Evaluation of epigenetic age acceleration scores and their associations with CVD related phenotypes in a population cohort

Olga Chervova[1], Elizabeth Chernysheva[2], Kseniia Panteleeva[1], Tyas Arum Widayati[1], Vladimir Maximov[3], Andrew Ryabikov[3], Taavi Tillmann[4], Hynek Pikhart[5], Martin Bobak[5], Vitaly Voloshin[6], Sofia Malyutina[3], and Stephan Beck[1]

[1]UCL Cancer Institute, University College London, London, UK
[2]Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand
[3]Institute of Internal and Preventive Medicine–Branch of Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia
[4]Institute of Family Medicine and Public Health, University of Tartu, Tartu, Estonia
[5]Institute of Epidemiology and Health Care, University College London, London, UK
[6]Royal Botanical Gardens Kew, London, UK

July 6, 2022

Abstract

Background: Epigenetic age acceleration (EAA) is a measure that can be used to investigate the relationship between molecular, clinical and phenotypic data. However, which EAA score is best suited for which phenotype(s) is an open question. To address this issue, we have conducted a comprehensive comparison of multiple EAA scores using currently understudied Eastern European ageing population cohort (HAPIEE) which is richly annotated for diverse phenotypes. Our analysis was based on a subset (n = 306; aged between 45 and 69 years) of samples with available DNA methylation (DNAm) and phenotypic data.

Results: We calculated nine established EAA measures and performed statistical hypothesis testing to investigate associations between these scores and 18 cardiometabolic phenotypes. This was implemented by splitting the data into groups with positive and negative EAAs. We observed strong association between all EAA scores and sex, suggesting that in any analysis EAAs should be adjusted by sex. We found that some sex-adjusted EAA scores were significantly associated with several phenotypes (not necessarily the same phenotypes for different EAAs), indicating that some EAA scores are more phenotype-specific than others. We were able to replicate some of the associations in sex-specific subsets. Furthermore, the most of the EAA associations with cardio-vascular (except presence of Carotid Plaque (CP)) and lipids phenotypes were established in females but not males (and vice versa for CP and alcohol intake). This demonstrates that even after adjusting EAAs for sex, EAA-phenotype associations remain sex-specific, which should be taken into account in any downstream analysis involving EAAs. We observed that in some EAA-phenotype associations, negative EAA scores (i.e. epigenetic age below chronological age) indicated more harmful phenotype values, which is counter-intuitive. Overall, GrimAge was associated with more phenotypes than any of the other EAA scores.

Conclusions: EAAs are sex-specific and should be adjusted for sex in EAA-phenotypes association studies. Associations between EAAs and cardiometabolic phenotypes are sex-specific, even after adjusting for sex. For some EAAs, the direction of the association with phenotype is counter-intuitive. Our results can be used as a guidance on which EAA score to use for which phenotype(s).

Keywords: DNAm age, epigenetic clock, epigenetic age acceleration

Contents

1 Introduction

It has been more than a decade since the very first epigenetic age predictor was proposed [1], and since then dozens of DNA methylation (DNAm) based clocks were developed. The term "epigenetic age" (EA) usually refers to a score calculated from the DNAm measurements at particular loci by applying one of the DNAm clocks - models for predicting EA. Epigenetic age acceleration (EAA) is defined as the deviation of the estimated EA from the chronological age (CA), and usually derived as either difference between EA and CA or as a residual from regressing EA onto CA (EA $\sim$ CA).

At the beginning of the epigenetic clocks era, the first generation EA predictors (e.g. [1], [2], [3]) were aiming primarily on accurate age prediction. The new EAA measures, derived from the second generation of epigenetic clocks (e.g. [4], [5]) are more focused on capturing physiological dysregulation [6] while keeping strong links with chronological age [7].

Various measures of EAA are shown to be associated with different phenotypes and diseases (see e.g. reviews [8] and [9]). For example, deviations in EAA were found to be connected to cancer [10, 11], metabolic syndrome [12], and cognitive function decline [13]. All of these diseases or conditions are linked with ageing, which is a complex process that involves the changes throughout all organs, tissues and cells, and cannot be quantified by a single biological measure. Similarly, there is no single EAA measure that could be pronounced the best epigenetic marker of ageing.

In this study we investigate the relationship between several widely used EAA scores with the phenotypic data on cardio-vascular disease (CVD) related risk factors and conditions available for random population sample (n = 306), which is a part of the Siberian cohort [14] established in 2003 in the frame of multicentre epidemiological study of CVD in East and Central Europe (The HAPIEE Project) [14]. One of our aims was to investigate which EAA measures are "sensitive" to which phenotypes and health-related conditions. Another aim is to identify how EAAs are associated with clinical data in ageing Russian population.

2 Methods

2.1 Data collection

The study is based on the data generated for a subset of the Russian branch of the HAPIEE (Health, Alcohol, and Psychosocial Factors in Eastern Europe) cohort [14], which was established in Novosibirsk

(Russia) in 2003-2005 and followed up in 2006-2008 and 2015-2017. The protocol of baseline cohort examination included assessment of cardiovascular and other chronic diseases history, lifestyle habits and health, socio-economic circumstances, objective measurement of blood pressure (BP), anthropometric parameters, physical performance and instrumental measurement. The details of protocol are reported elsewhere [14].

The dataset for our study consists of $n = 306$ cohort's participants free from baseline CVD with available whole blood DNAm profiles, measured in accordance with manufacturer's recommended procedures using Illumina MethylationEPIC BeadChip (Illumina, San Diego, CA, USA), and was previously described in [15].

2.2 Variables description

Phenotypic data available for our study includes age, sex, systolic and diastolic blood pressure values (SBP and DBP, mmHg), anthropometric parameters - body mass index (BMI, kg/m^2) and waist-hip ratio (WHR, units), smoking status (ever smoked or never smoked), estimated annual alcohol intake (g of ethanol) and number of annual occasions. A person who smoked at least one cigarette a day was classified as a smoker. The amount of alcohol consumed was assessed using Graduated Frequency Questionnaire and converted to pure ethanol (g) [16]. The height and weight was measured with accuracy to 1 mm and 100 g, respectively. Blood pressure (BP) was measured three times (Omron M-5 tonometer) on the right arm in a sitting position after a 5 minute rest period with 2 minutes interval between measurements. The average of three BP measurements was calculated.

Fasting blood serum tests' results contain measured levels of total cholesterol (TC, mmol/l), triglycerides (TG, mmol/l), high-density lipoprotein cholesterol (HDL, mmol/l), gamma-glutamyl transferase (GGT, mmol/l) and plasma glucose (mmol/l). The levels of TC, TG, HDL, GGT and glucose in blood serum were measured enzymatically by KoneLab 300i autoanalyser (Thermo Fisher Scientific Inc., USA) using Thermo Fisher Scientific kits. Friedewald formula [17] was applied to calculate low-density lipoprotein cholesterol (LDL, mmol/l). Fasting plasma glucose (FPG) was calculated from the fasting serum glucose levels using European Association for the Study of Diabetes (EASD) formula [18]. Hypertension (HT) variable refers to SBP $\geq$ 140 mmHg or DBP $\geq$ 90 mmHg according to European Society of Cardiology/European Society of Hypertension (ESC/ESH) Guidelines [19] and/or antihypertensive medication intake within two weeks prior the blood draw. Presence of type 2 diabetes mellitus (T2DM) was defined as FPG $>$ 7.0 mmol/l or current treatment with insulin or oral hypoglycaemic medicines [20]. None of the participants included in our analysis had any history of CVD (myocardial infarction (MI), acute coronary syndrome (ACS), stroke) at the time of baseline examination and blood draw. Binary coronary heart disease (CHD) variable in our dataset codes the incident CHD events (MI/ACS) occurred within 15-year follow-up of cohort.

Carotid arteries were examined with high resolution ultrasound using the systems Vivid q or Vivid7 (GE HealthCare) with 7.5/10-mHz phased-array linear transducer. Device settings were adjusted in accordance with American Society of Echocardiography (ASE) recommendations [21]. Longitudinal and transversal scans were performed at the right and left common carotid arteries with branches to assess the anatomy and atherosclerotic lesions. The digital images were archived and the measurements were conducted off-line by one experienced researcher (A.R.) who was blinded to the participants' characteristics [22]. The plaques were defined in accordance with the Mannheim consensus [23]. For present analysis we used two phenotypes of atherosclerosis: presence of at least one carotid plaque (CP) and multiple plaques (MCP). The ultrasound variables are only available for a subset of samples ($n = 105$, 35% of all samples).

Individual phenotypes were also joined into five groups of phenotypes, which we define as follows:

1. **Anthropometric:** BMI and WHR;
2. **Lifestyle:** smoking status and annual alcohol consumption (intake and number of occasions);
3. **Metabolic:** GGT, T2DM and plasma glucose;
4. **Lipids:** TC, HDL, LDL, TG;
5. **Cardio-vascular:** SBP, DBP, HT, CHD, CP and MCP.

EAA	Clock	Info
HannumAA	Hannum [2]	Residuals from regressing EA on CA
HannumEEAA	Hannum [30]	Residual from regressing the weighted average of Hannum's EA and estimated measures of blood cells counts on CA
HannumIEAA	Hannum [30]	Residuals from regressing Hannum's EA on CA and various blood immune cell counts
HorvathAAd	Horvath [3]	Difference between EA and CA
HorvathAAr	Horvath [3]	Residuals from regressing EA on CA
HorvathIEAA	Horvath [30]	Residuals from regressing Horvath's EA on CA and various blood immune cell counts
SkinBloodAA	Skin and Blood [29]	Residuals from regressing EA on CA
PhenoAA	PhenoAge [4]	Residuals from regressing EA on CA
GrimAA	GrimAge [5]	Residuals from regressing EA on CA
GrimAA	GrimAge [5]	Residuals from regressing EA on CA

Table 1: **Summary of the EAA scores measured by DNA Methylation Online Calculator.** ***Abbreviations:*** ***CA*** *- chronological age,* ***EA*** *- epigenetic age,* ***EAA*** *- epigenetic age acceleration,* ***IEAA*** *- intrinsic epigenetic age acceleration,* ***EEAA*** *- extrinsic epigenetic age acceleration*

2.3 DNAm data quality control (QC) and preprocessing

In preprocessing raw DNAm data we mostly followed the procedures from [24] which are in line with manufacturer's recommended steps. In brief, we checked array control probes' metrics (Illumina Bead Control Reporter), signal detection p-values, bead count numbers for all available cytosine-phospate-guanine (CpG) probes. Furthermore, we compared actual and DNAm predicted sex data for each sample. Only samples with less than 1% CpGs with detection $p \geq 0.01$, and probes with bead count numbers ≥ 3 and detection $p < 0.01$ in at least 99% of samples in the dataset, were used in our analyses. Initial DNAm data processing and QC based data filtering were implemented using `R` v.4.1.0 [25] together with specialised `R` libraries `minfi` [26], `ChAMP` [27] and `ENmix` [28].

2.4 Epigenetic Age Acceleration

Epigenetic age acceleration (EAA) scores were calculated using DNA Methylation Online Calculator [3]. This web based tool gives nine EAAs based on five epigenetic scores, namely, on Horvath's [3], Hannum's [2], Skin and Blood [29], PhenoAge [4] and GrimAge [5] measures, see Table 1.

2.5 Grouping

Analysis of associations between EAAs and phenotypes in our study involves comparing the distributions of the phenotypic data in two groups. The grouping is based on binary split with respect the sign of EAA, and defined as follows:

$$\text{All samples} = \begin{cases} \text{EAA}^{+}, & \text{samples with non-negative EAA,} \\ \text{EAA}^{-}, & \text{samples with negative EAA.} \end{cases} \tag{1}$$

In other words, we use the definition (1) to split our cohort into two groups with $\text{EAA} < 0$ and $\text{EAA} \geq 0$ for each EAA, and study the differences in phenotypes' distribution between these groups.

2.6 Statistical Analysis

All statistical analyses were performed using `R` v.4.1.2. They include descriptive analysis of the available data using relevant techniques (uni- and multivariate analyses), statistical hypothesis testing (Welch's t-test [31] for continuous variables and Fisher's exact test [32] for binary data), linear regression based data adjustments. The significance level is defined as $\alpha = 0.05$. All the graphs presented in the paper were produced using `ggplot2` [33] and its extensions, `pheatmap` [34], `PerformanceAnalytics` [35] and base `R` functions.

3 Results

3.1 Associations between sex and phenotypes

Our dataset consists of ($n = 306$) samples (166 females and 140 males). Summaries of the dataset characteristics for all samples and for sex-specific groups are given in Tables S1 and S3. In particular, Table S1 contains descriptive statistics (range, mean and standard deviation) for the available continuous phenotype data and the corresponding Welch's t-test p-values and 95% confidence intervals. Table S3 includes count numbers and percentages for dichotomous variables, together with sex-specific odds ratios, 95% confidence intervals and p-values calculated by performing Fisher's exact statistical test.

In our dataset there is no significant difference between males and females in distributions of age, blood pressure values (both systolic and diastolic), incidence of acute CHD events or diagnoses of hypertension and diabetes, levels of triglycerides and fasting glucose. Among the significantly different in males vs. females phenotypes are anthropometric measures (BMI and WHR), lifestyle choices (alcohol consumption and smoking status), blood levels of gamma-glutamyl transferase (GGT) and lipids (both LDL and HDL). Interestingly, in our dataset there is no significant difference between male and female odds ratios of being diagnosed with CP, but the odds ratios of having MCP significantly differ between sexes.

