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Zhang D-Y, Martens DS, An D-W, Marrachelli VG, Yu Y-L, Stolarz-Skrzypek K, Rajzer M, Li Y, Verhamme P, Nawrot TS, Monleon D, Redón J, Staessen JA

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Plasma metabolomic signature predicting deviation from healthy vascular aging in two European cohorts

Short title: Metabolomic signature of vascular aging

Dong-Yan Zhang[a,b], Dries S. Martens[c], De-Wei An[a,b], Vannina González-Marrachelli[d,e], Yu-Ling Yu[b,f], Katarzyna Stolarz-Skrzypek[g], Marek Rajzer[g], Yan Li[a], Peter Verhamme[h], Tim S. Nawrot[c,i], Daniel Monleon[j], Josep Redón[e,k], Jan A. Staessen[a,b,g,l]

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Correspondence

Jan A. Staessen,
Alliance for the Promotion of Preventive Medicine, Leopoldstraat 59,
BE-2800 Mechelen, Belgium

Telephone: +32-47-632-4928 (mobile)
Facsimile: +32-15-41-4542
Email: jan.staessen@appremed.org
X @jasta49

Affiliations
aDepartment of Cardiovascular Medicine, Shanghai Institute of Hypertension, Shanghai Key Laboratory of Hypertension, National Research Centre for Translational Medicine, State Key Laboratory of Medical Genomics, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; bNon-Profit Research Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium; cCentre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium; dDepartment of Physiology, University of Valencia, Valencia, Spain; eCardiometabolic Renal Risk Research Group, INCLIVA Biomedical Research Institute, University of Valencia, Valencia, Spain; fDepartment of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden; gThe First Department of Cardiology, Interventional Electrocardiology and Hypertension, Jagiellonian University Medical College, Kraków, Poland; hCenter for Molecular and Vascular Biology, KU Leuven Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium; iResearch Unit Environment and Health, Department of Public Health and Primary Care, University of Leuven, Leuven, Belgium; jDepartment of Pathology, University of Valencia, Valencia, Spain; kCentro de Investigación Biomédica en Red (CIBERON), Madrid, Spain; lBiomedical Research Group, Faculty of Medicine, University of Leuven, Leuven, Belgium

Corresponding Author
Jan A. Staessen, MD, PhD, Non-Profit Research Alliance for the Promotion of Preventive Medicine (URL: www.appremed.org), Leopoldstraat 59, BE-2800 Mechelen, Belgium (jan.staessen@appremed.org).

Abstract

Introduction: Biological rather than chronological age (C-age) drives deviation from healthy aging. This study aimed to identify a plasma metabolomic signature indicative of age-related cardiovascular risk (pMTB-age) predicting vascular morbidity and mortality. ***Methods:*** Nuclear magnetic resonance identified 38 plasma metabolites in two population cohorts: the Flemish cohort examined as discovery (N = 719 [2005–2010]) and internal replication cohort (N = 580/719 [2009–2013]) and the external replication Spanish HORTEGA cohort (N = 811 [2001]). ***Results:*** The trained model (pMTB-age), relating C-age to the plasma metabolome derived by elastic net regression, included 18 metabolites (six amino-acids) and explained from 28.6% to 22.9% of C-age in the discovery and replication data. Feature importance of the retained metabolites derived by SHapley Additive exPlanation showed large interindividual variability in the relation between C-age and pMTB-age. In Flemish and Spanish, cardiovascular risk factors were significantly associated with C-age, pMTB-age and pMTB-age uncorrelated from C-age (pMTB-age-R). In Flemish (median follow-up 12.3 years) and Spanish (18.8 years) mortality and cardiovascular complications correlated with C-age and pMTB-age. In Flemish, cardiac endpoints (hazard ratio [95%CI] 1.28 [1.00-1.63]) and its components kept significance in relation to pMTB-age-R. The pathway analysis revealed overrepresentation of glycine, serine and threonine. ***Conclusions:*** pMTB-age is a multidimensional biomarker, which identifies individuals with accelerated vascular aging with high precision and combined with the pathway analysis highlights the role of amino-acids in vascular disease. Therefore, pMTB-age can guide risk stratification and the personalized and timely prevention and treatment tailored to an individual's unique pMTB-age profile.

Key words: aging, biomarker, morbidity, mortality, nuclear magnetic resonance, plasma metabolomics

Introduction

Aging is characterized by progressive cellular and physiological dysfunction and accrual of cardiovascular risk factors, which enhance the susceptibility to vascular disease and mortality [1,2]. Major age-related cardiovascular end points, such as myocardial infarction and stroke, are caused by arterial disease and reflect deviation from healthy vascular aging. Chronological age (C-age) does not capture the inter-individual variability in biological or vascular aging. For the same C-age, individuals show marked differences in vascular function, disease susceptibility and survival, underscoring the need for more precise indicators of biological age [3]. In response to this need, high-throughput omics technologies enabled the construction of aging clocks based on DNA methylation (epigenetic clocks) [4,5], gene expression (transcriptomic clocks) [6], proteomics [7] or metabolomics [8-16]. Among these approaches, metabolomics captures products downstream of genomic, transcriptomic, and proteomic activity, while simultaneously reflecting environmental exposures, diet, lifestyle, and the influence of the gut microbiome. To date, only three published studies [11,12,14] reported association of adverse health outcomes with metabolomic aging clocks, with replication in a different cohort, but only one [11] unrelated the metabolomic-derived aging clock from C-age, thereby highlighting its independent predictive value.

To increase generalizability and clinical applicability, there is a need to derive metabolomic-based biological aging clocks in representative population cohorts with a core set of metabolomic markers, such as produced by nuclear magnetic resonance (NMR), which facilitate interpretability compared to more complex approaches [4-16]. To implement this objective, 38 plasma metabolites (pMTB) were measured by NMR and related to cardiovascular risk factors, incident cardiovascular complications and mortality in the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) [17] with an internal replication within this cohort by re-examination of participants and with an external validation in the Spanish HORTEGA study [18].

Methods

Study design

The family-based FLEMENGHO study complies with the Helsinki declaration for research in humans, is registered with the Belgian Data Protection Authority (III 11/1234/13; Augustus 22, 2013). The ethics committee of the University Hospitals Leuven, Belgium, approved the secondary use of FLEMENGHO data (B32220083510) [17]. From August 20, 1985, to December 14, 1990, a random sample of the households living in a geographically defined area of northern Belgium was investigated with the goal to recruit an equal number of participants in each of six subgroups stratified by sex and age (20–39, 40–59, and ≥60 years). All household members aged 20 years or older were invited to take part, provided that the quota of their sex-age group had not yet been satisfied. From April 3, 1996, to May 12, 2007, recruitment of families continued, including young adolescents aged 10-19 years. Participants younger than 18 years provided informed assent and their parents or custodians gave informed consent. Of 4,286 people invited to participate in FLEMENGHO, 3343 consented (participation rate 78.0%). From May 30, 2005, until May 31, 2010, 828 participants were invited to a follow-up examination, if their last known address was within 15 kilometers of the local examination center (Eksel, Belgium). This invitation excluded individuals who had passed away (N = 26), had been institutionalized (N = 27), moved out of the catchment area (N = 100), or had withdrawn their informed consent in previous examination cycles (N = 227), leaving 828 participants for the current study. Of those, 95 individuals were excluded because they had no measurement of their plasma metabolomic signature and four because they were younger than 18 years and healthy. Thus, two datasets were derived for FLEMENGHO participants. The 729 individuals who had participated in the first examination cycle (2005–2010) constituted the derivation dataset. The 2009–

2013 data of the 580 individuals, who had participated in both examination cycles were analyzed as the time-shifted internal validation dataset. The 2005–2010 data constituted the baseline for the prospective follow-up of health outcomes until June 30, 2019 (online suppl. Fig. 1).
The Spanish HORTEGA study, which recruited individuals within the 15–85 age range [19], was used for external replication. In Spain, within defined geographical areas, local networks of primary care practices are linked to tertiary hospitals for referrals. This framework defined the catchment area of the Rio Hortega University Hospital in Valladolid. The HORTEGA study was initiated in 1997 (phase I) as a mailed survey of a random selection of health care beneficiaries residing in the University Hospital Rio Hortega's catchment area (N = 11,423), followed by a pilot examination (phase II) of 495 phase-I participants in 1999–2000. In March 2001, 1,512 individuals, including the 495 phase-II participants took part in the HORTEGA baseline examination (phase III). Phase-III participants were followed until 31 December, 2021 (online suppl. Fig. 1).

