

# 1 Associations of four biological age 2 markers with child development: A multi- 3 omic analysis in the European HELIX 4 cohort

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## 36 Abstract

### 37 Background:

38 While biological age in adults is often understood as representing general health and resilience, the  
39 conceptual interpretation of accelerated biological age in children and its relationship to  
40 development remains unclear. We aimed to clarify the relationship of accelerated biological age,  
41 assessed through two established biological age indicators, telomere length and DNA methylation  
42 age, and two novel candidate biological age indicators , to child developmental outcomes, including  
43 growth and adiposity, cognition, behaviour, lung function and onset of puberty, among European  
44 school-age children participating in the HELIX exposome cohort.

### 45 Methods:

46 The study population included up to 1,173 children, aged between 5 and 12 years, from study  
47 centres in the UK, France, Spain, Norway, Lithuania, and Greece. Telomere length was measured  
48 through qPCR, blood DNA methylation and gene expression was measured using microarray, and  
49 proteins and metabolites were measured by a range of targeted assays. DNA methylation age was  
50 assessed using Horvath's skin and blood clock, while novel blood transcriptome and  
51 "immunometabolic" (based on plasma protein and urinary and serum metabolite data) clocks were  
52 derived and tested in a subset of children assessed six months after the main follow-up visit.  
53 Associations between biological age indicators with child developmental measures as well as health  
54 risk factors were estimated using linear regression, adjusted for chronological age, sex, ethnicity and  
55 study centre. The clock derived markers were expressed as  $\Delta$  age (i.e., predicted minus chronological  
56 age).

### 57 Results:

58 Transcriptome and immunometabolic clocks predicted chronological age well in the test set ( $r= 0.93$   
59 and  $r= 0.84$  respectively). Generally, weak correlations were observed, after adjustment for  
60 chronological age, between the biological age indicators.

61 Among associations with health risk factors, higher birthweight was associated with greater  
62 immunometabolic  $\Delta$  age, smoke exposure with greater DNA methylation  $\Delta$  age and high family  
63 affluence with longer telomere length.

64 Among associations with child developmental measures, all biological age markers were associated  
65 with greater BMI and fat mass, and all markers except telomere length were associated with greater  
66 height, at least at nominal significance ( $p<0.05$ ). Immunometabolic  $\Delta$  age was associated with better  
67 working memory ( $p = 4e^{-3}$ ) and reduced inattentiveness ( $p= 4e^{-4}$ ), while DNA methylation  $\Delta$  age  
68 was associated with greater inattentiveness ( $p=0.03$ ) and poorer externalizing behaviours ( $p= 0.01$ ).  
69 Shorter telomere length was also associated with poorer externalizing behaviours ( $p=0.03$ ).

70 Conclusions:

71 In children, as in adults, biological ageing appears to be a multi-faceted process and adiposity is an  
72 important correlate of accelerated biological ageing. Patterns of associations suggested that  
73 accelerated immunometabolic age may be beneficial for some aspects of child development while  
74 accelerated DNA methylation age and telomere attrition may reflect early detrimental aspects of  
75 biological ageing, apparent even in children.

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## 78 Introduction

79 The field of geroscience proposes that biological ageing, a set of interrelated molecular and cellular  
80 changes associated with ageing, drive the physiological deterioration that is the root of multiple age-  
81 related health conditions [1]. Understanding the process of biological ageing and developing markers  
82 to accurately assess biological age in individuals, holds great promise for public health and  
83 biomedical research in general to develop interventions, even in childhood and early life, that slow  
84 physiological decline and reduce the risk of chronic disease and disability in later life.

85 Telomere length, which shortens with age, is one of the most widely applied biological age markers  
86 primarily as it directly assesses a primary Hallmark of Ageing [2, 3]. More recently, high-throughput  
87 ‘omic’ methods, which provide simultaneous quantification of thousands of epigenetic marks,  
88 transcripts, proteins and metabolites, have been used to develop ‘biological clocks’ that provide a  
89 global measure of changes with age at the molecular level [4]. While biological clocks have been  
90 primarily trained on chronological age, “age acceleration”, commonly defined as the difference  
91 between clock-predicted age and chronological age, has been associated with age-related  
92 phenotypes and mortality [5-11], indicating their utility as biological age markers. DNA methylation-  
93 based clocks, such as the clock of Horvath [12], have been extensively applied in large-scale studies  
94 and remain a research field under active development, with “second generation” clocks further  
95 incorporating clinical biomarker and mortality information to improve their clinical utility [13, 14].  
96 Further clocks have been developed using transcriptome [8], metabolome [15] and proteome [9]  
97 data, including those that specifically target immune-system related proteins [16]. Generally, clocks  
98 have been found to be only weakly correlated with each other, suggesting that each clock captures  
99 different facets of biological ageing [17, 18].

100 While biological age in adults is intuitively understood as an overall indicator of general health and  
101 resilience, the conceptual interpretation of biological age acceleration in children is much less clear.  
102 Child development and ageing may at first be considered opposing processes, representing growth

103 and decay respectively. However, various related theoretical frameworks link the two processes:  
104 Under the developmental origin of health and disease hypothesis, the early life environment is a key  
105 determinant of ageing trajectories and disease risk in later life. Life-course models of ageing,  
106 supported by measures of physical and cognitive capability, view the childhood developmental  
107 phase as key to building up “biological capital” and to determining how long capabilities and disease  
108 risk remain above critical thresholds in later years following the gradual decline phase of adult life  
109 [19]. Horvath’s DNA methylation clock is currently the only clock trained to predict age throughout  
110 the lifespan, and many of the clock’s CpGs are in genomic regions known to regulate development  
111 and differentiation [12]. However, unlike life-course models of physical function, the level of DNA  
112 methylation at the clock’s CpGs changes in a predictable, unidirectional manner throughout the life-  
113 course, albeit at a much faster rate during childhood. This continuous molecular readout suggests  
114 that processes directing development are at least indirectly related to detrimental process in later-  
115 life and is consistent with quasi-programmed theories of ageing such as antagonistic pleiotropy [20],  
116 whereby molecular functions that promote development, inadvertently lead to ageing in later life  
117 [21]. Therefore, some authors have suggested that DNA methylation-based age acceleration may be  
118 beneficial during childhood [21, 22], reflecting greater physical maturity and build-up of biological  
119 capital.