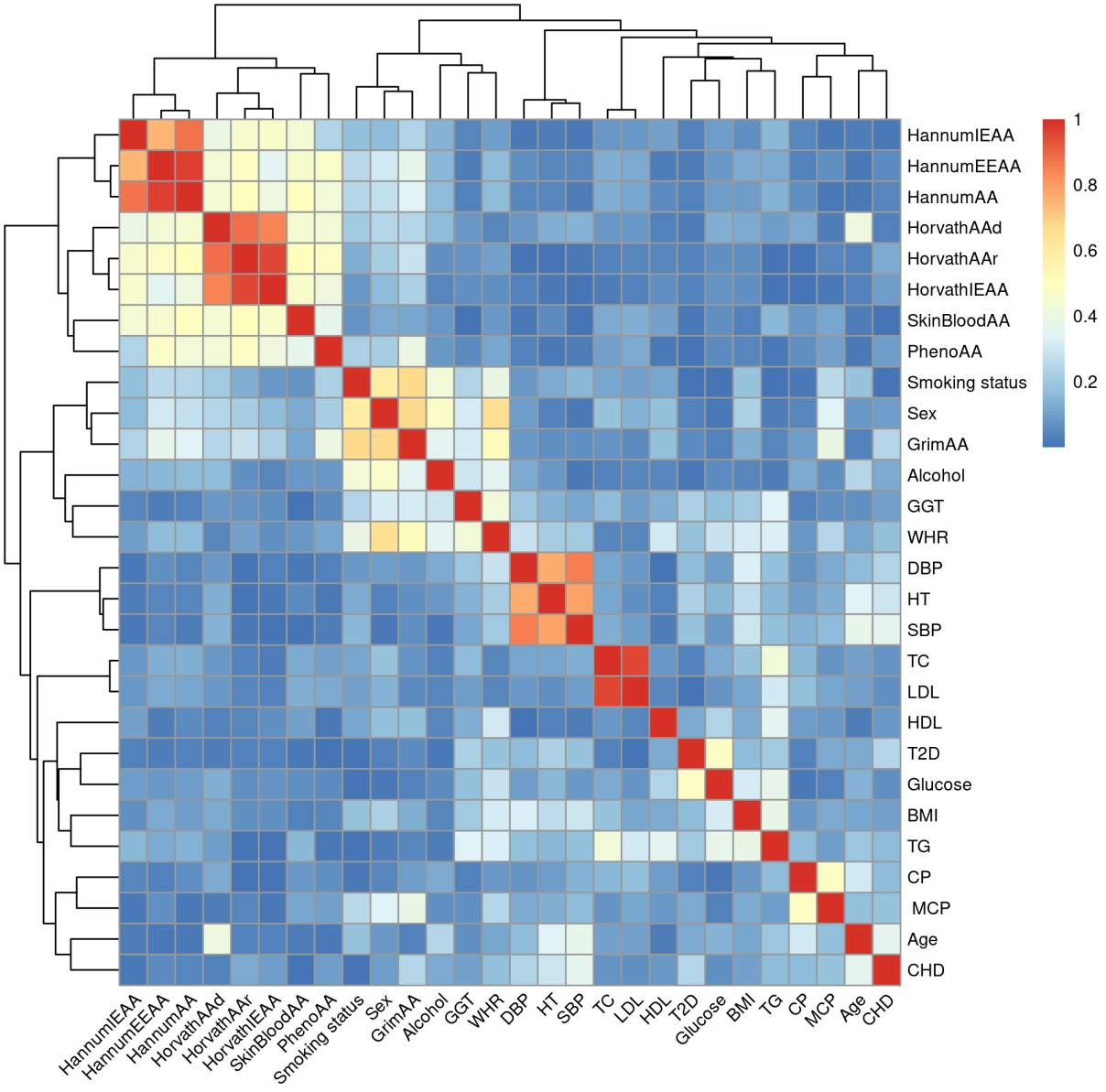

Figure 1: Heatmap of the correlations between all available traits

3.2 EAAs are associated with some phenotypes and have strong sex bias

Our EAA analyses are based on nine EAA scores (described in Section 2.4) which were obtained from the five different epigenetic clock models, with multiple EAA scores derived from Horvath's multi-tissue and Hannum's clocks (3 EAAs each). Correlation coefficients are higher among EAAs based on the same clock than among EAAs from different clocks, namely, Pearson correlation coefficients range between 0.78 and 0.97 within EAAs derived from Horvath's and Hannum's models, whilst the highest value for between the clocks correlations is $r = 0.57$, see correlation table on Figure S3. Note that neither of the EAAs is significantly correlated with chronological age apart from HorvathAAd, which is the only measure calculated without chronological age adjustment.

To explore the connections among the variables, we calculated correlation coefficients (Spearman correlation) and normalised entropy-based mutual information values for all the phenotypes and EAAs. Heatmaps for correlations (absolute values) and mutual information values, together with correlations-based network plot, are presented on corresponding Figures 1, S1 and S2 respectively. For both correlations and mutual information the EAAs clustered together apart from the GrimAA, which displays very strong associations with sex and smoking status.

We further investigated the relationship between phenotypes and EAAs by splitting dataset into EAA^+ and EAA^- groups using (1), and, subsequently, testing the phenotype data distribution using t-test (for continuous variables), and Fisher's exact test (for binary variables). The corresponding statistical testing results are presented in Table S5.

We noted that for the whole dataset the sizes of EAA^+ and EAA^- groups for different EAA measures were within 45%-55% range, apart from the HorvathAAd (32% EAA^+ samples vs. 68% EAA^- samples and GrimAA (61% EAA^+ samples vs. 39% EAA^- samples. Sex specific group splitting was found to be very unbalanced for all the EAAs for both sexes with an exception of HorvathIEAA, see Table S4. Furthermore, we observed significant differences in distributions of all nine EAA measures in our data between males and females, the corresponding data along with descriptive statistics are presented in Table S2. Taking into account strong associations between sex and phenotypes, the obtained significant results on EAA-phenotype associations might be confounded by sex.

3.3 Sex-adjusted EAAs are associated with various phenotypes

In order to eliminate the unwanted bias we adjusted all the EAA scores by sex and repeated the analysis described in previous section based on the calculated adjusted EAAs (adjEAA). Splitting the data into EAA^+ and EAA^- resulted in balanced group sizes for all the adjEAAs, all the groupings are within 44%-56%, see Table S4, and the medians of the adjEAAs for sex-specific subsets located closer to 0 compared to the medians of unadjusted EAAs, see Figure 2**A**.

The results on testing the differences in phenotype distribution between EAA^+ and EAA^- groups are given in Table 2. For all the available samples only four adjEAAs (GrimAA, PhenoAA, Horvath's residuals and IEAA) demonstrated statistically significant results for six phenotypes, with 4 phenotypes highlighted by the GrimAA, and one phenotype each by the rest of the adjAAs (7 phenotype-EAA combinations in total). Among the differently distributed phenotypes are blood levels of GGT and LDL, smoking status and annual alcohol consumption, diagnosed MCP and incident CHD status, with the latter being the only phenotype that was tested significantly different by multiple adjEAAs (GrimAA and Horvath's differences). Interestingly, for the GrimAA incident CHD and smoking status stayed significantly different for both males-only and females-only subsets, whilst GGT was not significantly different in any sex-specific groups.

Out of seven statistically significant for all samples phenotype-EAA combinations, five remained significant in male and three in female subsets. In males significant differences between EAA^+ and EAA^- groups were confirmed by four EAAs (same as for all the samples) and 7 phenotypes (10 phenotype-EAA combinations). Significant results for females feature seven EAAs (no Horvath's and HannumIEAA) and 10 traits (21 phenotype-EAA combinations). Nearly half (10 out of 21) of the results for female subgroup presented in Table 2 relate to the blood lipids measures (TG and lipoproteins, including both LDL and HDL), another 6 results for females relate to presence of hypertension diagnosis and blood pressure values (SBP and DBP). Neither lipids nor blood pressure related phenotypes were associated with EAA^+/EAA^- grouping in males, unlike presence of (M)CP diagnosis. Anthropometric parameters were also found to be statistically different in both sex-specific groups (BMI in males and WHR in females), but not for the combined dataset.

Phenotype	EAA	p, all	95% CI, all	p, F	95% CI, F	p, M	95% CI, M
Anthropometric							
BMI	GrimAA					0.039	(-3.079, -0.079)
WHR	HorvathAAd			0.004	(-0.048, -0.009)		
	GrimAA			0.010	(0.006, 0.046)		
Lifestyle							
Smoking status	GrimAA	< 0.001	(1.799, 4.895)	0.026	(1.077, 8.931)	< 0.001	(4.5, 58.7)
	HorvathAAd			0.016	(1.140, 9.454)		
	PhenoAA					0.004	(1.360, 8.319)
Alcohol, annual intake	HorvathIEAA	0.028	(-2832, -163)			0.023	(-5522, -422)
	GrimAA					0.049	(16, 5370)
Metabolic							
GGT	GrimAA	0.023	(0.728, 9.699)				
	HorvathAAr					0.030	(0.738, 14.5)
Lipids							
TC	HannumAA			0.009	(-0.947, -0.141)		
	GrimAA			0.046	(0.008, 0.818)		
	PhenoAA			0.010	(-0.919, -0.127)		
	HannumEEAA			0.003	(-1.004, -0.203)		
TG	GrimAA			0.015	(0.070, 0.632)		
HDL	HorvathAAr			0.013	(0.026, 0.219)		
	SkinBloodAA			0.027	(0.012, 0.205)		
LDL	PhenoAA	0.037	(-0.523, -0.016)	0.004	(-0.840, -0.157)		
	HannumAA			0.010	(-0.811, -0.112)		
	HannumEEAA			0.002	(-0.904, -0.215)		
Cardio-vascular							
CHD	GrimAA	< 0.001	(1.518, 4.060)	0.001	(1.458, 5.955)	0.042	(1.020, 4.389)
	HorvathAAr	0.006	(1.187, 3.139)			0.018	(1.150, 4.995)
CP	GrimAA					0.009	(1.367, 22.723)
MCP	GrimAA	0.004	(1.584, 26.779)			0.009	(1.401, 33.864)
HT	HorvathAAd			0.005	(0.202, 0.781)		
	GrimAA			0.043	(0.987, 3.764)		
SBP	HorvathAAd			0.008	(-20.1, -3.2)		
	GrimAA			0.024	(1.3, 18.4)		
DBP	HorvathAAd			0.003	(-10.626, -2.144)		
	GrimAA			0.039	(0.228, 8.832)		

Table 2: **Significant differences between $\mathbf{EAA^+}$ and $\mathbf{EAA^-}$ groups.**
Significance level $\alpha = 0.05$. Welch's t-test (**H_0:** Mean value of given variable in EAA^+ and EAA^- groups are not different) was used for continuous variables, Fisher's exact test (**H_0:** Classifications of a given binary variable in EAA^+ and EAA^- groups are not different) was used for binary variables.

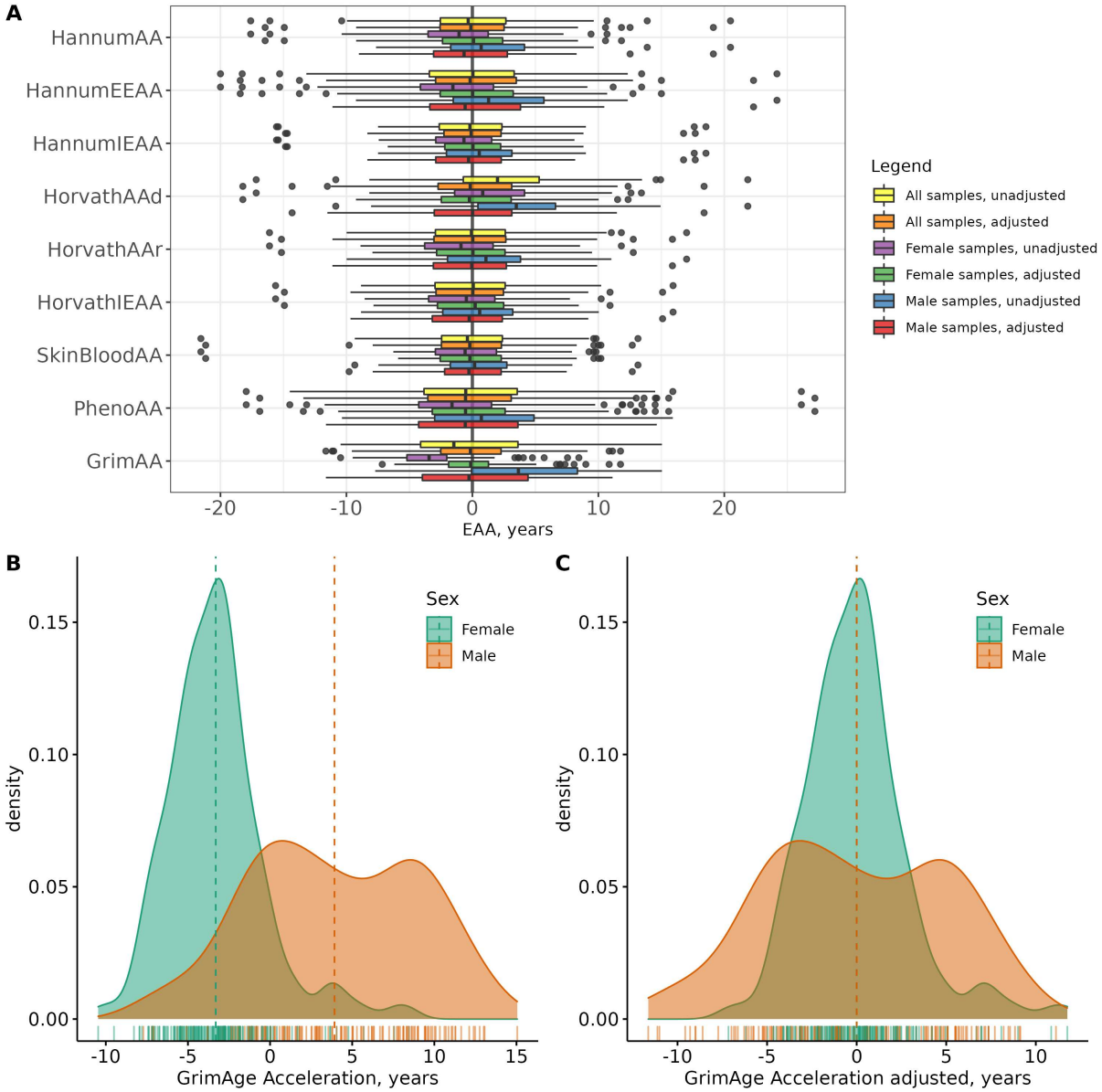

Figure 2: Boxplots of EAAs and sex-adjusted EAAs for all samples and sex-specific subsets **A**. Sex-specific GrimAA distribution before **B** and after **C** adjustment for sex data.

3.4 Directions of some EAA-phenotype associations in sex-specific subsets are different

Results presented in Tables 2 and 3 summarise the significant outcomes of statistical hypotheses testing of out data based on grouping (1). Some of the phenotypes in sex-specific groups were highlighted by multiple EAAs, e.g. female TC (HannumAA, PhenoAA, GrimAA and EEAA) and male alcohol consumption (IEAA and GrimAA), but the signs of the groups' mean differences are not consistent.

For instance, in Table 2 the confidence intervals for female WHR are of different sides of 0 for GrimAA and HorvathAAd. Further investigation revealed that EAA^+ group have higher mean WHR than EAA^- for GrimAA, but opposite true for HorvathAAd, see Figure S7. Furthermore, the mean WHR values were higher in EAA^- group for all three EAAs derived from Horvath's clock, together with HannumIEAA and SkinBloodAA. Similar trends were observed in male annual alcohol consumption, see Figure S4, and in female levels of TC, HDL, LDL, and blood pressure values (SBP and DBP), see Figures S10, S9, S8, S13 and S12 respectively.