Measurements

In FLEMENGHO, all clinical and vascular measurements, information on risk factors and use of medications, and blood samples for biochemistry and pMTB analysis were collected on the same examination day. Participants fasted for 8 hours before venipuncture and the aliquoted serum and plasma samples were stored –80°C until analyzed. Blood pressure was the average of five consecutive auscultatory readings obtained after participants had rested for 5 minutes in the seated position. Study nurses administered a standardized questionnaire to update each participant's medical history, smoking and drinking habits, and intake of medications, including antihypertensive medicines, oral and parenteral antidiabetic agents, and lipid-lowering drugs (fibrates, ezetimibe, cholestyramine and statins). Antihypertensive drugs were categorized into diuretics (thiazides, loop diuretics and aldosterone antagonists),
β-blockers, inhibitors of the renin-angiotensin system (angiotensin converting-enzyme inhibitors and angiotensin receptor blockers, including or excluding β-blockers), and vasodilators (calcium-channel blockers and □-blockers). In the discovery sample, ankle-brachial pulse wave velocity and the ankle-brachial pressure ratio were measured using the VP-2000 device (Omron Healthcare Co. LTD, Kyoto, Japan). In HORTEGA phase III, participants completed a self-administered questionnaire providing information on anthropometric characteristics, educational attainment, smoking and drinking status, and the intake of antihypertensive, antidiabetic and lipid-lowering medications. Blood pressure was measured up to three times after participants had rested for 5 minutes in the sitting position, using validated devices. Blood samples were obtained after 3 hours (median) of fasting (range 0–17 hours). In both FLEMENGHO and HORTEGA, certified laboratories did the routine biochemistry, using quality controlled automated methods. The estimated glomerular filtration rate (eGFR) was computed from serum creatinine [20], measured by a modification of Jaffe's methods with isotope-dilution calibration [21]. The Framingham risk score for coronary heart disease was computed by the formula published in 2008 [22].

Mortality and morbidity

At annual intervals, the vital status of FLEMENGHO participants was ascertained by record linkage with the National Population Registry in Brussels, Belgium. The ICD codes for the cause of death were obtained from the Flemish Registry of Death Certificates. In FLEMENGHO, information on the incidence of nonfatal endpoints was collected by a standardized questionnaire at each examination cycle, via structured telephone interviews of participants or their next of kin, and by searching the participants' medical records at the four regional hospitals and the University Hospitals Leuven, all serving the FLEMENGHO catchment area. Experienced study nurses under supervision of the study physicians did the ICD coding of nonfatal endpoints, which were validated against the records of the

general practices in the catchment area and the referral hospitals. In HORTEGA, a highly experienced nosologist, searched the digital records of the general practices in the network and the Rio Hortega University Hospital in Valladolid and did the ICD coding.

All endpoint analyses addressed the first event within each category and the time of occurrence relative to the baseline. The censoring data in participants without any endpoint was 30 June, 2019, in FLEMENGHO and 31 December, 2021, in HORTEGA. The coprimary endpoints were total mortality and a composite cardiovascular endpoint consisting of cardiovascular mortality combined with nonfatal coronary endpoints, heart failure, valvular heart disease, cardiomyopathy, dysrhythmia and atrial fibrillation and stroke. Angina pectoris, chronic ischemic heart disease and transient ischemic attack were not included in the coprimary cardiovascular endpoint. Secondary endpoints included cause-specific mortality and the components of the coprimary cardiovascular endpoint. No participant was lost to follow-up. However, the cause of death was unknown in four FLEMENGHO and 20 HORTEGA participants.

Plasma metabolomics

The plasma metabolomic signature was measured by NMR according to methods described previously [17,18]. To study reproducibility of the NMR measurements, the complete aliphatic spectral region was split into 0.005 ppm buckets. The mean bucket difference for all aliphatic spectral regions was 5.1% with a maximum of 7.0% for the bucket containing the high-density apolipoprotein signal. NMR spectroscopy is fast and keeps samples separated from the instrument, but produces crowded spectra that cannot always be reliably deconvoluted into single metabolites. When two peaks contributed to a spectral region, the two metabolites were jointly reported. The FLEMENGHO plasma samples for measurement of the plasma metabolites were shipped on dry ice from Leuven, Belgium, arriving in Valencia, Spain, within 24 hours and kept at –80°C until assayed.

The NMR procedure detected 60 metabolites in FLEMENGHO and HORTEGA plasma samples. Due to the inherent nature of NMR spectroscopy, individual metabolites often generate multiple distinct resonance signals with different ppm values, reflecting the presence of chemically non-equivalent hydrogen atoms within the same molecule. A single metabolite may therefore generate several spectral features. In case of overlapping or redundant signals representing the same metabolite, the signal with the strongest correlation with C-age was analyzed, resulting in a panel of 38 metabolites (online suppl. Table 1).

Statistical analysis

Database management and statistical analysis were done using SAS, version 9.4 (maintenance level 5). The distributions of the circulating metabolomic biomarkers were rank normalized, by sorting measurements from the smallest to the highest value and then applying the inverse cumulative normal function. Between-group means were compared using the large-sample Z test. Proportions were compared by Fisher's exact test and longitudinal changes in proportions by McNemar's test. In FLEMENGHO, the intraclass correlation coefficients modeling aggregation of traits between related individuals were estimated, using analysis of variance with unrelated participants excluded from analysis. Statistical methods also included linear and proportional hazards regression.

The analysis was done according to predefined steps (Fig. 1). First, the association between C-age and plasma metabolites was investigated in the 2005–2010 Flemish derivation dataset (N = 729), with cumulative adjustment for sex, body mass index (BMI), mean arterial pressure (diastolic blood pressure plus 0.40 × (systolic minus diastolic blood pressure), smoking (categorical), diabetes, history of cardiovascular disease, and the use of antihypertensive and lipid-lowering drugs. The plasma metabolites (pMTBs) were highly inter-correlated (online suppl. Fig. 2), so that each test did not introduce an independent opportunity for a type-I error and a correction for multiple testing was

unnecessary [23]. The second analysis step involved pMTBs significantly associated with C-age in the first step. These metabolites were analyzed by elastic net regression to construct a model predicting age (pMTB-age). The L1 and L2 regression penalties were determined by 10-fold random cross-validation. This procedure was bootstrapped 1000 times to obtain the final estimates of L1 and L2 and the 95% confidence interval (CI) of the regression coefficients, linking C-age with each metabolite in the reduced set of pMTBs. Validation in the next analysis step was implemented by applying the pMTB-age prediction model to the internal FLEMENGHO and the external HORTEGA validation datasets. To study the associations of biomarkers and adverse health outcomes with molecular aging, as captured by pMTB-age, over and beyond C-age, the residual of pMTB-age regressed on C-age (pMTB-age-R) was computed (online suppl. Fig. 3). Furthermore, the SHapley Additive exPlanation (SHAP) method [24], as implemented in R version 4.4.1 (R Core Team, Vienna, Austria) was applied to rank the plasma metabolites according to their importance and to visualize their associations with pMTB-age globally and at the individual level. The final step in the analysis was the molecular pathway analysis involving the plasma metabolites making up pMTB-age, using the databases of Reactome and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Pathways were visualized, using R software to create dot plots with q-values corrected for the false discovery rate [25].

Results

Characteristics of participants

Compared to the 729 FLEMENGHO participants making up the derivation dataset (2005–2010), the 811 HORTEGA patients (2001), being followed up at a tertiary referral hospital, had a higher cardiovascular risk profile (Table 1), as evidenced by the greater frequency of smoking, habitual alcohol intake, history of cardiovascular disease, the higher 10-year Framingham risk score for coronary heart disease and the lower prevalence of antihypertensive and lipid-lowering treatment. The Spanish patients were 2.0 years older and had a 2.0 centimeter lower waist circumference than the Flemish at the 2005–2010 examination without differences in BMI, and blood pressure. With regard to the biochemical risk profile, eGFR was 7.4 mL/min/m2 higher and total serum cholesterol 0.92 mmol/L lower in HORTEGA patients, whereas plasma glucose was 0.17 mmol/L higher and high-density lipoprotein (HDL) serum cholesterol and the total-to-HDL serum cholesterol ratio were respectively 0.08 mmol/L and 0.52 lower in the HORTEGA patients compared to the baseline levels (2005–2010) in Flemish. Brachial-ankle pulse wave velocity and the ankle-brachial pressure ratio in the Flemish derivation dataset (2005–2010) averaged 14.0 m/s and 1.10, respectively. Vascular measurements were not available in the HORTEGA participants.

The time-shifted internal replication FLEMENGHO dataset (2009–2013) consisted of 580 individuals, who had participated in the baseline and follow-up examinations (Fig. 1 and online suppl. Fig. 1). The median time interval between the two examination cycles was 4.75 years (5th-95th percentile interval 3.71 to 5.40 years). Aging from baseline to follow-up (Table 1) was accompanied by worsening of the risk profile, mainly related to unfavorable trends in the anthropometric characteristics, blood pressure, the prevalence of hypertension, eGFR and the serum lipid profile, although the frequency of antihypertensive and lipid-lowering treatment increased by 8.1% and 10.5%, respectively, and the prevalence of smoking decreased by 4.1%.

Information on the effects of study attrition was available for the FLEMENGHO participants included in the derivation dataset (N = 729) compared to the 1219 participants not included in current analysis, because they had passed away, were institutionalized, had moved away from the study area, declined further participation, or were excluded from analysis (online suppl. Table 2). In the1996–2005 examination cycle, participants included in derivation dataset were 1.9 years younger,

had 2.9 mL/min/1.73 m2 lower eGFR, 0.14 mmol/L lower plasma glucose, and 0.37 higher total-to-HDL serum cholesterol ratio with no differences in blood pressure or BMI.

