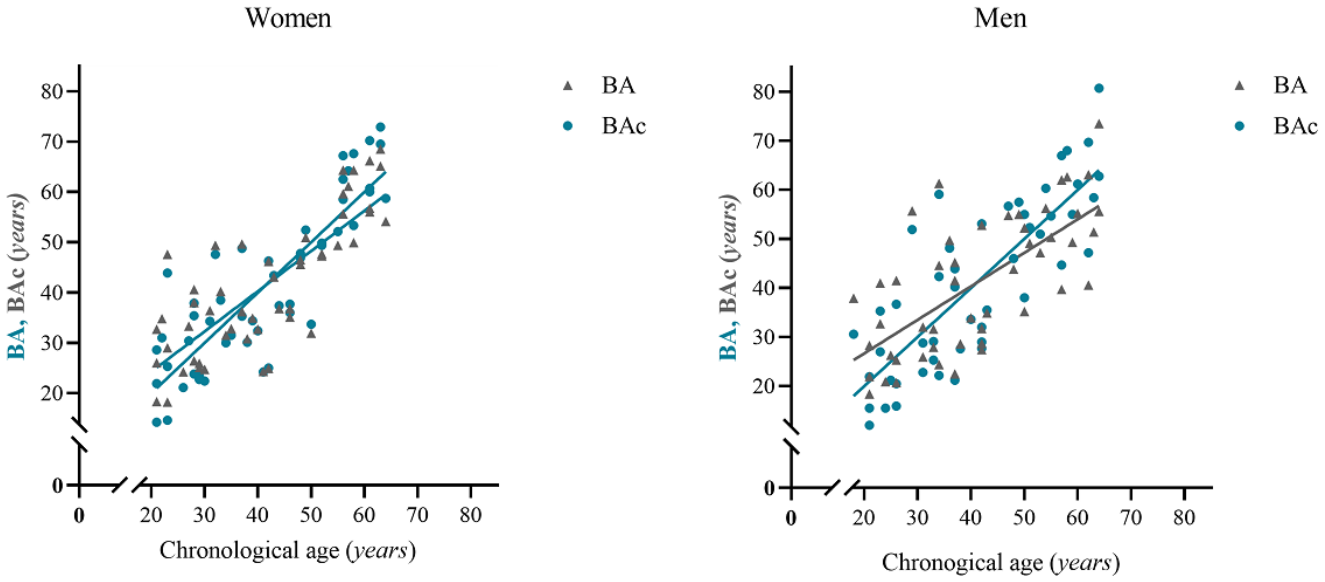


Figure 2. Regression lines before (BA) and after (BAc) correction for women and men, respectively.

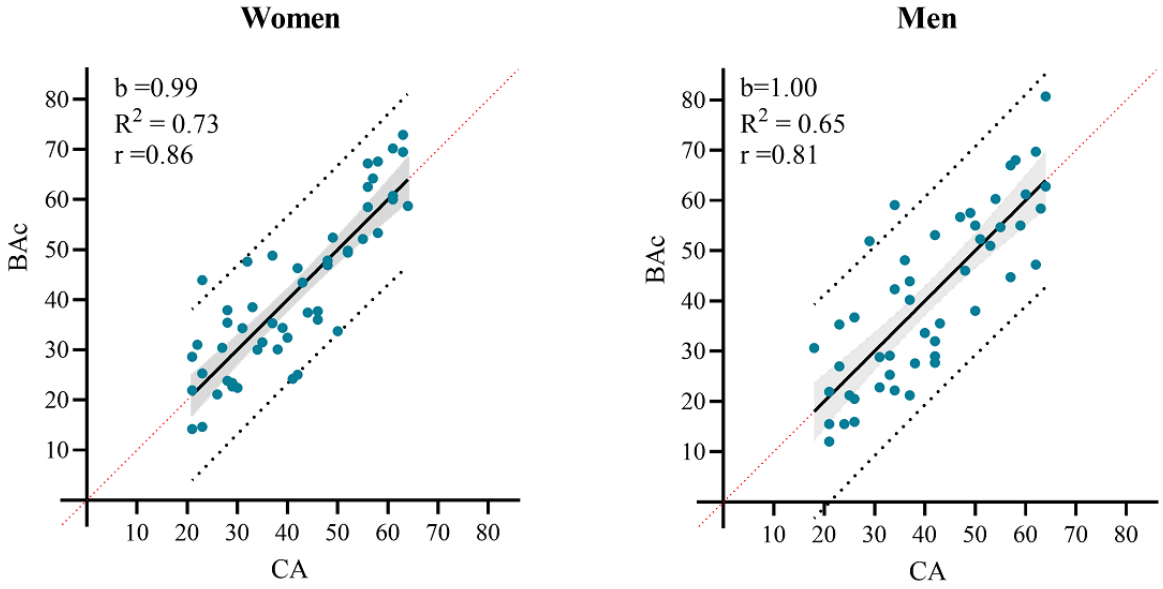


Figure 3. The BAc regression lines for women and men, respectively with 95% confidence intervals (shaded area), 95% prediction intervals (black dotted line) and line of identity (red dotted line). Slope (b), correlation coefficient (r) and coefficient of determination (R^2).

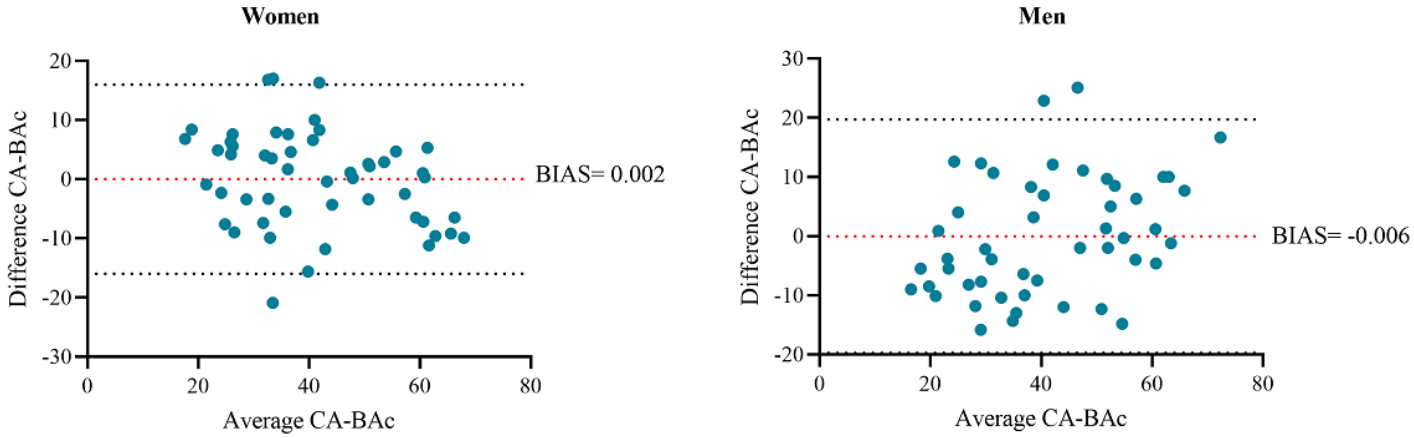


Figure 4. Bland Altman plot for women and men, respectively with BIAS (red dotted line), upper and lower limits of agreement (black dotted lines).

Discussion

In this study we aimed to develop a biological age model, able to measure healthy aging trajectory, using simple, clinically biomarkers that would respond to changes in health behavior. We selected 9 biomarkers listed in Table 4 and applied PCA to estimate individual BA. The nine biomarkers represent metabolic health (HDL, total cholesterol and adiponectin) and bodily functions (FEV1, MAP, suPAR), and include very important clinical age-related variables (VO2max, HbA1c and WC) (28). We found no difference between BAc and CA in the healthy reference group of women and men, and the BA model for both women and men showed a high linear relationship with CA. The disagreement between CA and BAc was low and unbiased. A higher variation in the BA model for men resulted in a lower coefficient of determination (R^2=0.65, $P < .001$) compared to the BA model for women (R^2 =0.73, $P < .001$).