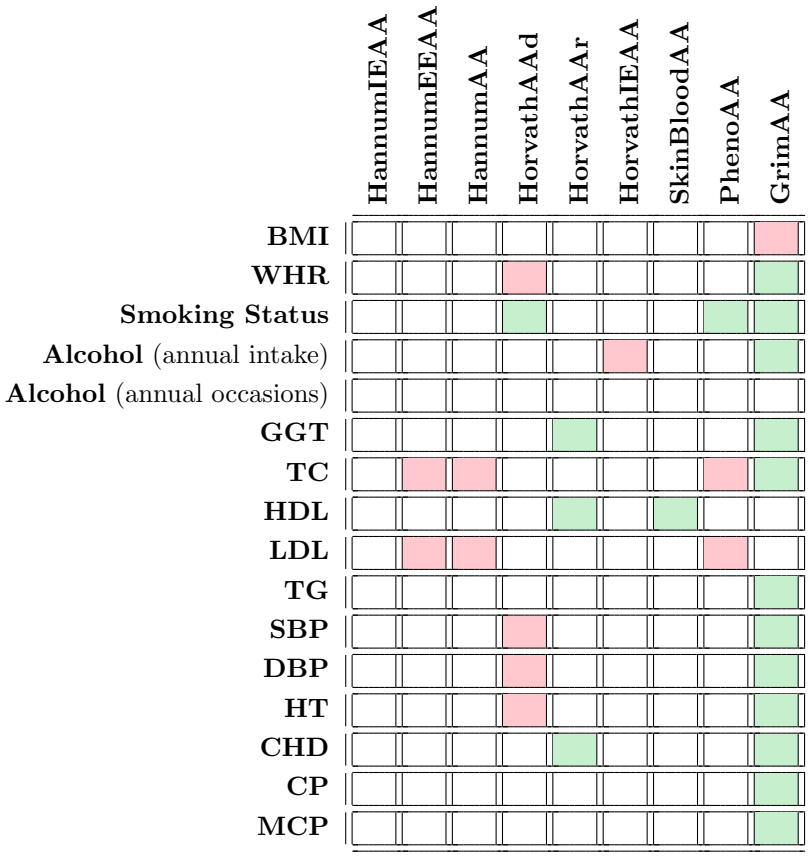

Table 3: **EAA-Phenotype association table.** White colour indicates no significant association, green/red colour indicate significantly higher/lower values of phenotype measures (higher odds ratios) in EAA^+ group compared to EAA^- for continuous (binary) phenotypes.

4 Discussion

Question of choosing the "best" or "most suitable" EAA to study particular phenotypes is yet to be answered. For our data we decided to take into consideration all the EAA measures that could be calculated using DNA Methylation Online Calculator [3], which is an amazing and relatively easy to use open access tool. Epigenetic clocks included into the Online Calculator are featured in vast majority of studies in EAA-phenotype/disease associations (see e.g. [36, 37, 38, 39]) and in benchmarking the newly developed DNAm based clocks' performance (see e.g.[40]).

Ability of the considered nine EAAs to reflect the differences in phenotype distribution was investigated by splitting the data based on the sign of the EAA scores. Similar grouping was also used in [41], where the risk of CHD was studied by splitting HorvathAAd and HannumAA to positive and negative groups. In recent paper [36], the authors used the positive/negative GrimAA and PhenoAA split to study the incident diabetes in the Coronary Artery Risk Development in Young Adults (CARDIA) cohort.

All the considered EAAs were independent (apart from HorvathAAd) of chronological age, but clearly sex-biased (Table S2), with generally lower EAA values for females. It was particularly obvious for the GrimAA scores, with distribution profiles separated for males and females, see Figure 2 **B-C**. As we pointed out in Section 3.3, splitting the dataset into EAA^+ and EAA^- groups revealed big variation in group sizes for different scores (Table S4), which became particularly extreme for sex-specific subsets. To avoid unwanted confounding, for our analyses we adjusted EAAs by sex and proceeded with adjEAA values. This step resulted in more balanced EAA^+/EAA^- group split for all adjEAAs. Of course, adjusting EAAs for sex did not affect the actual differences in phenotypes distributions between male and female subjects (see Tables S3 and S1). As a result, several phenotype-EAA combinations, which have previously demonstrated statistically significant results, did not persist after the adjustment (see Table S5).

Due to some phenotype show sex-specific behaviour, see e.g. [42, 43, 44], we presented the results for males and females separately, alongside the results for the entire dataset. In one of the recent reviews [45], the authors pointed out the lack of sex-specific results involving EAAs and recommended splitting data by sex in downstream analyses. Our results confirm the importance of using EAAs in sex-specific groups. We observed that the most phenotypes are reflected by some EAAs in one sex-specific group only.

We found that in our dataset both BMI and WHR were significantly different in males and females. Without adjusting for sex, multiple EAAs groupings highlighted significant differences in both BMI and WHR for all samples, but none of those associations replicated after adjustment (see Table S5). It is known [42], that in females WHR is associated with risks of CHD regardless of BMI, whilst in males WHR was found to be associated with incidence of CHD only for subjects with normal BMI measures. Our analysis found the anthropometric parameters to be statistically different in both sex-specific groups (BMI (GrimAA) in males and WHR (GrimAA and HorvathAA) difference in females), which is in line with results reported in large-scale US Sisters study [46] and Taiwan Biobank [47] cohorts. Interestingly (and opposite to findings in [42]), in [47] the authors report significant associations between WHR and EAAs (PhenoAge and GrimAge) in males, and between BMI and EAAs (PhenoAge and GrimAge) in females, which is the other way round in our dataset (WHR in females and BMI in males). We would like to point out, that GrimAA grouping revealed higher mean WHR in female EAA^+, but lower male mean BMI in the same group with positive EAAs (see Figures S7 and S6).

Lifestyle habits, including diet, smoking and alcohol consumption, are known to impact DNAm and being associated with epigenetic age in multiple studies, see e.g. [48, 49, 50]. Some DNAm clocks were specifically developed to be sensitive to smoking status, like, for example, GrimAge [5]. GrimAA was the only score associated with smoking status in the entire dataset, and the associations replicated in sex-specific subsets (Table 2). PhenoAA and HorvathAAd were also found to be significantly associated in male and female subgroups respectively. HorvathIEAA was significantly associated with annual alcohol consumption for all the samples and this association persisted in males, together with GrimAA, but not in females. Interestingly, the mean annual alcohol volume was higher in EAA^+ group for GrimAA, but lower in EAA^+ group for IEAA (Figure S4), which is not in line with the current state of the art in alcohol-ageing relationship, see e.g. review [51].

Previous publications suggest that EAAs are associated with diabetes and/or glucose levels [52, 53, 9]. It was also found that positive GrimAA (but not PhenoAA) is associated with higher 5-10 years incidence of type 2 diabetes, particularly for obese individuals [36]. In analysing our dataset we have not observed any significant associations of the considered EAAs with prevalent type 2 diabetes mellitus (T2DM) status and/or fasting blood glucose values. This might be attributed to the small proportion (11%) of

the diabetics in our data compared to other studies (e.g. nearly 20% in [53]). Blood levels of GGT are associated with many dismetabolic conditions, including fatty liver, excessive alcohol consumption, increased risks of CHD and T2DM [54, 55], and is known to be different in men and women [43], with no unified reference values. For the entire dataset, GrimAA EAA^+/EAA^- grouping demonstrated significant difference in serum GGT measures. This result was not replicated in sex-specific subsets, but at the same time, in male subgroup GGT level difference was detected in HorvathAAr split (see Figure S5).

Blood levels of total cholesterol, TG and lipoproteins (HDL and LDL) are known to be sex-specific and associated with risk of developing CVD in both sexes, see e.g. [56]. Changes in lipids concentrations are also shown to be reflected in age-related changes in DNAm following dietary interventions [57]. Furthermore, associations of EAAs and lipids levels were confirmed in several studies [58, 59]. In our entire dataset, among all available lipids data, only mean LDL levels in EAA^+ with PhenoAA grouping were significantly lower than in EAA^-, and this result persisted in female subset. No significant differences in mean lipids concentrations were highlighted by any EAA split for the male subgroup, whilst ten EAA-lipids phenotypes associations were highlighted in females. In particular, in female subset GrimAA grouping demonstrated significantly higher group mean levels of total cholesterol and TG in EAA^+ compared to EAA^- (see Table 2, Figures S10 and S11). At the same time mean TC and LDL concentrations (Figure S8) were significantly lower in EAA^+ group in PhenoAA, HannumAA and EEAA splits. Female HDL levels associations were picked up in SkinBloodAA and HorvathAAr groupings, with higher lipoprotein concentration in EAA^+ group (see Figure S9). Remarkably, for all four considered lipids-related measures, known CVD risk factors (high TC, LDL, TG, and low HDL) were associated (not all significantly) with positive age acceleration only for GrimAA grouping, whilst the opposite was demonstrated in all the significant (and vast majority of insignificant) EAAs-lipids associations based on other EAA splits (see Figures S8, S9, S10 and S11). In view of recently published age-related sex-specific trends in lipid levels [60] and hypertension prevalence [44], it would be interesting to conduct an extended sex-specific analyses on EAA-lipids and hypertension associations for the particular age groups to see whether EAA values reflect the observed age-related patterns.

Data on carotid atherosclerosis and advanced atherosclerosis, which are defined in our study as the presence of at least a single (CP) and multiple carotid plaques (MCP) respectively, was available for only 34% of the participants, with 50/23/14 and 55/23/2 total/CP/MCP samples available for males and females. Only GrimAA grouping was significantly associated with CP in males and MCP in the entire dataset and its male only subset. In female specific subset blood pressure values (both SBP and DBP) and hypertension status were significantly associated with HorvathAAd and GrimAA groupings. Interestingly, in case of GrimAA group split, mean values of SBP and DBP were higher in EAA^+ group, which might indicate the increased risk of CVD [61]. This is the opposite to the corresponding results of HorvathAAd grouping. None of these phenotypes were highlighted in the entire dataset and male subset. Two groupings, GrimAA and HorvathAAr, were significantly associated with incident CHD for all available samples. The results persisted in male subset for both groupings and in female subset for GrimAA split only. Similar results were also described in Genetic Epidemiology Network of Arteriopathy (GENOA) dataset study [53], where the authors reported significant connections not only between GrimAA and incident CVD, but also between GrimAA and time to the CVD event.

Notably, while higher odds of CHD were associated with EAA^+ for both GrimAA and HorvathAAr, only GrimAA EAA^+ was consistently associated with more harmful phenotypes values, indicating higher risk of CHD. All other EAA splits demonstrated mostly the opposite behaviour regarding available risk factors (lipids, anthropometric, lifestyle and cardio-vascular).

5 Conclusions

Our study conducted on a subset of HAPIEE cohort shows that EAAs are sex-specific and should be adjusted for sex in EAA-phenotypes association studies. Moreover, even after adjusting for sex, the associations between EAAs and considered 18 cardiometabolic phenotypes are sex-specific. The only two phenotype-EAA associations persisted through the entire dataset and both male and female subsets are incident CHD and smoking status.

Among all considered epigenetic clocks, GrimAge was significantly associated with more phenotypes than any other EA scores. Furthermore, for some EAAs, the direction of the association with phenotype is counter-intuitive, i.e. lower EAA scores corresponded to more harmful values of the phenotypes.

The obtained results could be used as a guidance on which EAA score to use for which phenotype(s).

6 Declarations

6.1 Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethics committee of IIPM—Branch of IC&G SB RAS (Institute of Internal and Preventive Medicine—Branch of Federal State Budgeted Research Institution, "Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences"), Protocol no. 1 from 14 March 2002 and Protocol no. 12 from 8 December 2020. All study participants provided informed consent.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and materials

Raw DNA methylation IDAT files are in the process of being deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS00001006390. Further information about EGA can be found on `https://ega-archive.org` "The European Genome-phenome Archive of human data consented for biomedical research" [62].

6.4 Competing interests

The authors declare that they have no competing interests.

6.5 Funding

The baseline HAPIEE study was funded by the Welcome Trust (WT064947, WT081081, 106554/Z/14/Z), the US National Institute of Aging (1RO1AG23522). O. Chervova and S. Beck were supported by grants from the Frances and Augustus Newman Foundation (172074). O. Chervova, S. Beck, M. Bobak and H. Pikhart were supported by EU-H2020 Project "CETOCOEN Excellence" (857560). T.A. Widayati was funded by Indonesian Endowment Fund (Lembaga Pengelola Dana Pendidikan).

6.6 Authors' contributions

OC drafted the manuscript with the input from all the authors. EC, KP and TAW equally contributed to all aspects of data analysis and draft preparation. AR, VM, MB, HP, SM, and SB participated in data generation and analyses design. VV, SM and SB supervised the study. All authors read and approved the manuscript.

Abbreviations

ACS Acute coronary syndrome

adjEAA Adjusted epigenetic age acceleration

ASE American Society of Echocardiography

BMI Body mass index

BP Blood pressure

CA Chronological age

CARDIA Coronary Artery Risk Development in Young Adults

CHD Coronary heart disease

CP Carotid plaque

CpG Cytosine-phospate-guanine

CVD Cardio-vascular disease

DBP Diastolic blood pressure

DNAm DNA methylation

EA Epigenetic age

EAA Epigenetic age acceleration

EASD European Association for the Study of Diabetes

EEAA Extrinsic epigenetic age acceleration

EGA European Genome-phenome Archive

ESC European Society of Cardiology

ESH European Society of Hypertension

FPG Fasting plasma glucose

GENOA Genetic Epidemiology Network of Arteriopathy

GGT Gamma-glutamyl transferase

HAPIEE Health, Alcohol, and Psychosocial Factors in Eastern Europe

HDL High-density lipoprotein

HT Hypertension

IEAA Intrinsic epigenetic age acceleration

IIPM Institute of Internal and Preventive Medicine

LDL Low-density lipoprotein

MCP Multiple carotid plaques

MI Myocardial infarction

QC Quality control

SBP Systolic blood pressure

T2DM Type 2 diabetes mellitus

TC Total cholesterol

TG Triglycerides

WHR Waist-hip ratio

References

[1] Bocklandt S, Lin W, Sehl ME, Sánchez FJ, Sinsheimer JS, Horvath S, et al. Epigenetic predictor of age. PloS one. 2011;6(6):e14821.

[2] Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular Cell. 2013;49(2):359-67.

[3] Horvath S. DNA methylation age of human tissues and cell types. Genome Biology. 2013;14(10):1-20.

[4] Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, et al. An epigenetic biomarker of aging for lifespan and healthspan. Aging (Albany NY). 2018;10(4):573.

[5] Lu AT, Quach A, Wilson JG, Reiner AP, Aviv A, Raj K, et al. DNA methylation GrimAge strongly predicts lifespan and healthspan. Aging (Albany NY). 2019;11(2):303.

[6] Ecker S, Beck S. The epigenetic clock: a molecular crystal ball for human aging? Aging (Albany NY). 2019;11(2):833.

[7] Jylhävä J, Pedersen NL, Hägg S. Biological age predictors. EBioMedicine. 2017;21:29-36.

[8] Horvath S, Raj K. DNAmethylation-based biomarkers and the epigenetic clock theory of ageing. Nature Reviews Genetics. 2018;19(6):371-84.

[9] Oblak L, van der Zaag J, Higgins-Chen AT, Levine ME, Boks MP. A systematic review of biological, social and environmental factors associated with epigenetic clock acceleration. Ageing research reviews. 2021;69:101348.

[10] Perna L, Zhang Y, Mons U, Holleczek B, Saum KU, Brenner H. Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. Clinical epigenetics. 2016;8(1):1-7.

[11] Qin N, Li Z, Song N, Wilson CL, Easton J, Mulder H, et al. Epigenetic age acceleration and chronic health conditions among adult survivors of childhood cancer. JNCI: Journal of the National Cancer Institute. 2021;113(5):597-605.

[12] Nannini DR, Joyce BT, Zheng Y, Gao T, Liu L, Yoon G, et al. Epigenetic age acceleration and metabolic syndrome in the coronary artery risk development in young adults study. Clinical epigenetics. 2019;11(1):1-9.

