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RESEARCH PAPER

Methylation of FKBP5 is associated with accelerated DNA methylation ageing and cardiometabolic risk: replication in young-adult and middle-aged Black Americans

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ABSTRACT

Methylation of *FKBP5* is involved in the regulation of the stress response and is influenced by early stress exposure. Two CpG sites, cg20813374 and cg00130530, have been identified as potential reporters of early stress. We examined whether *FKBP5* methylation was associated with accelerated DNA methylation ageing and indirectly predicted poorer cardiovascular health among both young adult and middle-aged Black Americans. Four hundred and forty-nine young adults, with a mean age of 28.67 and N = 469 middle-age parents and their current partners with a mean age of 57.21, provided self-reports, biometric assessments, and blood draws. Methylation values were obtained using the Illumina Epic Array. Cardiometabolic risk was calculated by summing the standardized log-transformed scores for the body mass index, mean arterial blood pressure, and HbA1c. We also used a more standard index of risk, the Framingham 10-year cardiometabolic risk index, as an alternative measure of cardiometabolic risk. To measure accelerated ageing, four widely used indices of accelerated, DNA methylation-based ageing were used controlling sex, age, other variation in FKBP5, and cell-type. Exposure to community danger was associated with demethylation of *FKBP5*. *FKBP5* methylation was significantly associated with accelerated ageing for both young-adult and middle-aged samples, with significant indirect effects from *FKBP5* methylation to cardiometabolic risk through accelerated ageing for both. Early exposure to danger may influence *FKBP5* methylation. In turn, *FKBP5* methylation may help explain intrinsic accelerated ageing and elevated cardiometabolic risk in adulthood for Black Americans.

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Black Americans are at considerably elevated risk for cardiometabolic illness and have increased risk for morbidity and mortality [1]. They also are more likely to have elevated scores on several cardiometabolic risk factors including elevated BMI and diabetes [1,2], and elevated blood pressure [3–6]. These factors account for much of the elevated risk for morbidity and mortality due to cardiometabolic illness experienced by Black Americans relative to white Americans [5–7]. However, Black Americans are not elevated on all cardiometabolic risk factors. For example, they do not have elevated rates of smoking [8], despite having elevated health consequences associated with smoking [9–11]. In addition, the association of blood lipids with CMR for Black Americans is complex [12], with Black Americans typically demonstrating a healthier lipid profile than whites, i.e., lower triglycerides and higher high-density lipoprotein [13,14]. These background factors have led us to focus on blood pressure, BMI, and HbA1c as likely useful indicators of cardiovascular risk factors that place Black Americans at increased relative risk compared to white Americans [15]. However, alternative characterizations of cardiovascular risk that include additional risk factors are common, particular those incorporating smoking and correction for blood pressure medication (e.g [16].). In either case, elevated cardiometabolic risk is thought to be a biological process associated with the whole organism that ultimately leads to the onset of chronic illness [17–

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19]). According to guidance from the WHO, risk is thought to be potentially modifiable with a focus on factors associated with BMI, HbA1c, and hypertension, such as physical inactivity, unhealthy diet, unhealthy alcohol consumption, and smoking.

Why use indices of accelerated ageing to explain cardiometabolic risk?

Despite widespread consensus regarding the likely importance of early experiences for later health in general [20] and the likely early origins of risk for cardiometabolic illnesses in particular, identifying and testing pathways to adult illness remains challenging due to the need for longitudinal, lifespan data to better map hypothesized processes that may begin early in life. Indices of healthy ageing, such as recently developed measures of accelerated ageing that capture early warning signs of problematic developmental processes, may address this need by allowing for examination of relevant biological changes well in advance of the emergence of illness phenotypes. In particular, examination of the extent to which DNA methylation-based measures of ageing are accelerated relative to an individual's chronological age, resulting in 'accelerated aging' (AA), provides a useful and flexible tool for better examining developmental processes unfolding across the lifespan.

Using DNA methylation-based indices of ageing to determine 'accelerated aging' and examine its association with cardiometabolic risk has been facilitated by the emergence of several different indices of accelerated ageing over the past several years, resulting in several largely independent indices now being widely available. Because independent information is provided by each of the indices of AA in widespread use, they sometimes show different patterns of effects and correlates [21,22]. Accordingly, it is important to briefly consider different types of DNA methylation-based indices of ageing that have been developed, the way they have been characterized in the broader literature, and whether they might be usefully combined to examine the general construct of 'accelerated aging.'

Indices of accelerated ageing

Briefly, early exemplars of DNA methylation-based indices of ageing used chronological age as the criterion variable to be predicted. For example, pioneering work by Horvath focused on the way in which methylation patterns across tissues followed a regular pattern of change with chronological age [23,24]. Using a similar approach, but focused on peripheral blood only, Hannum and colleagues devised an additional DNA methylation-based index focused on prediction of chronological age, resulting in a measure that focused on a largely non-overlapping set of CpG sites from those that Horvath used [25]. More recently, DNA methylation-based indices of ageing have been developed using disease phenotypes as the criterion. In particular, the 'PhenoAge' DNA methylation index developed by Levine et al. [26] provides a useful objective marker of elevated risk for early onset morbidity and chronic illness. Using a somewhat different strategy, Lu et al. (2019) developed a DNA methylation-based measure of predicted lifespan, focusing on the prediction of time to death due to all-cause mortality [27]. The resulting index provides a mortality risk estimate called 'DNAm GrimAge.' For each of the four indices, the DNA methylation-based ageing index can be used to estimate Accelerated Ageing (AA) by using the residual from the regression of the index on chronological age. It is accelerated ageing, i.e., ageing more quickly or slowly than expected, that is thought to predict cardiometabolic risk, and so in all cases our analyses use 'accelerated' ageing as the intermediate variable between our proxy for environmental exposures (FKBP5) and Cardiometabolic Risk outcomes.

Intrinsic DNA methylation based ageing

Associations between accelerated ageing and outcomes sometimes may be due to variation in the cell-type distributions that change with age instead of changes in patterns of methylation across tissues. This suggests the value of measures of DNA methylation based accelerated ageing (AA) that control for cell-type variation. These are referred to as 'intrinsic' indices of accelerated ageing (i.e., cell-type variation controlled). Intrinsic AA

indices are anticipated to better capture accelerated ageing patterns that are reflected across a variety of tissue types and so may be most appropriate as predictors of outcomes that affect several tissue types such as cardiometabolic risk.

Why combine these four separate DNA methylation based indices into a single measure of accelerated ageing?

Jointly, the four widely used indices of DNA methylation-based ageing described above provide several perspectives from which to estimate degree of 'accelerated aging' and this is hypothesized to predict both later biological problems and chronic illness. Levine et al. (2018) [26], for example, hypothesized that widespread changes in methylation across the genome may influence later health and be captured by DNA methylation-based measures of accelerated ageing (AA). Likewise, Beach et al. (2016) [28] found that childhood SES-risk was associated with widespread changes in methylation in young adulthood. Focusing more specifically on cardiometabolic health outcomes, it was reported recently that better cardiovascular health practices were associated with decreases in age acceleration on indices developed by Horvath [24] and Hannum [25], and effects were robust to calculations of intrinsic AA for both clocks [29].

Using the same four indices of accelerated ageing used in the current investigation, Ammous et al. (2021) [30], found that they were associated cross-sectionally with cardiometabolic markers of hypertension, insulin resistance, and dyslipidemia for Black Americans, and that change in GRIM improved prediction of future CVD events over clinical risk scores alone. Other authors have also reported associations of GRIM with cardiometabolic outcomes. In particular, McCrory et al. (2020) [31] examined several indices of accelerated ageing (AA) and found that GRIM-AA outperformed other indices of AA in predicting ageing related outcomes in the Irish Longitudinal Study on Ageing (TILDA) sample. Replicating and partially explaining these results, Fohr et al. (2021 [32] noted that GRIM-AA was less affected by genetic confounds than Horvath-AA, but that controlling for smoking, which is known to alter DNAm levels and is built into the GRIM-AA algorithm, attenuated the association between Grim-AA and all-cause mortality risk.

In the current investigation, we examined four widely used indices of intrinsic accelerated ageing as independent indices of a common underlying process, combining them into a single scale to better capture the shared underlying dimension that each is hypothesized to assess. Psychometric theory and simulations [33,34] suggest that using several indicators of a construct to create a multi-item scale typically results in greater predictive validity than using single indicators [33]. This is particularly likely when the individual indicators are only moderately correlated as is the case for indices of accelerated ageing. At the same time, given the relative newness of these measures, it also will be important to provide information to identify areas in which the scales perform differently. For example, there are differences in sensitivity of DNA methylation-based ageing indices to smoking (Lei et al., 2020), with GRIM more strongly associated with smoking [22], than other indices of accelerated ageing.

Is FKBP5 methylation an epigenetic marker of childhood early exposure to danger?