Sex differences were also observed in the relative contribution of each biomarker to the BA estimate. This indicates that some biomarkers of aging are influenced by sexual dimorphism (53). HDL for example contributes 15.8% in women and a negligible 0.04% in men. HDL levels are higher in women than in men of the same age (54). However, during menopause HDL levels decrease (and LDL increase), thereby increasing the cumulative risk of CVD (55). In general, the multifaceted effects of menopause on metabolism may imply that further development of the model should evaluate if separate models for pre-and post-menopausal women are required. Waist circumference contributed the most (24.1%) in the estimation of BA for men but only 3.0% in the BA estimation for women. This agrees well with the sex difference in fat distribution— men have a relatively more central distribution of fat with aging also in the absence of weight gain (56). On the other hand, a similar deterioration between sexes of VO_2max and FEV_1 is expected

(53). This was not the case in our study, as VO_2max and FEV_1 contributed more to the BA model for men. This difference may be balanced by normalizing VO_2max and FEV_1 to lean mass and height, respectively. In addition, the small sample size should be mentioned as a limitation in these observations.

The BA model is based on a healthy reference adult subsample of the population. However, in 7.8% (*n*=4) of the women and 16.3% (*n*=8) of the men the Agedif (BAc-CA) was more than +10 years (Fig. 2E+F). One of these women and seven of these men stand out by having a BMI between 25 and 36 kg/m^2. Because BMI is causally related to morbidity and mortality (57), it could be argued that individuals with a BMI> 24.9 are not suitable to be included in this study representing a healthy aging reference group. However, cardiorespiratory fitness (VO_2max) may be an even better predictor for CVD and premature all-cause mortality (58). Also, a better VO_2max was found to attenuate the risks related to overweight and obesity (59, 60). The majority (80.4% and 93.9% of women and men, respectively) of the study participants adhered to the recommendations of a minimum of 150 min/week of moderate to physical activity and had a moderate to high cardiorespiratory fitness level (28). Therefore, we did not use high BMI as exclusion criteria. Within this consideration, also lies an effort to recruit a subsample of the population representing normal healthy aging instead of an extremely healthy and active subsample often more prone to participate.

Comparison with previous work

In our dataset, the highest correlated biomarker with CA was MAP (r = 0.51, *P*< .001). MAP reflects vascular resistance and blood pressure measurements are commonly used biomarkers in BA studies (1, 61-64). However, in contrast to our study, pulmonary function (FEV_1 and FVC) consistently appears as the most significant parameter related to chronological age in these former studies (1, 61-64).In our study, FEV_1 only appears as the third most correlated biomarker (r=-0.38, *P* < .001). A possible explanation is that the biomarkers used for BA estimations rely on register-based data collected in the mid-and late 20th century, primarily representing individuals from Asia and USA. Thus, it reflects a certain time-era and population behavior, e.g., regarding smoking prevalence which has decreased since then (65). Finally, it is important also to take the difference in health behavior seen between ethnic groups into account.

To estimate BA, we used the first principal component as a general aging factor. In the field of BA prediction models, PCA is considered an improvement compared to multiple linear regression (48). Even so, PCA is still a linear model thereby assuming that biomarkers change linearly throughout the age span (66). While many biomarkers are assumed to decline with a slope of 1% per year (67), some biomarkers may deviate from this linearity especially towards the higher end of the age span. The proportions of total variance explained by the PCA in our study (31% and 25% women and men, respectively) were similar to

those found in other studies using the first principal component varying from 23-42% (63, 68-70) in women and 20-37% in men (61, 62, 68-71). These studies found that using PCA was valid and clinically useful. However, more recent studies comparing different algorithms, found that the less frequently used algorithm by Klemera and Doubal (KDM) (72, 73) are more stable and better at predicting mortality outcomes (5, 37, 74). Keeping in mind that these results also depend on the specific set of biomarkers included, the algorithm by KMD should be included in future research on the present BA estimation.

Future research

This is a first-generation model why this work should be used to initiate further research to understand the interpretation of the model fully. A larger sample size is necessary to do a proper sensitivity analysis on how changes in each biomarker affect the BA estimate. In addition, a larger sample size would improve the validity of the selected biomarkers. In this study, the biomarkers were selected based on their significant correlation with CA in a cross-sectional analysis. Using cross-sectional data provides information on the age difference in the biomarkers at a specific point in time. To improve the statistical validity of the measures selected as biomarkers, a significant longitudinal correlation with CA should be investigated. This way the age difference in the biomarkers can be assessed over time (9).

Applying the BA model to longitudinal data is an important future investigation, to see if a relative high BA is a predictor of poor health outcomes like type 2 diabetes, cardiovascular disease and mortality. Furthermore, investigating the BA model in health-related interventions will provide evidence if the BA model can be used as a valid clinical tool for measuring disease risks. Our study has strength in its reproducibility— a key element for biological age applicability. The nine biomarkers are common measurements in the clinic and in science, where standard quantitative techniques are used. Thus, quantifying BA by the combination of these nine biomarkers has the advantage of being less susceptible to artefactual variations related to the method of measurement and being accessible from stored plasma samples and databases in national health registers.

Conclusions

The nine physiological variables identified in this study as aging biomarkers are highly relevant to assess age-related changes affecting the risk of disease and physical capacity. We consider the BA model appropriate for clinical use, due to low technical difficulty and minimally invasive techniques. Estimation of BA has potential as an outcome measure in health-promoting interventions and as a pedagogical aid. Future research is required to investigate how the model will work in populations deviating from the healthy aging spectrum (e.g., individuals with diabetes, or CVD or low cardiorespiratory fitness). We expect

that the indicator of being biologically old is easy to understand, as a risk of disease and premature mortality, why this indicator might drive individual motivation towards a healthier lifestyle. However, work remains to be done to improve the validity as a clinical tool and the model's predictive abilities including, but not restricted to re-analysis of the model in a much larger sample size, test-retest reliability and assessment of the longitudinal stability of the biomarkers.

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Author contributions

KH and JWH conceptualized the study and in collaboration with ABK, K-ÅH, HBDS and JCBJ the study was designed. KH, MF, PH and AB collected the data. KH and ABK did the formal analysis. KH wrote the first draft, and ABK, K-ÅH, JCBJ, HBDS, FD and JWH revised and edited the manuscript. This work was supported by the Copenhagen Center for Health Technology, the Center for Healthy Aging and the University College Copenhagen. The sponsors had no involvement in the study design, writing of the manuscript or choice of publication.

Conflicts of Interest

None declared.