[13] Beydoun MA, Shaked D, Tajuddin SM, Weiss J, Evans MK, Zonderman AB. Accelerated epigenetic age and cognitive decline among urban-dwelling adults. Neurology. 2020;94(6):e613-25.

[14] Peasey A, Bobak M, Kubinova R, Malyutina S, Pajak A, Tamosiunas A, et al. Determinants of cardiovascular disease and other non-communicable diseases in Central and Eastern Europe: rationale and design of the HAPIEE study. BMC Public Health. 2006;6(1):1-10.

[15] Malyutina S, Chervova O, Tillmann T, Maximov V, Ryabikov A, Gafarov V, et al. The Relationship between Epigenetic Age and Myocardial Infarction/Acute Coronary Syndrome in a Population-Based Nested Case-Control Study. Journal of Personalized Medicine. 2022;12(1):110.

[16] Hilton ME. A comparison of a prospective diary and two summary recall techniques for recording alcohol consumption. British journal of addiction. 1989;84(9):1085-92.

[17] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry. 1972;18(6):499-502.

[18] Ryden L, Standl E, Bartnik M, Van den Berghe G, Betteridge J, De Boer MJ, et al. Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary: The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD). European heart journal. 2007;28(1):88-136.

[19] Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). European heart journal. 2018;39(33):3021-104.

[20] Rydén L, Grant P, Anker S, Berne C, Cosentino F, Danchin N, et al. Authors/Task Force Members; ESC Committee for Practice Guidelines (CPG); Document Reviewers. ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD: the Task Force on diabetes, pre-diabetes, and cardiovascular diseases of the European Society of Cardiology (ESC) and developed in collaboration with the European Association for the Study of Diabetes (EASD). European heart journal. 2013;34(39):3035-87.

[21] Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force endorsed by the Society for Vascular Medicine. Journal of the American Society of echocardiography. 2008;21(2):93-111.

[22] Ryabikov A, Malyutina S, Halcox J, Nikitin Y, Marmot M, Bobak M. Prevalence and predictors of carotid wall triple line pattern in a general population sample. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(7):1682-8.

[23] Touboul PJ, Hennerici M, Meairs S, Adams H, Amarenco P, Bornstein N, et al. Mannheim carotid intima-media thickness and plaque consensus (2004–2006–2011). Cerebrovascular diseases. 2012;34(4):290-6.

[24] Chervova O, Conde L, Guerra-Assunção JA, Moghul I, Webster AP, Berner A, et al. The Personal Genome Project-UK, an open access resource of human multi-omics data. Scientific Data. 2019;6(1):1-10.

[25] R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria; 2021. Available from: `https://www.R-project.org/`.

[26] Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30(10):1363-9.

[27] Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A, et al. ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics. 2017;33(24):3982-4.

[28] Xu Z, Niu L, Li L, Taylor JA. ENmix: a novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic acids research. 2016;44(3):e20-0.

[29] Horvath S, Oshima J, Martin GM, Lu AT, Quach A, Cohen H, et al. Epigenetic clock for skin and blood cells applied to Hutchinson Gilford Progeria Syndrome and ex vivo studies. Aging (Albany NY). 2018;10(7):1758.

[30] Horvath S, Gurven M, Levine ME, Trumble BC, Kaplan H, Allayee H, et al. An epigenetic clock analysis of race/ethnicity, sex, and coronary heart disease. Genome Biology. 2016;17(1):1-23.

[31] Welch BL. The generalization of 'STUDENT'S'problem when several different population varlances are involved. Biometrika. 1947;34(1-2):28-35.

[32] Fisher RA. On the interpretation of χ^2 from contingency tables, and the calculation of P. Journal of the Royal Statistical Society. 1922;85(1):87-94.

[33] Hadley W. Ggplot2: Elegrant graphics for data analysis. Springer; 2016.

[34] Kolde R. Package 'pheatmap'. R package. 2015;1(7):790.

[35] Peterson BG, Carl P, Boudt K, Bennett R, Ulrich J, Zivot E, et al. Package 'performanceanalytics'. R Team Cooperation. 2018;3:13-4.

[36] Kim K, Joyce BT, Zheng Y, Schreiner PJ, Jacobs Jr DR, Catov JM, et al. DNA Methylation GrimAge and Incident Diabetes: The Coronary Artery Risk Development in Young Adults (CARDIA) Study. Diabetes. 2021;70(6):1404-13.

[37] Roshandel D, Chen Z, Canty AJ, Bull SB, Natarajan R, Paterson AD. DNA methylation age calculators reveal association with diabetic neuropathy in type 1 diabetes. Clinical epigenetics. 2020;12(1):1-16.

[38] Wang C, Ni W, Yao Y, Just A, Heiss J, Wei Y, et al. DNA methylation-based biomarkers of age acceleration and all-cause death, myocardial infarction, stroke, and cancer in two cohorts: the NAS, and KORA F4. EBioMedicine. 2021;63:103151.

[39] Roetker NS, Pankow JS, Bressler J, Morrison AC, Boerwinkle E. Prospective study of epigenetic age acceleration and incidence of cardiovascular disease outcomes in the ARIC study (Atherosclerosis Risk in Communities). Circulation: Genomic and Precision Medicine. 2018;11(3):e001937.

[40] Snir S, Farrell C, Pellegrini M. Human epigenetic ageing is logarithmic with time across the entire lifespan. Epigenetics. 2019;14(9):912-26.

[41] Lind L, Ingelsson E, Sundström J, Siegbahn A, Lampa E. Methylation-based estimated biological age and cardiovascular disease. European Journal of Clinical Investigation. 2018;48(2):e12872.

[42] Li C, Engström G, Hedblad B, Calling S, Berglund G, Janzon L. Sex differences in the relationships between BMI, WHR and incidence of cardiovascular disease: a population-based cohort study. International journal of obesity. 2006;30(12):1775-81.

[43] Ha KH, Kim HC, Park S, Ihm SH, Lee HY. Gender differences in the association between serum γ-Glutamyltransferase and blood pressure change: a prospective community-based cohort study. Journal of Korean medical science. 2014;29(10):1379-84.

[44] Ramirez LA, Sullivan JC. Sex differences in hypertension: where we have been and where we are going. American journal of hypertension. 2018;31(12):1247-54.

[45] Fransquet PD, Wrigglesworth J, Woods RL, Ernst ME, Ryan J. The epigenetic clock as a predictor of disease and mortality risk: a systematic review and meta-analysis. Clinical epigenetics. 2019;11(1):1-17.

[46] Kresovich JK, Garval EL, Martinez Lopez AM, Xu Z, Niehoff NM, White AJ, et al. Associations of body composition and physical activity level with multiple measures of epigenetic age acceleration. American journal of epidemiology. 2021;190(6):984-93.

[47] Lin WY, Wang YC, Teng IH, Liu C, Lou XY. Associations of five obesity metrics with epigenetic age acceleration: Evidence from 2,474 Taiwan Biobank participants. Obesity. 2021;29(10):1731-8.

[48] Liu C, Marioni RE, Hedman ÅK, Pfeiffer L, Tsai PC, Reynolds LM, et al. A DNA methylation biomarker of alcohol consumption. Molecular psychiatry. 2018;23(2):422-33.

[49] Ryan J, Wrigglesworth J, Loong J, Fransquet PD, Woods RL. A systematic review and meta-analysis of environmental, lifestyle, and health factors associated with DNA methylation age. The Journals of Gerontology: Series A. 2020;75(3):481-94.

[50] Quach A, Levine ME, Tanaka T, Lu AT, Chen BH, Ferrucci L, et al. Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. Aging (Albany NY). 2017;9(2):419.

[51] Adamson SS, Brace LE, Kennedy BK. Alcohol and aging: From epidemiology to mechanism. Translational Medicine of Aging. 2017;1:18-23.

[52] Shiau S, Wang L, Liu H, Zheng Y, Drong A, Joyce BT, et al. Prenatal gestational diabetes mellitus exposure and accelerated offspring DNA methylation age in early childhood. Epigenetics. 2021;16(2):186-95.

[53] Ammous F, Zhao W, Ratliff SM, Mosley TH, Bielak LF, Zhou X, et al. Epigenetic age acceleration is associated with cardiometabolic risk factors and clinical cardiovascular disease risk scores in African Americans. Clinical epigenetics. 2021;13(1):1-13.

[54] Emdin M, Passino C, Pompella A, Paolicchi A. Gamma-glutamyltransferase as a cardiovascular risk factor. Oxford University Press; 2006.

[55] Whitfield J. Gamma glutamyl transferase. Critical reviews in clinical laboratory sciences. 2001;38(4):263-355.

[56] Bartlett J, Predazzi IM, Williams SM, Bush WS, Kim Y, Havas S, et al. Is isolated low high-density lipoprotein cholesterol a cardiovascular disease risk factor? New insights from the Framingham offspring study. Circulation: Cardiovascular Quality and Outcomes. 2016;9(3):206-12.

[57] Hahn O, Grönke S, Stubbs TM, Ficz G, Hendrich O, Krueger F, et al. Dietary restriction protects from age-associated DNA methylation and induces epigenetic reprogramming of lipid metabolism. Genome biology. 2017;18(1):1-18.

[58] Irvin MR, Aslibekyan S, Do A, Zhi D, Hidalgo B, Claas SA, et al. Metabolic and inflammatory biomarkers are associated with epigenetic aging acceleration estimates in the GOLDN study. Clinical epigenetics. 2018;10(1):1-9.

[59] Gao T, Wilkins JT, Zheng Y, Joyce BT, Jacobs DR, Schreiner PJ, et al. Plasma lipid profiles in early adulthood are associated with epigenetic aging in the Coronary Artery Risk Development in Young Adults (CARDIA) Study. Clinical epigenetics. 2022;14(1):1-10.

[60] Feng L, Nian S, Tong Z, Zhu Y, Li Y, Zhang C, et al. Age-related trends in lipid levels: a large-scale cross-sectional study of the general Chinese population. BMJ open. 2020;10(3):e034226.

[61] Stevens SL, Wood S, Koshiaris C, Law K, Glasziou P, Stevens RJ, et al. Blood pressure variability and cardiovascular disease: systematic review and meta-analysis. bmj. 2016;354.

[62] Lappalainen I, Almeida-King J, Kumanduri V, Senf A, Spalding JD, Saunders G, et al. The European Genome-phenome Archive of human data consented for biomedical research. Nature genetics. 2015;47(7):692-5.

Supplementary materials

Phenotype	**Sex**	**(min,max)**	**Mean** (SD)	p**-value**	**95% CI**
Age, years	All	(44.78, 70.37)	56.78 (7.13)	0.16	(-0.46, 2.75)
	F	(44.78, 70.11)	57.3 (7.28)		
	M	(45.15, 70.37)	56.15 (6.92)		
BMI, kg/m^2	All	(16.76, 53.62)	28.06 (5.3)	< 0.001	(1.18, 3.48)
	F	(16.76, 53.62)	29.12 (5.66)		
	M	(18.37, 43.27)	26.8 (4.54)		
WHR, units	All	(0.69, 1.13)	0.88 (0.08)	< 0.001	(-0.12, -0.09)
	F	(0.69, 1.01)	0.84 (0.06)		
	M	(0.79, 1.13)	0.94 (0.07)		
Alcohol (annual intake), g	All	(0, 43530)	2696.73 (5936.95)	< 0.001	(-6129.87, -3453.23)
	F	(0, 14850)	504.52 (1323.39)		
	M	(0, 43530)	5296.07 (7919.46)		
Alcohol (annual occasions), n	All	(0, 365)	47 (75.56)	< 0.001	(-77.54, -44)
	F	(0, 198)	19.19 (34.44)		
	M	(0, 365)	79.96 (95.4)		
GGT, mmol/l	All	(10, 140)	31.26 (19.92)	< 0.001	(-12.69, -3.77)
	F	(10, 140)	27.5 (18.34)		
	M	(12, 130)	35.73 (20.85)		
Glucose, mmol/l	All	(4.11, 17.11)	6.04 (1.65)	0.766	(-0.44, 0.32)
	F	(4.11, 16)	6.02 (1.6)		
	M	(4.56, 17.11)	6.07 (1.72)		
TC, mmol/l	All	(4.04, 11.06)	6.5 (1.28)	0.001	(0.19, 0.75)
	F	(4.14, 11.06)	6.72 (1.33)		
	M	(4.14, 11.06)	6.25 (1.17)		
TG, mmol/l	All	(0.56, 5.56)	1.62 (0.84)	0.189	(-0.06, 0.31)
	F	(0.56, 5.56)	1.67 (0.92)		
	M	(0.63, 4.59)	1.55 (0.71)		
HDL, mmol/l	All	(0.7, 3.29)	1.54 (0.33)	0.015	(0.02, 0.17)
	F	(0.93, 2.46)	1.59 (0.32)		
	M	(0.7, 3.29)	1.49 (0.35)		
LDL, mmol/l	All	(1.46, 8.3)	4.22 (1.14)	0.013	(0.07, 0.58)
	F	(1.94, 8.09)	4.37 (1.15)		
	M	(1.46, 8.3)	4.05 (1.1)		
SBP, mmHg	All	(93.33, 247)	140.95 (25.75)	0.861	(-5.21, 6.23)
	F	(93.33, 227.67)	141.18 (28.19)		
	M	(99.67, 247)	140.67 (22.62)		
DBP, mmHg	All	(54.33, 135.33)	88.65 (13.76)	0.275	(-4.8, 1.37)
	F	(59, 135)	87.87 (14.15)		
	M	(54.33, 135.33)	89.59 (13.26)		

Table S1: **Summary of the continuous phenotype data for males and females.** Sample size is $n = 306$, 166/140 female/male. Glucose measurements are only available for $n = 298$, 159/139 female/male samples. p-values obtained from the Welch's t-test testing difference between male and female groups for each variable ($\mathbf{H_0}$**:** Mean value of the variable is the same for male and female groups).