Identifying specific facets of the childhood environment that may contribute to cardiometabolic risk via biological mechanisms is also important and has the potential to provide substantial conceptual and practical benefits, potentially identifying areas that may account for health disparities and novel targets for preventive intervention, informing the development of preventive intervention programmes. Psychosocial processes leading to elevated cardiometabolic risk (CMR) are thought to begin in childhood in the context of elevated exposure to a range of early stressors [35–37], and contribute to the development of elevated cardiometabolic risk among Black Americans [38]. Some researchers have proposed that, beginning in childhood, stressors may become biologically embedded in young people's bodies via epigenetic memory [39–41]. At the beginning of the biological cascade leading to ill-health, there appears to be an initial disruption of HPA-axis regulation. This has led researchers to focus on specific regulator genes that are associated with early stress

exposures and also with subsequent HPA-axis dysregulation [41]. *FKBP5* is particularly promising because it regulates expression of the FK506-binding protein 51 (FKBP51), which has numerous regulatory effects, and has consequences for inter-related, stress-responsive biological processes [42–46]. Indeed, *FKBP5* is a plausible regulator of, and contributor to, long-term stress effects [47]. In particular, *FKBP5* up-regulation has been demonstrated in response to stress exposure and glucocorticoid stimulation, and predicts increased risk for future physical illness [45,48,49], as well as risk for cardiovascular disease [50,51]. Ortiz et al. (2018) [52] suggest that FKBP5 expression may be linked to chronic exposure to glucocorticoids as well as cardiovascular and metabolic dysfunction.

Recent developmental theory suggests that one facet of childhood adversity that may be of particular interest due to its potential to remodel a variety of health-related phenotypes, is elevated exposure to danger in childhood [53]. Based on animal models, elevated exposure to danger in childhood is hypothesized to be linked to hyperactivation of the HPA axis, leading to dysregulation of glucocorticoid and cortisol responses to stress [50], which may, in turn, have implications for FKBP5 methylation [54], accelerated ageing and cardiometabolic risk [55,56]. In particular, two CpG sites on *FKBP5* (cg20813374 and cg00130530) have shown associations with transcription of FKBP51, demethylation with increasing age, demethylation in response to exposure to stress, and also show demethylation in response to administration of glucocorticoids in vitro [50]. In addition, these effects were observed across several informative data sets comprising multiple racial groups including African Americans [50]. The two age-related CpG sites on *FKBP5* also lie in close proximity to each other, have methylation levels that are correlated, and are close to the *FKBP5* transcription start site. It also appears that demethylation of cg20813374 and cg00130530 (FKBP5-2) can influence HPA system response and the subsequent level of glucocorticoids (e.g., cortisol) which then cross the blood–brain barrier to affect multiple system throughout the body as well as in the brain [57]. Accordingly, this mechanism has the potential, over time, to result in broad and coordinated epigenetic reprogramming across the genome as well as the potential to affect cells in peripheral blood along with other tissues throughout the body.

If methylation of these two FKBP5 loci are a reflection of biological embedding of early risk [35–38,58,59], and continue to exert an influence across the lifespan, we would expect to observe associations between their methylation levels and DNA methylation-based indices of accelerated ageing, which in turn would lead to elevated CMR (e.g [30]. Ammous et al., 2021). Such effects could also be expected to influence DNA-methylation-based indices of accelerated ageing using measures derived from whole blood, and to exert long-term effects on increased risk for CMR observable for both younger and older cohorts. EWAS findings also support a possible association between level of methylation of these two loci and both BMI [60] and overall cardiovascular risk [50].

The hypothesis that differences in methylation of two key CpG sites on *FKBP5* (i.e., FKBP5-2) may initiate broader changes in indices of accelerated ageing (AA) [47], and set the stage for later elevated risk for cardiovascular illness can be tested in multiple ways. An initial step is see whether there is prospective evidence that elevated exposure to danger in childhood is associated with methylation of these loci. A second step is to examine indirect effect models to test expected patterns of association between FKBP5-2 methylation, Accelerated Ageing (AA), and CMR, controlling for potential confounders, such as sex, chronological age, and cell-type variation as well as the influence of overall variation in methylation across *FKBP5* (i.e., the first principal component of variation in methylation across *FKBP5*; FKBP5-PC1) to ensure that effects are attributable to FKBP5-2.

In sum, using measures of accelerated ageing (AA) allows examination of hypotheses about mechanisms affecting cardiometabolic risk across the lifespan, enabling examination of associations of cardiometabolic risk with AA in young adulthood as well as in later middle-age. Accordingly, AA measures reduce the follow-up time needed to assess the likely impact of early experiences on adult health. Likewise, because AA can be examined at different ages, use of AA measures

facilitates comparison of risk factors across the lifespan. In addition, DNA methylation-based ageing indices have been shown to be useful predictors of a variety of chronic illnesses as well as time to death [26,27,51], and have been used successfully in the past to examine how social conditions and relationships may influence an individual's speed of biological ageing [61–65]. These considerations suggest that examining the association of FKBP5-2 with epigenetic indices of AA may provide useful information about the way that exposure to adverse, stressful events can become biologically embedded and influence healthy ageing, morbidity, and mortality in early adulthood, middle-age, and beyond.

Hypotheses

To examine the hypothesis that FKBP5-2 demethylation is a marker of early exposure to elevated danger, and contributes to accelerated ageing (AA) and increased cardiometabolic risk, we proposed to examine the following four sets of hypotheses in a young adult and a middle-age sample.

We hypothesized that:

H1: Elevated exposure to danger in childhood will be related to FKBP5-2 methylation in the young adult sample.

H2. a. There will be significant associations among FKBP5-2, PC1, DNA-methylation-based indices of accelerated ageing (AA), and CMR in a young adult sample.

H2. b. For the young adult sample, effects of variation in methylation at FKBP5-2 on cardiometabolic risk (CMR; FCMR10) will be indirect through associations with the composite index of intrinsic AA (i.e., indices of DNA-methylation-based ageing controlling for sex, age, cell-type variation, and FKBP5-PC1). There will not be significant differences in patterns observed for males vs. females.

H2. c. There will be similar patterns for each of the individual indices of intrinsic AA, replicating the pattern observed with the composite index of intrinsic accelerated ageing for young adults.

H3. a. There will be significant correlations among FKBP5-2, PC1, DNA-methylation-based indices of accelerated ageing, and CMR in the middle-aged sample.

H3. b. For the middle-aged sample the effect of variation in methylation at FKBP5-2 on cardiometabolic risk (CMR; FCMR10) also will be indirect through associations with intrinsic AA (i.e., indices of DNA-methylation-based ageing controlling for sex, age, cell-type variation, and FKBP5-PC1). There will not be significant differences in patterns observed for males vs. females.

H3. c. For middle-aged participants there will be similar patterns for each of the individual indices of intrinsic AA, replicating the pattern observed with the composite index of intrinsic accelerated ageing.

H4. Because of its association with chronological age, FKBP5-2 methylation level will be significantly lower in the middle-age sample than in the young-adult sample.

Methods

Sample

The current study used data from the Family and Community Health Study (FACHS), comprising longitudinal assessments of 889 Black American families (children and their primary and secondary caregivers) in 1997. All the families had a fifth grader at study inception. The sampling strategy was designed to recruit families representing a range of socioeconomic statuses and neighbourhood settings. Additional details regarding recruitment are described by Gibbons and colleagues [66] and Simons and colleagues [67]. At Wave 1, about half of the sample resided in Georgia (n = 422) and the other half in Iowa (n = 467). The current investigation examines the association of FKBP5-2 methylation with accelerated ageing (AA) and cardiometabolic risk for both the young adult portion of this sample and

the middle-aged portion of the sample (i.e., parents and parents' partners). Analyses use data collected from the young-adult portion of the FACHS sample, who were re-interviewed in 2015–2016, retaining 62.5% of the original sample, and yielding N = 449 young adults (172 men and 277 women), and who provided both self-report and a blood sample. Young adults participating were, on average, aged 28.67 (SD = .792) for the 2015–2016 data collection. Average annual income of young adults was $23,007 (SD = 17794.14). Data used for the middle-aged, parent/caregiver plus romantic partner analyses, were obtained in 2018–2019, providing N = 469 (124 men and 345 women) middle-aged participants who provided both self-report and a blood sample. Middle-aged participants in the current study were, on average, age 57.21 (SD = 6.75). Average per capita annual income of middle-aged participants was $19,548 (SD = 17900.21).

For both the young adult and middle-aged samples, because the data collections included blood draws, only those residing in Georgia, Iowa, or a contiguous state were eligible for inclusion due to cost and difficulty of out-of-state blood draws. After excluding those who were deceased, incarcerated, or otherwise unreachable or out-of-state, our potential pool of young adults was N = 556 individuals, 470 (182 men and 288 women) of whom provided blood. Of these, 449 (95.5%) were successfully assayed and provided interview data, and comprise the sample for the current analyses of young adults. Likewise, for Middle-aged participants, after excluding deceased, incarcerated, or otherwise unreachable or out-of-state participants, we were able to interview and collect blood from N = 480, of whom 469 (97.7%) were successfully assayed, and they comprise the sample for the current analyses of Middle-aged participants.

All study protocols and procedures for Middle-aged participants were approved by the Institutional Review Board at the University of Georgia (Title: FACHS weathering – Protocol study number 00006152).

Procedures

For all participants, the phlebotomist drew four tubes of blood (30 mL); these were shipped on the same day to a laboratory at the University of Iowa for preparation. Whole blood DNA was prepared using cold protein precipitation [68], quantified with a NanoDrop photometer (Thermofisher, Waltham, MA, USA) and stored at −20°C until used [68].