Associations of age with plasma metabolites

In the FLEMENGHO derivation dataset, with adjustments applied for sex, BMI, mean arterial pressure, smoking, diabetes, history of cardiovascular disease, and the use of antihypertensive and lipid-lowering drugs, C-age was significantly associated with 21 metabolites, of which 16 were confirmed in the time-shifted FLEMENGHO replication dataset and 18 in the external replication HORTEGA data (online suppl. Fig. 4).

Of the 21 metabolites associated with C-age in the FLEMENGHO derivation dataset, the elastic net regression procedure retained 18 in pMTB-age with partial regression coefficients (weights) ranging from –8.01 (95% CI –10.5 to 5.16) for glutamate to 8.14 (95% CI 3.89 to 12.5) for creatinine (online suppl. Table 3). pMTB-age explained 28.6% of C-age in the derivation dataset (r = 0.54), with similar estimates in women (28.4% [r = 0.54]) and men (28.5% [r = 0.54]), 24.6% (r=0.50) in the time-shifted internal replication FLEMENGHO dataset and 22.9% (r = 0.48) in the external replication HORTEGA data (Fig. 2). C-age and pMTB-age were similar in the FLEMENGHO derivation dataset (C-age 51.3 years [SE 0.57]) *vs* pMTB-age 51.3 years [0.31], p=0.96). In the time-shifted FLEMENGHO and the HORTEGA datasets, C-age was higher than pMTB-age ($p \leq 0.0011$): 55.7 years (SE 0.60) *vs* 51.3 years (SE 0.33) and 53.3 years (SE 0.66) *vs* 51.3 years (SE 0.42), respectively. Furthermore, compared with the derivation dataset (Fig. 2A), the regression slopes of C-age on pMTB-age were similar in the time-shifted FLEMENGHO (Fig. 2B; p = 0.35) and the HORTEGA (Fig. 2C; p = 0.14). The estimates of the increases in C-age per 10-year increment in pMTB-age (95%CI) were 9.92 years (8.78–11.1 years) in the derivation dataset, 9.04 years (7.75–10.3 years) in the time-shifted FLEMENGHO dataset, and 7.48 years (6.54–8.43 years) in HORTEGA. Fig. 3 shows the feature importance of the 18 metabolites in the FLEMENGHO participants overall (panel A) and in individual patients (panel B) in their relation with C-age with adjustments applied for sex, BMI, mean arterial pressure, smoking, diabetes, history of cardiovascular disease, and the use of antihypertensive and lipid-lowering drugs. Given the intercept and weights of the 18 plasma metabolites (online suppl. Table 3), there was large interindividual variability in the association of C-age with pMTB-age (Fig. 3). For instance, in two men with the same C-age: 28.0 years, pMTB-age was 37.3 and 22.1 years (online suppl. Fig. 5). Similarly, in two women with the same C-age: 56.0 years, pMTB-age was 64.1 and 49.9 years (online suppl. Fig. 5). In the pathway analysis (Fig. 4), the metabolism of glycine, serine and threonine were overrepresented (p-value with Benjamini-Hochberg correction 0.0054).

Associations of age with risk factors

In FLEMENGHO and HORTEGA, blood pressure, plasma glucose, BMI, and waist circumference were positively and eGFR inversely correlated with C-age (Table 2). These correlations remained highly significant if C-age was replaced by pMTB-age except for diastolic blood pressure in FLEMENGHO participants. pMTB-age-R, an index reflecting accelerated aging independent of C-age, was significantly and inversely correlated with eGFR and positively with the other risk factors with the exception of blood pressure (Table 2).

In FLEMENGO participants, the brachial-ankle pulse wave velocity and the ankle-brachial pressure ratio were positively and significantly correlated with C-age and pMTB-age. The correlation between the ankle-brachial pressure ratio with pMTB-age-R retained significance, whereas this was not the case for the brachial-ankle pulse wave velocity. The FLEMENGHO study population consisted of 112 unrelated participants and 617 related participants, belonging to 16 single-generation families and 61 multigeneration pedigrees. The intraclass correlation coefficients (95% CI) among related FLEMENGHO participants were 0.06 (–0.021 to 0.15; p = 0.14) for C-age, 0.13 (0.047 to 0.21; p <

0.0023) for pMTB-age and 0.28 (0.20 to 0.36; p < 0.0001) for pMTB-age-R. Adjustment for family clusters produced (online suppl. Table 4) consistent results, but removed the significance of the associations of eGFR, waist circumference, the brachial-ankle pulse wave velocity and the brachial ankle pressure ratio with pMTB-age-R. In HORTEGA participants, all tabulated risk factors (Table 2) with the exception of eGFR were significantly associated with pMTB-age-R. Use of medications in the treatment of age-related chronic disease (online suppl. Table 5) was associated with a significantly higher C-age and pMTB-age (p ≤ 0.022). These medications included the major classes of antihypertensive drugs, given as monotherapy or in combination, non-steroidal anti-inflammatory drugs and lipid-lowering agents. However, pMTB-age-R was not different between users and non-users of antihypertensive and non-steroidal anti-inflammatory drugs, while pMTB-age-R was slightly but significantly lower in users than in non-users of lipid-lowering agents.

Mortality and morbidity

Median follow-up (IQR) was 12.3 (10.7–13.2) years in FLEMENGHO and 18.8 (17.4–18.9) in HORTEGA. In both cohorts (Table 3), total mortality and the coprimary cardiovascular endpoint and its components significantly increased with C-age and pMTB-age. Per 10-year age increment, in FLEMENGO, the HRs (95% CI) were 3.44 (2.72–4.34) and 2.76 (2.31–3.28) for total mortality and the coprimary cardiovascular endpoint in relation to C-age and 2.55 (1.97–3.30) and 2.33 (1.84–2.95) in relation to pMTB-age. In HORTEGA (Table 3), the corresponding HRs for total mortality and the coprimary cardiovascular endpoint were 3.49 (2.99–4.06) and 3.12 (2.62–3.71) in relation to C-age and 1.58 (1.42–1.75) and 1.64 (1.45–1.87) in relation to pMTB-age. Excluding 20 of 811 HORTEGA participants (2.47%) lost to follow-up with unknown cause of death did not materially alter the findings. Indeed, the HRs for total mortality in relation to C-age, pMTB-age and pMTB-age-R were 3.66 (3.10–4.32), 1.57 (1.41–1.75), and 0.93 (0.80–1.07), respectively.

In both cohorts, the HRs for cause-specific mortality, for the combined fatal and nonfatal cardiac, coronary and cerebrovascular endpoints and for nonfatal myocardial infarction and atrial fibrillation were confirmatory for their association with C-age and pMTB-age (Table 3). The associations of cardiac and coronary endpoints, myocardial infarction and atrial fibrillation with pMTB-age-R kept significance in FLEMENGHO (Table 3), but the associations of endpoints with pMTB-age-R did not reach significance (p ≥ 0.16) in HORTEGA.

Adjustment of the outcome results for clustering within families in 617 related FLEMENGHO participants (online suppl. Table 6) confirmed the results in the whole cohort also including singletons. Among related FLEMENGHO participants, the HR (95% CI) for the coprimary cardiovascular endpoint with pMTB-age-R was 1.68 (1.01–2.80) and reached significance (p = 0.044).

Fig. 5 shows the heat plots illustrating the multivariable-adjusted 10-year risk of an adverse health outcome in relation to C-age and pMTB-age in 729 FLEMENGHO participants. The heat plots were constructed for the outcomes for which pMTB-age-R was significant (Table 3), including all cardiovascular, cardiac and coronary endpoints, nonfatal myocardial infarction and new-onset atrial fibrillation. The 10-year risks significantly increased with C-age (along the horizontal axis) and pMTB-age (along the vertical axis).

Discussion

The main finding of this study was that C-age was associated with a specific change in the plasma metabolomic signature, as captured by pMTB-age. The relation of C-age with pMTB-age was derived in the FLEMENGHO discovery dataset and subsequently confirmed in the time-shifted FLEMENGHO and the external HORTEGA replication data (Fig. 2). Compared to C-age in the derivation dataset (51.7 years), pMTB-age was up to 4.4 years higher in the replication datasets and per 10-year increment in C-age, the estimated pMTB-age was from 2.44 to 0.88 years lower. The mean SHAP

values of the 18 metabolites making up pMTB-age in the FLEMENGHO discovery dataset ranged from 0.13 for HDL apolipoproteins to 8.14 for creatinine, but large interindividual variability in the associations between pMTB-age and C-age explained why for the same C-age pMTB-age can be substantially different (Fig. 3 and online suppl. Fig. 5). In FLEMENGHO and HORTEGA, common cardiovascular risk factors were significantly associated with C-age and pMTB-age and with the exception of blood pressure and the brachial-ankle pulse wave velocity in FLEMENGHO and eGFR in HORTEGA also with pMTB-age-R (Table 2). In FLEMENGHO, the use of medications to treat age-related chronic diseases, analyzed as proxy for unhealthy aging, were significantly correlated with C-age and pMTB-age and the use of lipid-lowering drugs also with pMTB-age-R (online suppl. Table 5). In FLEMENGO and HORTEGA, C-age and pMTB-age predicted mortality and cardiovascular endpoints (Table 3). In FLEMENGHO, cardiac endpoints and its components were also related to pMTB-age-R (Table 3 and Fig. 5). This was not the case in HORTEGA, probably because the Spanish participants had been referred a tertiary hospital, which may have resulted in a higher disease burden and a study group less representative of the general population. Additionally, in HORTEGA compared to FLEMENGHO (Fig. 2), the correlation coefficient between C-age and pMTB-age was weaker (0.48 *vs* 0.54) and the slope of C-age on pMTB-age was shallower (0.99 *vs* 0.75).