EAA	Sex	(min, max)	Mean (SD)	Median (IQR)	p-value	95% CI
HannumAA	All	(-17.6, 20.49)	0 (4.35)	-0.33 (5.2)	< 0.001	(-3.46, -1.56)
	F	(-17.6, 10.69)	-1.15 (4.06)	-1.06 (4.77)		
	M	(-7.65, 20.49)	1.36 (4.32)	0.7 (5.85)		
HannumEEAA	All	(-20, 24.18)	0 (5.42)	0.04 (6.73)	< 0.001	(-4.59, -2.27)
	F	(-20, 13.44)	-1.57 (5.15)	-1.55 (5.8)		
	M	(-9.24, 24.18)	1.86 (5.16)	1.29 (7.2)		
HannumIEAA	All	(-15.55, 18.54)	0 (3.91)	-0.19 (4.98)	0.001	(-2.44, -0.69)
	F	(-15.55, 8.11)	-0.72 (3.57)	-0.67 (4.43)		
	M	(-7.49, 18.54)	0.85 (4.13)	0.55 (5.19)		
HorvathAAd	All	(-17.16, 21.87)	2.16 (4.95)	2 (6.02)	< 0.001	(-3.51, -1.31)
	F	(-17.16, 13.42)	1.06 (4.58)	0.82 (5.55)		
	M	(-10.84, 21.87)	3.47 (5.07)	3.49 (6.15)		
HorvathAAr	All	(-16.11, 17.01)	0 (4.49)	-0.07 (5.55)	< 0.001	(-3.07, -1.08)
	F	(-16.11, 11.83)	-0.95 (4.13)	-0.9 (5.43)		
	M	(-9.98, 17.01)	1.13 (4.65)	1.08 (5.79)		
HorvathIEAA	All	(-15.62, 15.93)	0 (4.32)	0.07 (5.57)	0.002	(-2.51, -0.56)
	F	(-15.62, 10.23)	-0.7 (4.07)	-0.48 (5.26)		
	M	(-8.84, 15.93)	0.83 (4.48)	0.58 (5.56)		
SkinBloodAA	All	(-21.55, 13.15)	0 (3.68)	-0.39 (4.82)	0.048	(-1.66, -0.01)
	F	(-21.55, 9.82)	-0.38 (3.73)	-0.58 (4.85)		
	M	(-9.33, 13.15)	0.45 (3.58)	0.18 (4.48)		
PhenoAA	All	(-17.95, 26.12)	0 (5.74)	-0.53 (7.4)	< 0.001	(-3.62, -1.08)
	F	(-17.95, 26.12)	-1.08 (5.68)	-1.62 (5.78)		
	M	(-10.35, 15.91)	1.27 (5.56)	0.71 (7.88)		
GrimAA	All	(-10.46, 15.05)	0 (5.44)	-1.47 (7.73)	< 0.001	(-8.19, -6.26)
	F	(-10.46, 8.45)	-3.31 (2.94)	-3.44 (3.16)		
	M	(-7.71, 15.05)	3.92 (5.12)	3.66 (8.39)		

Table S2: **Summary of EAAs scores data for males and females.** EAA scores before adjustment for sex. p-values obtained from the Welch's t-test testing difference between male and female groups for each EAA ($\mathbf{H_0}$**:** Mean value of the EAA is the same for male and female groups).

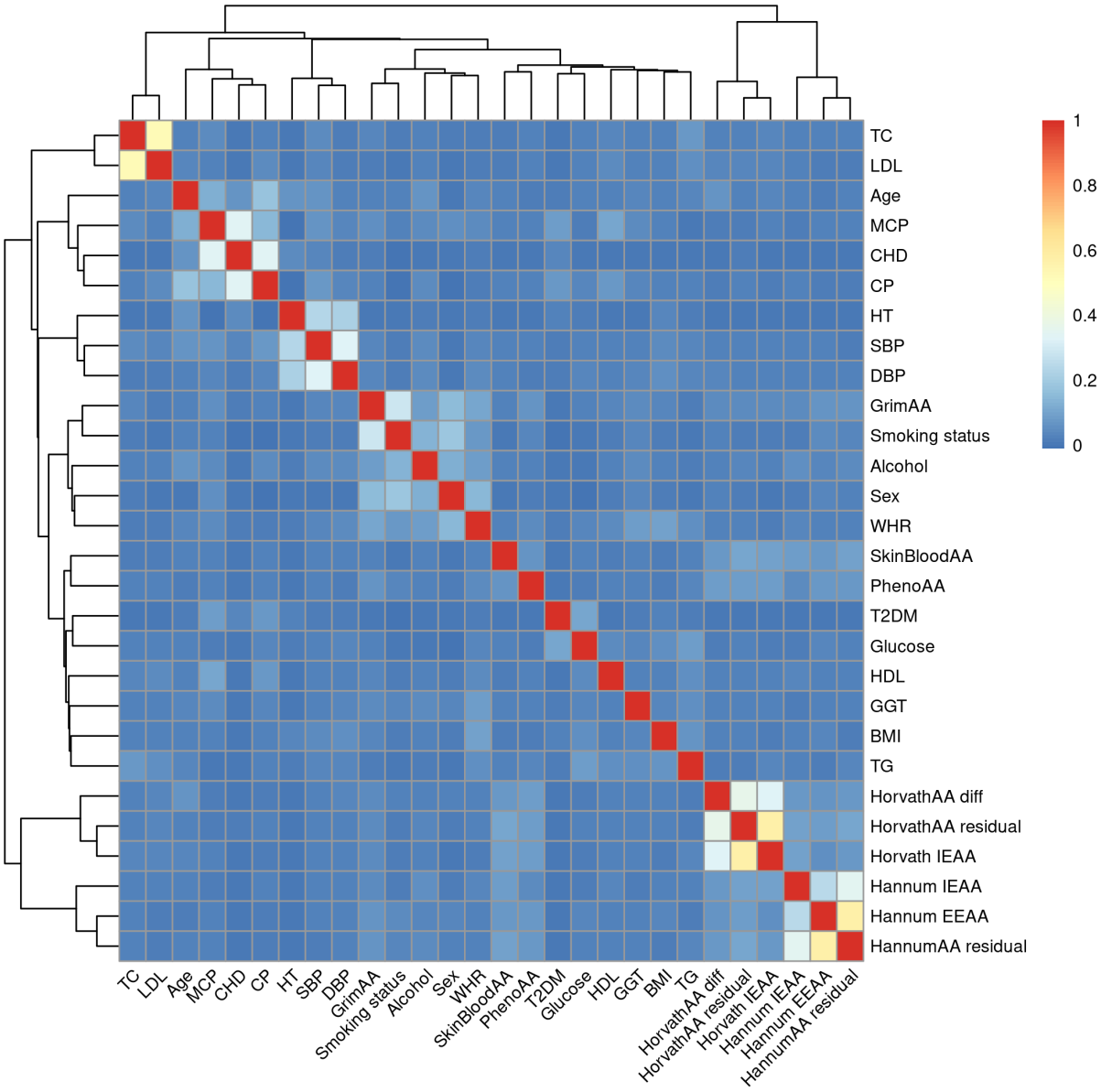

Figure S1: Normalised mutual information heatmap.

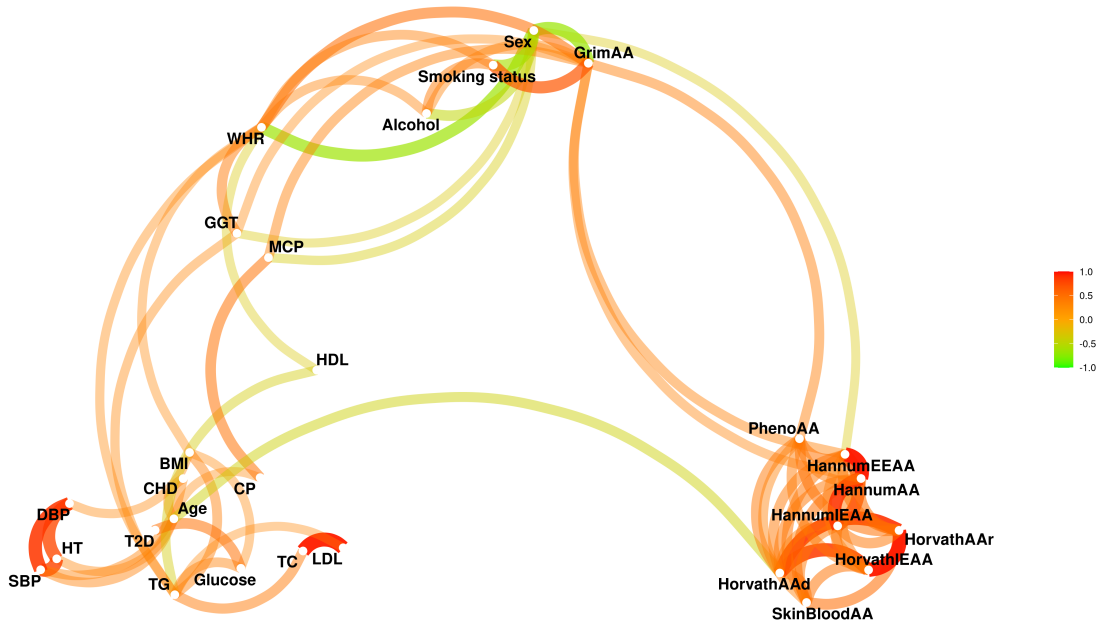

Figure S2: Network plot of of the connections among the phenotypes and EAAs in the dataset. Based on Spearman correlation coefficients with absolute values above 0.3

Phenotype	Sex	Samples, n	Cases, n (%)	OR (95%CI)	p-value
Smoking status	All	306	126 (41.18%)		< 0.001
	F	166	24 (14.46%)	0.06 (0.03, 0.12)	
	M	140	102 (72.86%)	15.69 (8.67, 29.34)	
T2DM	All	306	34 (11.11%)		0.466
	F	166	16 (9.64%)	0.72 (0.33, 1.57)	
	M	140	18 (12.86%)	1.38 (0.64, 3.03)	
CHD	All	306	130 (42.48%)		0.083
	F	166	63 (37.95%)	0.67 (0.41, 1.08)	
	M	140	67 (47.86%)	1.5 (0.93, 2.43)	
CP	All	105	46 (43.81%)		0.698
	F	55	23 (41.82%)	0.85 (0.36, 1.96)	
	M	50	23 (46%)	1.18 (0.51, 2.75)	
MCP	All	105	16 (15.24%)		0.001
	F	55	2 (3.64%)	0.1 (0.01, 0.47)	
	M	50	14 (28%)	10.1 (2.12, 96.93)	
HT	All	306	176 (57.52%)		0.643
	F	166	93 (56.02%)	0.88 (0.54, 1.41)	
	M	140	83 (59.29%)	1.14 (0.71, 1.85)	

Table S3: **Summary of binary phenotypes.** Being case means having phenotype. Smoking status cases correspond to current or former smokers. p-values and odds ratios (with corresponding 95% confidence intervals) obtained from the Fisher's exact test testing difference in ratio of each class of the variable between male and female groups ($\mathbf{H_0}$**:** Classification for given binary variable for male and female groups are not different).