120 Biological ageing is conceived as continuous balance of cellular damage, caused by both extrinsic  
121 environmental factors and by normal physiological processes, and resiliency mechanisms that  
122 protect against and compensate for this damage [23]. An alternative “wear and tear” model would  
123 view cellular damage to occur continuously from birth and, since the epigenetic clock has been  
124 proposed to reflect the epigenomic maintenance system, a resiliency mechanism, DNA methylation  
125 age acceleration in children may, as in adults, represent a greater accumulation of epigenetic  
126 instability and therefore reduced biological capital. However so far only a handful of studies have  
127 examined associations with developmental maturity in children [24-26]. Telomere length attrition is  
128 more rapid in early childhood during rapid somatic growth and more gradual in adulthood, with

129 those with a shorter telomere length in childhood maintaining a lower telomere length into  
130 adulthood [27]. While telomere length may serve as both a mitotic-clock and as a mediator of  
131 cellular stress [28], the associations reported between environmental stress in childhood and shorter  
132 telomere length suggest it reflects early-life cellular damage that may be carried into adulthood.

133 Little is known regarding the interpretation of biological age in children assessed at the  
134 transcriptome, proteomic and metabolomic levels, since few biological clocks are available for this  
135 age range using these data. To the best of our knowledge, only the study of Giallourou et al [29] has  
136 applied metabolomic data to provide a multivariate model of age in children, finding that growth  
137 constrained infants lag in their metabolic maturity relative to their healthier peers. It is possible that  
138 biological clocks constructed using these data, particularly proteomic and metabolomics, support the  
139 life-course ageing framework, where age acceleration in children represents a buildup of biological  
140 capital, since they are closer to the phenotype than the DNA-based epigenetic clocks and telomere  
141 length.

142 We hypothesized that age acceleration would be associated with child development. To test this and  
143 assess whether age acceleration is associated with beneficial or detrimental effects on child  
144 development, we have performed a comparative analysis of two established and two candidate  
145 assessments of biological age within the pan-European Human Early Life Exposome (HELIX) cohort of  
146 children aged between 5 and 12 years. We systematically compared associations with  
147 developmental endpoints, including growth and adiposity, cognition, behaviour, lung function and  
148 pubertal development, and common health risk factors, for telomere length, DNA methylation age,  
149 and two newly derived clocks: transcriptome age and immunometabolic age. Through this analysis,  
150 we aimed to clarify the interpretation of age acceleration in children, and more broadly develop new  
151 biological markers of overall developmental staging in children.

## 152 Materials and Methods

### 153 Study population

154 This study population included children recruited into the European population-based HELIX  
155 exposome cohort [30, 31], which was based on six on-going longitudinal population-based birth  
156 cohorts established in six countries across different parts of Europe (Born in Bradford [BiB; UK] [32],  
157 Étude des Déterminants Pré et Postnatals du Développement et de la Santé de l'Enfant [EDEN;  
158 France] [33], Infancia y Medio Ambiente [INMA; Spain] [34], Kaunas Cohort [KANC; Lithuania] [35],  
159 Norwegian Mother, Father and Child Cohort Study [MoBa; Norway] [36], and Mother-Child Cohort in  
160 Crete [RHEA; Greece] [37]) covering singleton deliveries from 2003 to 2008. All children participated  
161 in a harmonized 'HELIX subcohort' clinical examination in their respective study centres during 2014-  
162 2015, where biological samples were collected. A subset of children (from all study centres apart  
163 from MoBa), attended a second clinical examination, as part of the 'HELIX panel study'  
164 approximately 6 months after the first 'HELIX subcohort' examination, where a similar suite of  
165 biological samples were collected. A full description of the HELIX follow-up methods and study  
166 population, including eligibility criteria and sample size calculations are available in [60, 61]. In the  
167 current study we included all children with available molecular data (Figure 1).

168 Prior to the start of HELIX, all six cohorts had undergone the required evaluation by national ethics  
169 committees and obtained all the required permissions for their cohort recruitment and follow-up  
170 visits. Each cohort also confirmed that relevant informed consent and approval were in place for  
171 secondary use of data from pre-existing data. The work in HELIX was covered by new ethical  
172 approvals in each country and at enrolment in the new follow-up, participants were asked to sign a  
173 new informed consent form. Additionally, the current study was approved by the Imperial College  
174 Research Ethics Committee (Reference: 19IC5567).

## 175 Biological sample collection and processing

176 Blood was collected at the end of the clinical examination of the child to ensure an approximate 3  
177 hours (median = 3.5 hours, SD = 1.1 hour) fasting time since the last meal. Blood samples were  
178 collected using a 'butterfly' vacuum clip and local anaesthetic and processed into a variety of sample  
179 matrices, including plasma, whole blood for RNA extraction (Tempus tubes - Life Technologies, USA),  
180 red cells, and buffy coat for DNA extraction. These samples were frozen at -80°C under optimized  
181 and standardized procedures until analysis.

182 DNA was obtained from children's peripheral blood (buffy coat) collected in EDTA tubes. DNA was  
183 extracted using the Chemagen kit (Perkin Elmer, USA) in batches of 12 samples within each cohort.  
184 DNA concentration was determined in a Nanodrop 1000 UV-Vis Spectrophotometer (Thermo Fisher  
185 Scientific, USA) and also with Quant-iT™ PicoGreen dsDNA Assay Kit (Life Technologies, USA). DNA  
186 extraction was repeated in around 8% of the blood samples as the DNA quantity or quality of the  
187 first extraction was low. Less than 1.5% of the samples were finally excluded due to low quality.

188 RNA was extracted from whole blood samples collected in Tempus tubes (Thermo Fisher Scientific,  
189 USA) using MagMAX for Stabilized Blood Tubes RNA Isolation Kit. The quality of RNA was evaluated  
190 with a 2100 Bioanalyzer (Agilent Technologies, USA) and the concentration with a NanoDrop 1000  
191 UV-Vis Spectrophotometer. Samples classified as good RNA quality (78.67%) had a similar RNA  
192 pattern at visual inspection in the Bioanalyzer, a RNA Integrity Number (RIN) >5 and a concentration  
193 >10 ng/ul. Mean values (standard deviation, SD) for the RIN, concentration (ng/ul), Nanodrop  
194 260/280 ratio and Nanodrop 260/230 ratio were: 7.05 (0.72), 109.07 (57.63), 2.15 (0.16) and 0.61  
195 (0.41).

196 During the clinical examination, two spot urine samples (one before bedtime and one first morning  
197 void) were brought by the participants to the research centre in cool packs and stored at 4°C until  
198 processing. Urine samples of the night before the visit and the first morning void on the day of the  
199 visit were combined to provide Two urine samples, representing last night-time and first morning



200 voids, were collected on the evening and morning before the clinical examination and were  
201 subsequently pooled to generate a more representative sample of the last 24 h for metabolomic  
202 analysis (n = 1107). Either the night-time void (n = 37) or morning void (n = 48) sample was analysed  
203 in cases where a pooled sample was missing [30].