Abbreviations

BA: biological age

BAS: biologcial age score

Bac: corrected biological age

CA: chronological age

PCA: principal component analysis

1PC: first principal component

VO_2max: maximal oxygen consumption

FBG: fasting blood glucose

HbA1c: glycated hemoglobin

HDL: high density lipoprotein

LDL: low density lipoprotein

TC: total cholesterol

suPAR: soluble urokinase plasminogen activator receptor

SBP: systolic blood pressure

DBP: diastolic blood pressure

MAP: mean arterial pressure

FEV1: forced expiratory volume within the 1. second

FVC: forced vital capacity

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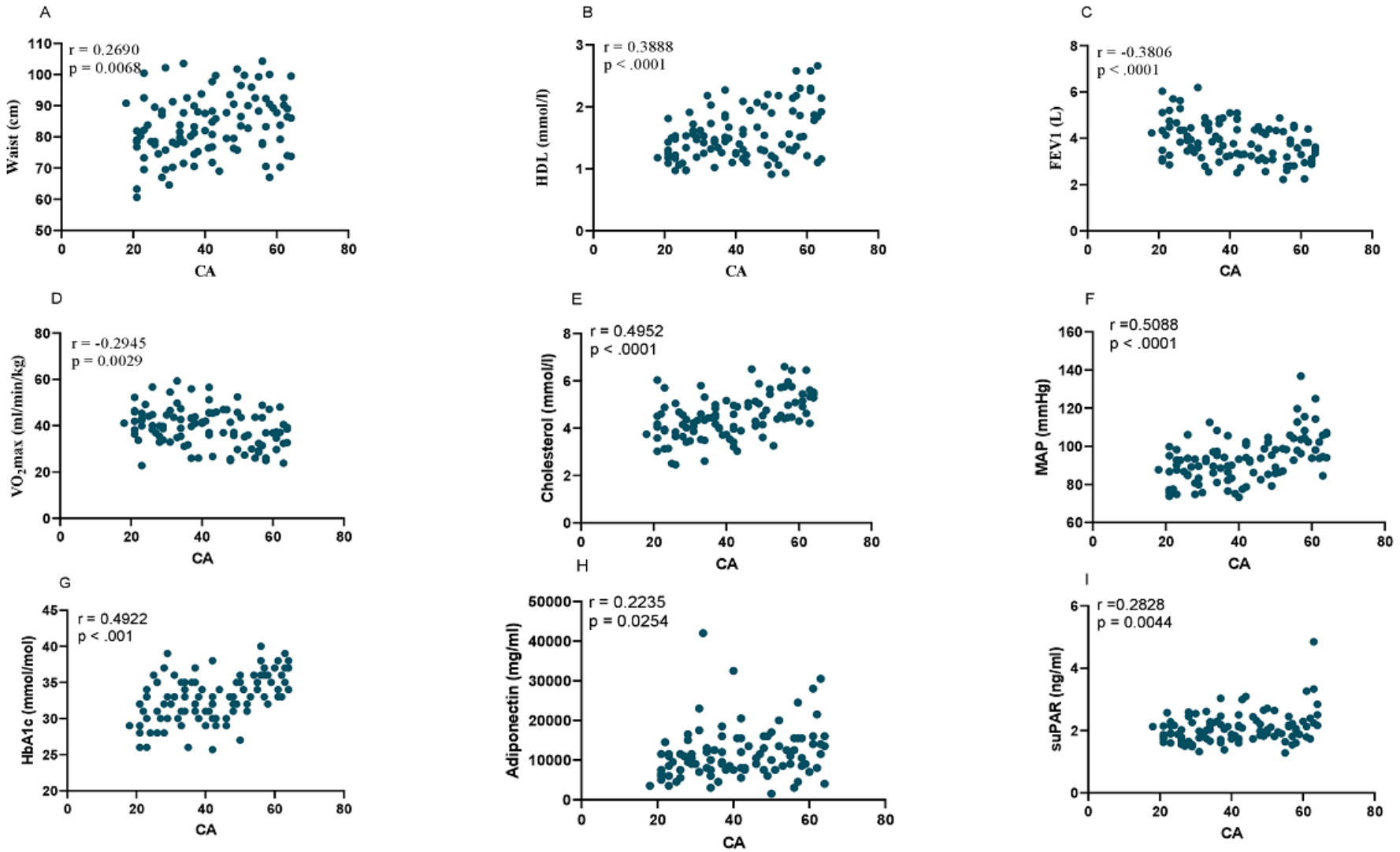
Suppl. **Table 1.** Candidate biomarkers measured in the study participants (n=100) and their correlation with chronological age

Biomarkers	Pearson's r	*P*
Body composition		
(1) Weight, *kg*	0.028	.79
(2) Waist circumference, *cm*	0.269	.007
(3) Hip circumference, *cm*	-0.003	.98
(4) Waist/Hip ratio	0.365	.0002
(5) Fat mass, *%*	0.156	.12
(6) Muscle mass, *kg*	-0.068	.50
Metabolic health		
(7) Fasting blood glucose, *mmol/l*	0.315	.001
(8) HbA1c, *mmol/mol*	0.492	<.0001
(9) Insulin, *pmol/l*	0.026	.79
(10) Triglycerides, *mmol/l*	0.077	.44
(11) Free fatty acids, *µmol/l*	0.152	.13
(12) Leptin, pn/mL	-0.086	.40
(13) Adiponectin, *mg/ml*	0.224	.03
(14) HDL, *mmol/l*	0.389	<.0001
(15) LDL, *mmol/l*	0.370	.0002
(16) CHOL, *mmol/l*	0.495	<.0001
(17) CHOL/HDL ratio	0.044	.66
Immune function		
(18) CRP, *mg/l*	-0.169	0.12
(19) suPAR *ng/ml*	0.283	.004
Cell blood count		
(20) Hemoglobin, *mmol/l*	0.070	0.49
(21) Hematocrit, *%*	0.112	0.28
Cardiorespiratory function		
(22) Diastolic BP	0.512	<.0001
(23) Systolic BP	0.451	<.0001
(24) FEV1, *L*	-0.381	<.0001
(25) FVC, *L*	-0.316	0.001
(26) FEV1/FVC, *%*	-0.305	0.002
Physical capacity		
(27) VO_2max, *ml/min/kg*	-0.294	0.003
(28) STS, *stands*	-0.176	0.08
(29) Handgrip strength, *kg*	-0.115	0.25
(30) Bicep strength, *kg*	-0.155	0.13
(31) Quadriceps strength, *Nm*	-0.174	0.03

Abbr.:HbA1c; g*lycosylated hemoglobin type A1c,* AGEs*; Advanced glycation end products,* HDL; *High density lipoprotein*, LDL; *Low density lipoprotein*, CHOL; *Total cholesterol*, CRP; *C-reactive protein,* suPAR; *soluble urokinase plasminogen activator receptor*, BP; *Blood pressure*, FEV1; *Forced expiratory volume in first second*, FVC; *Forced vital capacity*, STS; *30 sec. sit-to-stand chair rise*, VO_2max; *maximal oxygen consumption. Missing values was present in CRP (n=87), Hematocrit (n=97) and Hemoglobin (n=99) and Bicep strength (n=98).*



Suppl. Figure 1. Correlation coefficient with chronological age for the nine measurements included as biomarkers in the BA model. A: Waist circumference (cm), B: High Density Lipoprotein (mmol/L), C: Forced Expiratory Volume in the first second (L), D: Maximal oxygen consumption (ml/min/kg), E: Total cholesterol concentration (mmol/L), F: Mean Arterial Pressure (mmHg), G: Glycated hemoglobin (mmol/mol), H: Adiponectin (mg/ml), I: soluble urokinase plasminogen activator receptor (ng/ml).

Paper IV

Proof of concept and change in biological age following 15-weeks of lifestyle intervention.

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Abstract:

Introduction The global burden of chronic disease is expected to rise due to the combination of increasing life expectancy and obesity prevalence. Biological age (BA) can be used to identify individuals with a high risk of future incidence of chronic disease, and as a tool in health promotion. Based on a healthy aging reference population we have developed a BA model consisting of 9 biomarkers representing cardiorespiratory, cardiovascular, inflammatory and respiratory functions. This study serves as the first proof of concept in assessing the clinical utility of the BA-model.

Methods BA was measured in overweight and obese women and men participating in a 15-week lifestyle intervention. The intervention was carried out at Ubberup folk high school. BA of Ubberup participants was compared to the healthy aging reference group at baseline. Changes in weight, BA and single biomarkers after the intervention were analyzed, and the relationship between BA and established indicators of future risk of chronic disease was investigated (BMI and HOMA-IR).

Results Compared to the healthy aging reference group, BA was consistently higher across the age spectrum in women (p<0.0001). Both women and men had a clinically relevant weight loss (*W:* 9% IQR: 7% to 10% *M*: 10% IQR: 5% to 13%) and a decrease in BA of -4.1 years (95% CI: -2.1 to -6.1; p= 0.0006) and -16.4 years (95% CI: -23.4 to -9.3; p=0.0007) in women and men, respectively. We found that BA and BMI were associated (r=0.5, p=0.01), and BA increased 1.5 years (95% CI: 0.4 to 2.7) for every unit

increase in BMI. This can, conversely, be interpreted as an improvement in BA of 1.5 years with one unit reduction in BA i.e., a weight loss, in individuals with overweight or obesity.

Conclusions This study show initial evidence that the BA-model is a useful measure for the assessment of lifestyle interventions and to predict risk of future chronic disease in young and old individuals.

Introduction

In general, the global increase in life expectancy is a success, reflecting less mortality due to infectious diseases, malnutrition and accidents (1). Because aging, however, is the primary risk factor for chronic disease the increase in life expectancy will impact the prevalence of individuals living with cardiovascular disease, type 2 diabetes, cancer and osteoarthritis to mention a few (2). Unfortunately, healthy life expectancy has not increased at the same pace as life expectancy, and disability related to chronic diseases has not reduced markedly (3). Studying longevity, the WHO stated that from 2000 to 2019 life expectancy has increased by 6.6 years compared to healthy life expectancy that increased 5.4 years worldwide (4). Unfortunately, the increase in healthy life expectancy was primarily driven by declining mortality rates instead of fewer years lived without chronic disease and disabilities (4). Presumably, the fast-growing obesity epidemic contributes to this discrepancy, as obesity also increases the risk of chronic disease and disability (5). As a result, chronic diseases have been and still are a major target in biomedical sciences (6).

In order to decrease the global burden of chronic disease, risk stratification tools is used to identify those individuals at high risk for future manifestation of disease (7). BA is a concept utilizing the healthy aging trajectory as the basis of comparison, to assess individual general health, risk of disease and predict life expectancy (8). The considerations for using biological age in health care are: 1) it explores the heterogeneity in the aging process and the risk of disease on a continuous scale 2) it provides an intuitively meaningful outcome that is easily translated into the risk of disease and mortality 3) it can be

used to evaluate health-promoting interventions 4) it can be used to motivate to adhere to risk-reducing behaviour.