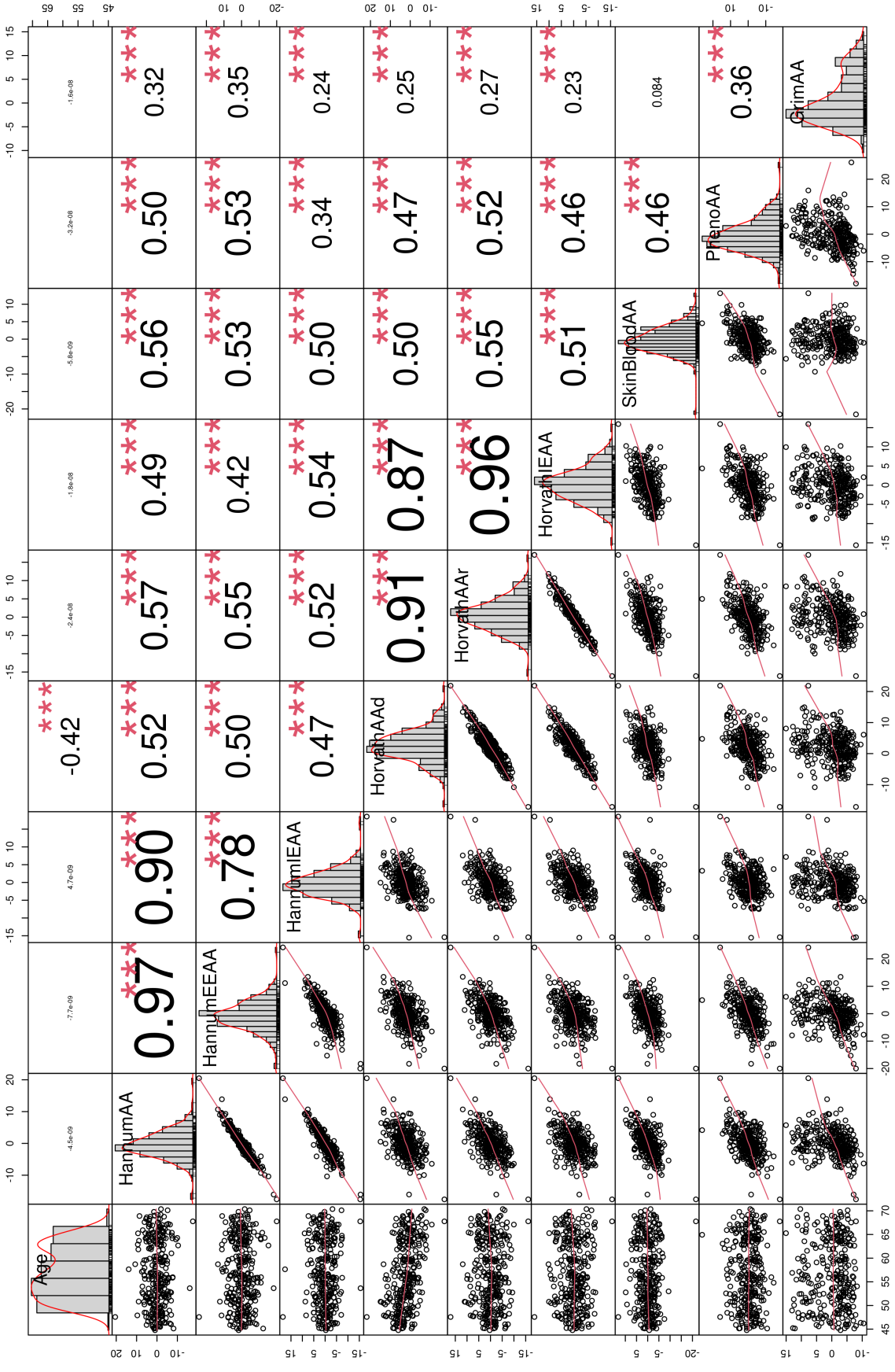

Figure S3: Correlation table for EAAs

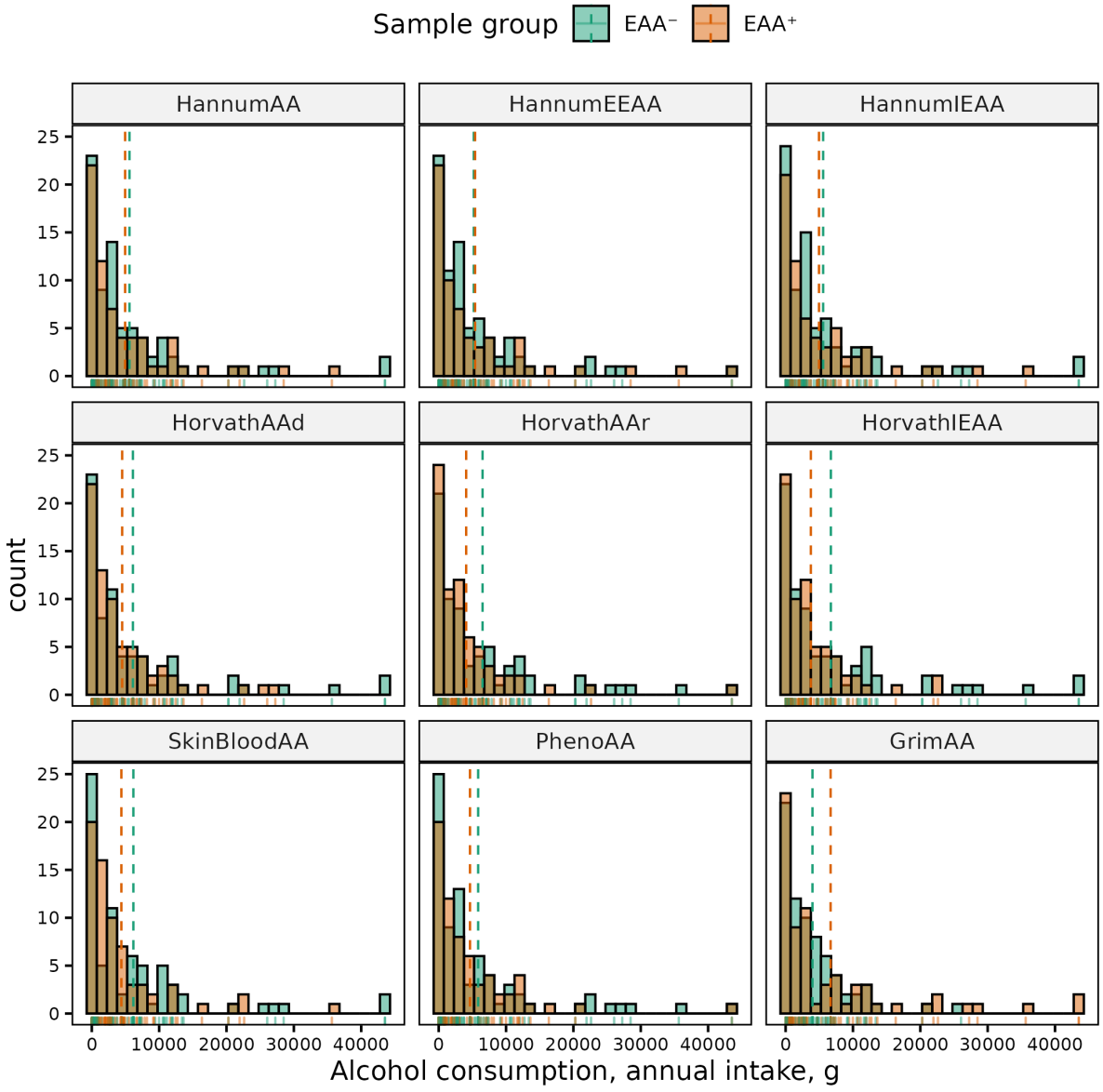

Figure S4: Histograms of distributions of annual alcohol consumption in males in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

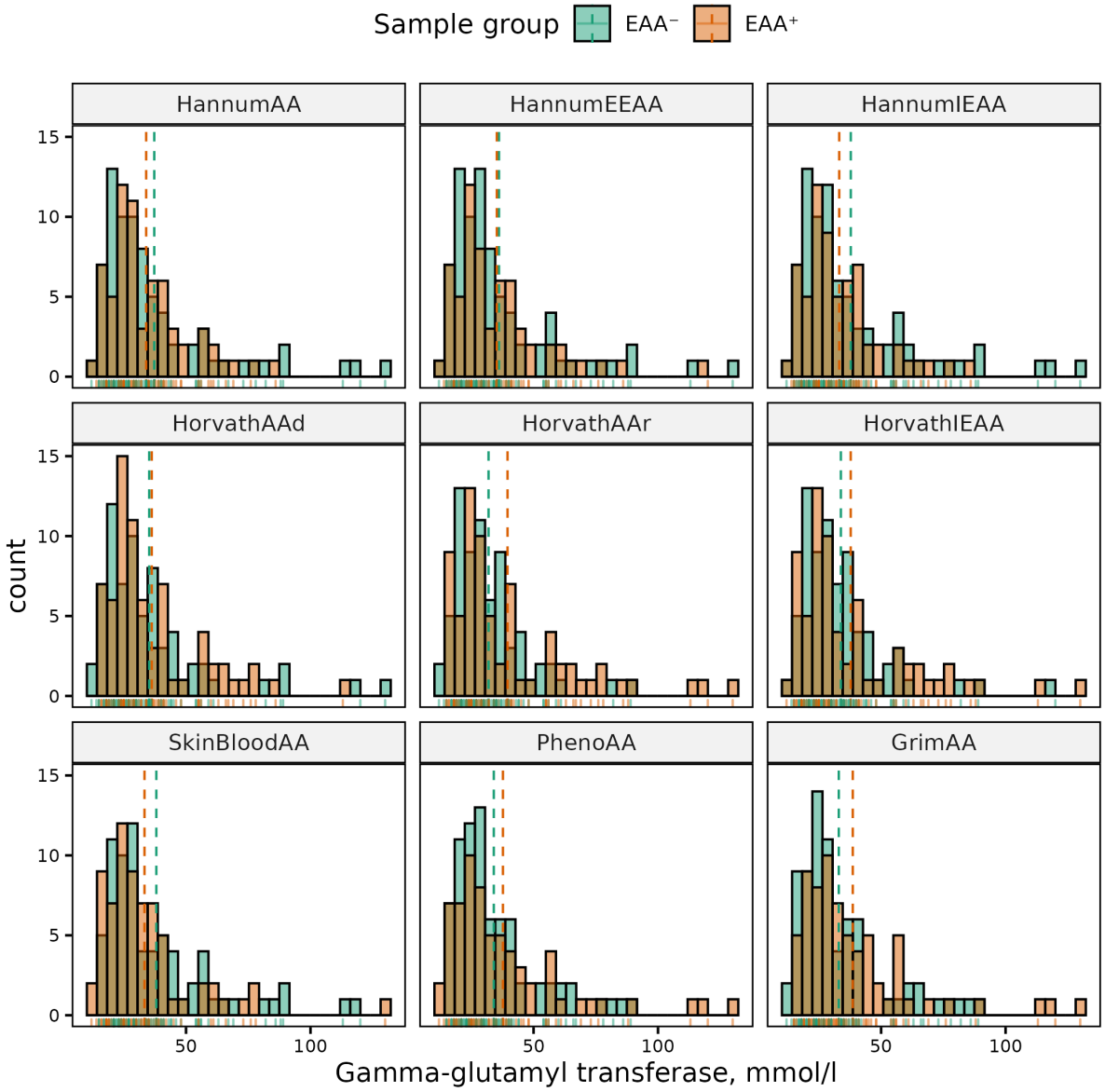

Figure S5: Histograms of distributions of GGT levels in males in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

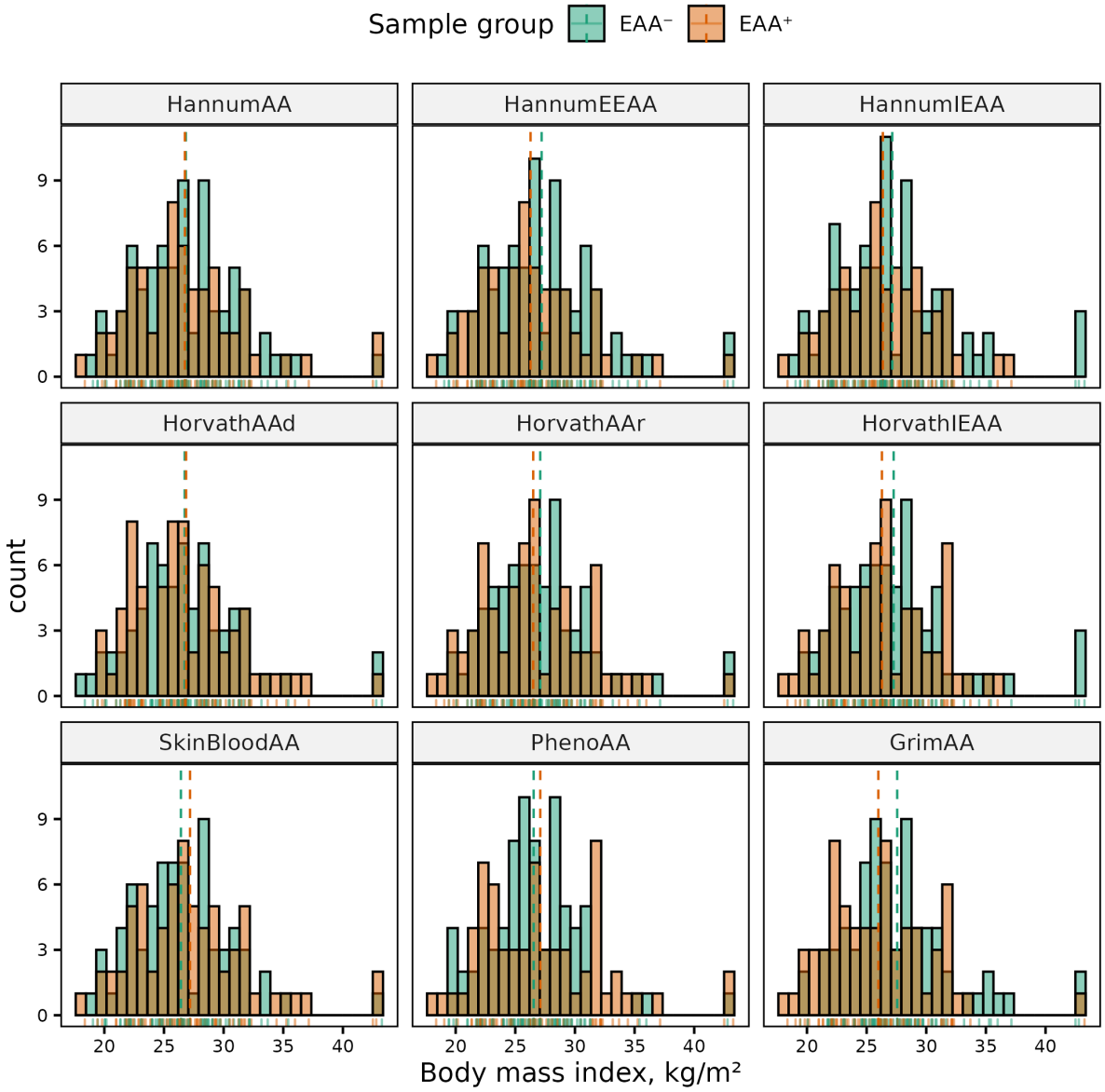

Figure S6: Histograms of distributions of BMI in males in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

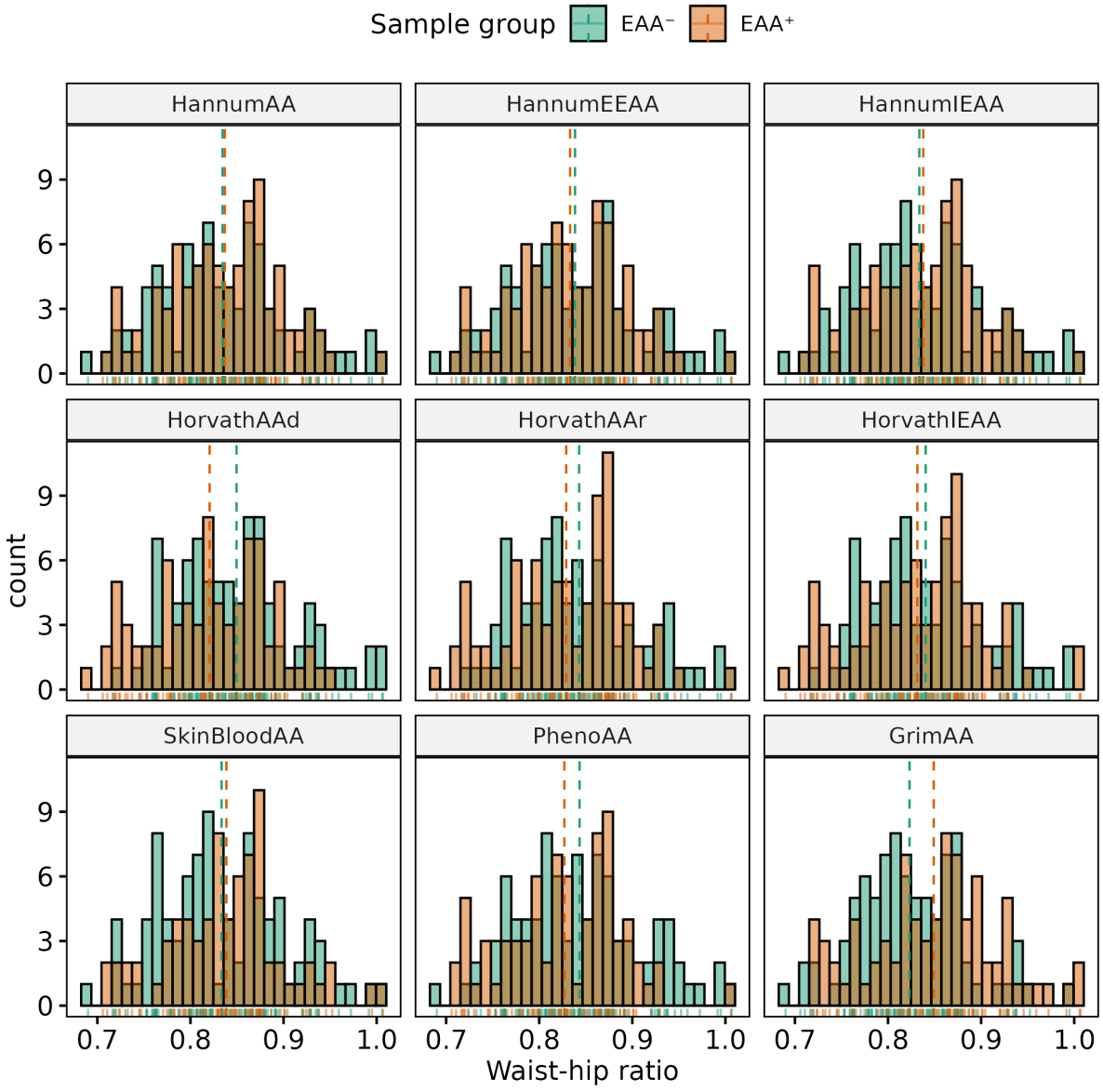

Figure S7: Histograms of distributions of waist-hip ratio in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