A PubMed search updated until June 30, 2025, revealed that seven publications are relevant to the current findings, because they examined the associations of biological aging with metabolomic signatures and the potential for risk stratification in the general population. In spite of substantial methodological differences, common design characteristics consisted of single-omics approach in five articles, using nuclear magnetic resonance (NMR) spectroscopy in three studies [13-15] and liquid chromatography-mass spectrometry (LC-MS) in two [9,11]. Two studies applied a multi-omics approach by integrating DNA methylation and metabolomic data [12,16]. Only three articles [11,12,14] included an external replication of the associations of adverse health outcomes with metabolomic age and only one [11] assessed the contribution of metabolomic age independent of C-age, but focused on lipidomic data, ignoring the critical role of amino-acids, carbohydrates, lipids and energy-metabolism intermediates in biological aging. Features of the present study adding to the existing literature include the long-term follow-up for adverse vascular health outcomes over and beyond mortality as the only endpoint in most studies, the assessment of the association of cardiovascular risk factors and drug use with pMTB-age, uncorrelation of pMTB-age from C-age, and the replication of the findings in a subsample of the discovery cohort examined approximately 5 years later and in an external cohort with different lifestyle and dietary habits.

Of the 18 metabolites making up pMTB-age, six were amino-acids (online suppl. Table 3). The overrepresentation of the metabolism of glycine, serine and threonine in the pathway analysis contributes to the growing insight that amino-acids play a pivotal role in the regulation of aging, extending their physiological significance far beyond their traditional function as the building blocks of proteins. Specific amino-acids act as metabolic and signaling modulators that influence key cellular processes involved in aging and age-related diseases. Selective limitation of the dietary intake of certain amino-acids, such as methionine and tryptophan, trigger evolutionarily conserved stress-response pathways, notably involving mTOR and GCN2 signaling [26]. These pathways contribute to enhanced protein homeostasis, reduced oxidative stress, and improved mitochondrial function, collectively promoting longevity in model organisms, such as *C. elegans*, yeast, and mice [26]. Furthermore, the metabolism of glycine, serine, and threonine acts as a central axis associated with improved healthy lifespan and longevity in mice on calorie-restricted diets and during time-restricted feeding patterns [27]. This multi-omics experimental study suggested that metabolic remodeling of these amino-acid pathways under fasting conditions supports liver function, redox balance, and

energy homeostasis, thereby delaying the onset of age-related pathologies, such as fatty liver disease and cancer [27]. Further adding to this complexity, amino-acid metabolism plays a role in vascular aging, particularly via the metabolism of arginine and its downstream products, such as homoarginine and polyamines [28]. These metabolites influence vascular health through mechanisms, such as nitric oxide (NO) production, autophagy regulation, and macrophage polarization. Dysregulation of these pathways is linked to atherosclerosis and systemic inflammation, underscoring the relevance of amino-acid homeostasis in preventing age-associated vascular disease. Taken together, these findings suggest that the balance between amino-acids and their metabolism are key determinants of aging and longevity. Targeted dietary manipulation of specific amino-acids, or pharmacological modulation of their metabolic pathways, may offer promising strategies for promoting healthy aging and preventing age-related diseases across multiple organ systems [29]. Indeed, glycine is a significant component of bile acids secreted into the lumen of the small intestine that is necessary for the digestion of dietary fat and the absorption of long-chain fatty acids [30]. Glycine plays an important role in metabolic regulation and anti-oxidative reactions. Thus, this nutrient has been used to prevent tissue injury; to enhance anti-oxidative capacity; to promote protein synthesis and wound healing; to improve immunity [30]; and to treat metabolic disorders in obesity, diabetes, cardiovascular disease, ischemia-reperfusion injuries, cancers [31], and various inflammatory diseases [29].

pMTB-age was strongly associated with metabolic traits, including glucose and lipid biomarkers, independent of C-age as evidenced by the significant associations with pMTB-age-R (Table 2). These findings imply that pMTB-age may serve as a sensitive indicator of subtle metabolic dysregulation, offering additional utility in identifying individuals at risk of metabolic diseases. The longitudinal analyses further supported the clinical relevance of pMTB-age, in terms of the increased risk of death and cardiovascular complications, in particular cardiac events and its components in the Flemish discovery cohort (Table 3 and Fig. 5). Previous studies demonstrated that metabolomic profiles can predict specific health outcomes independent of C-age, such as cardiovascular endpoints and all-cause mortality [11,14]. Furthermore, the present finding that pMTB-age-R selectively predicts myocardial infarction and atrial fibrillation aligns with the work by Wang and coworkers [11], which suggests that metabolomic aging clocks may capture organ-specific or pathway-specific mechanisms of biological aging, reinforcing the need for domain-specific biomarkers of biological aging. Unlike models based on mass spectrometry, the NMR-based pMTB-age offers a reproducible and scalable platform more suitable for large population studies [32].

Strengths and limitations

Strengths of the current study are the internal and external replication of the pMTB-age prediction model, the demonstration of the clinical relevance of pMTB-age and pMTB-age-R in relation to common risk factors in FLEMENGHO and HORTEGA, and more importantly, in relation to prospectively collected cardiac endpoints in FLEMENGHO, including both fatal and nonfatal events. The pMTB-age model is unbiased, because it does not depend on predefined markers but only on metabolites detectable by NMR. The current study was population-based (FLEMENGHO) or community-based (HORTEGA) and at baseline did not include severely ill patients as a proxy of aging and covered a wide age range. Notwithstanding these strong points, the present study must also be interpreted within the context of its limitations. First, NMR spectroscopy produces crowded spectra that cannot always be reliably deconvoluted into single metabolites. However, the mean bucket difference for all aliphatic spectral regions was 5.1% with a maximum of 7.0% for the bucket containing the high-density apolipoprotein signal [17,18]. Second, using NMR spectroscopy limited the number of unambiguously identified plasma metabolites to 38, whereas other platforms, such as

liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) or matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) provide a much wider or focused range of plasma metabolites. However, given the large interindividual variability in the association between aging clocks and C-age (Fig. 4 and online suppl. Fig. 5), more *vs* less metabolites do not necessarily increase precision and reproducibility. Third, as is the case in all long-term cohort studies, attrition occurred. Using 1996–2005 data as reference, FLEMENGHO participants analyzed were approximately 2 years younger than those not analyzed. Declining further participation and seeking higher education or job opportunities outside the catchment area were the main drivers of attrition in FLEMENGHO. In the HORTEGO cohort, 20 participants (2.47%) were lost to follow-up. Excluding these participants with undocumented cause of death did not alter the associations of total mortality with C-age, pMTB-age or pMTB-age-R. Fourth, the FLEMENGHO and HORTEGA cohorts were recruited in high-income countries, while low- and middle-income countries are transitioning rapidly from a disease burden dominated by communicable, maternal, neonatal, and nutritional causes to non-communicable diseases with vascular complication in the lead position. Fifth, whether the metabolomic aging signature improved risk prediction over and beyond established cardiovascular risk factors was not assessed, using discrimination or reclassification metrics. The justification was that metabolomic risk profiling is not readily available in day-to-day clinical practice, requires complex instrumentation and expertise to interpret the crowded spectra [32], and that the goal of the current study was gaining insight in vascular aging rather than predicting outcome. However, all models were adjusted for traditional risk factors. Finally, although the study focused on healthy vascular aging, direct vascular measurements were only available in the FLEMENGHO cohort but not in the HORTEGA cohort. Therefore, the concept of vascular aging in this study was partly inferred from cardiovascular outcomes and metabolomic signatures rather than fully characterized by arterial stiffness and hemodynamic parameters.

Conclusions

Aging is associated with a specific and reproducible shift in the plasma metabolomic signature captured by the multidimensional biomarker pMTB-age. With replication in two European cohorts, pMTB-age reflects biological aging at the individual level. pMTB-age was strongly associated with metabolic traits, including glucose and lipid biomarkers and included six amino-acids. The overrepresentation of glycine, serine and threonine in the pathway analysis underscores the relevance of amino-acid homeostasis in preventing age-associated vascular disease. Clinically, this supports the utility of pMTB-age as a personalized tool for identifying individuals with accelerated vascular aging, over and beyond what C-age can provide as information. Therefore, pMTB-age can guide risk stratification and the personalized and timely prevention and treatment tailored to an individual's unique pMTB-age profile.