#### 204 [Telomere length measurement](#)

205 Blood average relative telomere length was measured by a modified qPCR protocol as described  
206 previously [38]. Telomere and single copy-gene reaction mixture and PCR cycles used can be found  
207 in Martens et al. [39]. All measurements were performed in triplicate on a 7900HT Fast Real-Time  
208 PCR System (Applied Biosystems) in a 384-well format. On each run, a 6-point serial dilution of  
209 pooled DNA was run to assess PCR efficiency as well as eight inter-run calibrators to account for the  
210 inter-run variability. Relative telomere lengths were calculated using qBase software (Biogazelle,  
211 Zwijnaarde, Belgium) and were expressed as the ratio of telomere copy number to single-copy gene  
212 number (T/S) relative to the average T/S ratio of the entire sample set. We achieved CV's within  
213 triplicates of the telomere runs, single-copy gene runs, and T/S ratios of 0.84%, 0.43%, and 6.4%,  
214 respectively.

#### 215 [DNA methylation](#)

216 Blood DNA methylation was assessed with the Infinium HumanMethylatio450 beadchip (Illumina,  
217 USA) at the University of Santiago de Compostela – Spanish National Genotyping Center (CeGen-  
218 USC) (Spain). 700 ng of DNA were bisulfite-converted using the EZ 96-DNA kit (Zymo Research, USA)  
219 following the manufacturer's standard protocol. All samples of the study were randomized  
220 considering sex and cohort. In addition, each plate contained a HapMap control sample and 24 HELIX  
221 inter-plate duplicates were included.

222 After an initial inspection of the quality of the methylation data with the MethylAid package[40],  
223 probes with a call rate <95% based on a detection p-value of 1e-16 and samples with a call rate  
224 <98% were removed [41]. Samples with discordant sex were eliminated from the study as well as

225 duplicates with inconsistent genotypes and samples with inconsistent genotypes respect to existing  
226 genome-wide genotyping array data. Methylation data was normalized using the functional  
227 normalization method with prior background correction with Noob [42]. Then, some probes were  
228 filtered out: control probes, probes to detect single nucleotide polymorphisms (SNPs), probes to  
229 detect methylation in non-CpG sites, probes located in sexual chromosomes, cross hybridizing  
230 probes [43], probes containing a SNP at any position of the sequence with a minor allele frequency  
231 (MAF) >5% and probes with a SNP at the CpG site or at the single base extension (SBE) at any MAF in  
232 the combined population from 1000 Genomes Project (Supplementary file 1). Batch effect (slide)  
233 was corrected using the ComBat R package [44]. CpGs were annotated with the  
234 IlluminaHumanMethylation450kanno.ilmn12.hg19 R package [45].

### 235 Transcriptome analysis

236 Gene expression was assessed using the GeneChip® Human Transcriptome Array 2.0 (HTA 2.0) from  
237 Affymetrix (USA) at the University of Santiago de Compostela (USC) (Spain). Briefly, RNA samples  
238 were concentrated or evaporated in order to reach the required RNA input concentration (200 ng of  
239 total RNA). Amplified and biotinylated sense-strand DNA targets were generated from total RNA.  
240 Microarrays were hybridized according to the Affymetrix recommendations using the Affymetrix  
241 labeling and hybridization kits. All samples were randomized within each batch considering sex and  
242 cohort. Two different types of control RNA samples (HeLa and FirstChoice® Human Brain Reference  
243 RNA (Thermo Fisher Scientific, USA)) were included in each batch, but they were hybridized only in  
244 the first batches.

245 Raw data were extracted with the Affymetrix AGCC software and normalized with the GCCN (SST-  
246 RMA) algorithm at the gene level ([http://tools.thermofisher.com/content/sfs/brochures/sst\\_gccn\\_whitepaper.pdf](http://tools.thermofisher.com/content/sfs/brochures/sst_gccn_whitepaper.pdf)). Annotation of transcripts clusters (TCs) to genes was done with the  
247 Affymetrix Expression Console software using the HTA-2\_0 Transcript Cluster Annotations Release  
248 na36 (hg19). A transcript cluster is defined as a group of one or more probes covering a region of the  
249 genome reflecting all the exonic transcription evidence known for the region and corresponding to a  
250

251 known or putative gene. Four samples with discordant sex were detected with the MassiR R package  
252 [46] and excluded. Control probes, and TCs in sexual chromosomes and without chromosome  
253 information were filtered out. Batch effect (slide) was corrected using the ComBat R package [44]. To  
254 determine TC call rate, 10 constitutive or best probes based on probe scoring and cross-hybridization  
255 potential were selected per TC. Probe Detection Above Background (DABG) p-values were computed  
256 based on the rank order against the background probe set intensities. Probe level p-values were  
257 combined into a TC level p-value using the Fisher equation. TCs with a DABG p-value <0.05 were  
258 defined as detected. Three samples with low call rate (<40%) as well as TCs with a call rate <1% were  
259 excluded from the dataset (Supplementary file 1). Gene expression values were log<sub>2</sub> transformed.

## 260 Proteome analysis

261 Plasma protein levels were assessed using the antibody-based multiplexed platform from Luminex.  
262 Three kits targeting 43 unique candidate proteins were selected (Thermo Fisher Scientifics, USA):  
263 Cytokines 30-plex (Catalog Number (CN): LHC6003M), Apolipoprotein 5-plex (CN: LHP0001M) and  
264 Adipokine 15-plex (CN: LHC0017M).

265 All samples were randomized and blocked by cohort prior measurement. For quantification, an 8-  
266 point calibration curve per plate was performed with protein standards provided in the Luminex kit  
267 and following procedures described by the vendor. Commercial heat inactivated, sterile-filtered  
268 plasma from human male AB plasma (Sigma-Aldrich, USA) was used as constant samples to control  
269 for intra- and inter-plate variability. Four control samples were added per plate. All samples,  
270 including controls, were diluted ½ for the 30-plex kit, ¼ for the 15-plex kit and 1/2500 for the 5-plex  
271 kit.

272 Raw intensities obtained with the xMAP and Luminex system for each plasma sample were  
273 converted to pg/ml using the calculated standard curves of each plate and accounting for the  
274 dilutions made prior measurement. The percentages of coefficients of variation (CV%) for each  
275 protein by plate ranged from 3% to 36%. The limit of detection (LOD) and the lower and upper limit  
276 of quantification (LOQ1 and LOQ2, respectively) were estimated by plate, and then averaged. Only

277 proteins with >30% of measurements in the linear range of quantification were kept in the database  
278 and the others were removed. Seven proteins were measured twice (in two different multiplex kits).  
279 We kept the measure with higher quality. The 36 proteins that passed the quality control criteria  
280 mentioned above were log<sub>2</sub> transformed [47]. Then, the plate batch effect was corrected by  
281 subtracting the plate specific average for each protein minus the overall average of all plates for that  
282 protein. After that, values below the LOQ1 and above the LOQ2 were imputed using a truncated  
283 normal distribution implemented in the `truncdist` R package [48]. Twenty samples were excluded  
284 due to having ten or more proteins out of the linear range of quantification (Supplementary file 1).