Questionnaires were administered on laptop computers in the respondent's home and took on average about 2 h to complete. In an effort to further enhance anonymity, the questionnaires were administered using audio-enhanced, computer-assisted, self-administered interviews (ACASI). Using this procedure, the respondent sat in front of a computer and responded to questions as they were presented both visually on the screen and auditorily via earphones.

DNA methylation procedures

DNA methylation-based assessments were conducted with the Illumina Infinium (Sequenom, Inc., San Diego, CA, USA) HumanMethylationEPIC 850 BeadChip. We randomized samples with respect to slide and position on arrays to minimize potential batch effects as recommended by the Illumina Infinium Protocol Guide. Prior to normalization, DNA methylation data were filtered based on these criteria: (a) samples were examined to identify any 'poor quality samples' containing 1% or more of CpG sites with detection $p < 0.05$ (but, no samples were found to fail this criterion), (b) sites were removed if a bead count of <3 was present in 5% of samples.

The beta value at each CpG site was calculated as the ratio of the intensity of the methylated probe to the sum of intensities of the methylated and unmethylated probes. Quantile normalization was used, with separate normalization of the Type I and Type II probes used in the HumanMethylationEPIC array, as this approach has been found to produce marked improvement for the Illumina array in detection of relationships by correcting distributional problems inherent in the manufacturers default method for calculating beta values [69].

Measures

Prospective measures of childhood adversity (Danger, Family Conflict, Discrimination, and Family SES)

Youth were asked about a number of circumstances when they were 10 years old. Their responses were used to create scales reflecting key stressors during childhood. To assess perceived exposure to *community level danger*, youth were asked three questions about their perception of dangerous activities in their neighbourhood, assessing over the past 6 months how often 1) there was a fight in your neighbourhood in which a weapon like a gun or knife was used; 2) there was a sexual assault or rape; 3) a robbery or mugging. Responses were on a scale from 1 = *never* to 3 = *often*. Exposure was gauged by taking the mean of the items (mean of 1.40, Sd = .50). Alpha = .614. To assess perceived exposure to *family conflict*, youth were asked four questions about perceived parental hostility over the past 12 months including: 1) your parent got angry at you, 2) got so mad he/she broke or threw things, 3) Criticized your ideas, or 4) Insulted or swore at you. Responses were on a scale from 1 = *never* to 4 = *always*. Exposure was gauged by taking the mean of the items (mean of 1.52, SD = .43). Alpha = .462. To assess perceived exposure to *Discrimination* [70], youth were asked four questions: Just because you were African American 1) someone said something insulting, 2) someone ignored you or excluded you, 3) yelled a racial slur or racial insult at you, or 4) threatened to harm you physically. Responses were on a scale from 1 = *never*, 2 = *once or twice*, 3 = *a few times*, 4 = *several times* (mean of 1.65, SD = .64). Exposure was gauged by taking the mean of the items. Alpha = .681. Exposure to different levels of *SES* related risk was assessed using parent report of family SES when youth were age 10 was used. Caregiver reports across six indicators were used to create a measure of socio-economic risk. Risk indicators were (a) family poverty, defined as being below the poverty level, taking into account both family income and number of family members; (b) primary caregiver non-completion of high school or an equivalent; (c) primary caregiver unemployment; (d) single-parent family structure; (e) family receipt of Temporary Assistance for Needy Families; and (f) income rated by the primary caregiver as not adequate to meet all needs. Each indicator was scored dichotomously (0 if absent, 1 if present). SES risk was defined as the number of SES-related indicators, summing items to form an index with a theoretical range of 0 to 6 (M = 1.81, SD = 1.52), with larger numbers indicating greater SES risk (i.e., lower SES).

DNA methylation-based Accelerated Ageing (AA)

We assessed DNA methylation-based ageing using established procedures to calculate each of the previously established and widely used DNA methylation-based measures of accelerated ageing including the Hannum index [25], the Horvath index [23], the PhenoAge index [26], and the GrimAge index [27]. All indices were analysed using the online 'New Methylation Age Calculator' (https://dnamage.genetics.ucla.edu/) with the Advanced Analysis option and the normalize data option. The Hannum index used 71 CpG sites. The Horvath method used 353 CpG sites. The accelerated phenotypic ageing (PhenoAge) used 513 CpG sites that reflect several known ageing pathways [24]. Finally, the GrimAge index used 1030 sites. The four indicators of accelerated ageing were combined into a single index (AA) by summing the standardized scores and dividing by 4. Positive values indicated accelerated ageing relative to the sample as a whole and so elevated risk for morbidity and mortality, whereas negative values indicated decelerated ageing relative to the sample as a whole.

Cardiometabolic risk was assessed in two ways, first using three indicators known to place Black Americans at increased risk (CMR) and second using these three indictors as well as smoking status and blood pressure medication status (FCMR10).

CMR

First, we created an index of those factors on which Black Americans are elevated relative to whites by combining three biomarkers. (1) Each person's body mass index (BMI) score was calculated as weight in kilograms divided by the square of height in metres, with mean BMI at age 29 for young adults of 31.43 (*SD* = 8.36), and mean BMI

for middle-aged adults of 34.26 (SD = 8.70). (2) Resting diastolic and systolic blood pressure (BP) was monitored with Dinamap Pro 100 while the participants sat reading quietly. Three readings were taken, one every 2 minutes, and the average of the last two readings was used as the resting index. Mean arterial BP (MAP) was calculated according to the following formula: [(systolic BP) + (2 × diastolic BP)]/3. For young adults, mean MAP in the current sample was 93.49 (*SD* = 11.88). For middle-aged adults MAP = 103.80 (SD = 14.19). (3) Haemoglobin A1c (HbA1c) was assessed at the University of Iowa using antecubital serum samples drawn by certified phlebotomists. HbA1c provides an indication of average blood glucose concentrations over the preceding 2 to 3 months. For young adults, mean HbA1c was 5.35 (*SD* = .82), with 2.7% of the sample having HbA1c above 6.5, the cut-off for type II diabetes (The International Expert Committee). For middle-aged adults, mean HbA1c was 6.20 (*SD* = 1.36), with 21.9% of the sample having HbA1c above 6.5. Given that these three biomarkers are characterized by a skewed distribution, we applied a log transformation to normalize the distribution for each age group separately. CVD risk was calculated by summing the standardized log-transformed scores of BMI, MAP, and HbA1c.

FCMR10

We also examined effects on cardiometabolic risk following the gender-specific Framingham algorithm proposed by D'Agostino and colleagues (publicly available online tool: https://framinghamheartstudy.org/fhs-risk-functions/cardiovascular-disease-10-year-risk/). To estimate 10-year cardiometabolic risk (FCMR10), the Framingham algorithm uses systolic blood pressure (SBP), body mass index (BMI), and diabetes (HbA1C > 6 or taking diabetes medication), plus it adjusts for an individual's chronological age and gender, and whether they currently smoke (young adults: 1 = yes, 24.3%; middle-age adults: 1 = yes, 27.1%) and whether they are taking antihypertensive medication (young adults: 1 = yes, 7.8%; middle-age adults: 1 = yes, 65.5%). This measure has been shown to have high validity and reliability [16] and has been commonly used by physicians to monitor their patient's health condition [17–19,71–73].

FKBP5-2

To provide an index of methylation level for the two methylation cites on FKBP5 previously shown to be related to childhood exposures, we examined level of methylation at cg20813374 and cg00130530. The two CpG sites were correlated r = .480, p < .001. and r = .577, p < .001, for young adults and middle-aged adults respectively, supporting previous work indicating that they covary and could be combined into a meaningful index. To create a single index, we took the mean of the quantile-normalized beta values at each CpG site, resulting in a single methylation index of likely early exposure. This approach allowed for meaningful comparison of FKBP5-2 levels for different age groups.

FKBP5-PC1

Due to the correlated nature of CpG sites within *FKBP5*, in addition to examining the two CpG sites expected to be implicated in long-term biological effects, we also employed a principal component data reduction approach to characterize broadly the level of methylation across FKBP5, excluding the two CpG sites included in FKBP5-2 (cg20813374 and cg00130530). The first principal component was extracted and a standardized factor score was computed for each participant, allowing us to control for background variation in methylation of *FKBP5* and so more stringently test the role of FKBP5-2. Loadings for CpG sites on FKBP5-PC1 are provided in Supplemental Table 1 (for young adults) and Supplemental Table 2 (for middle-aged adults).

Cell-type variation

We also controlled for cell-type variation to adjust for the cellular heterogeneity that can affect methylation-based scores. Specifically the 'EstimateCellCounts' function in the minfi Bioconductor package was performed to assess individual differences in the distribution of cell types. This statistical package is based on the reference-based and regression calibration methods developed by Houseman and colleagues (2012). The peripheral white blood cell contribution was

subclassified into five different cell types. Using this approach, we estimated cell-type proportions for CD4 + T cells, CD8 + T cells, Natural Killer cells, B cells, and monocytes, and controlled these in all analyses to examine 'intrinsic' indices of DNA-methylation-based ageing. That is, because controlling cell-type variation yields DNA-methylation-based index values that are relatively free of influences from age-related or other individual differences in cell-type variation, they are described as 'intrinsic' measures of ageing.