Article information

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Statement of Ethics

The family-based FLEMENGHO study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki (2013 revision). The database was registered with the Belgian Data Protection Authority (III 11/1234/13; Augustus 22, 2013). The ethics committee of the University Hospitals Leuven, Belgium, approved the secondary use of FLEMENGHO data (B32220083510). The HORTEGA study was conducted in accordance with the ethical principles of the Declaration of Helsinki (2013 revision). Ethical approval was granted by the Comité Ético de Investigación Clínica (CEIC) of the Hospital Universitario Río Hortega, Área de Salud Valladolid Oeste on March 7, 2017 (Internal Code: CEIC: 21/17). All FLEMENGHO and RIO HORTEGA participants provided written informed consent.

Conflict of Interest Statement

All authors completed the ICMJE Form for Disclosure of Potential Conflicts of Interest. Yan Li was a member of the journal's Editorial Board at the time of submission. All other authors declare no competing interests.

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The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Author Contributions

DYZ, DSM, TSN and JAS conceptualized the study. JAS coordinated the FLEMENGHO study (1985-2019) and constructed the FLEMENGHO database. VGM, DM and JR provided the de-identified HORTEGA data (2001-2021) and supervised the NMR measurement of the plasma metabolites in FLEMENGHO and HORTEGA participants. DYZ, DSM, YLY, DWA and JAS did the statistical analyses. DSM did the pathway analysis. KSS, MR, YL and PV provided expertise in evaluating the epidemiological and pathophysiological implications of the study results. All authors interpreted the results, commented on successive drafts of the manuscript, and approved the final version. All authors had full access to all of the data in the study and shared the responsibility for the decision to submit for publication.

Data Availability Statement

The data that support the findings of this study are not publicly available due to privacy and ethical restrictions related to participant confidentiality. However, anonymized data will be made available upon request directed to the corresponding author. Each request will undergo a formal review process by the corresponding author and co-investigators. After approval of a proposal and ethics clearance, a data access and confidentiality agreement will be signed. Data will be shared via a

secure online platform. The applicant can use shared data for a maximum of 3 years after concluding the data sharing agreement.

ORCID numbers

Dong-Yan Zhang: 0000-0003-0551-971X
Dries S. Martens: 0000-0001-7893-3642
De-Wei An: 0000-0002-1395-7050
Vannina González-Marrachelli: 0000-0002-4097-3312
Yu-Ling Yu: 0000-0002-8255-3770
Katarzyna Stolarz-Skrzypek: 0000-0002-7057-7302
Marek Rajzer: 0000-0001-7074-7263
Yan Li: 0000-0002-5825-5968
Peter Verhamme: 0000-0001-8698-2858
Tim S. Nawrot: 0000-0002-3583-3593
Daniel Monleon: 0000-0002-4803-1573
Josep Redón: 0000-0001-8777-6773
Jan A. Staessen: 0000-0002-3026-1637

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Legends to figures

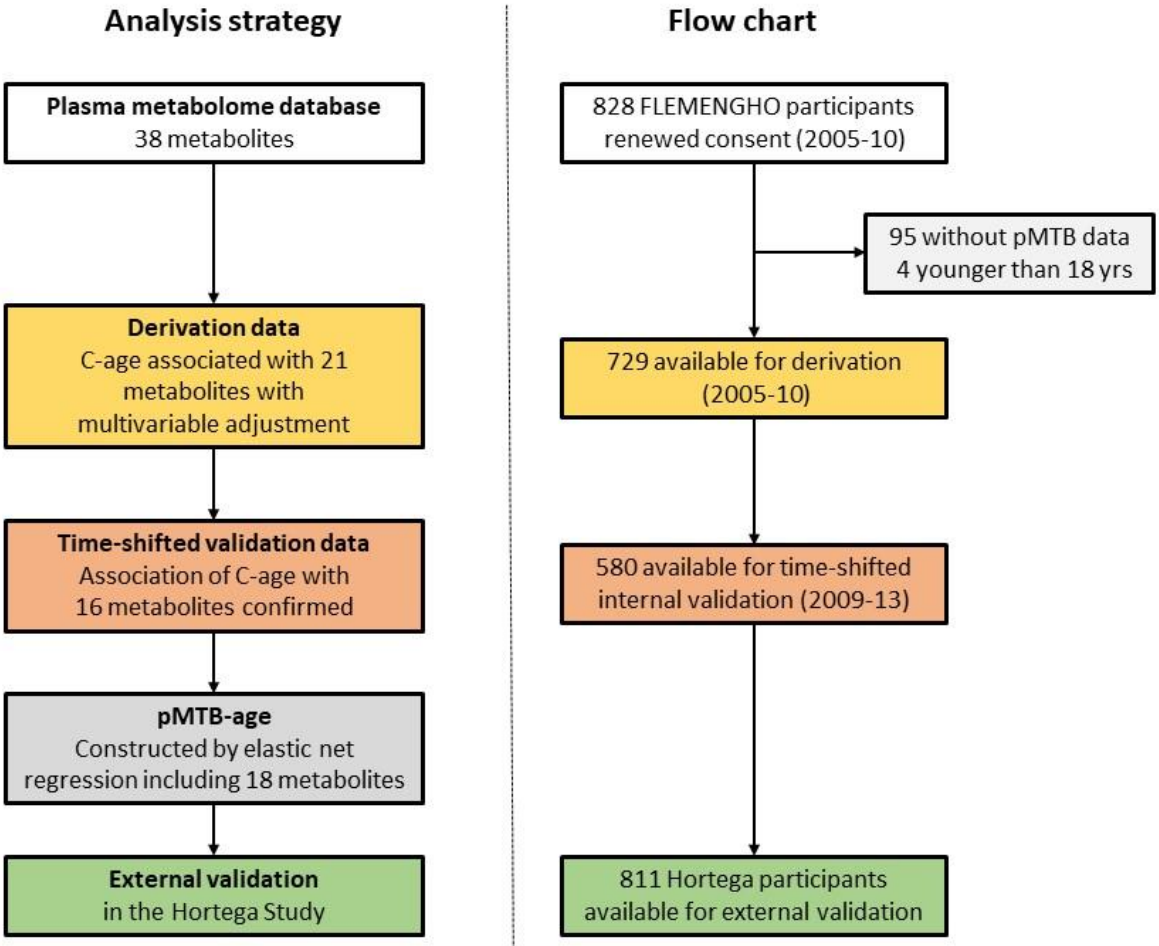
Fig. 1. Chart describing the flow of the analysis
FLEMENGHO=Flemish Study on Environment, Genes, and Health Outcomes. pMTB=plasma metabolomic profile.

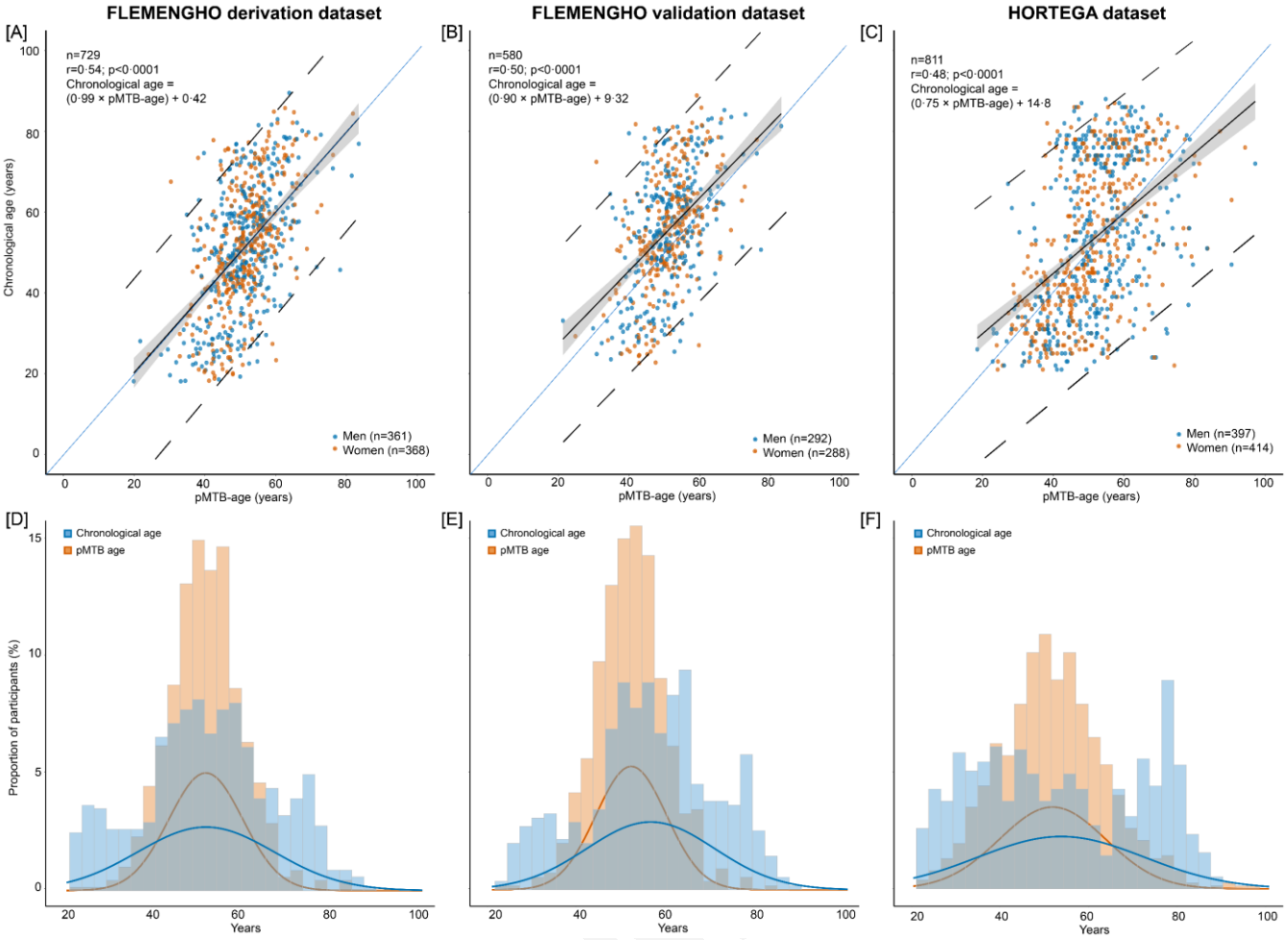
Fig. 2. Correlations between observed chronological age and age predicted by the plasma metabolome
Correlations for the FLEMENGHO derivation dataset (2005–2010; left), the time-shifted internal FLEMENGHO replication dataset (2009–2013; middle) and the external replication HORTEGA dataset (2001; right) are presented in panels A-C. The regression lines (solid black) are given with 95% confidence intervals for the predicted mean of chronological age (grey band) and the predicted chronological age of individual participants (dotted lines). The blue line in the correlation plots is the identity line. The corresponding distributions of chronological age and pMTB age are given in panels D-F. FLEMENGHO=Flemish Study on Environment, Genes, and Health Outcomes. pMTB=plasma metabolomic profile. pMTB-age=age as predicted by the plasma metabolome.