285

## 286 [Metabolomic analysis](#)

287 The AbsoluteID<sup>Q</sup>TM p180 kit was chosen for serum analysis as it is a standardised, targeted LC-  
288 MS/MS assay, widely used for large-scale epidemiology studies and its inter-laboratory  
289 reproducibility has been demonstrated by several independent laboratories [49]. Serum samples  
290 were quantified using the AbsoluteID<sup>Q</sup>TM p180 kit following the manufacturer's protocol (User  
291 Manual UM\_p180\_AB\_SCIEX\_9, Biocrates Life Sciences AG) using LC-MS/MS; an Agilent HPLC 1100  
292 liquid chromatography coupled to a SCIEX QTRAP 6500 triple quadrupole mass spectrometer. A full  
293 description of the HELIX metabolomics methods and data can be found elsewhere [50].

294 Briefly, the kit allows for the targeted analysis of 188 metabolites in the classes of amino acids,  
295 biogenic amines, acylcarnitines, glycerophospholipids, sphingolipids and sum of hexoses, covering a  
296 wide range of analytes and metabolic pathways in one targeted assay. The kit consists of a single  
297 sample processing procedure, with two separate analytical runs, a combination of liquid  
298 chromatography (LC) and flow injection analysis (FIA) coupled to tandem mass spectrometry  
299 (MS/MS). Isotopically labelled and chemically homologous internal standards were used for  
300 quantification. The AbsoluteID<sup>Q</sup> p180 data of serum samples were acquired in 18 batches. Every  
301 analytical batch, in a 96-well plate format, included up to 76 randomised cohort samples. Also in  
302 every analytical batch, three sets of quality control samples were included, the NIST SRM 1950

303 plasma reference material (in 4 replicates), a commercial available serum QC material (CQC in 2  
304 replicates, SeraLab, S-123-M-27485) and the QCs provided by the manufacturer in three  
305 concentration levels. The NIST SRM 1950 reference was used as the main quality control sample for  
306 the LC-MS/MS analysis. Coefficients of variation (CVs) for each metabolite were calculated based on  
307 the NIST SRM 1950 and also the limits of detection (LODs) were also used to assess the analytical  
308 performance of individual metabolites. Metabolite exclusion was based on a metabolite variable  
309 meeting two conditions: (1) CV of over 30% and (2) over 30% of the data are below LOD. Eleven out  
310 of the 188 serum metabolites detected were excluded as a result, leaving 177 serum metabolites to  
311 be used for further statistical analysis (Supplementary file 1). The mean coefficient of variation  
312 across the 177 LC-MS/MS detected serum metabolites was 16%. We also excluded one HELIX  
313 sample, which was hemolyzed.

314 Urinary metabolic profiles were acquired using <sup>1</sup>H NMR spectroscopy according to (Lau et al., 2018).  
315 In brief one-dimensional 600 MHz <sup>1</sup>H NMR spectra of urine samples from each cohort were acquired  
316 on the same Bruker Avance III spectrometer operating at 14.1 Tesla within a period of 1 month. The  
317 spectrometer was equipped with a Bruker SampleJet system, and a 5-mm broad-band inverse  
318 configuration probe maintained at 300K. Prior to analysis, cohort samples were randomised.  
319 Deuterated 3-(trimethylsilyl)-[2,2,3,3-d<sub>4</sub>]-propionic acid sodium salt (TSP) was used as internal  
320 reference. Aliquots of the study pooled quality control (QC) sample were used to monitor analytical  
321 performance throughout the run and were analysed at an interval of every 23 samples (i.e. 4 QC  
322 samples per well plate). The <sup>1</sup>H NMR spectra were acquired using a standard one-dimensional  
323 solvent suppression pulse sequence. 44 metabolites were identified and quantified as described (Lau  
324 et al., 2018). The urinary NMR showed excellent analytical performance, the mean coefficient of  
325 variation across the 44 NMR detected urinary metabolites was 11%. Data was normalized using the  
326 median fold change normalization method [51], which takes into account the distribution of relative  
327 levels of all 44 metabolites compared to the reference sample in determining the most probable  
328 dilution factor. An offset of ½ of the minimal value was applied and then concentration levels were

329 expressed as log<sub>2</sub>.

330

### 331 Building biological clocks

332 Child epigenetic age was calculated based on Horvath's Skin and Blood clock [52] using the  
333 methylclock R package [53].

334 New transcriptome and immunometabolic clocks were trained against chronological age on  
335 transcriptome data and concatenated proteomic and metabolomic data respectively, from the HELIX  
336 subcohort children through elastic net regression, using the *glmnet* R package [54]. All 'omic data  
337 was first mean centred and univariate scaled. To tune hyperparameters alpha and lambda, we  
338 performed a line search for alpha between 0 and 1, in 0.1 increments, and each time found the  
339 optimal value of lambda based on minimization of cross-validated mean squared error, using the  
340 *cvfits* function and 10-fold cross-validation. The best performing combination of alpha and lambda  
341 was reserved for fitting the final model.

342 Transcriptome data and concatenated proteomic and metabolomic data from the HELIX panel study  
343 children, was reserved for testing performance (Pearson's *r* and mean absolute error with  
344 chronological age) of the derived clocks. Paired, one-tailed t-tests were used to test if biological age  
345 measures increased between the HELIX subcohort and subsequent HELIX panel clinical examinations.

### 346 Developmental measurements

347 During the HELIX subcohort examination, height and weight were measured using regularly  
348 calibrated instruments and converted to BMI and height age-and-sex-standardized z-scores (zBMI  
349 and zHeight) using the international World Health Organization (WHO) reference curves [55].

350 Bioelectric impedance analyses were performed with the Bodystat 1500 (Bodystat Ltd.) equipment  
351 after 5 min of lying down. The proportion of fat mass was calculated using published age- and race-  
352 specific equations validated for use in children [56].