Statistical analyses

After examining zero-order correlations along with means and SDs for all primary study variables, we also examined the association of early perceived exposure to dangerous community contexts and other potential sources of childhood stress to examine whether FKBP5-2 is a plausible indicator of childhood exposure. We next examined hypothesized indirect-effects models from FKBP5-2 to CMR and FCMR10 through a composite index of intrinsic DNA Methylation-Based Accelerated Ageing (AA) that controlled age and gender as well as FKBP5-PC1 and cell-type variation. We tested for any differences between the proposed indirect-effects models from FKBP5-2 to CMR through AA for female vs. male participants, and for younger vs. older samples, using the multiple group analysis option in *Mplus*. All analyses were conducted using *Mplus Version 8* [74]. To characterize goodness-of-fit of each model, standardized root-mean-square residual (good fit SRMR < .05) and the comparative fit index (good fit CFI >.90) along with Chi-square and degrees of freedom were reported. Indirect effects between FKBP5-2, DNA methylation-based epigenetic accelerated ageing (AA), and CMR were estimated and reported in the text, along with the 95% confidence interval (CI) estimated using bias-corrected and accelerated bootstrapping with 1,000 resamples.

Models for young adults and their middle-aged parents were analysed separately, except in the analysis where we compared them to each other to test for age-related differences in level of FKBP5-2. In addition, to better examine variability across different indices of intrinsic accelerated ageing (AA), we also examined all indirect effect models for each individual index of intrinsic AA (Hannum, Horvath, PhenoAge, and GRIM). This resulted in two models using the intrinsic composite accelerated ageing indices for both young adults and middle-aged adults, and then eight follow-up models each for young adults and middle-aged adults. Direct and indirect effects for the follow-up models using individual indices of accelerated ageing are summarized in tabular form and the full models are provided as figures in the supplemental material.

Results

Effects for targets

H1: Elevated exposure to danger in childhood will be related to demethylation of FKBP5-2 in the young adult sample. Table 1 presents correlations between youth report of exposure to community danger, family conflict, and discrimination at age

Table 1. Partial correlations for young-adults, along with means and sd's, showing the level and association of FKBP5-2, cg20813374, cg00130530, FKBP5-PC1, exposure to community danger, family conflict, and discrimination at age 10, and parent report of family SES when youth were age 10, controlling age and gender. (N = 449).

	1	2	3	4	5	6	7	8
1. FKBP5-2	–							
2. Cg20813374	.885**	–						
3. Cg00130530	.831**	.477**	–					
4. PC1	.353**	.416**	.170**	–				
5. Danger	−.108*	−.081†	−.108*	.019	–			
6. Family Conflict	−.029	−.052	.006	−.003	.196**	–		
7. Discrimination	−.042	−.047	−.024	−.023	.268**	.141**	–	
8. Family SES	−.007	.004	−.018	−.014	.140**	.078†	.053	–
Mean	.505	.418	.590	.000	1.397	1.523	1.654	1.809
SD	.031	.039	.03	1.000	.500	.425	.641	1.519

† $p < 0.1$, * $p < 0.05$, ** $p < 0.01$.
PC1 = FKBP5-PC1; Danger = Exposure to community danger.

10, parent report of family SES when youth were age 10, FKBP5-PC1, FKBP5-2, and the two CpGs it comprises (cg20813374; cg00130530), controlling sex and age. To test whether childhood environment reported prospectively at age 10 by the young-adult sample was associated with FKBP5-2 or either of its component CpG sites, we examined four facets of the environment that would be expected to be associated with some hardship: SES, discrimination, family harshness, and exposure to community danger. As can be seen in Table 1, we found that perceived exposure to elevated levels of danger in the community was associated with FKBP5-2, and the two CpG sites in the predicted direction (greater exposure to danger was associated decreased methylation of FKBP5-2), $r = -.108$, $p = .023$; $r = -.081$, $p = .089$; $r = -.108$, $p = .023$, for FKBP5-2, cg20813374 and cg00130530, respectively. These associations were not diminished by controlling for the first principal component of FKBP5 (FKBP5-PC1), yielding correlations of $r = -.123$, $p = .010$; $r = -.097$, $p = .040$; $r = -.113$, $p = .017$, for FKBP5-2, cg20813374 and cg00130530, respectively. Conversely, other common measures of childhood difficulty such as family SES, family harshness, and discrimination were not associated with FKBP5-2 methylation. Further, when the four facets of prospectively measured childhood difficulty were examined jointly, controlling age sex, and FKBP5-PC1 only greater exposure to danger was significantly associated with FKBP5-2 methylation, b = −.632, p = .040; all other b's NS.

H2. a. There will be significant associations among FKBP5-2, PC1, DNA-methylation-based indices of accelerated ageing (AA), and CMR in a young adult sample. Table 2 presents intercorrelations for young adults (N = 449) below the diagonal. Means, medians, and standard deviations are presented below the correlations. As shown, there was a significant correlation for targets between FKBP5-2 and the composite index of accelerated ageing (AA), $r = -.304$, $p < .001$ and between AA and cardiometabolic risk (CMR) $r = .165$, $p < .001$, as well as between AA and the Framingham FCMR10, $r = .233$, $p = .001$. At the same time there were significant associations of FKBP5-PC1 with FKBP5-2, AA, and CMR,

Table 2. Correlations for young adults are presented below the diagonal. Correlations for middle-aged adults are above the diagonal. Correlations and level are provided for FKBP5-2, a composite methylation-based index of extrinsic accelerated ageing (AA), a three-indicator measure of cardiometabolic risk (CMR), the Framingham cardiometabolic 10-year risk score (FCMR10), BMI, Blood Pressure (BP), HbA1C, Smoking, FKBP5-PC1, five cell-types, as well as sex (N = 449 for young adults; N = 469 for middle-aged adults). Medians, Means and SDs are shown below the correlations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. FKBP5-2	–	−.34**	.04	−.23**	.08	−.01	−.00	−.11*	.12**	−.04	.07	−.09†	−.23**	−.14**	−.23**
2. AA	−.30**	–	.12**	.25**	.09*	.04	.11*	.28**	−.30**	−.19**	−.31**	.05	.00	.24**	.28**
3. CMR	−.11*	.17**	–	.46**	.65**	.58**	.57**	−.13*	−.12*	−.07	−.13**	−.02	−.04	.05	−.05
4. FCMR10	−.13**	.23**	.55**	–	.08†	.42**	.33**	.31**	−.17**	−.01	−.19**	.07	−.02	.12*	.44**
5. BMI	−.02	.11*	.71**	.25**	–	.08†	.09*	−.21**	−.08	−.10*	−.10*	−.02	−.06	.07	−.17**
6. BP	−.15**	.16**	.67**	.57**	.23**	–	−.03	.07	−.01	.02	−.10*	.05	−.01	.04	.11*
7. HbA1C	−.06	.09†	.67**	.36**	.20**	.19**	–	−.05	−.14**	−.06	−.05	−.07	−.02	−.04	−.02
8. Smoking	−.00	.04	−.12*	.39**	−.11*	−.04	−.09	–	−.03	−.01	.02	−.04	.04	−.03	.16**
9. PC1	.34**	−.37**	−.11**	−.07	−.07	−.13**	−.03	−.03	–	.58**	.37**	.37**	.41**	−.36**	−.06
10. CD8T	−.07	−.23**	−.11*	.03	−.20**	.00	−.02	.00	.62**	–	−.07	.33**	−.08	−.14**	.07
11. CD4T	.15**	−.41**	−.01	−.04	.06	−.10*	−.01	.15**	.44**	.15**	–	−.13**	.18**	−.50**	−.13**
12. NK	−.07	.07	−.01	.07	−.10*	.06	.02	−.07	.33**	.21**	−.12*	–	−.06	−.04	.08
13. Bcell	.15**	−.25**	−.05	−.08	.02	−.14**	.00	−.02	.50**	.14**	.36**	−.05	–	−.24**	.00
14. Mono	−.12*	−.33**	.04	.12*	.03	.08	−.01	−.10*	−.29**	−.17**	−.51**	.06	−.31**	–	.21*
15. Sex	−.13**	−.18**	−.02	.45**	−.23**	.15**	.04	.00	.08	.18**	−.15**	.25**	.05	.19**	–
Young adults															
Median	.51	−.04	−.22	.02	29.70	92.50	5.30	.00	−.06	.09	.15	.00	.04	.05	.00
Mean	.51	.00	.04	.02	31.44	94.47	5.35	.24	.00	1.00	.15	.01	.04	.06	.38
SD	.03	.69	2.06	.01	8.36	11.90	.82	.43	1.00	.05	.05	.02	.03	.02	.49
Middle-aged															
Median	.42	.00	.01	.21	33.00	102.67	5.80	.00	−.03	.11	.11	.00	.02	.06	.00
Mean	.42	.00	−.01	.27	34.26	103.79	6.20	.27	.00	.12	.13	.01	.03	.06	.26
SD	.03	.69	1.83	1.95	8.70	14.19	1.36	.45	1.00	.05	.06	.03	.05	.03	.44

AA = The composite methylation-based ageing; CMR = Cardiometabolic Risk; FCMR10 = Framingham Cardiometabolic Risk 10 years; BMI = Body Mass Index; BP = High Blood Pressure; PC1 = FKBP5-PC1.

† $p < 0.1$, * $p < 0.05$, ** $p < 0.01$.

suggesting the need to control for FKBP5-PC1 in the examination of the hypothesized indirect effect models.