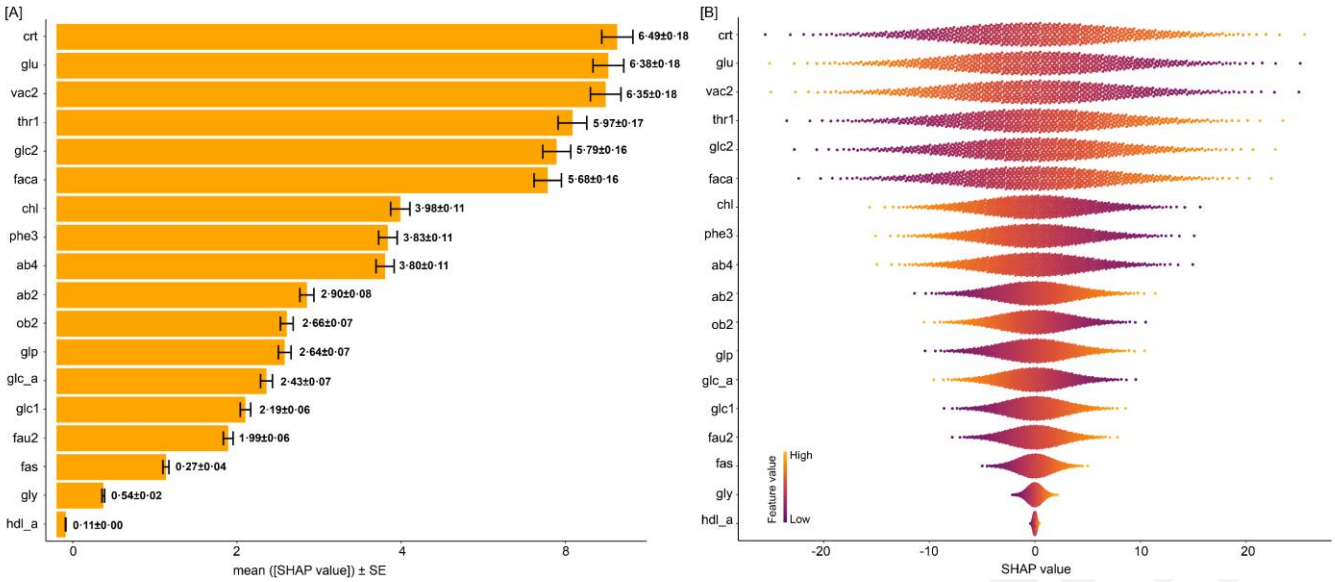
Fig. 3. Importance of the plasma metabolites in their association with pMTB-age overall and individually
The analysis conducted according to the SHapley Additive exPlanation method includes the FLEMENGHO derivation dataset (N = 729) and the 18 metabolites significantly associated with age with adjustment for sex, body mass index, mean arterial pressure, smoking, diabetes, history of cardiovascular disease, and the use of antihypertensive and lipid-lowering drugs. Panel A ranks the plasma metabolites according to their importance and Panel B shows the individual data points. SHAP values indicate how much each metabolite contributes to pMTB-age, facilitating an interpretable understanding of the model output. For the abbreviations of the metabolites, see online suppl. Table 1. pMTB=plasma metabolomic profile. pMTB-age=age as predicted by the pMTB.

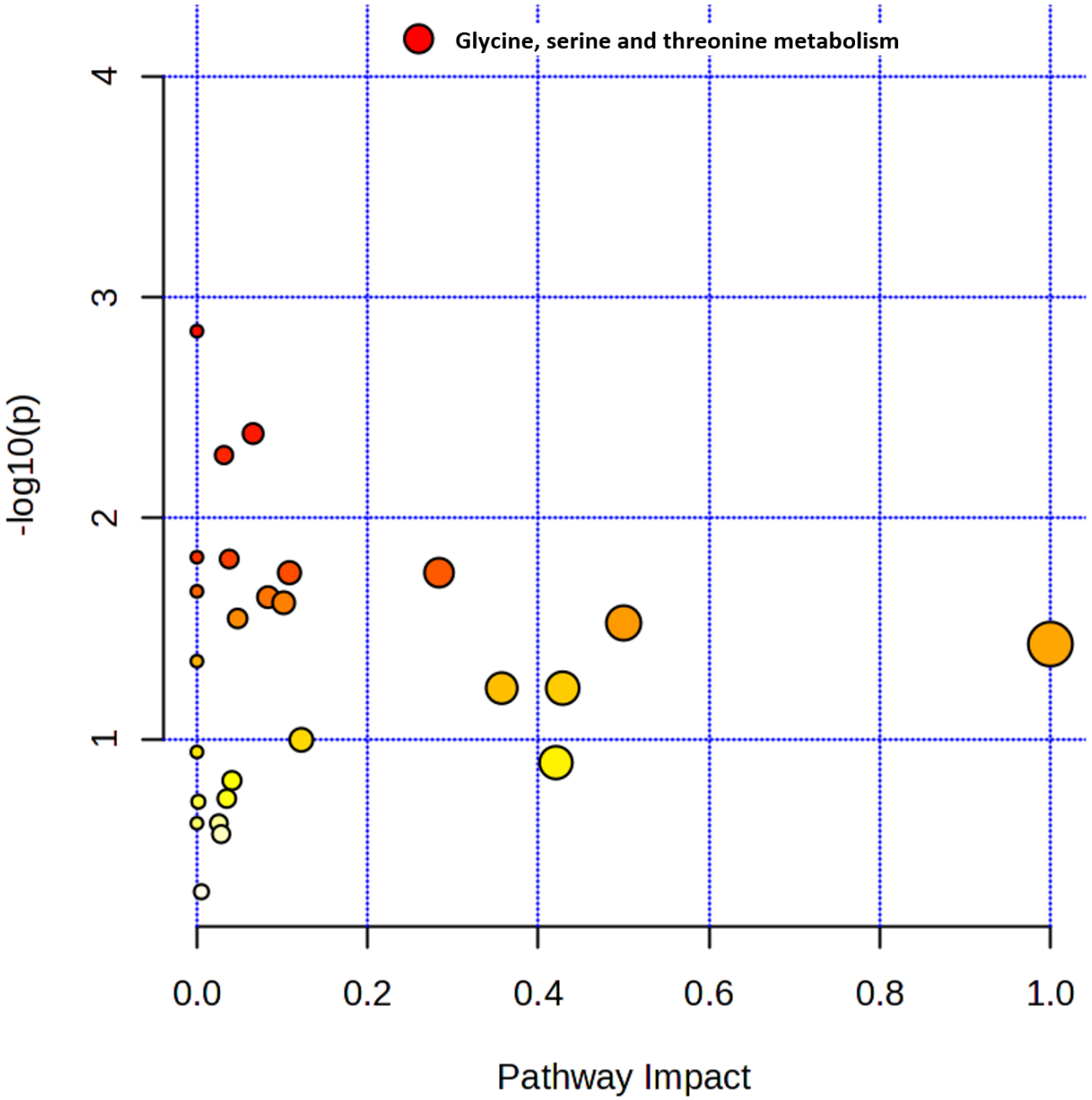
Fig. 4.
Pathway analysis of 18 metabolites making up pMTB-age
The metabolism of glycine, serine and threonine (p-value with Benjamini-Hochberg correction 0.0054) was the highest ranking and only significant enriched KEGG pathway). The size of the plotted points is proportional to the impact of the pathway.

