Author’s response to reviews

Title: Intrinsic and Extrinsic Epigenetic Age Acceleration are associated with Hypertensive Target Organ Damage in Older African Americans

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Author’s response to reviews:

We thank the Editor for the opportunity to revise and resubmit this manuscript to BMC Medical Genomics. We also thank the Reviewers for helpful comments that have substantively improved this work. Below, we provide our responses to the Reviewer comments. Any text directly from the revised manuscript is in quotations. We also provide a revised copy of the manuscript.

Please also note that we have added a co-author to the manuscript, Sharon L.R. Kardia. Dr. Kardia was involved in the data collection for all phases of the GENOA study, and substantially contributed to the manuscript revisions. All co-authors have approved the addition of Dr. Kardia to the author list.

Reviewer reports:

Morgan E. Levine (Reviewer 1): The study utilized the GENOA study to evaluate the association between two epigenetic age measures and target organ damage. Overall, the aims of the study are well conceived, however there are some major concerns in regards to the statistical analysis, literature review, and interpretation of results.
Major Concerns

1) Not enough was done to ensure that the use of two different arrays did not bias results. The authors state that for the 100 people measured on both the 450k and the EPIC array, the correlation between IEAA was only $r=0.7$, which in this case, I would not consider a strong correlation (assuming this is from the same blood draw). Can the authors provide a plot of the estimate from 450k against that from EPIC. Also, I think the array type should at the very least be considered a covariate in all models. Going a step further, the authors should perform sensitivity analysis in which they rerun their models using just the estimates from the EPIC array. It appears that they would only lose about 200 observations. Finally, at the very least, it should be mentioned that the EPIC array does not include all the CpGs used to calculate either IEAA or EEAA. While this has been demonstrated to not be a major problem, it should be mentioned.

We agree with the Reviewer that the two different arrays may introduce bias. It is also concerning that EPIC array is missing 19 CpG sites (out of 353 CpG sites) that should be used to estimate Horvath DNAm age and 6 CpG sites (out of 71 CpG sites) that should be used to estimate Hannum DNAm age. Thus, we investigated the effect of the array on DNAm age estimates from 102 duplicated samples from the same blood draw on both the 450K and the EPIC arrays. As shown in Figure S1 (A) and (B), DNAm age estimates using both the Horvath and Hannum methods have decent correlation across arrays ($r = 0.88$ and $0.95$, respectively), which is comparable to correlations observed in other studies that compare DNAm age estimates using these same arrays (e.g., McEwen, 2018). However, the Horvath DNAm age estimate was shifted by an average of 2 years between platforms in our study. This is also unsurprising, as McEwen and colleagues observed that processing differences between the 450K and EPIC arrays can lead to systematic bias as reflected by a median shift up to 3.1 years. However, although there may be a shift in the median/mean, the age acceleration residual, which reflects the relative differences among the measured population, should be minimally affected and may still be combined across platforms. As shown in Figure S1 (C) and (D), IEAA and EEAA had relatively high correlations in our study as well ($r=0.70$ and 0.84). In addition to adding Figure S1 to the manuscript, we have addressed this issue in the methods section as follows:

“We note that the EPIC array does not include all of the CpG sites that were originally used to construct the Horvath DNAm age (EPIC is missing 19 sites) and Hannum DNAm age (EPIC is missing 6 sites). Previous studies have demonstrated that this does not substantively compromise the performance of the DNAm age predictors [35]. Further, epigenetic age acceleration measurements such as EEAA and IEAA, which reflect the relative differences among the measured population, are especially robust to this difference in calculation.” (p.8)

However, we do note that these are not perfect correlations. Thus, we agree with the Reviewer’s suggestion to add array type as a covariate and/or perform a sensitivity analysis in which only those with EPIC data are used. We re-ran the analysis using both suggested approaches, and the results for Model 1 are shown in Table S3. We have added this sensitivity analysis to the manuscript, as follows, and presented the relevant results in the supplementary materials.