Table 3 presents the intercorrelations of PC1, cell-type variability, CMR, FCMR10, age, and sex, along with each of the individual indices of accelerated ageing and each of the individual cardiometabolic risk factors (see Table 3). As can be seen, there were significant associations between each of the individual indices of accelerated ageing and CMR, except in the case of GRIM*(r* = .123; *p* = .009); *(r* = .137; *p* = .004); *(r* = .193; *p* = .001); *(r* = .003; *p* = .948) for Hannum, Horvath, PhenoAge, and GRIM, respectively. Likewise, there were significant associations between each of the individual indices of accelerated ageing and FCMR10, except in the case of PhenoAge for which the association was marginal, with*(r* = .154; *p* = .001); *(r* = .135; *p* = .004); *(r* = .084; *p* = .075); *(r* = .269; *p* = .001) for Hannum, Horvath, PhenoAge, and GRIM, respectively. Cell-type variability was also associated with indices of AA, indicating the utility of examining 'intrinsic' AA.

H2. b. For young adults, the effect of variation in methylation at FKBP5-2 on cardiometabolic risk (CMR; FCMR10) will be primarily indirect through associations with DNA-methylation-based measures of intrinsic accelerated ageing (AA). We examined the model portrayed in Figure 1 to characterize direct and indirect effects of FKBP5-2 on CMR using the composite measures of intrinsic AA in young adults. As shown in Figure 1 we found good fit for the model (CFI = 1.000, and SRMR = 0.010; Chi-square = 3.319, *df* = 6, *p* = .7679) As can be seen in Figure 1, there was no significant direct effect from FKBP5-2 to CMR. However, there was a significant association of FKBP5-2 with intrinsic AA (*b* = −.239; *p* = .001), and a significant association of intrinsic AA to CMR (*b* = .178; *p* = .001), setting the stage for a possible significant indirect effect of FKBP5-2 on CMR.

Indirect effects were examined using 1,000 bias-corrected bootstrapped sampling with 95% confidence intervals (CIs) involving standardized parameter estimates given the non-normal distribution of the product term comprising the indirect effect (Hayes, 2009). We estimated that the indirect pathway from FKBP5-2 to CMR through intrinsic Accelerated Ageing and it was IE = −.043, and the

Table 3. Correlations for young adults are presented below the diagonal. Correlations for middle-aged adults are above the diagonal. Correlation and level are provided for FKBP5-2, a three-indicator measure of cardiometabolic risk (CMR), the Framingham cardiometabolic 10-year risk score (FCMR10), four methylation-based indices of extrinsic accelerated ageing (AA), FKBP5-PC1, five cell-types, as well as covariates of sex and age (N = 449 for young adults; N = 469 for middle-aged adults). Medians, Means and SDs are shown below the correlations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. FKBP5-2	–	.04	−.23**	−.34**	−.25**	−.20**	−.17**	.12**	−.04	.07	−.09†	−.23**	−.14**	−.23**	−.29**
2. CMR	−.11*	–	.46**	.10*	.12*	.15**	−.04	−.12*	−.07	−.13**	−.02	−.04	.05	−.05	−.04
3. FCMR10	−.13**	.55**	–	.14**	.09†	.13*	.32**	−.17**	−.01	−.19**	.07	−.02	.12*	.44**	.48**
4. Hannum-AA	−.29**	.12**	.15**	–	.42**	.46**	.24**	−.22**	−.16**	−.40**	.15**	.04	.26**	.29**	−.01
5. Horvath-AA	−.24**	.14**	.14**	.36**	–	.35**	.02	−.06	.03	−.08	.13*	.10*	.00	.12*	−.02
6. PhenoAge-AA	−.17**	.19**	.08	.52**	.36**	–	.34**	−.33**	−.27**	−.23**	−.06	−.02	.20**	−.00	−.02
7. Grim-AA	−.14**	.00	.27**	.24**	.01	.30**	–	−.21**	−.12**	−.14**	−.08	−.12**	.19**	.35**	.01
8. PC1	.34**	−.11*	−.07	−.34**	−.15**	−.34**	−.19**	–	.58**	.37**	.37**	.42**	−.36**	−.06	−.14**
9. CD8T	−.06	−.11*	.03	−.30**	.09	−.27**	−.17**	.62**	–	−.07	.33**	−.08†	−.14**	.07	−.01
10. CD4T	.15**	−.01	−.04	−.45**	−.11*	−.39**	−.18**	.44**	.15**	–	−.13**	.18**	−.50**	−.13**	−.15**
11. NK	−.07	−.01	.07	.16**	.09	−.01	−.06	.33**	.21**	−.12*	–	−.06	−.04	.08	.13**
12. Bcell	.15**	−.04	−.08	−.23**	−.10*	−.22**	−.14**	.50**	.14*	.36**	−.05	–	−.24**	.00	.01
13. Mono	−.12*	.03	.12*	.36**	.12*	.28**	.17**	−.29**	−.17**	−.51**	.06	−.31**	–	.21**	.06
14. Sex	−.13**	−.02	.45**	.18**	.17**	−.12*	.26**	.08	.18**	−.15**	.25**	.05	.18**	–	.07
15. Age	−.05	.01	.08	.01	−.00	.03	−.01	−.03	−.04	−.01	−.02	−.01	−.00	−.05	–
Young adults															
Median	.51	−.22	.02	.28	−.01	−.27	−.18	−.06	.09	.15	.00	.04	.05	.00	29.00
Mean	.51	.04	.02	.00	.00	.00	.00	.00	1.00	.15	.01	.04	.06	.38	28.67
SD	.03	2.06	.01	3.41	4.03	5.43	4.22	1.00	.05	.05	.02	.03	.02	.49	.79
Middle-aged															
Median	.42	.01	.21	.03	−.32	−.18	−1.00	−.03	.11	.12	.00	.02	.06	.00	56.17
Mean	.42	−.01	.27	−.01	.03	−.06	−.04	.00	.12	.13	.01	.03	.06	.26	57.21
SD	.03	1.83	1.95	3.65	4.41	4.82	4.66	1.00	.05	.06	.03	.05	.03	.44	6.75

CMR = Cardiometabolic Risk; FCMR10 = Framingham Cardiometabolic Risk 10 years; PC1 = FKBP5-PC1.
† $p < 0.1$, * $p < 0.05$, ** $p < 0.01$.

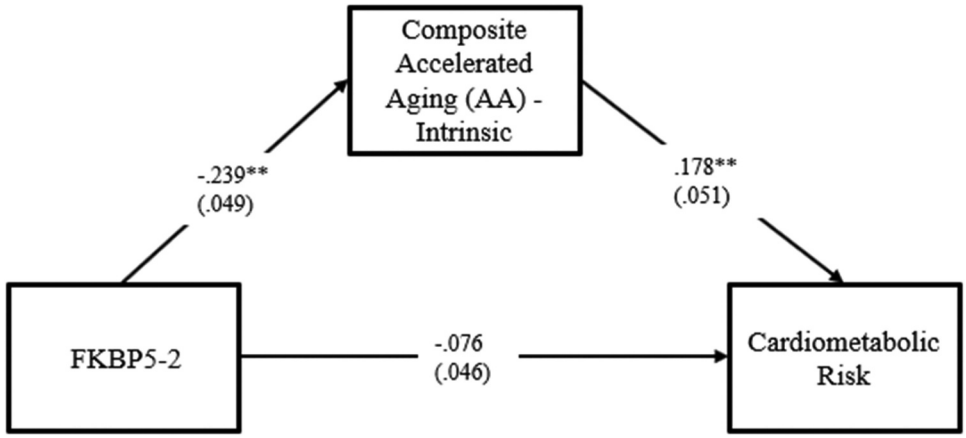

Figure 1. Indirect effect of FKB5-2 on CMR through the composite of the four intrinsic Accelerated Ageing (AA) Indices for young-adults. N = 449. Values are standardized parameter estimates and standard errors are in parentheses. Gender, FKBP5-Pc1, chronological age, and cell-types are controlled in the analyses. Model fit: CFI = 1.000 and SRMR = 0.010; Chi-square = 3.319, *df* = 6, p = .7679. Indirect pathway: IE = −.043, 95% CI = [−.082, −.018].
*$p < .05$, **$p < .01$ (two-tailed tests).

95% confidence interval did not include 0, 95% CI = [−.082, −.018], rejecting the null hypothesis. The total effect from FKBP5-2 to CMR is the sum of the standardized direct and indirect effects presented in Table 4, and so is −.119. Accordingly, the indirect effect accounted for 36% of the variance in the total effect.

We examined the possibility of different patterns of effects for males and females by constraining all pathways in model to be equal for males and females. This did not result in a significant deterioration in model fit, indicating that observed effects were not significantly different for males and females. ($\Delta\ \chi^2$ (2) = 1.833, p = .400).

We also examined the indirect pathway from FKBP5-2 to CMR through a composite of extrinsic Accelerated Ageing indices (i.e., controlling age, but not cell type; see Supplemental Figure S5), replicating the pattern observed for the composite of intrinsic indices, with good Model fit, CFI = 1.000, and SRMR = 0.004; Chi-square = 0.263, *df* = 2, p = .8768, an Indirect pathway estimate IE = −.026, with a 95% CI = [−.059, −.008] that does not contain zero.