Fig. 5.
Heat maps relating the 10-year risk of endpoints to C-age and pMTB-age in the FLEMENGHO derivation dataset constructed by multivariable-adjusted proportional hazards regression. Numbers in the grids in Panel A represent the percent of participants within each cross-classification category between C-age and pMTB age. Endpoints with a significant association with pMTB-age-R (Table 3) are plotted: cardiovascular mortality in panel B, all cardiac and coronary endpoints in panels C and D, nonfatal myocardial infarction in panel E and new-onset atrial fibrillation in panel F.









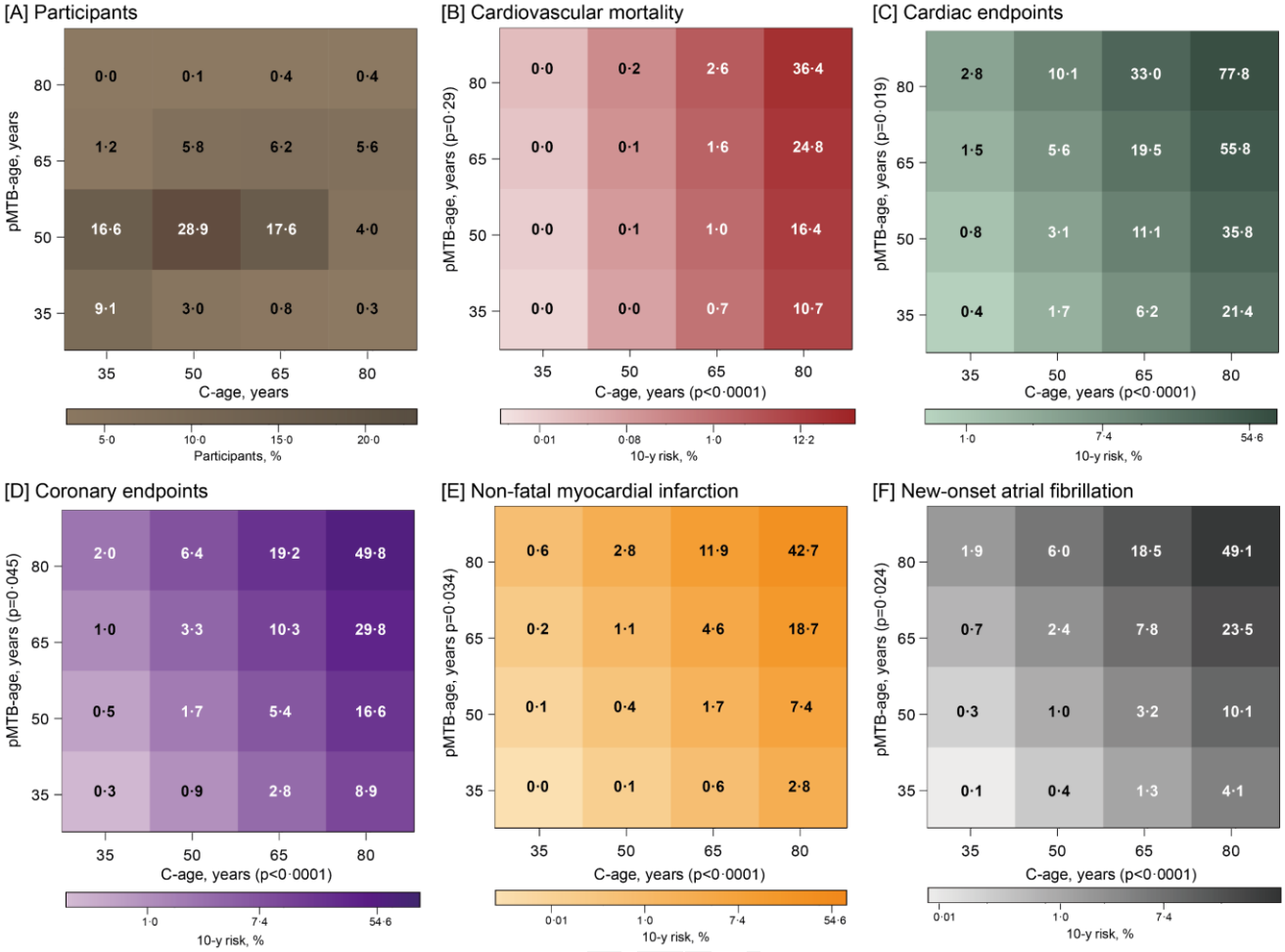


Table 1. **Characteristics of FLEMENGHO and HORTEGA participants**

Characteristic	FLEMENGHO				HORTEGA	
	Derivation	Internal time-shifted validation			External validation	
	2005-2010 (baseline)	2005-2010 (baseline)	2009-2013 (follow-up)	p value	2001	p value
Number in group	729	580			811	
Sex						
Women	368 (50.5)	288 (49.7)		…	414	0.82
Men	361 (49.5)	292 (50.3)			397	
Risk factors						
Active smokers	148 (20.3)	108 (18.6)	84 (14.5)	<0.0001	173 (23.4)	<0.0001
Alcohol intake	287 (39.4)	238 (41.0)	219 (37.8)	0.069	431 (53.1)	<0.0001
Hypertension	301 (41.3)	236 (40.7)	303 (52.2)	<0.0001	320 (43.0)	0.52
Body mass index ≥30 kg/m^2	136 (18.7)	105 (18.1)	139 (24.0)	<0.0001	142 (17.5)	0.56
Waist circumference ≥88/102 cm (♀/♂)	245 (33.6)	189 (32.6)	303 (52.2)	<0.0001	244 (30.1)	0.14
Diabetes	33 (4.5)	23 (3.97)	27 (4.66)	0.046	57 (7.7)	0.038
Previous cardiovascular disease	36 (4.94)	27 (4.66)	34 (5.86)	0.32	83 (10.2)	0.0001
10-year Framingham risk score	7.76 (3.00-16.2)	7.64 (3.07-15.0)	8.87 (4.02-17.8)	0.017	9.85 (2.55-26.9)	0.0033
Use of prescription drugs						
Antihypertensive drugs	186 (25.5)	142 (24.5)	189 (32.6)	<0.0001	133 (17.9)	0.0004
Lipid-lowering drugs	95 (13.0)	77 (13.3)	138 (23.8)	<0.0001	48 (6.44)	<0.0001
Clinical measurements						
Age, y	51.3 (15.4)	51.0 (14.5)	55.7 (14.4)	<0.0001	53.3 (18.7)	0.029
Body mass index, kg/m^2	26.6 (4.37)	26.7 (4.4)	27.4 (4.41)	<0.0001	26.5 (3.98)	0.53

Table1. **Characteristics of FLEMENGHO and HORTEGA participants (cont.)**

Characteristic	FLEMENGHO				HORTEGA	
	Derivation	Internal time-shifted validation			External validation	
	2005-2010 (baseline)	2005-2010 (baseline)	2009-2013 (follow-up)	p value	2001	p value
Waist circumference	90.6 (12.6)	90.9 (12.4)	96.3 (12.6)	<0.0001	88.6 (12.2)	0.0017
Women, cm	86.3 (12.4)	86.6 (12.6)	92.7 (13.2)	<0.0001	83.1 (12.1)	0.0004
Men, cm	95.0 (11.2)	95.2 (12.6)	99.7 (10.8)	<0.0001	94.3 (9.4)	0.36
Systolic pressure, mm Hg	129.4 (17.1)	128.7 (16.2)	132.2 (16.4)	<0.0001	130.4 (19.6)	0.30
Diastolic pressure, mm Hg	80.0 (9.3)	80.0 (9.3)	82.6 (9.70)	<0.0001	79.6 (10.1)	0.43
Mean arterial pressure, mm Hg	96.4 (10.4)	96.2 (10.1)	99.1 (9.99)	<0.0001	96.5 (12.0)	0.92
Heart rate, bpm	63.8 (9.70)	63.2 (9.4)	62.5 (9.76)	0.085	…	…
Arterial measurements						
Brachial-ankle pulse wave velocity, m/s	14.0 (2.8)	14.0 (2.8)	…	…	…	…
Ankle-brachial pressure ratio	1.10 (0.07)	1.10 (0.07)	…	…	…	…
Biochemical measurements						
Serum creatinine, μmol/L	86.6 (15.4)	86.6 (15.8)	90.3 (23.6)	<0.0001	75.1 (17.3)	<0.0001
eGFR, mL/min/1.73 m^2	79.8 (15.5)	80.0 (14.8)	74.8 (15.5)	<0.0001	87.2 (22.0)	<0.0001
Plasma glucose, mmol/L	4.94 (0.76)	4.93 (0.76)	4.97 (0.79)	0.35	5.11 (0.96)	0.0001
Total serum cholesterol, mmol/L	5.25 (0.94)	5.25 (0.93)	5.03 (0.93)	<0.0001	4.33 (0.88)	<0.0001
HDL serum cholesterol, mmol/L	1.42 (0.35)	1.42 (0.36)	1.44 (0.38)	0.19	1.34 (0.29)	<0.0001
Total-to-HDL serum cholesterol ratio	3.88 (1.04)	3.87 (1.02)	3.71 (1.27)	0.0006	3.36 (0.91)	<0.0001