Methods:

“Given that the full sample included individuals with epigenetic age acceleration estimated from either the EPIC or the 450K array, we evaluated whether adding array type as a covariate and/or limiting our sample to just those individuals with EPIC data (N=1,074) substantively influenced our major findings.” (p.12)

Results:

“Adding array type as a covariate did not have any impact on effect estimates or p-values for any of the associations. When we conducted analysis with the smaller sample measured on the EPIC array only (N=1,074), there are no substantive changes, except that some previously significant signals are slightly attenuated (UACR and IEAA, p=0.059; LVMI and IEAA, p=0.062, ABI and IEAA, p=0.107), which may be due to the smaller sample size. Also, the association between WMH and EEAA attained significance (p=0.04) when it had previously been marginally significant (p=0.068). Therefore, we conclude that combining two array types did not substantively influence the results.” (pp.15-16)

2) There are some inaccuracies or misconceptions in the authors discussion of the literature. Examples:

a. The authors state, "Epigenetic age acceleration is significantly associated with a variety of age-related conditions including …cardiovascular risk factors and cardiovascular mortality." However, the paper they cite found that, "CHD risk factors bear little or no relationship with IEAA" and "IEAA is not associated with CHD in the WHI".

b. The authors state that only IEAA has robust relationships across tissues. This is inaccurate for two reasons: 1) IEAA is a blood specific measure because it adjusts for blood cell counts; 2)
both Horvath and Hannum DNAmAge show robust (and very comparable) age correlations in nearly every cell tissue tested.

c. The description of the calculation of IEAA versus EEAA is somewhat misleading. They state, "EEAA is calculated as the residual from a model that regresses the weighted average of IEAA and the blood cell components on chronological age." EEAA is not calculate from IEAA. IEAA is based on Horvath DNAmAge and EEAA is based on Hannum DNAmAge. They are different measures that use different CpGs. EEAA is estimated by: 1) calculating the Hannum 71 CpG DNAmAge measure; 2) combining this measure with only 3 cell count estimates (naïve cytotoxic T cells, exhausted cytotoxic T cells, and plasmablasts) using the algorithm proposed by Klemera and Doubal; and 3) calculating the age residual from a linear model in which the cell amplified Hannum DNAmAge measure is regressed on age.

We thank the reviewer, and have now corrected and clarified these points, as follows:

a. We have removed the term “cardiovascular risk factors,” from the introduction (p.5) and have more thoroughly discussed the relationship between epigenetic age and cardiovascular risk factors (smoking, diabetes, blood pressure, triglycerides, etc.) in the discussion. Please see our response to Reviewer 1, Question 3.

b. We have removed the phrase “that are consistent across multiple tissues types,” from the description of IEAA. (p.21) We have also removed the following sentence from the discussion, “This may be particularly true for IEAA, which shows the most robust relationships across organ systems.” (p.18).

c. We have clarified the calculation of IEAA and EEAA in the methods section, as follows:

“Intrinsic epigenetic age acceleration (IEAA) is the residual from a multivariable regression of Horvath DNAm age, estimated using 353 CpGs specified in Horvath, et al (2013) [13], on chronological age and blood cell count estimates. This metric is independent of age-related changes in blood cell composition that are characteristic of immune system aging. It captures cell-intrinsic properties of aging with preservation across differing cell types and organs that likely indicates a fundamental aging process [33, 34].

To calculate extrinsic epigenetic age acceleration (EEAA), 71 CpGs specified in Hannum, et al. (2013) [14] are used to calculate Hannum DNAm age, which is then combined with three blood cell components (naïve cytotoxic T cells, exhausted cytotoxic T cells, and plasmablasts) to form an aggregate measure (enhanced Hannum DNAm age). EEAA is the residual from a regression of enhanced Hannum DNAm age onto chorological age. This measure captures both intrinsic epigenetic age as well as the weighted average of age-related characteristic changes in blood cell composition such as decreases in naive CD8+ T cells and increases in memory or exhausted CD8+ T cells [19]. Whereas IEAA is designed to be independent of blood cell counts, EEAA incorporates them, and is thus a measure of immune system aging.” (pp.7-8)
3) The authors also need to do a better job putting their findings in the context of previously published studies. For instance, there is substantial literature on the associations between epigenetic age and: SBP, obesity, diabetes, smoking, and heritability analysis. Given that most of the participants are AA and the authors discuss how this may be a more vulnerable population, they may want to mention the race/ethnic findings for epigenetic age. For instance, neither of these two measures reflect racial disparities, and in fact IEAA is lower in non-Hispanic black compared to non-Hispanic whites.