We next examined the model portrayed in Figure 2 to characterize direct and indirect effects of FKBP5-2 on the Framingham measure of cardiometabolic risk (FCMR10) using the composite measures of intrinsic AA in young adults. As shown in Figure 2 we found good fit for the model (CFI = 1.000, and SRMR = .003; Chi-square = 1.959, *df* = 2, p = .3756) As can be seen in

Table 4. Table of direct effects from FKBP5-2 to cardiometabolic outcomes and indirect effects through composite and individual indices of intrinsic accelerated ageing for young adults. Cell-type variation, sex, and age are controlled in all analyses. Effect size of IE = ratio of the indirect effect to the total effect.

Paths	Direct Effect	Indirect Effect	95% CI	Effect Size of IE
FKBP5-2 →Accelerated Ageing → CMR				
FKBP5-2 → Composite AA → CMR	−.076	−.043*	[−.082, −.018]	0.361
FKBP5-2 → Hannum-AA → CMR	−.091	−.036*	[−.071, −.010]	0.283
FKBP5-2 → Horvath-AA → CMR	−.092*	−.010	[−.031, .000]	0.098
FKBP5-2→ PhenoAge-AA → CMR	−.091*	−.031*	[−.067, −.010]	0.254
FKBP5-2→ Grim-AA → CMR	−.119*	.001	[−.015, .018]	0.008
FKBP5-2 →Accelerated Ageing → FCMR10				
FKBP5-2 → Composite AA → FCMR10	−.021	−.042*	[−.078, −.021]	0.667
FKBP5-2 → Hannum-AA → FCMR10	−.053	−.026	[−.058, .002]	0.329
FKBP5-2 → Horvath-AA → FCMR10	−.063	−.004	[−.020, .001]	0.060
FKBP5-2 → PhenoAge-AA → FCMR10	−.050	−.026*	[−.057, −.009]	0.342
FKBP5-2 → Grim-AA → FCMR10	−.059	−.024*	[−.052, −.007]	0.289

**$p \le .01$; *$p \le .05$ (two-tailed tests), n = 449.

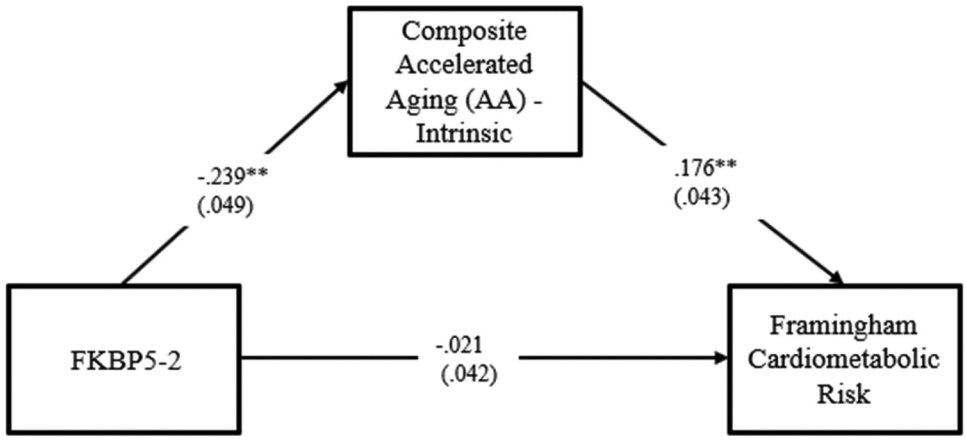

Figure 2. Indirect effect of FKB5-2 on FCMR10 through the composite of the four intrinsic Accelerated Ageing (AA) Indices for young-adults. N = 449. Values are standardized parameter estimates and standard errors are in parentheses. Gender, FKBP5-PC1, chronological age, and cell-types are controlled in the analyses. Model fit: CFI = 1.000 and SRMR = 0.003; Chi-square = 1.959, *df* = 2, *p* = .3756. Indirect pathway: IE = −.042, 95% CI = [−.078, −.021].

*$p < .05$, **$p < .01$ (two-tailed tests).

Figure 2, there was no significant direct effect from FKBP5-2 to FCMR10. However, there was a significant association of FKBP5-2 to AA ($b = -.239$; $p = .001$), and a significant association of AA to CMR ($b = .176$; $p = .001$), setting the stage for a possible indirect effect of FKBP5-2 on FCMR10.

Indirect effects were examined using 1,000 bias-corrected bootstrapped sampling with 95% confidence intervals (CIs) involving standardized parameter estimates given the non-normal distribution of the product term comprising the indirect effect (Hayes, 2009). We estimated that the indirect pathway from FKBP5-2 to FCMR10 through intrinsic Accelerated Ageing was IE = −.042, and that the 95% confidence interval did not include 0, 95% CI = [−.078, −.021], rejecting the null hypothesis. The total effect from FKBP5-2 to FCMR10 is the sum of standardized direct and indirect effects presented in Table 4, and so is −.063. Accordingly, the indirect effect accounted for 67% of the variance in the total effect.

We also examined the indirect pathway from FKBP5-2 to FCMR10 through a composite of extrinsic Accelerated Ageing indices (i.e., controlling age, but not cell type; see Supplemental Figure S6), replicating the pattern observed for the composite of intrinsic indices, with good Model fit, CFI = 1.000, and SRMR = 0.008; Chi-square = 1.291, *df* = 2 *p* = .524, and an Indirect pathway estimate of IE = −.025, and a 95% CI = [−.056, −.008] that does not contain zero.

We next examined the possibility of different patterns of effects for males and females by constraining the pathways in the indirect pathway of the model to be equal for males and females. This did not result in a significant deterioration in model fit, indicating that observed effects were not significantly different for males and females. ($\Delta\ \chi^2\ (2) = .352$, $p = .837$).

H2. c. There will be similar patterns for each of the individual indices of intrinsic AA, replicating the pattern observed with the composite index of intrinsic accelerated ageing. The model portrayed in Figure 1 was next examined using each individual index of accelerated ageing separately in place of the composite index. Direct and indirect effects for each model are summarized in Table 4, and the full models are provided in supplemental Figures S1a-d for intrinsic indices of Hannum, Horvath, PhenoAge, and Grim respectively. In all cases model fit was good (CFI = 1.000, 0.965, 1.000, 0.983 using CMR as the outcome) and patterns of significant associations were similar to that obtained for the composite index. In all cases, except for Horvath and GRIM, which was not significantly associated with CMR, there was a negative indirect effect from FKBP5-2 to CRM (IEs = −.036; −.010; −.031; .001) for Hannum, Horvath, PhenoAge,

and no effect for GRIM, respectively. There were significant direct effects from FKBP5-2 to CRM for three of the models (Horvath = −.092, PhenoAge = −.091, Grim = −.119), indicating that some of the association between the FKBP5-2 and CMR was not mediated by these indices of AA. In Table 4, the effect size for the indirect effect in each model is provided, with effect sizes uniformly smaller than what was observed for the composite index.

Analyses for young adults using individual indices of intrinsic accelerated ageing with FCMR10 as the outcome also provided a similar pattern of results, except that because GRIM was significantly associated with FCRM10 there was also a negative indirect effect from FKBP5-2 to increased cardiometabolic risk through GRIM. Direct and indirect effects for each model are summarized in Table 4, and the full models are provided in supplemental Figures S2a-d. Using FCMR10 as the outcome, in all cases there was a negative indirect effect from FKBP5-2 to FCMR10 (IEs = −.026; −.004; −.026; −.024). There was no significant direct effect from FKBP5-2 to FCMR10 for any of the individual indices of AA. In Table 4, the effect size for the indirect effect in each model using FCRM10 as the outcome is provided, with effect sizes uniformly smaller than what was observed for the composite index.

Replication with middle-aged sample

H3. a. There also will be significant correlations among FKBP5-2, PC1, DNA methylation-based indices of accelerated ageing, and CMR in a middle-aged sample. Table 2 presents intercorrelations for the middle-aged sample (N = 469) above the diagonal, showing associations for FKBP5-2, the Framingham the composite index of DNA methylation-based ageing (AA), the index of cardiometabolic risk (CMR), as well as FKBP5-PC1, sex and age. Means, medians, and standard deviations are presented below the correlations. As can be seen in Table 2, patterns of correlations for middle-aged adults were similar to those observed for young adults, including significant correlations between FKBP5-2 and each of the indices of AA. In addition, there was a correlation of FKBP5-2 with the overall index of and AA, r = −.344, p < .001 and between AA and CMR r = .116, p < .012. There were also significant correlations of study variables with FKBP5-PC1 and sex, showing the importance of controlling these in subsequent analyses.

Table 3 presents the intercorrelations of PC1, cell-type variability, CMR, FCMR10, age, and sex, along with each of the individual indices of accelerated ageing and each of the individual cardiometabolic risk factors (see Table 3). As can be seen, there were significant associations between each of the individual indices of accelerated ageing and CMR(r = .096; p = .038); (r = .116; p = .012); (r = .151; p = .001); (r = −.042; p = .368) for Hannum, Horvath, PhenoAge, and GRIM, respectively. Likewise, there were significant associations between each of the individual indices of accelerated ageing and FCMR10, with(r = .142; p = .002); (r = .087; p = .060); (r = .133; p = .004); (r = .316; p = .001) for Hannum, Horvath, PhenoAge, and GRIM, respectively. Cell-type variability was also associated with indices of AA, indicating the utility of examining 'intrinsic' AA.