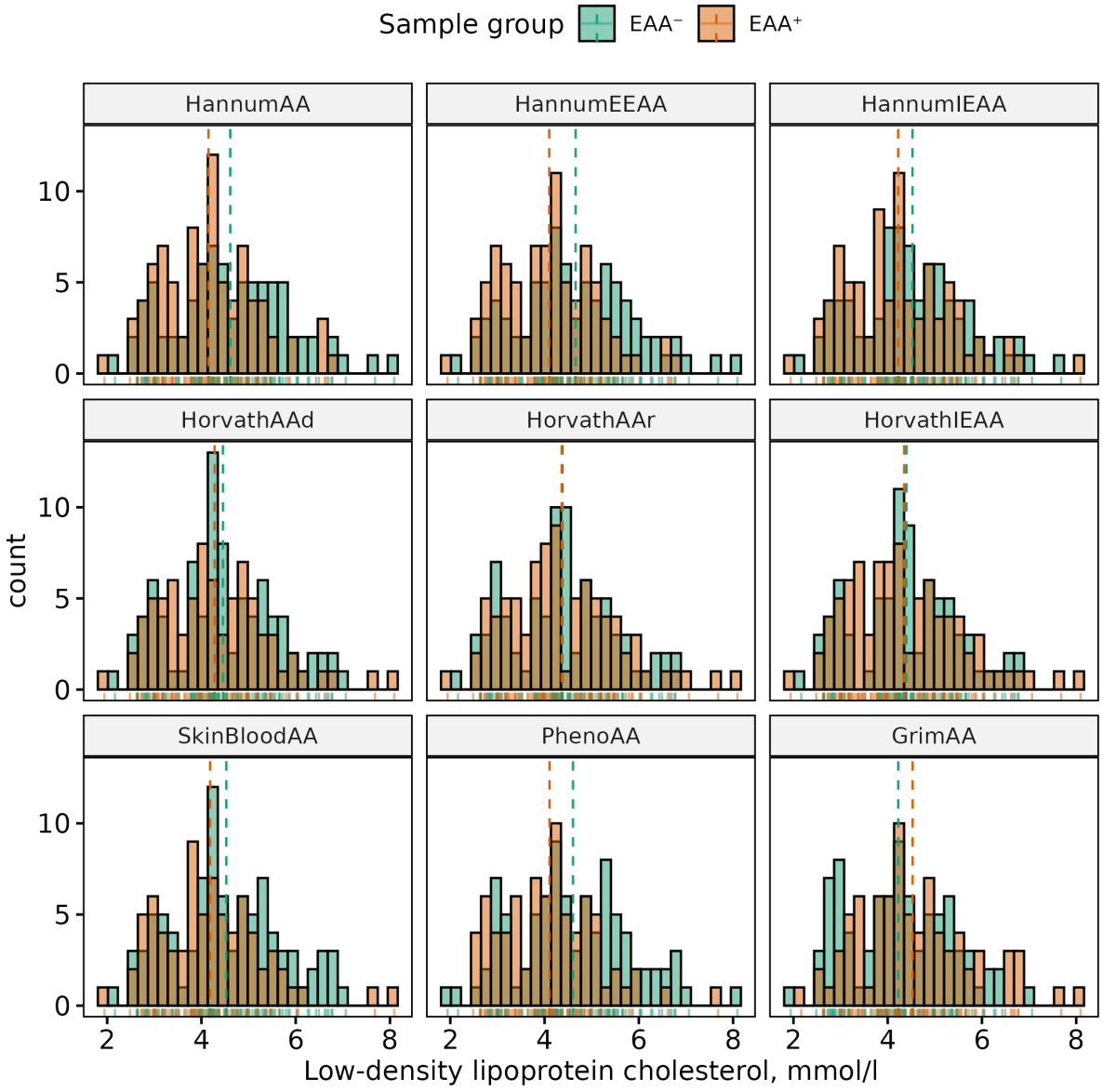

Figure S8: Histograms of distributions of LDL levels in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

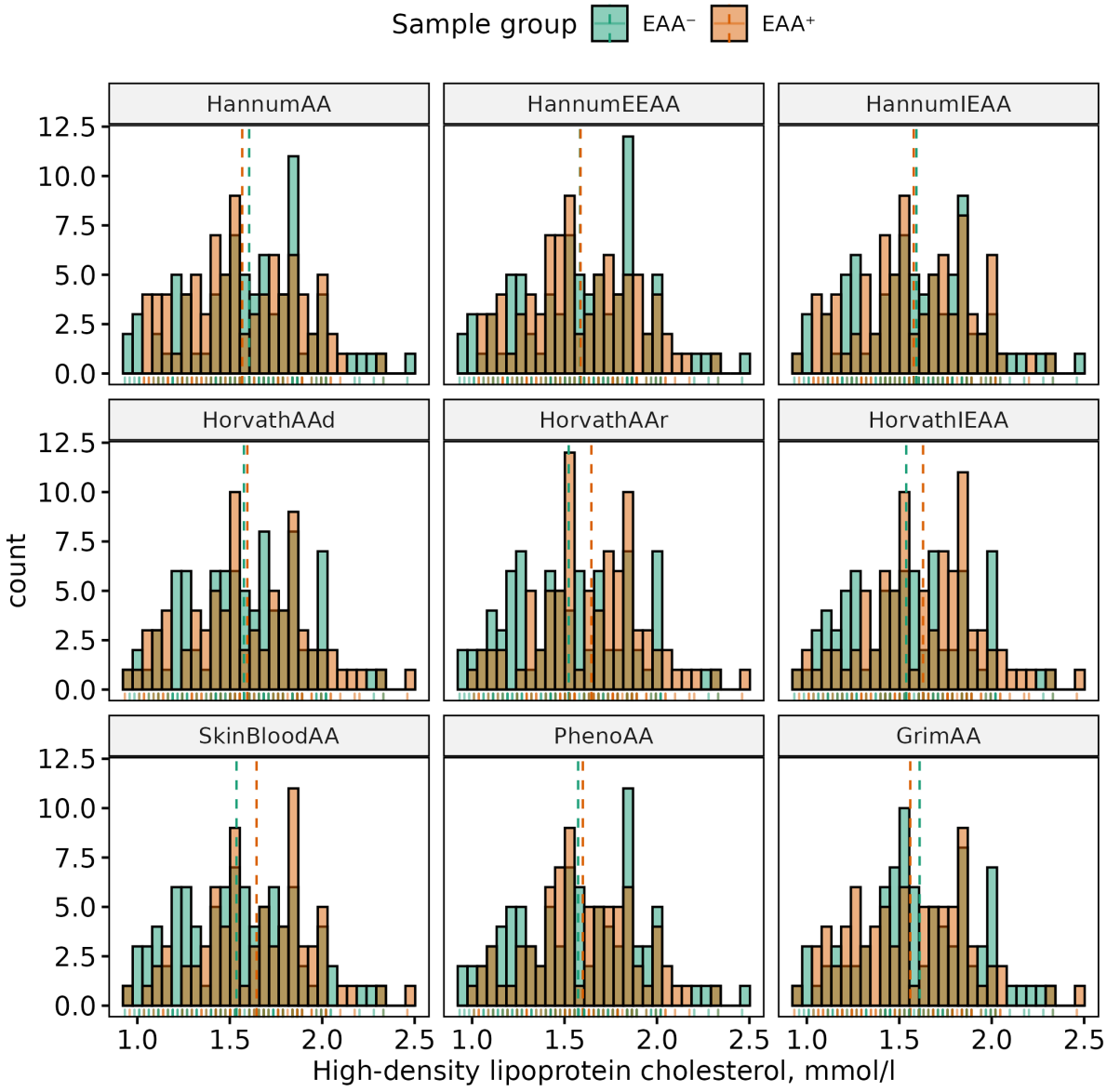

Figure S9: Histograms of distributions of HDL levels in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

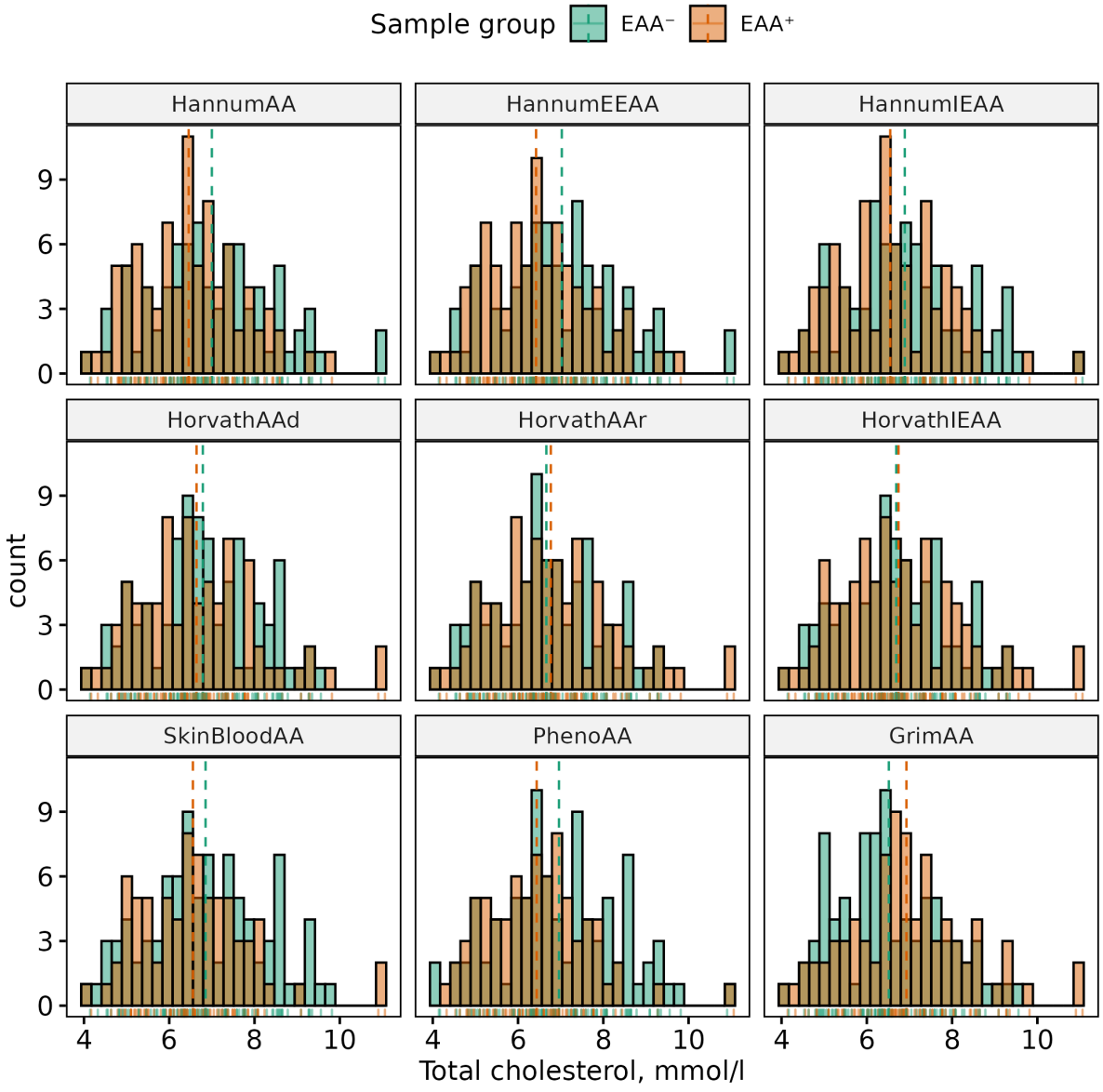

Figure S10: Histograms of distributions of TC levels in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

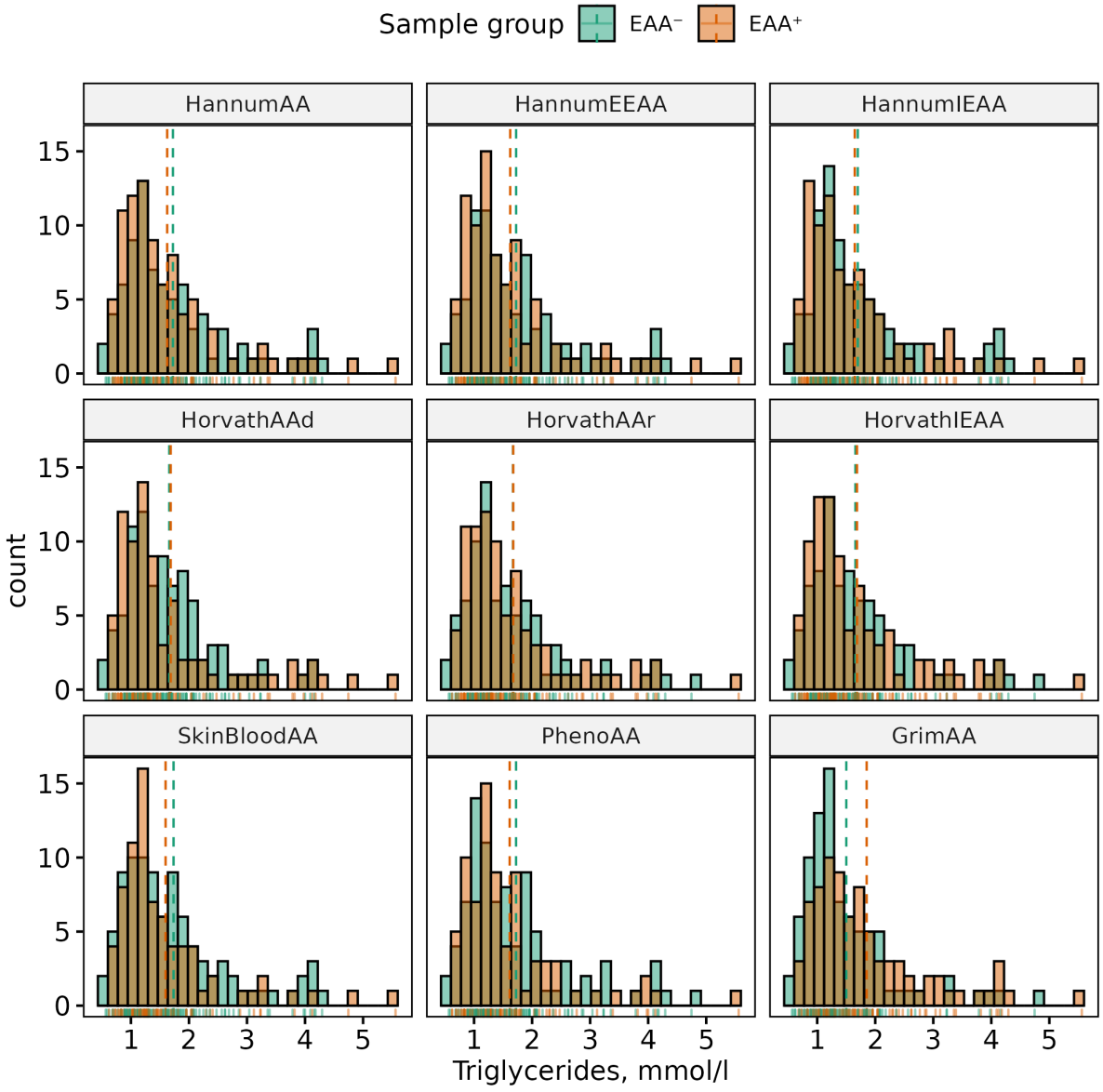

Figure S11: Histograms of distributions of TG levels in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