In a previous study, we developed a BA model based on biomarkers representing central mechanisms of the age-related physiological changes in bodily functions, essential in the maintenance of health and prevention of chronic disease, technically simple to measure, easy to reproduce and minimally invasive (Husted et al., JMIR Aging, 2022, *Currently in peer-review*). The model combines 9 biomarkers including waist circumference, glycated hemoglobin (HbA1c), mean arterial blood pressure (Map), High-Density Lipoprotein cholesterol (HDL), Total Cholesterol (TC), Adiponectin, soluble urokinase Plasminogen Activator Receptor (suPAR), Forced Expiratory Volume in the 1 second (FEV1) and relative maximal oxygen uptake (VO_2max).

Waist circumference is a valid surrogate measure for visceral adipose tissue (VAT) (9). Individuals with high VAT are more prone to chronic low-grade inflammation and dyslipidemia including low levels of HDL-C (10). Chronic low-grade inflammation plays an important role in the development of CVD, cancer and T2D (11). Among other things, chronic low-grade inflammation suppresses adiponectin (12) and an inverse relationship between obesity, aging and plasma levels of adiponectin has been found (13). Adiponectin mediates insulin sensitivity by increasing fat oxidation by skeletal muscle thereby decreasing circulating plasma FFA (14). The amount of VAT is causally related to inactivity (15). With age physical inactivity increases, enhancing the age-related decline in maximal oxygen uptake due to decreased cardiac output and muscle arterial-venous oxygen uptake (16, 17). In addition, low physical activity enhance the age-realted loss of muscle mass and quality leading to sarcopenia (18). Thus, physical activity is one of the main strategies for maintenance of physical function and avoiding chronic diseases with increasing age (19). In addition, physical activity has positive effects in general ,but not always, on blood pressure (20, 21).

Altogether, we would expect that the BA model is sensitive towards lifestyle interventions focusing on weight loss through physical activity and healthy dieting. Therefore, this study aimed to provide the first concept of proof that the BA model can be used to identify high-risk individuals and evaluate the clinical relevance of biological age using a 15-week lifestyle intervention as a model for a health enhancing intervention.

We hypothesize that 1) baseline BA of the course participants is higher compared to the reference group and 2) BA will decrease after the 15-week lifestyle intervention.

Methods

The intervention

The lifestyle intervention took place at Ubberup Højskole, a Danish folk school, where the participants stay in residence throughout the 15 weeks with the opportunity to go home for the weekends.
The course participants paid 200-350 € /week, plus loss of income albeit some was supported by social welfare. A daily program was scheduled from early morning 7.00 AM until 4.00 PM. The program consisted of three core elements: 1) supervised training (1-3 hours/day) mainly including aerobic exercise (walking, cycling, dancing, ballgames) but also some resistance training (circuit training); 2) class-based theoretical teaching on behavioural changes and effects of a healthy lifestyle and 3) individual cognitive therapy. In addition, all meals were provided for and based on a healthy mixed diet following an estimate of average macronutrients as percent of energy as follows: 55-60 E% carbohydrates, 15-20 E% proteins and <30 E% fat. In terms of weight loss, course goal is, to reach a weight loss of approximately 10% from baseline.

Participants

We recruited participants among the 80 women and men signed up for the 15-week lifestyle intervention. Inclusion criteria was age between 18-65 years. Based on the purpose of the stay, lifestyle diseases were expected and medication for those accepted, except for the use of beta-blockers. Pregnancy also excluded participation. We informed participants orally and in writing about the study protocol and potential risks, before written consent was obtained. The study was approved by the local research ethics committee, Copenhagen, Denmark (H-19073643; Clinical Trial Number: NCT04279366) and performed in accordance with the Helsinki declaration.

The biological age test protocol

We carried out the test protocol at the site of the folk high school.

Blood pressure

Lying on the back, blood pressure was measured three times using an automatic monitor (BoSo Medicus Control, BOSCH + SOHN GmbH) with two minute between each measurement. We used mean systolic and diastolic blood pressure (SBP and DBP) to calculate MAP:

$$MAP = (^{2}/_{3} * DBP) + (^{1}/_{3} * SBP)$$

Blood sample

For the analysis of adiponectin, TC, HDL, triglycerides, fasting glucose and suPAR a venous blood sample was collected. Blood samples were obtained in the morning after an overnight fast.. The participants were asked to restrain from smoking and from physical activity in the morning before the blood sample.

Glycated hemoglobin (HbA1c) was analyzed on site on whole blood (Bayer DCA 2000+ , Bayer Healthcare, Elkhart, IN, USA). At the same time, blood samples was centrifuged at 2500 G at 4°C for 10 minutes and plasma was collected and stored at -80°C for later analysis. Plasma glucose, triglycerides

and total cholesterol concentrations were analyzed on COBAS (COBAS 6000, C 501, Roche Diagnostics, Mannheim, Germany). Adiponectin concentrations will be analyzed using RIA kit (Millipore, MA, USA). Plasma concentrations of suPAR were analyzed using the commercially available suPARnostic® kit in accordance with the manufacturer's instructions (ViroGates, Copenhagen, Denmark).

Waist circumference

Waist circumference was measured twice, using a tape measure, at the narrowest place between the lowest ribbon and the crista iliaca at the end of an expiration.

Spirometri

Sitting on a chair wearing a nose clip, participants breathed normally into a handheld spirometer (VyntusTM SPIRO spirometer, North Riverwoods, USA), before performing a maximal inspiration immediately followed by an expiration with maximal effort. The expiration continued until exhaustion while maintaining an upright position. This was repeated a minimum of three times and a maximum of seven, to obtain the highest forced vital capacity (FVC) and forced expiratory volume in the first second (FEV1). To assess the validity of the tests the VyntusTM SPIRO software (SentrySuite) automatically assessed the repeatability, acceptability and usability criteria defined by the American Thoracic Society and European Respiratory Society (22).

Graded exercise test.

A graded exercise test was performed on an electromagnetically braked bicycle (Monarch 839E, Varberg, Sweden) to determine maximal oxygen consumption (VO_2max) using continuous gas exchange measurements obtained breath by breath and sampled into 10 seconds intervals by an automated online system (Quark PFT, Cosmed). Participants were instructed to perform an all-out effort and were cheered on in the final stages of the test. The protocol began with a 4 min warm-up period of 30 and 50 Watt for women and men, respectively. This was followed by a 20/25 W (women/men) increase in load

every minute until voluntary exhaustion. The protocol was designed to reach exhaustion after 8-12 minutes. Throughout the exercise test, heart rate was continuously monitored (Garmin Vivoactive, Garmin International Inc., Olathe, Kansas, USA) and perceived rate of exhaustion (RPE) was noted at the end of each workload (23). Plateauing of VO_2 (≤ 150 ml O_2/min increase between two workloads) was used as the primary criteria and respiratory exchange ratio ≥ 1.15 (CO_2 expired/ O_2consumed) and maximal HR (≤±10 beat from predicted maximal HR (220-age)) were used as secondary criteria to assess the validity of the test. The highest VO_2 value measured over 30 consecutive seconds determined VO_2max (ml/min/kg).

Healthy aging trajectory

Reference group

BA of the reference group serve as the base of comparison and constitutes a sample of 100 women (n=51) and men (N=49) in the age range 18-65 years free from disease. Across gender and age categories relative VO_2max was moderate to high and mean BMI was 24 kg/m^2 (± 4 kg/m^2 SD) and 25 (± 3 kg/m^2 SD) in women and men, respectively (24). Medication use constituted: birth control pills n=10 and allergy medication n= 3. Measurements of the nine biomarkers were obtained in the reference group (Table 1).

Table 1. Biomarkers included in the BA model

Biomarkers	**Units**
Body composition	
Waist circumference	*cm*
Metabolic health	
HbA1c	*%*
MAP	*mmHg*
HDL-C	*mmol/L*
TC	*mmol/L*
Adiponectin	*mg/mL*
Inflammation	
suPAR	*ng/ml*
Cardiorespiratory function	
FEV1	*L*
VO_2max	*ml/min/kg*

Abbr.: *HbA1c*: glycated hemoglobin; *MAP*: mean arterial pressure; *HDL-C*: high-density lipoprotein cholesterol; *TC*: total tholesterol; *suPAR*: soluble urokinase plasminogen activator receptor; *FEV1*: forced expiratory volume in the 1 second; *VO_2max*: maximal oxygen uptake.