We agree with the Reviewer and have now more thoroughly discussed our results in the context of previous literature. In the discussion section, we have now added the text below. In addition, we have discussed the relationship between diabetes, glucose, triglycerides, and epigenetic age acceleration (please see Reviewer 2, Comment 4).

“On these points, our study is consistent with prior literature in two ways. First, that there has been little to no association observed between hypertension or blood pressure and IEAA or EEAA after controlling for BMI and lifestyle factors [17, 49]. Second, like our study, previous studies have demonstrated relationships between hypertension-related endpoints such as WMH [24] and cardiovascular mortality [50] after controlling for blood pressure level.” (p.18)

“The relationship between IEAA and ABI was attenuated after adjusting for smoking, which is a very strong risk factor for ABI. Even though self-reported smoking was not found to be associated with epigenetic age acceleration in previous literature [49, 57-59], many smoking-associated CpG sites were associated with methylation age acceleration [59, 60]. It has been hypothesized that biological indicators of smoking may represent susceptibility to more generalized environmental factors, including alcohol consumption and lifestyle factors, which may be partially responsible for the apparent mediating effect of smoking on the relationship between IEAA and ABI observed in this study.” (pp.19-20)

“Previous studies have found that EEAA rates are lower for African Americans than non-Hispanic whites, but that IEAA rates are similar [17]. This is contrary to what would be expected given that African Americans experience higher rates of many age-related chronic diseases. Recently, a new DNAm age predictor has been developed that better captures the racial differences in aging that would be expected based on the disparities in health outcomes across race/ethnic groups (that is, African Americans have higher rates of epigenetic aging) [61, 62]. Future studies examining this new DNAm age predictor are warranted.” (p.22)

“The heritability estimates of epigenetic age acceleration vary by age group, ranging from 0.39 to 0.74 in adults [13, 57, 63]. The estimated heritabilities in this study were similar (0.48 to 0.60).” (p.22)
4) There is no adjustment for multiple comparisons, or even acknowledgements of this. Based on my understanding, just in "Model 1", there were 12 separate models tested. It is true that the variables are correlated and thus a strict Bonferroni correction may be too conservative; however, the multiple testing bias should at least be discussed.

We acknowledge that multiple testing correction is a concern when multiple outcomes are tested. Indeed, the six outcomes we evaluated are all hypertension-related target organ damage traits and are correlated. Additionally, the two predictors (IEAA and EEAA) are highly correlated. As the Reviewer indicates, applying a Bonferroni correction would be too conservative. Given the small number of tests that were conducted, using a false discovery rate is also not appropriate in this case. However, we agree with the Reviewer that multiple testing should be at least discussed. Therefore, we added the following sentences to the methods, results and discussion sections to address the issue.

Methods

“Since we tested six related target organ damage traits with a prior hypothesis for each of them, we were interested in significant results at both a nominal p-value (p<0.05) as well as a Bonferroni corrected p-value (0.0083). We note that the Bonferroni approach is conservative in this setting, since the measures of target organ damage as well as the measures of epigenetic age acceleration are not independent.” (p. 12)

Results

“Among the five associations we observed for Model 1, three (UACR and IEAA, LVMI and IEAA, LVMI and EEAA) remained significant after Bonferroni correction for multiple testing.” (p. 15)

Discussion

“However, the associations between RWT and IEAA as well as ABI and IEAA were not significant after Bonferroni correction for multiple testing (Model 1); thus these results should be interpreted with caution.” (p.17)

5) Relatedly, the authors over-state their findings. Most of the associations were very weak and nearly all were eliminated when adjusting for confounders in subsequent models (Model 3). Given the multiple tests, I would consider most of these associations as "suggestive".
Nevertheless, the authors make statements like, "These results may help to lay the foundation for a precision medicine approach that better predicts individuals who are at risk of specific types of target organ damage." Also, paragraph 1 only discusses the results from Models 1 and 2, while the attenuation of the associations is not discussed until paragraph 3.