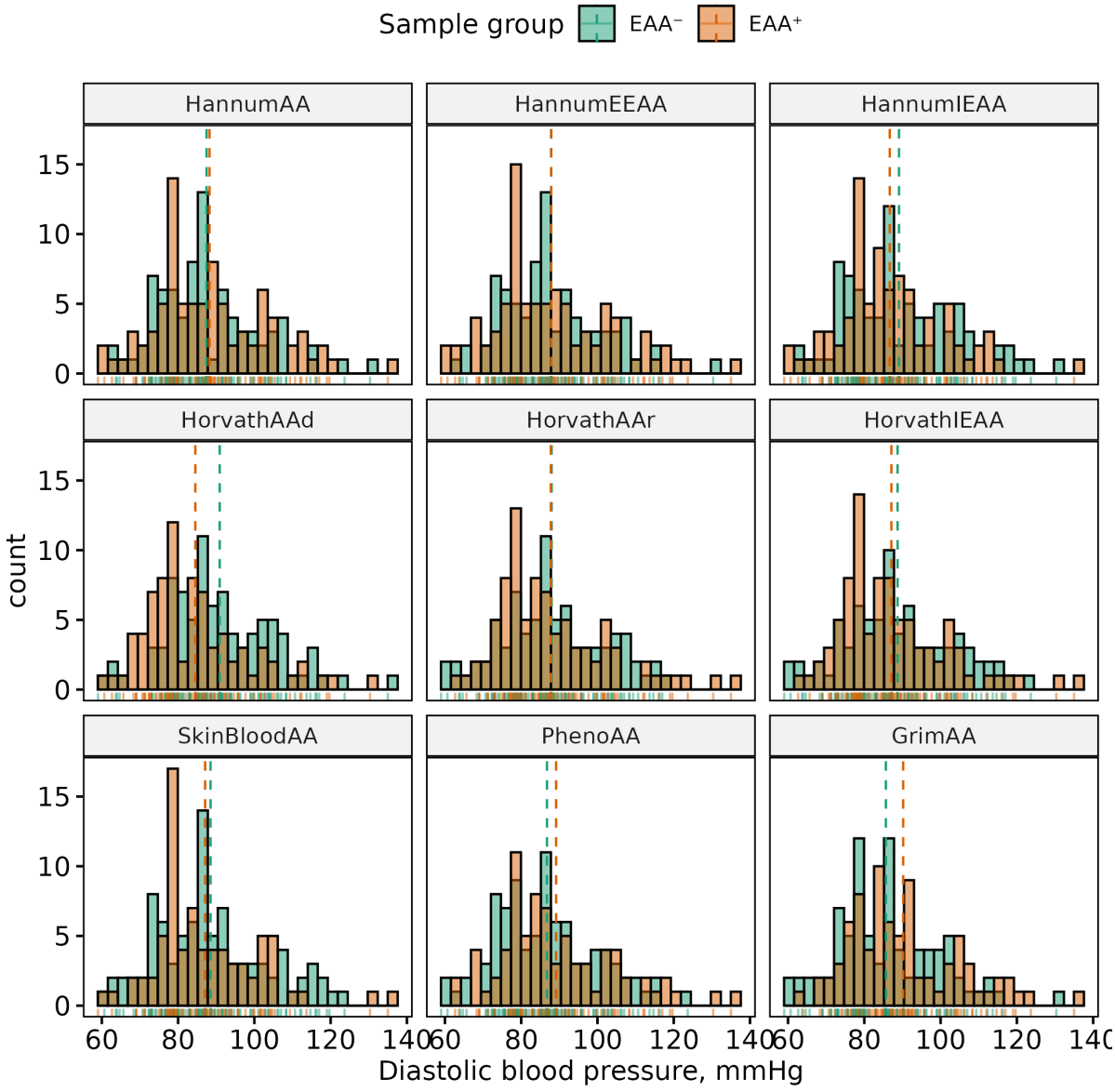

Figure S12: Histograms of distributions of DBP levels in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

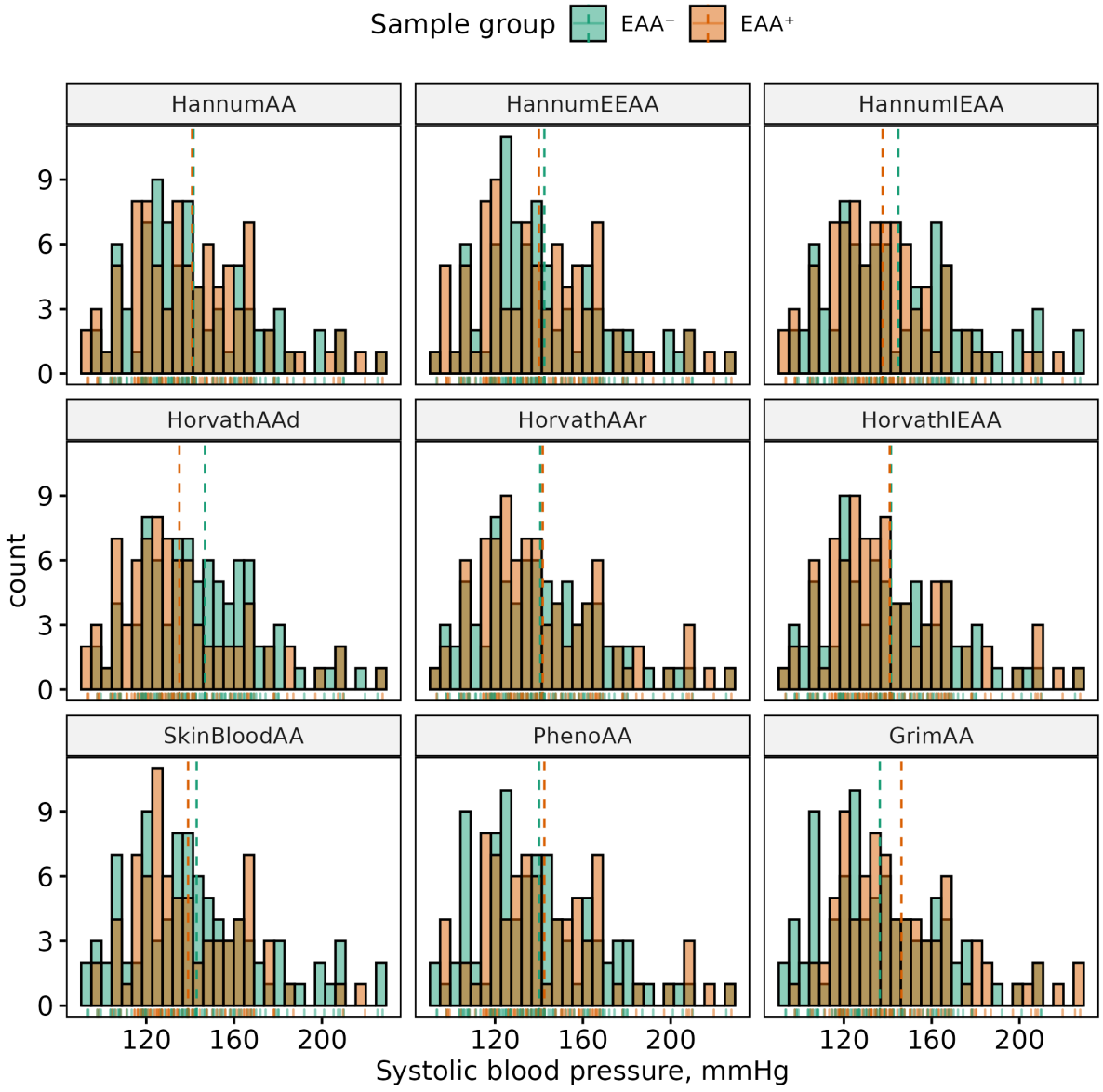

Figure S13: Histograms of distributions of SBP levels in females in EAA^+ and EAA^- groups. Dashed lines correspond to the group means.

EAA	**Sex**	**EAA group size, n (%)**		**adjEAA group size, n (%)**	
		EAA< 0	**EAA**≥ 0	**adjEAA**< 0	**adjEAA**≥ 0
HannumAA	All	162 (53%)	144 (47%)	155 (51%)	151 (49%)
	F	104 (63%)	62 (37%)	80 (48%)	86 (52%)
	M	58 (41%)	82 (59%)	75 (54%)	65 (46%)
HannumEEAA	All	150 (49%)	156 (51%)	159 (52%)	147 (48%)
	F	100 (60%)	66 (40%)	81 (49%)	85 (51%)
	M	50 (36%)	90 (64%)	78 (56%)	62 (44%)
HannumIEAA	All	160 (52%)	146 (48%)	159 (52%)	147 (48%)
	F	95 (57%)	71 (43%)	82 (49%)	84 (51%)
	M	65 (46%)	75 (54%)	77 (55%)	63 (45%)
HorvathAAd	All	98 (32%)	208 (68%)	156 (51%)	150 (49%)
	F	68 (41%)	98 (59%)	87 (52%)	79 (48%)
	M	30 (21%)	110 (79%)	69 (49%)	71 (51%)
HorvathAAr	All	155 (51%)	151 (49%)	151 (49%)	155 (51%)
	F	98 (59%)	68 (41%)	81 (49%)	85 (51%)
	M	57 (41%)	83 (59%)	70 (50%)	70 (50%)
HorvathIEAA	All	150 (49%)	156 (51%)	152 (50%)	154 (50%)
	F	89 (54%)	77 (46%)	79 (48%)	87 (52%)
	M	61 (44%)	79 (56%)	73 (52%)	67 (48%)
SkinBloodAA	All	163 (53%)	143 (47%)	162 (53%)	144 (47%)
	F	96 (58%)	70 (42%)	91 (55%)	75 (45%)
	M	67 (48%)	73 (52%)	71 (51%)	69 (49%)
PhenoAA	All	168 (55%)	138 (45%)	164 (54%)	142 (46%)
	F	105 (63%)	61 (37%)	89 (54%)	77 (46%)
	M	63 (45%)	77 (55%)	75 (54%)	65 (46%)
GrimAA	All	186 (61%)	120 (39%)	157 (51%)	149 (49%)
	F	151 (91%)	15 (9%)	85 (51%)	81 (49%)
	M	35 (25%)	105 (75%)	72 (51%)	68 (49%)

Table S4: **Number of samples in EAA$^-$ and EAA$^+$ groups for unadjusted and adjusted for sex EAA scores.**

Table S5: **Significant differences in EAA$^-$ and EAA$^+$ groups for unadjusted and adjusted for sex EAA scores.**

Phenotype	EAA	Unadjusted						Adjusted for sex					
		All		Female		Male		All		Female		Male	
		p	95% CI	p	95% CI	p	95% CI	p	95% CI	p	95% CI	p	95% CI
Anthropometric													
BMI	GrimAA	0.011	(-2.767, -0.364)									0.039	(-3.079, -0.079)
	HorvathAAd	0.019	(-2.950, -0.267)	0.031	(-3.751, -0.188)								
WHR	HorvathAAd			0.007	(-0.048, -0.008)					0.004	(-0.048, -0.009)		
	GrimAA	<0.001	(0.069, 0.103)			0.046	(-0.0004, 0.042)			0.010	(0.006, 0.046)		
	SkinBloodAA	0.041	(-0.001,0.038)										
	HannumEEAA	0.016	(-0.004, 0.042)										
Lifestyle													
Smoking status	GrimAA	<0.001	(15.646, 61.134)	<0.001	(3.642, 52.054)	<0.001	(5.237, 39.030)	<0.001	(1.799, 4.895)	0.026	(1.077, 8.931)	<0.001	(4.515, 58.734)
	HorvathAAd	0.003	(1.294, 3.884)							0.016	(1.140, 9.454)		
	PhenoAA	0.002	(1.262, 3.359)			0.013	(1.201, 6.494)					0.004	(1.360, 8.319)
	HannumAAr	<0.001	(1.447, 3.873)										
	HannumIEAA	0.015	(1.112, 2.940)										
	HannumEEAA	<0.001	(1.717, 4.677)										
Alcohol (annual intake)	HorvathIEAA					0.009	(-6721.199, -1005.072)	0.028	(-2832.461, -163.529)			0.023	(-5521.650, -422.226)
	HorvathAAr					0.033	(-5976.624, -264.277)						
	GrimAA	<0.001	(2477.312, 5546.062)									0.049	(16.036, 5369.626)
	HannumAAr	0.028	(165.525, 2866.550)										
	HannumEEAA	0.009	(432.292, 3063.949)										
Alcohol (annual occasions)	HorvathIEAA					0.020	(-73.632, -6.503)						
	HorvathAAr					0.027	(4.412, 72.233)						
	HannumEEAA	0.015	(4.092, 37.637)										
	GrimAA	<0.001	(28.848, 66.692)										
	HannumAAr	0.016	(3.953,38.375)										
Metabolic													
GGT	HannumIEAA					0.029	(0.817, 15.130)						
	GrimAA	0.003	(2.518, 11.746)					0.023	(0.728, 9.699)				
	HorvathAAr											0.030	(0.738, 14.519)
Lipids													
TC	HannumAAr	0.011	(-0.652, -0.085)	0.014	(-0.904, -0.103)					0.009	(-0.947, -0.141)		
	GrimAA	0.025	(-0.630, -0.042)							0.046	(0.008, 0.818)		
	PhenoAA	0.010	(-0.653, -0.088)	0.004	(-0.993, -0.198)					0.010	(-0.919, -0.127)		
	HannumEEAA	0.012	(-0.653, -0.082)	0.012	(-0.904, -0.113)					0.003	(-1.004, -0.203)		
TG	GrimAA									0.015	(0.070, 0.632)		
	HannumIEAA	0.043	(-0.378, -0.006)			0.028	(-0.510, -0.030)						
	HannumAAr	0.023	(-0.403, -0.031)			0.034	(-0.522, -0.021)						
HDL	HorvathAAr			0.004	(0.046, 0.236)					0.013	(0.026, 0.219)		
	SkinBloodAA									0.027	(0.012, 0.205)		
	HorvathIEAA			0.002	(0.053, 0.241)								
	HannumIEAA					0.033	(0.010, 0.236)						
	HannumAAr					0.036	(0.008, 0.233)						
	GrimAA	0.004	(-0.192, -0.036)										
LDL	PhenoAA	0.012	(-0.575, -0.070)	0.002	(-0.891, -0.208)			0.037	(-0.523, -0.016)	0.004	(-0.840, -0.157)		
	HannumAAr	0.013	(-0.575, -0.068)	0.007	(-0.822, -0.129)					0.010	(-0.811, -0.112)		
	HannumEEAA	0.008	(-0.601, -0.093)	0.004	(-0.848, -0.165)					0.002	(-0.904, -0.215)		
Cardio-vascular													
CHD	GrimAA	0.003	(1.267, 3.408)			0.011	(1.220, 7.596)	<0.001	(1.518, 4.060)	0.001	(1.458, 5.955)	0.042	(1.020, 4.389)
	HorvathAAr	0.015	(1.106, 2.913)					0.006	(1.187, 3.139)			0.018	(1.150, 4.995)
CP	GrimAA											0.009	(1.367, 22.723)
MCP	GrimAA	0.001	(1.979, 46.687)					0.004	(1.584, 26.779)			0.009	(1.401, 33.864)
HT	HorvathAAd	0.009	(0.296, 0.867)	0.007	(0.195, 0.793)					0.005	(0.202, 0.781)		
	GrimAA									0.043	(0.987, 3.764)		
SBP	HorvathAAd	0.024	(-13.311, -0.964)	0.028	(-18.191, -1.045)					0.008	(-20.142, -3.153)		
	GrimAA									0.024	(1.294, 18.398)		
DBP	HorvathAAd			0.048	(-8.648, -0.031)					0.003	(-10.626, -2.144)		
	GrimAA									0.039	(0.228, 8.832)		