Estimation of biological age

The BA model was established through the reference population employing principal component analysis (PCA) to the nine biomarkers of aging. Employment of PCA revealed the individual contribution (expressed by factor loadings) of the nine biomarkers to the latent BA estimate. We observed that the factor loadings were different for women and men (Husted et al., JMIR Aging, 2022, *in review*). Based on this observation, together with the difference in life expectancy and the sexual dimorphisms especially related to menopause in women, we thus formulated the BA equations on sex-specific basis:

$$BAwomen = -56.67 + 0.27 \cdot MAP + 1.02 \cdot HbA1c + 0.1453 \cdot Waist - 2.03 \cdot FEV1 - 0.43 \cdot VO2\,\max + 0.0003 \cdot Adiponectin + 7.39 \cdot HDL + 4.06 \cdot CHOL + 3.24 \cdot suPAR + 0.20 \cdot CA$$

and

$$BAmen = -70.37 + 0.34 \cdot MAP + 0.95 \cdot HbA1c + 0.60 \cdot Waist - 3.96 \cdot FEV1 - 0.62 \cdot VO2\,\max - 9.73 \cdot 10^{-5} \cdot Adiponectin - 0.57 \cdot HDL + 4.06 \cdot CHOL + 7.61 \cdot suPAR + 0.32 \cdot CA$$

By these equations, BA in Ubberup participants is measured by multiplying individual clinically measured biomarkers to subgroups of specific coefficients. Because the specific coefficients are scaled to standard deviations of the related biomarkers, the relative contribution to the linear combination is not entirely transparent in the equations above. Therefore, we show the relative contribution of each biomarker in percentage in Table 2.

Table 2 The relative contribution of each biomarker to the BA estimation

Women		**Men**	
Biomarker	Contribution (%)	Biomarker	Contribution (%)
TC	21.8	Waist circumference	24.1
MAP	18.9	Maximal oxygen consumption	22.6
Glycated hemoglobin	16.7	Total cholesterol	14.5
High-density lipoprotein	15.2	Mean arterial blood pressure	12.2
Maximal oxygen consumption	11.6	Glycated hemoglobin	10.5
suPAR	5.7	Forced expiratory volume in 1. sec.	9.5
Adiponectin	5.2	suPAR	6.4
Waist circumference	3.0	Adiponectin	0.2
Forced expiratory volume in 1. sec.	1.9	High-density lipoprotein	0.04

Abbr.*: TC*: Total cholesterol, *MAP*: Mean arterial pressure, *HbA1c*: glycated hemoglobin, *HDL-C*: High-density lipoprotein cholesterol, Maximal oxygen consumption, *suPAR*: soluble urokinase plasminogen activator receptor, *FEV1*: Forced expiratory volume within the 1st second. Modified from Husted et al., JMIR Aging, 2022, *Currently in review.*

Additional measurements

Body composition

We measured fat percentage and muscle mass using 4-point bioelectrical impedance (Tanita, BC420s, Illinios, USA). In addition, Tanita MC780 was used to obtain MetabolicAge® (MA). The Tanita MC780 estimates individual MA by comparing basal metabolic rate (BMR) with the average BMR for people of the same age (25).

Strength

We measured grip strength by a handheld dynamometer (Handgrip, Takei Grip-D TKK5401, Japan). Participants stood with arms by the side and a bit away from the body. The dynamometer was held in the dominant hand and adjusted to individual hand size. Maximal compression was applied, and the highest value (kg) recorded.

Blood sample

Fasting plasma insulin and glucose concentrations were analyzed on COBAS (COBAS 6000, C 501, Roche Diagnostics, Mannheim, Germany) and used for the assessment of insulin resistance using the homeostatic model assessment of insulin resistance (HOMA-IR). HOMA-IR was calculated using fasting insulin concentration times fasting glucose concentration divided by 22.5. We used a conversion factor of 6 to convert insulin from pmol/L to mIU/L.

Statistics

Baseline comparison of BA between course participants and the reference group was tested by comparison of slopes and intercepts using ANCOVA and group-level differences by unpaired t-test. Comparison of pre/post differences between continuous variables was tested using paired t-test and Fisher's exact test was used to analyse changes in frequency of metabolic syndrome. Missing data include the lack of blood plasma samples from one woman and two men, why their biological age and HOMA-IR were not estimated.

During the 15 weeks, COVID19 escalated and extra precautions had to be taken at follow-up. Specifically, this resulted in harsh cleaning of the flowmeter (used for the online ventilatory flow measurements) in Rodalon between each test, followed by fast-drying procedure using a hairdresser and a filter between the flowmeter and mouthpiece. Unfortunately, these necessary precautions have made these follow-up measurements unreliable. Despite an increase in test time/maximal workload, we found no increase or even a decrease in oxygen consumption (Suppl. Fig. 1). Assuming that their cycling efficiency is the same or in any case better at follow-up, this observation is theoretically very unlikely (26). Therefore, we used the absolute measurements of VO_2max (ml/min) at baseline and the VO_2-work rate related oxygen consumption to estimate their VO_2max at follow-up. In 15 of the 28 baseline VO_2max test, we observed a plateau of oxygen uptake occurring between the two final workloads (< 150 ml O_2). In the 13 tests without a levelling off in oxygen consumption, we measured an RER value ≥ 1.15 and the remaining two fulfilled the criteria for maximal HR. Based on the baseline VO_2max measures, we used a theoretical VO_2-work rate relation of 9 ml/O_2/W/min corresponding to a work efficiency of approximately 25% to calculate VO_2max at follow-up (27).

Data are presented as means with standard deviations ±SD or medians with interquartile range (IQR) in case of skewed distribution. In the case of non-normality, data was transformed to normality by log10 transformation. If log transformation failed to normalize data Mann Whitney test was applied for the comparison of medians between unpaired data. A *p*-value of ≤ 0 .05 was set as the level of significance. Statstical analysis and graphical presentations were conducted in SAS Enterprise Guide 7.1 and GraphPad Prism 9.

Results:

Participant characteristics

At baseline, 40 individuals (21 women and 19 men) from the folk high school volunteered. At the end of the 15-week lifestyle course, 13 participants (5 women and 8 men) where lost to follow-up (Figure 1).

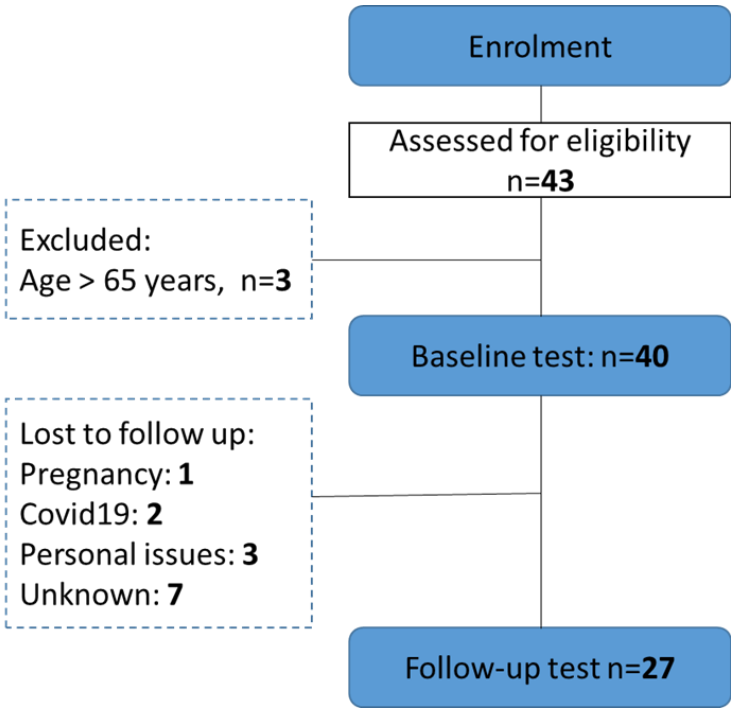

Fig. 1. Flow chart of the recruitment process

In total, 27 individuals (16 women and 11 men) completed the 15-week lifestyle course. Baseline characteristics for women and men included in the study are presented in Table 3. All participants were overweight (BMI ≥25 and ≤29.9, n=2) or obese (BMI≥30, n=25) but without type 2 diabetes (cut off criteria: HbA1c ≥ 6.5%) (28). Relative VO_2max was low for most participants (n=22). In three cases VO_2max was beneath what is considered necessary to obtain independent lifestyle and associated with high risk of mortality (VO_2max ≤17.5 ml/kg/min) (29, 30). Two women had a moderate VO_2max, and one man had a high VO_2max(31, 32). No indications of chronic obstructive pulmonary disease were observed at baseline, using FEV_1/FVC ≤ 70% as a clinical indicator of airway obstruction (33). Medications use constituted: birth control pills n=4, blood pressure lowering medication: n= 3, allergy medication: n= 4, obesity medication (liraglutide): n= 1, diabetes medication (metformin) n=1, cholesterol lowering medication: n=2, asthma medication n=7, anti-depressive medication: n=1 and ADHD medication: n= 1.