We agree with the Reviewer, and have modified the language describing our results, as follows:

Abstract

“These measures may act as subclinical biomarkers for damage to the kidney, heart, and peripheral vasculature; however more research is needed to determine whether these relationships remain independent of lifestyle factors and comorbidities.” (p.3)

Discussion

“Further, the majority of these findings were attenuated after adjusting for BMI, diabetes, and smoking, indicating that the associations between epigenetic age and target organ damage should be considered suggestive. [...] However, further research is needed to determine whether these relationships remain independent of lifestyle factors and comorbidities.” (pp.17-18)

“However, most of these associations were attenuated after adjusting for blood pressure levels, BMI, diabetes, and smoking. Further research is needed to determine whether epigenetic age acceleration may have potential clinical utility in helping to quantify risk of target organ damage across organ systems.” (pp.23-24)

We would also like to note that the first two paragraphs describing the associations between IEAA/EEAA and target organ damage do, in fact, discuss the results from all three Models (1-3). We did notice one omission, the discussion of Model 3 attenuation with respect to LVMI, which has now been added.

6) Why do the authors not adjust for education/SES?

We agree with the Reviewer that education/SES could be a potential confounder in the analysis. We performed a sensitivity analysis by adding education (< high school, high school, college or above) as a covariate in Models 1-3, and results are shown in Table S4. We have added this sensitivity analysis to the manuscript, as follows, and presented the relevant results in the supplementary materials.
Methods

“Next, since educational attainment or other markers of socioeconomic status may influence both the epigenome and the development of target organ damage, we evaluated the impact of adjusting for educational attainment (< high school, high school, college or above) in Models 1-3.” (pp.12-13)

Results

“Adding educational attainment as a covariate also did not substantively impact the findings, except for the slight attenuation of the relationship between ABI and IEAA in Model 2 (p=0.056, Table S4).” (p.16)

Reviewer 2 (Reviewer 2): PEER REVIEWER ASSESSMENTS:

GENERAL COMMENTS: This is an interesting and generally well-designed study although there is a part of the methodology (assessment of kidney damage) in which I disagree with the authors and it is in the interpretation of these results that I disagree with the authors.

The rest of the design and methodology I consider appropriate.

REQUESTED REVISIONS:

1) Design: 1) the authors have used clinical blood pressure to correlate EAA with the hemodynamic parameter. It is possible that the evaluation of the hypertensive population and its degree of control would have been more appropriate by 24 hours ambulatory blood pressure monitoring (ABPM). It is well known the possible presence of an alert reaction to blood pressure measurement in the office and also to the existence of other hemodynamic factors that influence the target organ damage such as nocturnal hypertension or blood pressure variability; 2) In relation to the study of renal function, there is an important limitation, since only a single determination of albuminuria was performed and its variability is well known (as the authors comment). Therefore, it is recommended to make at least two determinations. So the results obtained at this point should be taken with caution.

We appreciate this point, and have now added it to the discussion section, as follows:
“An important limitation in this study was that blood pressure and target organ damage traits were measured at only one study visit. Since blood pressure is known to be highly variable and elevated during clinical visits, a better approach for accurate blood pressure measures may be to 24-hour ambulatory blood pressure measures. These are available for only a subset of GENOA participants, however, so the sample size would have been prohibitory. Some of the measures of target organ damage, such as urinary albumin, are also highly variable and would be more accurate if measured on multiple days; however, UACR is a more stable biomarker than urinary albumin alone [64].” (p.23)

2) Execution: there was a significant percentage of patients who were lost between phases 1 and 2. Specifically more than 460, representing approximately 25% of the study population. There is no information in the manuscript about this loss. If some of the losses were due to cardiovascular death, it could have been important for interpreting the results.

We thank the Reviewer for this comment, and acknowledge that only approximately 75% of the participants enrolled at Phase 1 were included in the current study. This loss was for two reasons: 1) loss to follow-up (N=372), which may be related to cardiovascular mortality or cardiovascular risk factors, and 2) the participant had an examination at Phase 2 but did not have DNA methylation data (N=78).