353 Trained fieldwork technicians measured three cognitive domains in children using a battery of

354 computer-based tests: fluid intelligence (Raven Coloured Progressive Matrices Test [CPM]), attention  
355 function (Attention Network Test [ANT]) and working memory (N-Back task). Complete outcome  
356 descriptions are provided in [57]. The CPM comprised a total of 36 items and we used the total  
357 number of correct responses as the outcome. A higher CPM scoring indicates better fluid  
358 intelligence. Fluid intelligence is the ability to solve novel reasoning problems and depends only  
359 minimally on prior learning. For ANT, we used the outcome of hit reaction time standard error (HRT-  
360 SE), a measure of response speed consistency throughout the test. A high HRT-SE indicates highly  
361 variable reaction time during the attention task and is considered a measure of inattentiveness [58].  
362 As the main parameter of N-Back, we used d prime ( $d'$ ) from the 3-back colours test, a measure  
363 derived from signal detection theory calculated by subtracting the z-score of the false alarm rate  
364 from the z-score of the hit rate. A higher  $d'$  indicates more accurate test performance, i.e. better  
365 working memory [58]. All examiners were previously trained following a standardized assessment  
366 protocol by the study expert psychologist. Furthermore, during the pilot phase, a coordinator visited  
367 each cohort site and checked for any potential error committed by the previously trained examiners.  
368 Parents completed questionnaires related to child's behavior, including the Conner rating scale's (N  
369 = 1287) and child behavior checklist (CBCL, N = 1298), within a week before the follow-up visit at 6–  
370 11 years of age. The 99-item CBCL/6–18 version for school children was used to obtain standardized  
371 parent reports of children's problem behaviours, translated and validated in each native language of  
372 the participating six cohort populations [59]. The parents responded along a 3-point scale with the  
373 code of 0 if the item is not true of the child, 1 for sometimes true, and 2 for often true. The  
374 internalizing score includes the subscales of emotionally reactive and anxious/depressed symptoms,  
375 as well as somatic complaints and symptoms of being withdrawn. The externalizing score includes  
376 attention problems and aggressive behaviors.

377 Lung function was measured by a spirometry test (EasyOne spirometer; NDD [New Diagnostic  
378 Design], Zurich, Switzerland), by trained research technicians using a standardised protocol. The  
379 child, sitting straight and equipped with a nose clip, was asked to perform at least six manoeuvres (if

380 possible). Details of exclusion of unacceptable maneuvers and validation of acceptable spirometer  
381 curves is fully described in [60]. FEV1 percent predicted values were computed using the reference  
382 equations estimated by the Global Lung Initiative [61], standardised by age, height, sex, and  
383 ethnicity.

384 Parents of children aged 8 years or older completed an additional questionnaire based on the  
385 pubertal development scale (PDS) [62]. Boys were asked whether growth has not begun, barely  
386 begun, is definitely underway, or has finished on five dimensions: body hair, facial hair, voice change,  
387 skin change, and growth spurt. Girls were asked the same questions about body hair, skin change,  
388 breast development, and growth spurt. Responses were coded on 4-point scales (1 = no  
389 development and 4 = completed development). For girls, a yes-no question about onset of menarche  
390 is weighted more heavily (1 = no and 4 = yes). For both genders, ratings are then averaged to create  
391 an overall score for physical maturation. Due to the young age of participants, we took the average  
392 scores and created a binary variable, to define whether puberty had started ( $PDS > 1$ ) or not ( $PDS = 1$ ).

393

#### 394 Covariates

395 During pregnancy and in the childhood HELIX subcohort examination information on the following  
396 key covariates was collected: cohort study centre (BiB, EDEN, INMA, MoBa, KANC and RHEA), self-  
397 reported maternal education (primary school, secondary school and university degree or higher),  
398 self-reported ancestry (White European, Asian and Pakistani, or other), birth weight (continuous,  
399 kg), gestational age at delivery (continuous in weeks).

400 Information about the children's habitual diet was collected via a semi quantitative food-frequency  
401 questionnaire (FFQ) covering the child's habitual diet, which was filled in by the parent attending the  
402 examination appointment. The FFQ, covering the past year, was developed by the HELIX research  
403 group, translated and applied to all cohorts. For the Mediterranean Diet Quality Index (KIDMED  
404 index) [63], items positively associated with the Mediterranean diet pattern (11 items) were



405 assigned a value of +1, while those negatively associated with the Mediterranean diet pattern (4  
406 items) were assigned a value of -1. The scores for all 15 items were summed, resulting in a total  
407 KIDMED score ranging from -4 to 11, with higher scores reflecting greater adherence to a  
408 Mediterranean diet.

409 The smoking status of the mother at any point during pregnancy was categorised into “non-active  
410 smoker, or “active smoker”. Global exposure of the child to environmental tobacco smoke was  
411 defined based on the questionnaires completed by the parents into: "no exposure", no exposure at  
412 home neither in other places; “exposure”: exposure in at least one place, at home or outside.

413 Moderate-to-vigorous physical activity variable was created based on physical activity questionnaire  
414 developed by the HELIX research group. It was defined as the amount of time children spent doing  
415 physical activities with intensity above 3 metabolic equivalent tasks (METs) and is expressed in units  
416 of min/day.

417 Family Affluence Score (FAS) [64] was included based on questions from the subcohort  
418 questionnaire. A composite FAS score was calculated based on the responses to the next four items:  
419 (1) Does your family own a car, van or truck? (2) Do you have your own bedroom for yourself? (3)  
420 During the past 12 months, how many times did you travel away on holiday with your family? (4)  
421 How many computers does your family own? A three-point ordinal scale was used, where FAS low  
422 (score 0,1,2) indicates low affluence, FAS medium (score 3,4,5) indicates middle affluence, and FAS  
423 high (score 6,7,8,9) indicates high affluence FAS.

424 Family social capital-related questions were included in the HELIX questionnaire to capture different  
425 aspects of social capital, relating both to the cognitive (feelings about relationships) and structural  
426 (number of friends, number of organizations) dimensions and to bonding capital (close friends and  
427 family), bridging capital (neighbourhood connections, looser ties) and linking capital (ties across  
428 power levels; for example, political membership). Family social capital was categorized into low,  
429 medium and high based on terciles.

## 430 Statistical analysis

431 All statistical analyses described here were performed among the HELIX subcohort children only.

432 Since there were few missing covariate data (Supplementary file 2), complete-case analysis was  
433 performed. Correlations between biological age measures and chronological age were calculated  
434 using Pearson's correlations. Partial correlations, adjusted for chronological age and cohort study  
435 centre, were applied to assess correlations between biological age measures.