H3. b. For middle-aged adults, the effect of variation in methylation at FKBP5-2 on cardiometabolic risk (CMR) will be indirect through associations with DNA methylation-based measures of intrinsic accelerated ageing (AA). We found adequate model fit for the model examining the association of FKBP5-2 with CMR, using a composite measure of intrinsic AA as the intervening variable. This model is portrayed in Figure 3 (CFI = 0.902 and SRMR = 0.021; Chi-square = 23.789, df = 6, p = .0006), and an IE = −.032, with a 95% CI = [−.068, −.005] that did not include 0. However, direct and indirect effects were of opposite sign, making it impossible to compute a meaningful effect size for the indirect effect.

Using multiple-group comparison, and constraining all pathways in the indirect effects model to be equal across sex, did not result in a significant deterioration in model fit, indicating that patterns of the effect were not significantly different for males and females ($\Delta\ \chi^2$ (2) = .097 p = 0.9527.

We also examined the indirect pathway from FKBP5-2 to CMR through a composite of extrinsic

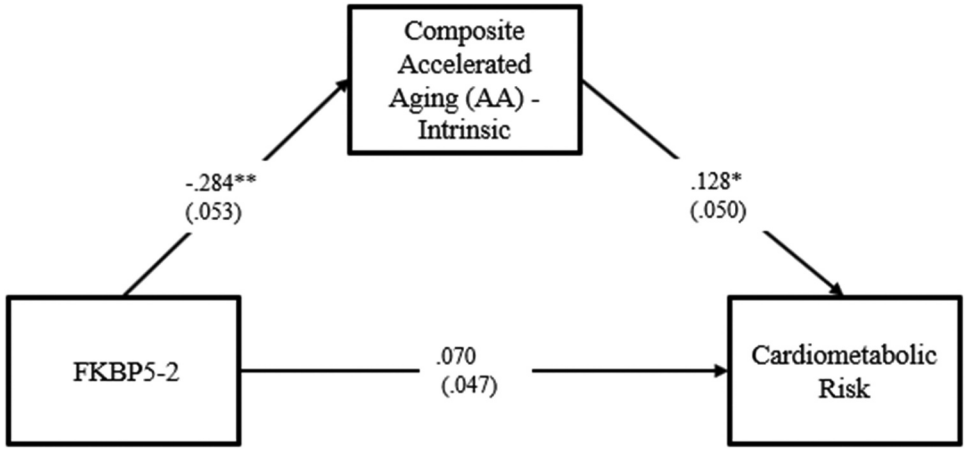

Figure 3. Indirect effect of FKB5-2 on CMR through the composite of the four intrinsic Accelerated Ageing (AA) Indices for young-adults. N = 469. Values are standardized parameter estimates and standard errors are in parentheses. Gender, FKBP5-PC1, chronological age, and cell types are controlled in the analyses. Model fit: CFI = 0.902, and SRMR = 0.021; Chi-square = 23.789, *df* = 6, *p* = .0006. Indirect pathway: IE = −.032, 95% CI = [−.068, −.005].

*$p < .05$, **$p < .01$ (two-tailed tests).

Accelerated Ageing indices (i.e., controlling age, but not cell type; see Supplemental Figure S7), replicating the pattern observed for the composite of intrinsic indices with middle-aged adults. We found adequate to good Model fit, CFI = 0.912 and SRMR = 0.027; Chi-square = 13.307, *df* = 1, *p* = .0003, and an Indirect pathway estimate, IE = −.036, with a 95% CI = [−.077, −.011] that did not contain zero.

Similar results were obtained for a model examining direct and indirect effects of FKBP5-2 on the Framingham measure of cardiometabolic risk (FCMR10) using the composite measures of intrinsic AA in middle-aged adults. As shown in Figure 4 we found good fit for this model (CFI = .952, and SRMR = .021; Chi-square = 21.366, *df* = 1, *p* = .001). As can be seen in Figure 4, there was no significant direct effect from FKBP5-2 to FCMR10. However, there was a significant association of FKBP5-2 to AA (b = −.284; p = .001), and a significant association of AA to CMR (b = . 150; p = .001), setting the stage for a possible indirect effect of FKBP5-2 on FCMR10 among middle-aged adults.

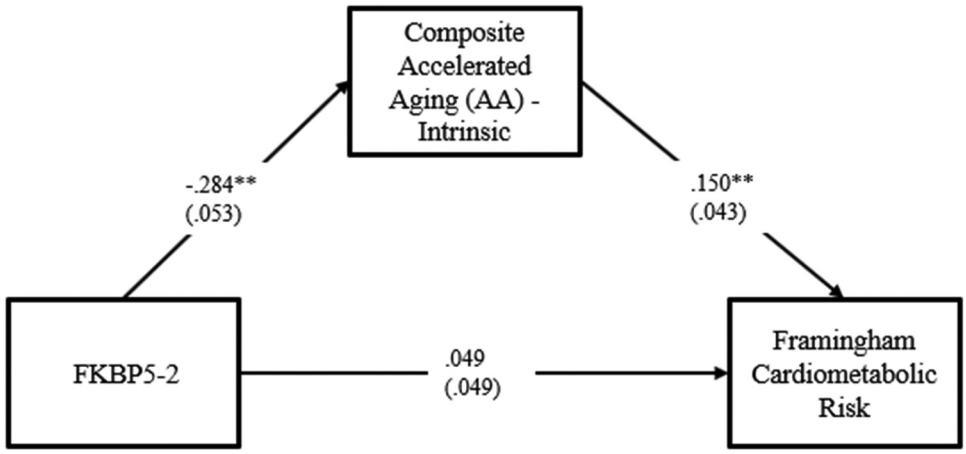

Figure 4. Indirect effect of FKB5-2 on FCMR10 through the composite of the four extrinsic Accelerated Ageing (AA) Indices for young-adults. N = 469. Values are standardized parameter estimates and standard errors are in parentheses. Gender, FKBP5-PC1, chronological age, and cell types are controlled in the analyses. Model fit: CFI = 0.952, and SRMR = 0.021; Chi-square = 21.366, *df* = 1, *p* = .0001. Indirect pathway: IE = −.043, 95% CI = [−.077, −.020].

*$p < .05$, **$p < .01$ (two-tailed tests).

Indirect effects were examined using 1,000 bias-corrected bootstrapped sampling with 95% confidence intervals (CIs) involving standardized parameter estimates given the non-normal distribution of the product term comprising the indirect effect (Hayes, 2009). We estimated that the indirect pathway from FKBP5-2 to FCMR10 through intrinsic Accelerated Ageing was IE = −.043, and that the 95% confidence interval did not include 0, 95% CI = [−.077, −.020], rejecting the null hypothesis. Again, direct and indirect effects were of opposite sign, making it impossible to compute a meaningful effect size for the indirect effect.

We next examined the possibility of different patterns of effects for males and females by constraining the pathways in the indirect pathway of the model to be equal for males and females. This did not result in a significant deterioration in model fit, indicating that observed effects were not significantly different for males and females. ($\Delta\ \chi^2$ (2) = 2.165, p = .3388).

We also examined the indirect pathway from FKBP5-2 to FCMR10 through a composite of extrinsic Accelerated Ageing indices (i.e., controlling age, but not cell type; see Supplemental Figure S8), replicating the pattern observed for the composite of intrinsic indices with middle-aged adults. We found good Model fit, CFI = 0.968 and SRMR = 0.030; Chi-square = 13.307, df = 1, p = .0003, and an Indirect pathway estimate, IE = −.040, with a 95% CI = [−.079, −.017].

H3. c. For middle-aged participants there will be similar patterns for each of the individual indices of intrinsic AA, replicating the pattern observed with the composite index of intrinsic accelerated ageing. We first examined each individual index of intrinsic AA used in the model portrayed in Figure 3. Direct and indirect effects for each model are summarized in Table 5, and the full models are provided in Supplemental Figure 3 (a-d) show the indirect effects of FKBP5-2 on CMR for intrinsic accelerated ageing indices of Hannum, Horvath, PhenoAge, and GRIM respectively. In all cases model fit was adequate to good (CFI = 0.909, 0.922, 0.953, 1.000 for Hannum, Horvath, PhenoAge, and GRIM, respectively) and patterns of significant associations were similar to that obtained for the composite index. As was the case for young adults, in all cases but one, i.e., for GRIM, there was a negative indirect effect from FKBP5-2 to CMR (IEs = −.024; −.017; −.024; .010). There was a significant effect from FKBP5-2 to GRIM, but GRIM was not associated with CMR, precluding a significant indirect effect. As can be seen in Table 5, for all models using individual indicators of AA and CMR as the outcome, there was no significant direct effect from FKBP5-2 to cardiometabolic outcomes among middle-aged participants. Again, because direct and indirect effects were of opposite signs it was not possible to compute an effect size for the indirect effects.