Table 3 Baseline characteristics.

	Women (n=16)	**Men (n=11)**
Age (years)	35 ±14	31 ±9
Weight (kg)	106 ±22.6	133 ±24
BMI (kg/m^2)	37 ±7	38 ±5
Fat mass (%)	43 ±5	36 ±6
Muscle mass (kg)	57 ±9	80 ±12
Waist circumference (cm)	110 ±16	125 ±17
Systolic blood pressure (mmHg)	122 ±18	130 ±13
Diastolic blood pressure (mmHg)	77 ±9	80 ±9
HbA1c %	5.3 ±0.3	5.1 ±0.3
FEV_1/FVC (%)	78.3 ±8.7	78.4 ±7.8
VO_2max (ml/min)	2542 ±544	3740 ±711
VO_2max (ml/min/kg)	26 ±6	29 ±9
Smoking (frequency)	7	5

Abbr.: *BMI*: Body Mass Index; *HbA1c*: glycated hemoglobin; *FEV1/FVC*: the ratio between forced expiratory volume in the first second and forced vital capacity; *VO_2max*: maximal oxygen uptake

Biological age at baseline

The individual BA estimates are scattered above the reference group regression line representing the healthy aging trajectory, irrespective of sex (Figure 2). Comparing the slopes and intercepts of the regression lines between Ubberup participants and the healthy aging reference group, we observed that the intercept was higher ($p < 0.0001$) in Ubberup women with no difference in slopes (p=0.87).

Regarding men, the direction of the regression line was non-comparable to the regression line of the healthy aging reference group.

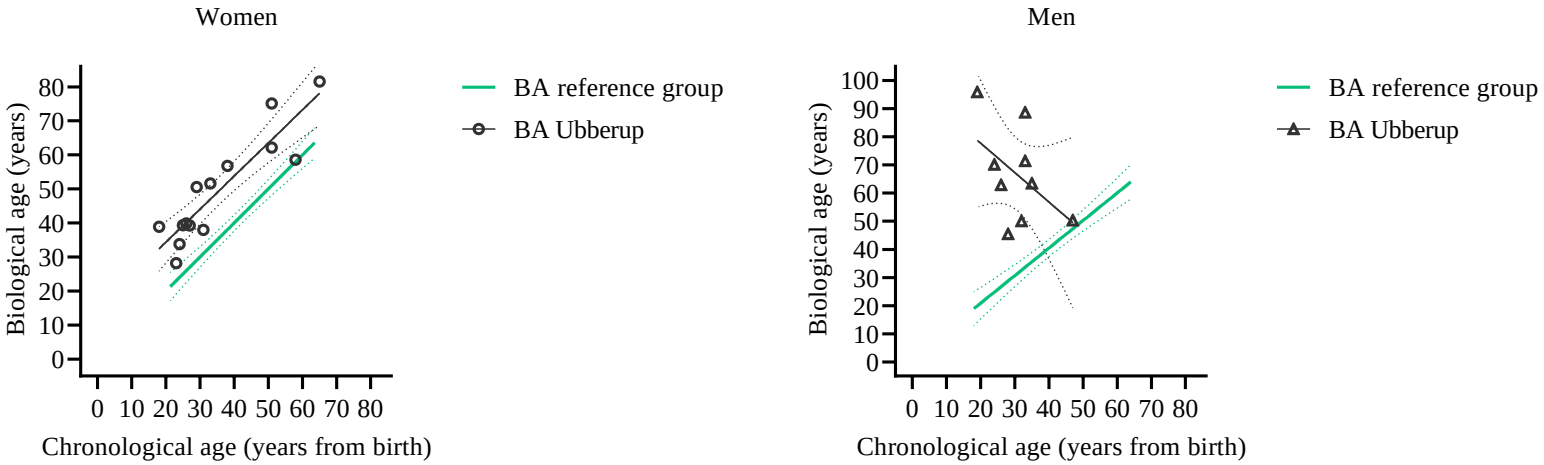

Fig.2. Scatterplot of individual biological age (BA) and the relation to chronological age. The green line represents the linear regression of the healthy aging trajectory, and the circle and triangles represent the baseline biological age values for women and men, respectively with related regression lines (black lines). Dashed lines represent 95% confidence intervals.

Effect of the intervention

At follow-up the participants achieved a weight-loss equivalent to 9% (median, IQR: 7% to 10%) and 10% (median, IQR: 5% to 13%) for women and men, respectively, concurrent with a decline in waist circumference and some loss of muscle mass. We observed that MAP decreased in men with no change in women. Both women and men improved VO_2max. Metabolically, only plasma TC decreased in men with no change in women (Table 4).

Table 4 Changes in weight, the 9 biomarkers for biological age estimation and grip strength, divided by sex.

	Women (n=16)			Men (n=11)		
	Baseline	**Follow-up**	*P*	**Baseline**	**Follow-up**	*P*
Weight (kg)	102 (88; 122)	93 (81; 109)	<.0001	130 (115; 152)	116 (109; 132)	0.0001[a]
Muscle mass (kg)	57 ± 9	56 ± 8	0.01	80 ± 12	78 ± 10	0.03
MAP (*mmHg*)	92.0 ± 11.8	92.0 ± 12.5	n.s.	97.0 ± 9.3	92.5 ± 8.4	0.002
HbA1c (*mmol/mol*)	33.9 ± 2.7	33.9 ± 2.7	n.s.	32.5 ± 3.1	32.5 ± 3.1	n.s.
Waist (*cm*)	110 ± 16	99 ± 14	<.0001	125 ± 17	109 ± 13	0.0004
FEV1 (L)	3.1 ± 0.7	3.0 ± 0.6	n.s.	4.4 (3.9 , 5.2)	4.3 (3.7, 5.1)	n.s.[a]
VO_2max (*ml/kg/min*)	25.5 ± 6.4	29.3 ± 6.7	<.0001	29.1 ± 8.8	33.8 ± 10.7	0.0006
Adiponectin (*mg/mL*)						
HDL-C (*mmol/L*)	1.1 (1.01; 1.32)	1.1 (0.96; 1.31)	n.s.[a]	1.2 (1.05; 1.27)	1.1 (0.9; 1.3)	n.s.[a]
TC (*mmol/L*)	4.5 ± 1.2	4.7 ± 1.0	n.s.	4.3 (4.2 - 4.8)	3.8 (3.2 - 4.5)	0.004[a]
suPAR (*ng/ml*)	2.9 ± 0.8	3.1 ± 1.0	n.s.	2.3 (2.1, 2.7)	2.3 (1.9, 3.6)	n.s.[a]
Grip strength (*kg*)	33 ± 5	33 ± 5	n.s.	49 ± 9	48 ± 9	n.s.
Metabolic syndrome (n)	7	4	n.s.	3	2	n.s.

Abbr.: *MAP*: Mean Arterial Pressure; *HbA1c*: Glycated Hemoglobin*; FEV1*: Forced Expiratory Volume in the 1. Second; *VO_2max*: maximal oxygen uptake; *HDL-C*: High-Density Lipoprotein Cholesterol; *TC:* Total Cholesterol; *suPAR*: soluble urokinase plasminogen activator receptor. Metabolic syndrome was diagnosed using the International Diabetes Federation definition.

Missing values W*omen (W)*: HDL-C n=14, TC n=14, *Men (M)*: suPAR: n=10, TC n=9, HDL n=9, Metabolic syndrome: *W* n=2, *M* n=2; Adiponectin *W* n=16, *M* n=11 [a] log10 transformation was applied. Normal distributed data are represented as Mean ± SD, and log-transformed data as Medians (IQR).

BA was improved with -4.1 years (95% CI: -2.1 to -6.1; *p*= 0.0006) and -16.4 years (95% CI: -23.4 to -9.3; *p*=0.0007) for women and men, respectively. In comparison, no change was observed for MA as only three women and two men had a change in their MA after the 15-week intervention (Figure 3).