436 In analysis with health risk factors and developmental outcomes, relative telomere length was  
437 multiplied by -1 to provide directions of effect consistent with the biological age clocks and  
438 univariate scaled to express effects in terms of SD change in telomere length. The markers derived  
439 from omic-based biological clocks were expressed as  $\Delta$  age (clock-predicted age – chronological age).  
440 Associations between the biological age markers and developmental measures were estimated using  
441 linear regression, or logistic regression for onset of puberty, with the developmental measure as the  
442 dependent variable. CBCL scores were log transformed to achieve an approximately normal  
443 distribution. Continuous outcomes, apart from the BMI and height z-scores, were mean centered  
444 and univariate scaled for the purposes of graphical representation. Associations between health risk  
445 factors and biological age markers were estimated using linear regression with the biological age  
446 marker as the dependent variable. All regression analyses were adjusted for chronological age, sex,  
447 ethnicity, and study centre.

448 We performed four sensitivity analyses: Firstly, we repeated analysis with health outcomes stratified  
449 by child sex, since the relationship between biological age and development may differ between  
450 boys and girls. Secondly, we further adjusted regression models for estimated cell counts (CD4T,  
451 CD8T, monocytes, B cells, NK cells, neutrophils and eosinophils), since it has been proposed for  
452 epigenetic clocks that cell proportion adjustments allow estimation of effects on the intrinsic cellular  
453 ageing rate, rather than the extrinsic rate outputted by blood based biological clocks, which may be  
454 partly determined by age-related changes in cell composition [65]. Blood cell type proportion was

455 estimated from DNA methylation data using the Reinius et al. [79] reference panel as implemented  
456 in meffil package [80]. Thirdly, we assessed the effects of further adjustment for health risk factors  
457 identified as associated with any of the biological age markers (family affluence and social capital,  
458 birthweight, maternal active smoking, and child passive smoking). In our main analysis, we have not  
459 adjusted for these factors as our assumption is that the effects of health risk factors on child  
460 development is mediated through biological age. However, an alternative assumption is that health  
461 risk factors exert independent effects on both biological age and developmental outcomes, which  
462 would require adjustment for these factors to estimate direct effects of biological age on  
463 developmental outcomes. Finally, we stratified by study centre to check consistency of effects  
464 among observed associations with developmental outcomes across study centres.

465 Due to the exploratory nature of the analysis, we report and discuss associations significant at the  
466 both the nominal significance threshold (unadjusted  $p < 0.05$ ) and after correction for 5% false  
467 discovery rate using the Benjamini and Hochberg [66] method, calculated across all computed  
468 associations (i.e. multiple testing corrected  $p$  value  $< 0.05$ ).

469 We performed overrepresentation analyses (ORA) among KEGG and REACTOME pathways and gene  
470 ontology (GO) sets of all transcripts contributing to the transcriptome clock using the  
471 ConsensuspathDB online tool (<http://consensuspathdb.org/>). A pathway or GO set was considered  
472 significantly enriched if FDR corrected  $p$ -values were smaller than 0.05 and included at least 3 genes.

473 Additionally, to assess concordance with gene expression changes with age in adults, we tested  
474 enrichment of all transcripts contributing to the transcriptome clock among age-associated  
475 transcripts reported by Peters et al. [8], using a hypergeometric test using the R “phyper” function.

476 All analyses were performed in R version 4.1.2.

## 477 Results

### 478 Sample characteristics

479 We used blood or urine derived measurements from the pan-European HELIX cohort. This included  
480 blood telomere length ( $N = 1,162$ ), blood DNA methylation ( $N = 1,173$ , 450K CpGs), blood gene  
481 expression ( $N = 1,007$ , 50K genes), and proteins and metabolites ( $N = 1,152$ , 36 plasma proteins, 177  
482 serum metabolites and 44 urinary metabolites), with 869 children overlapping across all  
483 measurements. Each subsample included around 55% boys, 89% children of white European  
484 ancestry, and a mean age of around 8 years (range 5-12 years). Around 51% of mothers of the HELIX  
485 children in each subsample had a high education level. The HELIX cohort included children from six  
486 study centres based in the UK, Spain, Greece, Lithuania, France, and Norway, with each centre  
487 contributing between 11 and 24% to each subsample (see Table 1 for sample characteristics).

### 488 Biological age marker performance

489 We included two established markers of biological age, telomere length, DNA methylation age, and  
490 developed two new candidate biological age markers, transcriptome age and 'immunometabolic'  
491 age (Figure 2). DNA methylation age was calculated using the published Skin and blood Horvath  
492 clock [52] to allow greater comparison to the wider literature, including in adults. We previously  
493 reported this epigenetic clock to show the best performance in chronological age prediction within  
494 the HELIX cohort [67]. Since no published applicable transcriptome, proteome or metabolome clocks  
495 were available for the age range of our sample, we trained two new biological clocks using these  
496 data in the HELIX cohort, through elastic net regression and cross-validation. We combined the  
497 proteome and metabolome data into a single immunometabolic age clock, since the available  
498 proteomic data included biomarkers targeting both metabolic and inflammatory functions, both  
499 omic types represent final products of gene regulation, and since the metabolic and immune  
500 systems are closely linked [68].

501 The correlation between telomere length and chronological age was weak but statistically significant  
502 ( $r = -0.07$ ,  $p = 0.02$ ). Correlations with chronological age were  $r = 0.85$  for DNA methylation age,  $r =$   
503  $0.94$  for transcriptome age, and  $r = 0.86$  for immunometabolic age (Figure 2).

504 We validated the transcriptome and immunometabolic clocks using cross-validation within the HELIX  
505 subcohort (cross-validated  $r$  of  $0.87$  and  $0.82$  respectively) and further tested in a subset of children  
506 who attended a second clinic visit approximately  $0.5$  years after the main follow-up visit (standard  
507 deviation (SD) =  $0.18$  years) as part of the HELIX panel study. Correlations in this test set were  $r = 0.93$   
508 for transcriptome age ( $N = 128$ ) and  $r = 0.84$  for immunometabolic age ( $N = 151$ ) (Figure 2). Predicted  
509 biological age increased by mean  $0.33$  years (SD =  $0.58$ ) for transcriptome age (t-test,  $p = 3 \times 10^{-5}$ ) and  
510 mean  $0.22$  years (SD:  $0.59$  years) for immunometabolic age (t-test,  $p = 2 \times 10^{-5}$ ) between the first and  
511 second visits (Figure 2-figure supplement 1). Correlations were significant ( $p < 0.05$ ) within each  
512 study centre for both clocks, except for immunometabolic age for children from the BiB (UK) cohort  
513 (Figure 2-figure supplement 2 and Figure 2-figure supplement 3).

514 The immunometabolic age clock was composed of  $135$  predictors including  $20$  proteins,  $79$  serum  
515 metabolites and  $36$  urinary metabolites (Supplementary file 3). The transcriptome clock was  
516 composed of  $1,445$  genes,  $652$  of which were annotated to Gene Symbols (Supplementary file 4).  
517 The transcriptome clock genes were enriched (false discovery rate (FDR)-corrected  $p < 0.05$ ) in  
518 'ribosome' and 'ribosome biogenesis' KEGG pathways (Supplementary file 5) and the following level  
519 2 Gene Ontology biological process terms: 'leukocyte activation', 'movement of cell or subcellular  
520 component', 'leukocyte migration', 'cell activation', and 'secretion by cell' (Supplementary file 6).