Follow-up analyses using FCMR10 as the outcome also provided a similar pattern of results, except that GRIM was a significant predictor of FCMR10, and so resulted in a significant indirect effect. Direct and indirect effects for each model are summarized in Table 5, and the full models are provided in Supplemental Figure 4(a-d). In all cases except for the Hannum measure of AA, there was a negative indirect effect from FKBP5-2 to FCRM 10 through intrinsic AA (IEs = .003; −.006; −.026; −.031 for Hannum, Horvath, PhenoAge, and GRIM respectively). There a not significant direct effect from FKBP5-2 to FCRM10

Table 5. Table of direct effects from FKBP5-2 to cardiometabolic outcomes, and indirect effects from FKBP5-2 to cardiometabolic outcomes through composite and individual indices of accelerated ageing for middle-aged adults.

Paths	Direct Effect	Indirect Effect	95% CI for IE
FKBP5-2 →Accelerated Ageing → CMR			
FKBP5-2 →; Composite AA → CMR	.070	−.036*	[−.072, −.011]
FKBP5-2 → Hannum-AA → CMR	.060	−.024	[−.060, .003]
FKBP5-2 → Horvath-AA → CMR	.067	−.017*	[−.045, −.003]
FKBP5-2→ PhenoAge-AA → CMR	.060	−.024*	[−.060, −.006]
FKBP5-2→ Grim-AA → CMR	.029	.010	[−.006, .038]
FKBP5-2 →Accelerated Ageing → FCMR10			
FKBP5-2 → Composite AA → FCMR10	.049	−.043*	[−.077, −.020]
FKBP5-2 → Hannum-AA → FCMR10	−.004	.003	[−.022, .033]
FKBP5-2 → Horvath-AA→ FCMR10	.007	−.006	[−.025, .002]
FKBP5-2 → PhenoAge-AA → FCMR10	.030	−.026*	[−.054, −.009]
FKBP5-2 → Grim-AA → FCMR10	.033	−.031*	[−.060, −.012]

**$p \leq .01$; *$p \leq .05$ (two-tailed tests), (N = 469).

for any of the individual indices of AA. Again, because direct and indirect effects were of opposite signs it was not possible to compute an effect size for the indirect effects.

H4: FKBP5-2 methylation level will be lower in the middle-age sample than in the young-adult sample. As can be seen in Table 2, the mean level of FKBP5-2 was substantially lower in the middle-age sample than in the young adult sample, as would be expected from prior research. We compared the full middle-age sample to the full young-adult sample using robust standard errors to correct for relatedness between some members of the sample. We found a significant difference $t(916) = -2.22$, $p = 0.0265$.

Discussion

Early adverse childhood events exert long lasting effects on health, and black youth and adults may be particularly susceptible to these effects because they are more likely to be chronically exposed to a range of contextual stressors [35–37], including elevated levels of neighbourhood violence and other threats. In addition, elevated stressful exposures for Black youth and adults continue across the lifespan. Prior work has shown that chronic exposures that stimulate prolonged HPA axis response may become biologically embedded via long-lasting epigenetic changes [26,39–41], and may be reflected in changes in methylation of two CpG sites on FKBP5. In the current investigation we examined reports of exposure to community danger, parental harshness, and experiences of discrimination provided by our young-adult sample when they were age 10, as well as family SES reported by a parent at that time. Consistent with hypotheses, we found that exposure to community danger was uniquely associated with demethylation of two CpG sites on *FKBP5* previously shown to be demethylated in response to stress and ageing.

Building on prior theorizing [41], we tested a model in which demethylation of the two CpG sites on *FKBP5* previously shown to be demethylated in response to stress and ageing, set in motion a broader set of biological changes captured by indices of accelerated ageing and ultimately lead to elevated risk for cardiometabolic illness [47]. In keeping with Levine et al. (2018) [26], and more recently Ammous et al. (2021) [30], we proposed that currently available DNA methylation-based indices of intrinsic accelerated ageing would potentially capture the impact of FKBP5-2 demethylation on a range of biological systems, and so constitute an intermediate step in the progression to elevated cardiometabolic risk (i.e., elevated blood pressure, elevated HbA1c, blood sugar, inflammation, BMI). These considerations led to our expectation that there would be a significant association of FKBP5-2 with accelerated ageing (AA), significant indirect effects from FKBP5-2 to two measures of cardiometabolic risk (CMR and FCMR10), and that similar patterns of association would be observed across the lifespan for both outcomes, using intrinsic measures of AA.

In keeping with the proposed model, we focused on FKBP5-2, i.e., two CPG sites of *FKBP5* that are responsive to stress, cortisol exposure, and age, as the starting point of the risk process, and examined accelerated ageing (AA) across multiple DNA methylation-based indices of ageing as the intermediate stage leading to elevated cardiometabolic risk. We found that FKBP5-2 was associated with AA even after controlling global variation in methylation across *FKBP5 (i.e.*, FKBP5-PC1), as well as age, sex, and cell-type variation, supporting hypotheses that these two CpG sites may be particularly important in forecasting longer-term outcomes. In addition, at the zero-order level each index of AA considered separately was also associated with FKBP5-2. Likewise, in analyses using the Framingham 10-year risk measure as the outcome similar patterns of effects were observed between FKBP5-2 and AA, and between AA and CMR. Adding weight to these findings, the predicted patterns of association were first shown for a young adult sample and then replicated in a middle-aged sample. In both young adult and middle-aged adult samples, the patterns were non-significantly different for males and females. As predicted, the older sample was more demethylated at FKBP5-2 than was the younger sample.

At a minimum, the current results provide support for the hypothesis that demethylation of FKBP5-2 is associated with broad indices of accelerated ageing [50], and that it has likely implications for multiple interrelated health outcomes,

particularly cardiometabolic health. Accordingly, the current findings support the value of additional work to further characterize the longitudinal antecedents of variation in methylation of *FKBP5*, and the longer-term sequelae of that variation. It is particularly important to examine this association among Black youth as well as younger and older adults and to better capture environmental variables that may vary with race or be unique to some social contexts. Results also suggest the need for identification of protective factors in childhood as well as potential ameliorative processes in adulthood. It is possible, that early exposures associated with demethylation of FKBP5-2 may lead to some changes that are not readily reversible, potentially creating vulnerabilities for future stressors or leaving a long legacy of health-related consequences. Future research examining this possibility also is required.

Interestingly, although the four indices of AA used in the current investigation (Hannum, Horvath, PhenoAge, and DNAm GrimAge) were intercorrelated for the most part as expected, Grim was not significantly associated with Horvath's index of AA or with CMR for either age group. In contrast, Grim was associated with the Framingham measure FCMR10. This suggests that observed associations between AA and CMR would have been more robust in the current study if DNAm GrimAge were *not* included in the overall index of AA for models predicting CMR. Conversely, GRIM *did* add to the robustness of the prediction of FCMR10. This also serves as a cautionary note that different indices of AA may not always be associated with exposures and illness in the same manner [22]. Understanding the different pattern shown by GRIM for CRM vs FCMR10 may be explained, in part, by an important difference between the CRM measure and the FCRM10 measures. Specifically, FCRM10 includes an additional important predictor of cardiovascular events: smoking. As has been shown previously GRIM preforms better than other DNA methylation-based indices of ageing in capturing the effect of smoking (Lei et al., 2020). Accordingly, it is not surprising that it would perform better when predicting a cardiometabolic risk index that included smoking status than when predicting an index that did not include smoking.

It is also interesting to note that use of a composite index of accelerated ageing provided a useful simplification of the association between FKBP5-2, AA, and cardiometabolic outcomes. It would have been harder to discern the overall pattern in these relationships using only individual indicators of AA one-by-one. The utility of composite indicators is due to its ability to define an underlying common dimension across multiple indicators, in this case, the dimension of accelerated ageing. That is, in line with standard psychometric practice, increasing the number of independent indicators will typically increase reliability of measurement and so its validity. By examining the composite index of accelerated ageing prior to looking at individual indices, we were able to provide an initial omnibus test of the overall hypothesis, increasing confidence that patterns observed across the individual clocks were not spurious or due to factors specific to each particular clock. At the same time, it is important to note the interesting and interpretable differences between GRIM and other indices of AA, highlighting the value of retaining examination of individual indices to compliment examination of composite indices.

Limitations should also be noted. Because indicators of FKBP5-2, AA, and CMR were all examined within a single time period for each age group, additional research examining relationships over time, and predictors of change over time, will be helpful in further clarifying and testing the proposed relationships. Currently it is not known whether there are optimal lag times for observing changes in these constructs or whether there are critical periods during which change is mostly likely to occur. Accordingly, future research on likely time lags for observing changes is needed. Because disruption of HPA-axis regulation could result in psychological and behavioural sequelae, future research should also examine whether FKBP5-2 predicts behavioural or emotional difficulties in response to stress [22], or serves to modulate substance use or exercise in response to stress. In addition, the current investigation did not include measures of lipid levels, and these might also be informative in future research. Likewise, it will be important for future research to map the role of health behaviours in mediating

the effects of early stress on variation in methylation of *FKBP5*.

Taken together, the results suggest a biologically plausible pathway from various stressful experiences to later health problems, and suggest that DNA methylation-based accelerated ageing can provide a window on biological changes that lead to adverse health outcomes. If supported in future research, this would suggest that DNA methylation-based accelerated ageing measures may provide a particularly useful tool for research on the delayed effects of childhood adversity on ageing and health in later life.

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Availability of data

The datasets supporting the conclusions of this article are included as supplemental files.

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