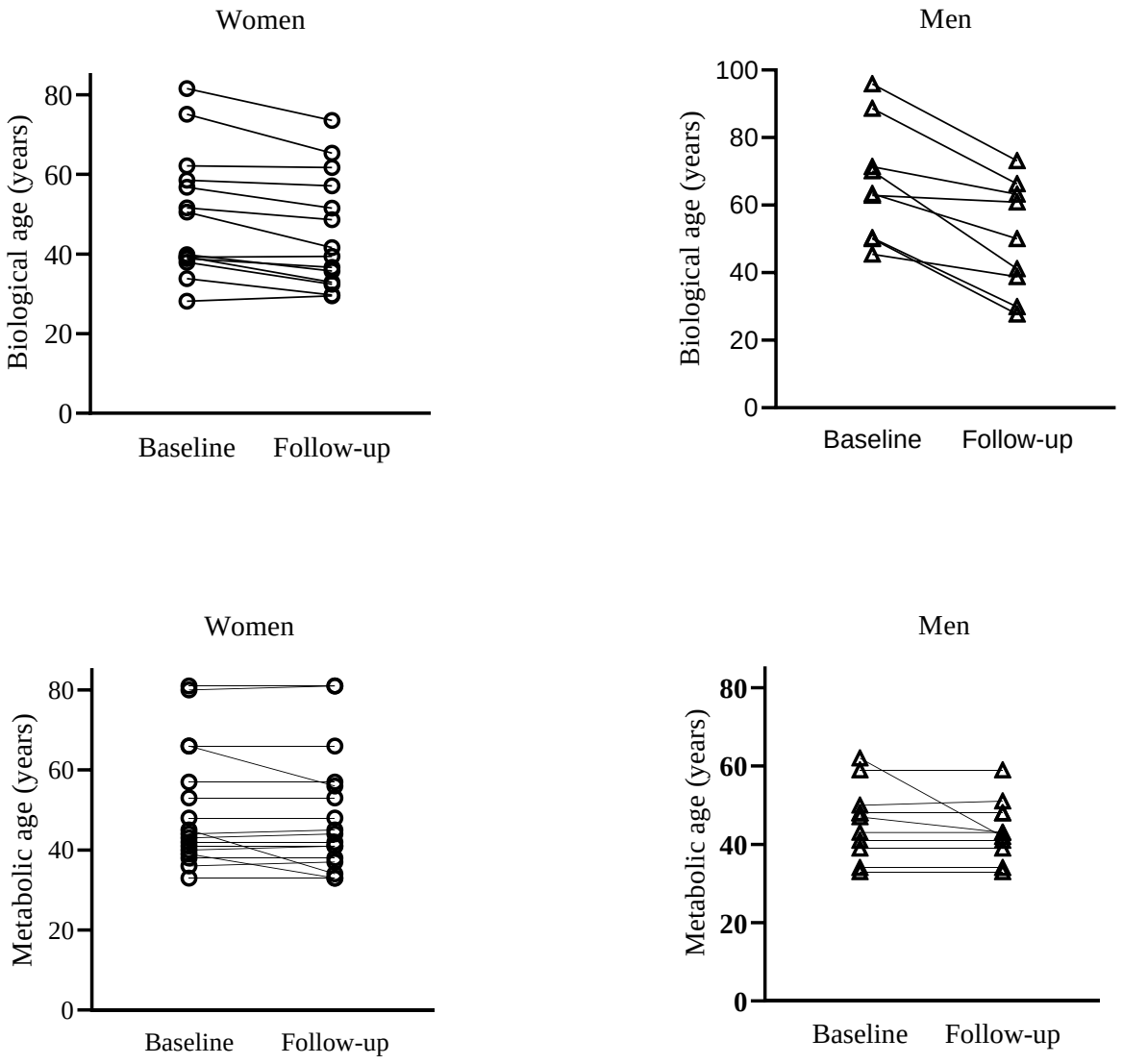

Fig.3. The difference in biological age (top row) and metabolic age (bottom row) at the beginning of the intervention (Baseline) and 15 weeks later (Follow-up).

Biological age and clinical relevance

We found a positive association between BA and BMI (r = 0.52, *p*=0.01). The linear regression indicates that for each BMI point increase above BMI of 25, BA increases by 1.5 years (95% CI of the slope: 0.4 to 2.7). A similar association was not found between BMI and chronological age (r=0.08 *p*=0.7) (Figure 4). A positive association is also observed between BA and HOMA-IR (r = 0.48, *p*=0.02) (Figure 5).

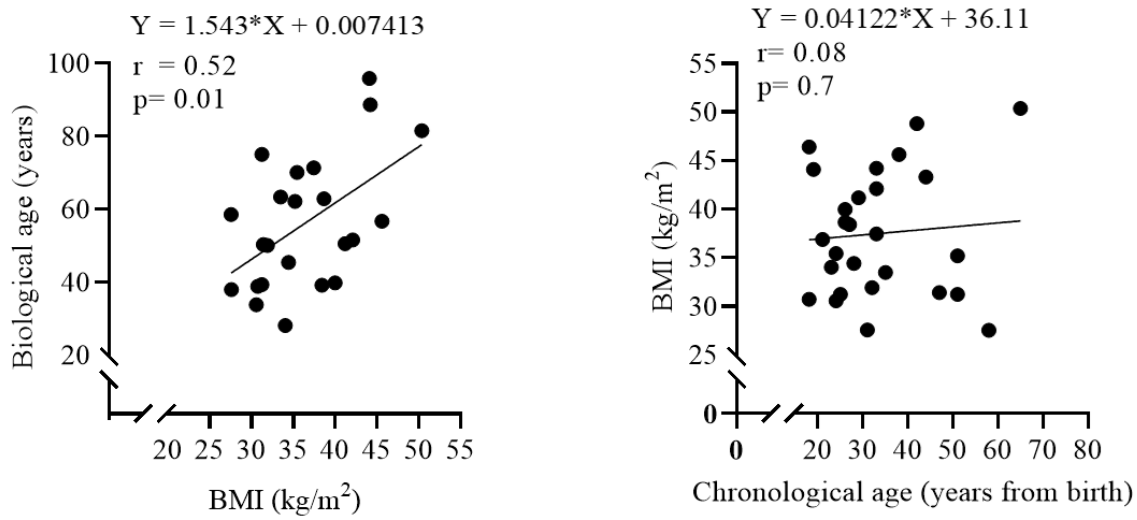

Fig.4. Linear regression and Pearson's correlation between A) biological age (BA) and body mass index (BMI) and B) BMI and chronological age (CA). Pooled analysis n=22

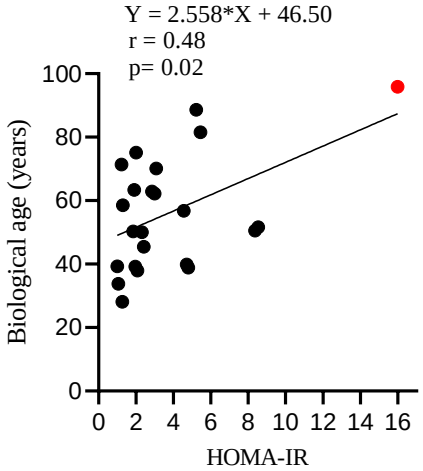

Fig. 5. Linear regression and Pearson's correlation between biological age (BA) and the homeostatic model assessment of insulin resistance (HOMA-IR). The red dot represents a highly influential observation to the correlation analysis. Pooled analysis n =22.

Discussion:

This is the first study assessing the clinical utility of the specific BA model. We found that BA could discriminate between healthy and high-risk individuals, and that BA improved following a clinically relevant weight loss. Finally, BMI and BA was highly related indicating the potential predictive ability and clinictal relevance of the BA model.

As hypothesized BA was scattered above the healthy aging trajectory both in women and men. Due to the limited sample size and age range (18-47 years) in men, comparison of regressions lines were only possible for women. The parallel upward shift in the regression line indicates an accelerated rate of aging in Ubberup women compared to healthy women of the same age (the reference group).

Principal findings

Following the National Heart, Lung and Blood Institute guidelines, a 10% weight loss is considered clinically relevant (34). In addition, smaller weight loss down to 5% in individuals with obesity have been shown to impact on risk factors for chronic diseases (35, 36). A clinically relevant weight loss induces improvements in blood pressure (37) and metabolic risk factors such as improved cholesterol profile (38), improved glucose tolerance (39, 40) as well as decreased levels of inflammatory markers such as C-reactive protein (41, 42). In this present study, the relatively novel inflammatory marker suPAR was used. suPAR have been shown to be a risk factor for CVD and associated with lifestyle behaviour (diet, physical activity and smoking) in population-based cohort studies (43-45).