521 We also tested enrichment of transcriptome clock predictors among genes reported by a large meta-  
522 analysis of age in adults [8]: among the  $1,406$  reported age-associated genes that could be matched  
523 to our measured genes,  $43$  were included in our transcriptome clock (hypergeometric enrichment  
524 test,  $p = 0.052$ ). We note that since a common definition of markers of biological age is that they  
525 should be associated with age-related disease and mortality [69] these new clocks may only

526 currently be considered “candidate” biological age markers. However, we have referred to both the  
527 established and candidate markers as biological age markers throughout to simplify presentation.

528 Figure 2 shows partial correlations, adjusted for chronological age and study centre, between the  
529 biological age markers. Only null to weak correlations were observed, with significant correlations  
530 between telomere length and DNA methylation age ( $r = -0.06$ ,  $p = 0.04$ ) and between transcriptome  
531 age and immunometabolic age ( $r = 0.08$ ,  $p = 0.01$ ).

### 532 Biological clock associations with health risk factors

533 Table 2 shows associations, adjusted for chronological age, sex, study centre and ethnicity, between  
534 health risk factors and the biological age markers. The markers derived from omic-based biological  
535 clocks are expressed as  $\Delta$  age (clock-predicted age – chronological age) and since the adjustment set  
536 included chronological age, effects can be interpreted as years of age acceleration as often defined  
537 [18].

538 Nominally significant associations were observed for the following health risk factors: Telomere  
539 length was longer among girls compared to boys ( $p = 3e -06$ ) and among children of high affluence  
540 families ( $p = 0.008$ ). DNA methylation  $\Delta$  age was higher among children of mothers who actively  
541 smoked during pregnancy ( $p = 0.018$ ) and children exposed to passive smoke ( $p = 0.023$ ), while DNA  
542 methylation  $\Delta$  age was lower among children from families with high social capital ( $p = 0.048$ ).  
543 Conversely, transcriptome  $\Delta$  age was positively associated with medium and high ( $p = 0.011$ ) family  
544 social capital. Immunometabolic  $\Delta$  age was associated with higher birthweight ( $p = 0.0075$ ). Only the  
545 association between longer telomere length and female sex passed FDR correction.

### 546 Biological age associations with development

547 Figure 4 and Table 3 shows associations, adjusted for chronological age, sex, study centre and  
548 ethnicity, between the biological age markers and developmental outcomes related to growth and  
549 adiposity, cognition, behaviour, lung function and onset of puberty.

550 Several developmental outcomes were associated with biological age markers after FDR correction:  
551 DNA methylation and immunometabolic  $\Delta$  age were associated with greater height z-score ( $p= 6e^{-6}$   
552 and  $p= 4e^{-11}$  respectively) and greater fat mass % ( $p= 0.0004$  and  $p= 5e^{-6}$  respectively). All  
553 biological age markers were associated with greater BMI z-score (telomere length  $p =8e^{-4}$ , DNA  
554 methylation  $\Delta$  age  $p = 8e^{-5}$ , transcriptome  $\Delta$  age  $p = 0.005$ , immunometabolic  $\Delta$  age  $p =4e^{-19}$ ).  
555 Furthermore, immunometabolic  $\Delta$  age was associated after FDR correction with better working  
556 memory ( $p = 0.0036$ ) and reduced inattentiveness ( $p= 5e^{-4}$ ).

557 Associations at the nominal significance ( $p<0.05$ ) level were observed for increases in height z-score  
558 with transcriptome  $\Delta$  age ( $p= 0.014$ ), shorter telomere length with increased fat mass % ( $p= 0.009$ ),  
559 and DNA methylation  $\Delta$  age with greater inattentiveness ( $p=0.03$ ). Both shorter telomere length and  
560 DNA methylation  $\Delta$  age were associated with greater externalizing behaviours ( $p= 0.032$  and  $p= 0.01$   
561 respectively). Among a smaller subset of children (table 1) aged over 8 years, we observed a  
562 nominally significant association between immunometabolic  $\Delta$  age and odds of onset of puberty  
563 (Odds Ratio: 1.41, 95% CI: 1.01, 1.97,  $p=0.046$ ).

564 No significant associations with lung function were observed, but like the patterns of associations  
565 observed with cognitive and behavioural outcomes, there was a trend for a negative association  
566 with DNA methylation  $\Delta$  age ( $p = 0.085$ ) and a positive association with immunometabolic  $\Delta$  age  
567 ( $p=0.16$ ).

## 568 Sensitivity Analysis

569 In sensitivity analysis. We firstly stratified by sex and generally observed similar associations among  
570 boys and girls, apart from the following differences (Figure 4-figure supplement 1): Associations  
571 between shorter telomere length and BMI z-score and adiposity were stronger among boys. For DNA  
572 methylation  $\Delta$  age, associations with poorer externalizing and internalizing behaviours were only  
573 apparent among boys. For transcriptome  $\Delta$  age, stronger associations among boys were observed  
574 with BMI z-score, adiposity and poorer externalizing and internalizing behaviours. Conversely, we

575 observed an association between transcriptome  $\Delta$  age and reduced inattentiveness among girls only.  
576 Immunometabolic  $\Delta$  age was more strongly associated with reduced inattentiveness among girls and  
577 also associated with greater odds of puberty onset among girls only.

578 Secondly, we additionally adjusted our models by estimated cell counts to determine the influence  
579 of cell composition on associations with developmental outcomes (Figure 4-figure supplement 2B,  
580 Figure 4-figure supplement 36B, Figure 4-figure supplement 4B and Figure 4-figure supplement 5B,  
581 Supplementary file 7). Associations were generally little changed: For DNA methylation  $\Delta$  age,  
582 associations were attenuated with adiposity and growth outcomes although all remained FDR  
583 significant and the association with externalizing behavior was slightly attenuated. For transcriptome  
584  $\Delta$  age associations with adiposity and growth outcomes and lung function increased slightly and the  
585 association with greater lung function became nominally significant.

586 Thirdly, we assessed the effects of further adjustment for health risk factors (family affluence and  
587 social capital, birthweight, maternal active smoking, and child passive smoking) since health risk  
588 factors could be independently associated with both biological age and developmental outcomes  
589 (Figure 4-figure supplement 2C, Figure 4-figure supplement 3C, Figure 4-figure supplement 4C and  
590 Figure 4-figure supplement 5C, Supplementary file 7). Associations were generally little changed,  
591 expect for an attenuation of the association between telomere length and externalizing behavior,  
592 while conversely the association between DNA methylation  $\Delta$  age and externalizing behavior was  
593 slightly strengthened.