We compared GENOA participants that had both Phase 1 and Phase 2 exams (N=1,482) to those who did not complete a Phase 2 exam (N=372). We found that those lost to follow up were more likely to be older (average = 1.2 years), male, and a current smoker (all p<0.05). On average, they also had a higher BMI (average = 0.58 kg/m2) and a lower eGFR (average = 6.0 mL/min/1.73m2, and were more likely to have hypertension and diabetes (all p<0.05). All of this indicates that loss to follow-up is greater in those with higher risk of cardiovascular disease. We have now acknowledged this in the discussion section, as follows:

“We also note that there was loss to follow-up between Phases 1 and 2 which was differential by cardiovascular risk. We found that those lost to follow-up were more likely to be older (average = 1.2 years), male, and a current smoker (all p<0.05). They also had higher BMI (average = 0.58 kg/m2) and lower eGFR (average = 6 mL/min/1.73m2), and were more likely to have hypertension and diabetes (all p<0.05). All of this indicates that loss to follow-up was greater in those with higher risk of cardiovascular disease, and thus our study sample may not include those with the highest risk of developing target organ damage in this population.” (p.21)

We also compared Phase 2 GENOA participants who had valid methylation data (N=1,404) to those who did not (N=78). We observed no significant differences in terms of age, sex, smoking, BMI, eGFR, hypertension status, or diabetes status.
3) There is also a certain bias in the population studied, since there is a high percentage of women. Therefore, I believe that the authors should include these points within a section of limitations of the study.

We acknowledge this and have now addressed it in the discussion section, as follows:

“Finally, over 2/3 of our sample consisted of women, so our results may be more relevant to this population; this is important to recognize since epigenetic aging rates in blood have been shown to be higher for men than women [17].” (p.21)

4) Interpretation: in relation to albuminuria there is a point of discussion that I do not agree with. The authors comment that in diabetes, before the decrease in filtration, there is a progressive increase in albuminuria. However, they point out that this does not happen in hypertension which is inaccurate (European HTA ref). From here they make an inadequate interpretation and point out that there may be a relationship between IEAA and albuminuria in diabetes and that IEAA may be a marker of diabetes. This point must be modified.

We apologize for the potential inaccuracy of our discussion with respect to diabetes, hypertension, and kidney disease. We removed a section of the discussion, and have modified the paragraph as follows:

“For target organ damage traits in the kidney (UACR) and heart (RWT), the relationship with IEAA was attenuated after adjusting for diabetes, although it remained marginally significant (p<0.1) for RWT. The two primary etiologies for chronic kidney disease (CKD) are diabetic nephropathy and hypertension [51], and kidney damage due to diabetic nephropathy is often first detected clinically through increased albuminuria (higher UACR) prior to declines in eGFR [52]. Likewise, diabetes is also an independent risk factor for higher LVMI and RWT in African Americans [53], so it is possible that the relationship between these traits and IEAA was due entirely to the relationship between diabetes and IEAA. Some previous studies have found that epigenetic age acceleration was not associated with glucose and/or diabetes status after adjusting for BMI and lifestyle risk factors, and was only weakly associated with other diabetes risk factors such as triglycerides [17, 49]. A recent longitudinal study, however, observed the association between epigenetic age acceleration and fasting glucose [54]. More studies are needed to elucidate the complicated interplay among epigenetic age acceleration, diabetes, and related target organ damage to the kidney and heart.” (pp.19)

5) In addition, although the authors take into account factors related to albuminuria, such as smoking, diabetes and obesity, dyslipidemia is not mentioned, which is also associated with the excretion of albumin in the urine.
We agree with the Reviewer, and we examined the relationship between dyslipidemia and UACR, RWT, LVMI, and ABI by evaluating the change in beta coefficient between IEAA and the target organ damage measure after adding dyslipidemia to the model (similar to analysis shown in Table S2). Dyslipidemia was defined as total cholesterol \( \geq 240 \), or LDL \( \geq 160 \), or triglycerides \( \geq 200 \), or being on a lipid lowering medication. For each of the associations, we observed less than a 4\% change in beta coefficient, indicating that dyslipidemia did not attenuate the observed relationships.

6) Finally, regarding antihypertensive treatment, there are drugs families that are more specific in controlling target organ damage such as the renin angiotensin system (RAS) inhibitors and albuminuria. The authors should comment on that.

We agree, and have now added the following to the discussion section of the manuscript.

“Finally, we note that antihypertensive medications have differential effects in adequately preventing or controlling target organ damage. For example, renin-angiotensin-aldosterone system-blocking agents (RAAS inhibitors) may be particularly effective in reducing damage to the kidneys and heart [64], so future studies may benefit from systematically evaluating the relationship between epigenetic age acceleration and target organ damage taking differences in antihypertensive drug classes into account.” (p.23)