In the present study, the 15-week intervention yielded a similar weight loss for women and men (p=0.8) of approximately 10%. This clinically relevant weight loss was accompanied by an improvement in BA, however, the impact on BA was 4-fold greater in men compared to women (-16.4 years versus -4.1 years, respectively). This is partly explained by the sex difference in the biomarker contribution to BA estimation. For example, both women and men reduced waist circumference and increased their

VO_2max. However, in the BA model for men VO_2max and waist circumference are the two most influential biomarkers contributing by 22.6% and 24.1%, respectively, whereas in the BA model for women the same biomarkers contribute by 11.6% and 3.0%, respectively. Only men improved their TC and MAP, additionally contributing to the difference in the BA change observed at at follow-up.

PCA was used as an objective method to weight the biomarkers against each other in the effort to combine the biomarkers into one BA measure. The physiological validity of the weighting can be discussed. Poor VO_2max is a measure of low cardiorespiratory fitness (CRF) and a risk factor for age-associated chronic diseases such as type 2 diabetes and particularly cardiovascular disease, in both sexes (46-48). In fact, poor VO_2max seems to outperform smoking and hypercholesterolemia as predictors of mortality (19). However, a lower relative CRF level in women compared to men have been shown to associated with the same absolute mortality risk (49). This indicates that women tolerate an absolute lower CRF level better compared to men and support the difference in the weighting of VO_2max in the present BA model. On the other hand, the VO_2max decline of 6-7% per decade, is expected to be similar between sexes (48). Altogether, a moderate difference between sexes in the contribution of VO_2max to the estimation of BA seem reasonable from a physiological point of view but should be investigated further in a larger sample size.

Waist circumference is a surrogate measure of VAT. As previously mentioned, VAT is an important risk factor for chronic disease development. The difference in waist circumference is relevant from an age-related sexual dimorphism point of view and in line with some BA models including waist circumference (50) but not in others (51). Due to the influence of sex hormones, the accumulation of adominal adipose tissue is more predominant in men and associated with the risk of CVD (9). Waist circumferences increase with age in both women and men, but more in men in combination with a weight gain (52).

Clinical relevance

The clinical relevance of the BA model is related with its ability to estimate risk. The continuous BA model does not provide an absolute risk directed at a specific outcome and separates from for example Framingham risk score predicting the absolute risk of CVD (53). An inherent issue with absolute risk prediction is that young adults have low absolute risk predictions despite a high relative risk (54). This is problematic as young adults with chronic disease risk factors (i.e., high relative risk) would benefit from early interventions to change health behaviour to reduce the absolute risk of chronic disease at an older age. Instead, risk prediction of younger adults with high relative risk might better be assessed and guided by their biological age. The reverse can be the case for older individuals predicted with a high absolute risk of future CVD, without consideration of for example physical activity level as a confounding factor. Here too, biological age might be a more useful way to assess health risk.

As an initial validation of the BA model, we assessed the relationship between BA and measures associated with CVD and type 2 diabetes. We demonstrated that the BA model was positively associated with the degree of overweight and obesity and insulin resistance. The association found between BA and HOMA-IR is, however, highly influenced by a single observation marked with red (Figure 4). The correlation found between BA and HOMA-IR disappears when excluding this observation from the dataset (r=0.2, p=0.37). The observation belongs to a young male with clear evidence of insulin resistance. This is not, however, an argument for excluding the observation in the analysis. Instead, the association between BA and HOMA-IR should be interpreted with caution and in any case, reproduced with a larger sample size to confirm the relationship.

The MA by Tanita is used by fitness and medical professionals to assess body composition and metabolic health (25). The personal at Ubberup uses it as a pedagogical aid to summarize the participant's risk related to their body composition and to assess the success of the lifestyle intervention. We demonstrate that in contrast to BA, the 15-week intervention resulting in a clinically significant weight

loss had no impact on MA. Besides age, sex, height and training status the estimation of BMR, and thereby MA, primarily relies on the amount of muscle mass (25). Specifically, a low muscle mass would result in an older MA, following the known age-related decrease in BMR, due to a decrease in fat-free mass and/or an increase in adipose tissue (55, 56). In the present study, BMI continued to be high after the weight loss (BMI range at follow-up: women: 26-45 kg/m^2 and men: 28-40 kg/m^2). We propose that MA did not change as a result of the intervention due to the small loss in muscle mass in combination with a consistent amount of fat mass. While recognizing the importance of preservation of muscle mass during weight loss (57), the results indicate that MA is not useful as a tool to assess the health effects of clinically relevant weight loss in individuals with high BMI.

Limitations and future research

This study is limited by sample size, and the strength of the relationship between BA and CA would benefit from more observations especially within the +40-year age category and particularly in the male category. AThis would also allow a more precise comparison with the regression line of the healthy aging reference group.

Some shortcomings are bound to be addressed before BA can be recommended to use in a clinical setting. To gain utility as a management tool, it is interesting to know how sensitive the BA measure is to a high/or low measurements in every single biomarker. Such sensitivity analysis should be performed in different age categories adjusted for sex and BMI.

Validation of the BA model should entail day-to-day reliability to ensure consistency of the BA measurement and test-retest reliability, to assess the stability of the BA measure during a short period of time where nothing is expected to impact BA. Finally, validation of BA estimation with hard outcomes such as CVD is wanted. While prospective data naturally is more difficult to obtain, as it requires several years of follow-up, applying the BA model on a register-based study is a study for future investigations.

Strengths

A strength of this study is that BA was improved after a lifestyle intervention carried out after normal procedures in a real-life setting. In this study, we followed golden standards measuring model biomarkers. The feasibility for health personal to obtain the measurements in a practice setting is, however, moderate. A finger prick test can replace the venous blood sample, and portable analyzers can measure blood lipids and HbA1c. Waist circumference requires a tape band, HbA1c, blood cholesterol, MAP and FEV1 is all possible to measure using standard bedside equipment easy to operate. Measurements of VO_2max can be obtained indirectly by submaximal test protocols (58, 59) or by future technology aids (60, 61). Employing such methodology entails reassessment of the BA-model reliability. Measuring suPAR and adiponectin turn out more difficult without biochemical laboratory analysis available. With no change in suPAR levels at follow-up and a close to negligible contribution to BA from adiponectin in men, exclusion of these biomarkers could be considered in future improvement of the BA model.

Conclusion

The results of this intervention study suggest that the BA model has clinical utility. We demonstrate that the BA model can detect the benefits related to a clinically relevant weight loss in women and men with overweight and obesity. The BA model enables risk stratification among young adults with high relative risk and have applicability as a tool in health-enhancing interventions. The BA model should, however, be validated further with a focus on reliability and absolute risk prediction validity.

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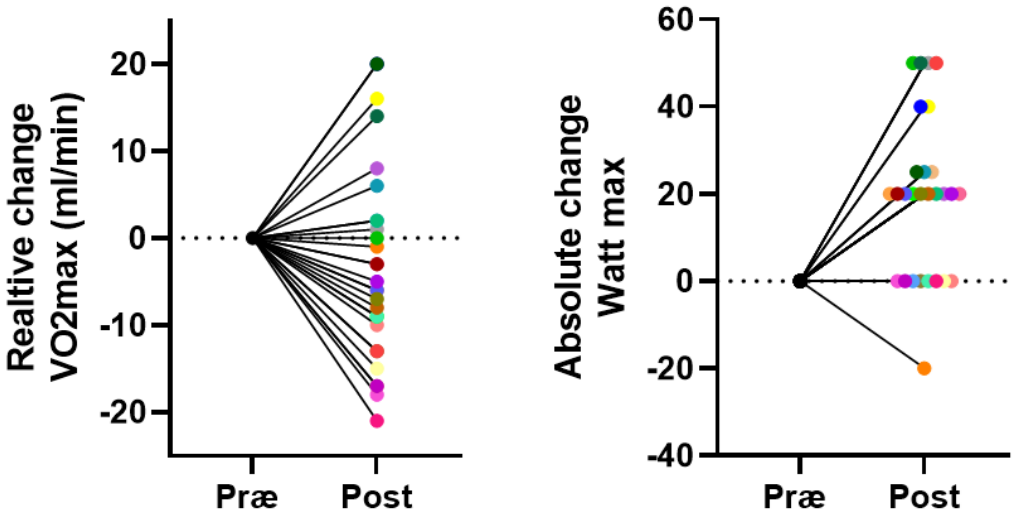

Suppl. Fig. 1. Individual plot of relative change (%) in absolute maximal oxygen uptake (VO2max) *left hand side* with corresponding changes in maximal work capacity (watt max) *right hand side*.