Data are number of participants (%), mean (SD), or median (IQR). Alcohol intake refers to the daily or weekly habitual consumption of alcoholic beverages. Hypertension is a blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic or use of antihypertensive drugs. Diabetes is a fasting plasma glucose of ≥7.0 mmol/L (126 mg/dL), a self-reported diagnosis, diabetes documented in practice or hospital records, or use of antidiabetic drugs. In HORTEGA, diabetes also included a random plasma glucose of ≥11.1 mmol/L (200 mg/dL) or HbA1c of ≥6.5%. eGFR=glomerular filtration rate derived from serum creatinine using the race-free Chronic Kidney Disease Epidemiology Collaboration equation. To convert eGFR from mL/min to mL/s per 1.73 m^2, multiply by 0.0167. HDL=high-density-lipoprotein. P-values denote the significance between baseline and follow-up in 580 FLEMENGHO participants or the difference between the FLEMENGHO and HORTEGA data. An ellipsis indicates not applicable or data unavailable.

Table 2. **Risk biomarkers in relation to age in FLEMENGHO and HORTEGA participants**

Risk markers	Chronological age		pMTB-age		pMTB-age-R	
	r	p value	r	p value	r	p value
FLEMENGHO 2005–2010 (N = 729)						
Systolic blood pressure	0.492	<0.0001	0.276	<0.0001	0.015	0.69
Diastolic blood pressure	0.133	0.0003	0.050	0.18	–0.025	0.50
Mean blood pressure	0.350	<0.0001	0.182	<0.0001	–0.007	0.85
eGFR	–0.696	<0.0001	–0.448	<0.0001	–0.089	0.016
Plasma glucose	0.260	<0.0001	0.535	<0.0001	0.468	<0.0001
Body mass index	0.229	<0.0001	0.216	<0.0001	0.111	0.0026
Waist circumference	0.289	<0.0001	0.225	<0.0001	0.084	0.024
Total serum cholesterol	0.217	<0.0001	0.407	<0.0001	0.344	<0.0001
Total-to-HDL serum cholesterol ratio	0.196	<0.0001	0.335	<0.0001	0.273	<0.0001
Brachial-ankle pulse wave velocity	0.747	<0.0001	0.391	<0.0001	-0.001	>0.99
Ankle-brachial pressure ratio	0.076	0.039	0.085	0.022	0.073	0.050
HORTEGA 2001 (N = 811)						
Systolic blood pressure	0.537	<0.0001	0.341	<0.0001	0.095	0.0069
Diastolic blood pressure	0.245	<0.0001	0.197	<0.0001	0.090	0.010
Mean blood pressure	0.432	<0.0001	0.297	<0.0001	0.103	0.0034
eGFR	–0.569	<0.0001	–0.326	<0.0001	–0.060	0.085
Plasma glucose	0.313	<0.0001	0.398	<0.0001	0.282	<0.0001
Body mass index	0.332	<0.0001	0.312	<0.0001	0.174	<0.0001
Waist circumference	0.422	<0.0001	0.365	<0.0001	0.185	<0.0001
Total serum cholesterol	0.160	<0.0001	0.159	<0.0001	0.094	0.0071
Total-to-HDL serum cholesterol ratio	0.168	<0.0001	0.382	<0.0001	0.344	<0.0001

r is the Pearson correlation coefficient. eGFR=glomerular filtration rate derived from serum creatinine. pMTB-age=age as predicted by the plasma metabolomic profile. pMTB-age-R=residual of the regression of pMTB-age on chronological age, reflecting accelerated aging as predicted by the pMTB-age, independent of chronological age.

Table 3. **Association of the primary endpoints and their components with age in FLEMENGHO and HORTEGA**

	n/N (%)	Chronological age		pMTB-age		pMTB-age-R	
		HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
FLEMENGHO							
Total mortality	68/729 (9.33)	3.44 (2.72–4.34)	<0.0001	2.55 (1.97–3.30)	<0.0001	1.13 (0.89–1.43)	0.33
CV	22/729 (3.02)	7.68 (4.43–13.3)	<0.0001	3.71 (2.42–5.67)	<0.0001	1.42 (0.94–2.14)	0.096
Non-CV	42/729 (5.76)	2.54 (1.95–3.29)	<0.0001	1.96 (1.38–2.77)	0.0001	0.96 (0.71–1.31)	0.80
Cancer	29/729 (3.98)	2.45 (1.80–3.35)	<0.0001	1.90 (1.25–2.89)	0.0026	0.95 (0.66–1.37)	0.78
All CV endpoints	97/729 (13.3)	2.76 (2.31–3.28)	<0.0001	2.33 (1.84–2.95)	<0.0001	1.05 (0.86–1.29)	0.64
Cardiac endpoints	66/729 (9.05)	2.73 (2.21–3.37)	<0.0001	2.81 (2.12–3.71)	<0.0001	1.28 (1.00–1.63)	0.049
Coronary heart disease	34/729 (4.66)	2.54 (1.90–3.39)	<0.0001	2.81 (1.96–4.03)	<0.0001	1.43 (1.03–1.99)	0.035
Stroke	20/729 (2.74)	3.09 (2.05–4.67)	<0.0001	1.80 (1.09–2.96)	0.021	0.80 (0.51–1.24)	0.31
Non-fatal CV events	89/729 (12.2)	2.44 (2.04–2.92)	<0.0001	2.06 (1.60–2.64)	<0.0001	1.02 (0.83–1.26)	0.85
Myocardial infarction	14/729 (1.92)	3.50 (2.06–5.93)	<0.0001	3.52 (2.13–5.82)	<0.0001	1.79 (1.12–2.86)	0.015
Atrial fibrillation	21/729 (2.88)	2.70 (1.84–3.96)	<0.0001	3.06 (1.98–4.75)	<0.0001	1.59 (1.06–2.37)	0.024
HORTEGA							
Total mortality	220/811 (3.02)	3.49 (2.99–4.06)	<0.0001	1.58 (1.42–1.75)	<0.0001	0.94 (0.82–1.07)	0.35
CV	64/811 (7.89)	5.63 (3.83–8.28)	<0.0001	1.50 (1.24–1.82)	<0.0001	0.79 (0.61–1.03)	0.79
Non-CV	102/811 (12.6)	2.71 (2.26–3.26)	<0.0001	1.61 (1.39–1.87)	<0.0001	1.04 (0.86–1.26)	0.68
Cancer	59/811 (7.27)	2.05 (1.69–2.49)	<0.0001	1.56 (1.28–1.90)	<0.0001	1.10 (0.86–1.41)	0.44
All CV endpoints	138/811 (17.0)	3.12 (2.62–3.71)	<0.0001	1.64 (1.45–1.87)	<0.0001	1.02 (0.87–1.21)	0.79
Cardiac endpoints	86/811 (10.6)	3.51 (2.75–4.50)	<0.0001	1.62 (1.37–1.90)	<0.0001	0.97 (0.78–1.20)	0.97
Coronary heart disease	29/811 (3.58)	2.46 (1.77–3.42)	<0.0001	1.54 (1.16–2.05)	0.0028	0.99 (0.69–1.44)	0.98
Stroke	45/811 (5.55)	3.38 (2.41–4.74)	<0.0001	1.70 (1.36–2.12)	<0.0001	1.07 (0.80–1.42)	0.67
Non-fatal CV events	111/811 (13.7)	2.89 (2.40–3.48)	<0.0001	1.72 (1.50–1.98)	<0.0001	1.14 (0.95–1.36)	0.16
Myocardial infarction	19/811 (2.34)	1.94 (1.37–2.75)	0.0002	1.61 (1.15–2.27)	0.0061	1.18 (0.77–1.81)	0.46
Atrial fibrillation	49/811 (6.04)	2.53 (1.93–3.31)	<0.0001	1.65 (1.34–2.04)	<0.0001	1.11 (0.84–1.45)	0.47

HRs, given with 95% confidence interval express the relative risk per 10-year increment in chronological age, pMTB-age, or pMTB-age-R. The number of cardiovascular (CV) and non-cardiovascular (non-CV) do not add up to total mortality, because the cause of death was unknown in 4 FLEMENGHO and 54 HORTEGA participants. HR=hazard ratio. n/N=number of incident endpoints per number of individuals at risk. pMTB-age=age as predicted by the plasma metabolomic profile. pMTB-age-R=residual of the regression of pMTB-age on chronological age, reflecting accelerated aging as predicted by the pMTB-age, independent of chronological age. CV=cardiovascular.