594 Finally, we stratified by cohort study centre, observing generally consistent directions of effect  
595 among developmental outcomes of at least at nominal significance ( $p < 0.05$ ) in the main pooled  
596 analysis (Figure 4-figure supplement 6).



## 597 Discussion

598 In a large sample of European children, we have analysed two established and two candidate  
599 measures of biological age, derived from molecular features at different levels of biological  
600 organization, in relation to developmental outcomes and health risk factors. We assessed two  
601 established biological age markers, telomere length and DNA methylation age, and derived two new  
602 measures, transcriptome age and immunometabolic age. Despite finding only null to weak  
603 correlations between the measures, we found all measures to be positively associated with greater  
604 BMI and adiposity, and both DNA methylation  $\Delta$  age and immunometabolic  $\Delta$  age were associated  
605 with taller height. While immunometabolic  $\Delta$  age was associated with greater cognitive maturity  
606 including greater working memory and attentiveness, conversely DNA methylation  $\Delta$  age was  
607 nominally associated with greater inattentiveness and both DNA methylation  $\Delta$  age and shorter  
608 telomere length were nominally associated with poorer externalizing behaviours.

609 BMI has consistently been associated with accelerated ageing in adults across a diverse range of  
610 biological age markers [8, 18, 70, 71] underlining the integral role of metabolism in ageing. Indeed, a  
611 recent large study of Dutch adults found BMI to be the only health risk factor tested associated with  
612 accelerated ageing across five biological age clocks, including telomere length, DNA methylation,  
613 transcriptome, proteome and metabolomic age markers [18]. Here we show that the link between  
614 BMI and multiple dimensions of accelerated ageing is also apparent in children. Energy and nutrient  
615 intake influence all Ageing Hallmarks and multiple lines of evidence link increased adiposity to  
616 shorter lifespan [72]. These effects appear to be partially mediated through evolutionarily conserved  
617 nutrient sensing systems such as the mTOR signaling pathway, which promote anti-ageing cellular  
618 repair mechanisms, at the expense of growth and metabolism, in response to lower nutrient  
619 availability [72]. Furthermore, excess adiposity increases generalized inflammation and oxidative  
620 stress [73, 74], which may have direct effects on age markers, particularly telomere length and DNA  
621 methylation age acceleration.

622 The observed associations between greater height with biological age may indicate developmental  
623 maturity. Height is generally considered reflective of a beneficial early-life environment [75],  
624 however, evidence for an association with lifespan is mixed [75, 76], with a recent meta-analysis  
625 suggesting a u-shaped relationship with all-cause mortality [77]. Greater comparative height at 10  
626 years was also inversely associated with longevity in a recent large-scale Medelian randomization  
627 study [78]. Furthermore, there is some evidence that the link between height and longevity may be  
628 mediated through the insulin-like growth factor-1 signaling pathway [76, 79]. The associations may  
629 also be interpreted as greater rates of growth and anabolism exerting greater “wear and tear” on  
630 cellular structures. Two other studies have also observed an association between height and DNA  
631 methylation age acceleration in children [25, 26].

632 Despite similarities in associations with growth and adiposity measures, patterns of association  
633 across cognitive and behavioral domains varied across biological age markers, underlying the view of  
634 biological ageing as a multi-faceted process. Immunometabolic  $\Delta$  age was associated with greater  
635 cognitive maturity, fitting the life-course model of greater accumulation of biological capital during  
636 the build-up phase of development. Immunometabolic  $\Delta$  age may be considered a phenotypic  
637 summary measure of metabolic and immune system maturity, and these cognitive developmental  
638 associations suggest that it may also be generalisable to overall developmental stage. On the other  
639 hand, DNA methylation  $\Delta$  age was related to relative immaturity in attentiveness and externalizing  
640 behaviour. A previous Finish study of children age between 11 and 13 years also reported  
641 associations between DNA methylation age acceleration and behavioural problems [26]. Similarly,  
642 shorter telomere length was associated here with greater externalizing behaviours, although not  
643 with any cognitive domains. Four other studies have examined the link between shorter telomere  
644 length and externalizing behaviours [80-83], with all, except one [83], also reporting an association.

645 Overall patterns of associations between risk factors and biological age measures also suggest the  
646 detrimental nature of accelerated ageing in children assessed through telomere length and DNA

647 methylation  $\Delta$  age, and potentially beneficial nature of advanced immunometabolic  $\Delta$  age. Both  
648 prenatal maternal active smoking and child passive smoking were associated with DNA methylation  
649  $\Delta$  age, while greater birthweight was associated with immunometabolic  $\Delta$  age. We examined  
650 maternal education level, family affluence, and social capital that broadly represent the three forms  
651 of interconvertible capital (cultural, economic, and social) proposed by Bourdieu [84]. It has been  
652 theorized that biological capital represents a fourth type of human capital, and that the conversion  
653 across these forms of capital underlies inequalities in ageing trajectories [85]. Nominally significant  
654 associations between higher family affluence with longer telomere length and high social capital  
655 with a younger DNA methylation age indicate that age acceleration assessed through these  
656 measures does not represent an accumulation of biological capital. Generally, directions of effect  
657 for immunometabolic  $\Delta$  age were in the opposite direction which may suggest it represents greater  
658 biological capital.

659 Girls were found to have longer telomere lengths than boys. Women have been consistently found  
660 to have longer telomere lengths [86] although the few generally smaller studies in children have  
661 been inconsistent [82, 87-89]. No other biological age markers were associated with sex, which  
662 contrasts with the study of Jansen et al [18] in adults which reported accelerated biological age in  
663 men across all measures tested except for proteomic age. Indeed, the phenomenon of accelerated  
664 DNA methylation age in men is well-established [21], consistent with lower life-expectancies for  
665 men. Although it is not known if these biological age differences are due to biological mechanisms or  
666 greater prevalence of disease risk factors among men, our data in children before divergence of risk  
667 factor prevalence could indicate a biological mechanism for telomere sex differences and a risk  
668 factor mediated mechanism for other biological age markers. Interestingly, we observed differences  
669 in associations between biological age and development between boys and girls, with some  
670 consistency across markers: Both shorter telomere length and transcriptome  $\Delta$  age and were more  
671 strongly associated with adiposity in boys, DNA methylation and transcriptome  $\Delta$  age showed  
672 stronger associations among boys with poorer behaviour, while in girls both transcriptome and

673 immunometabolic  $\Delta$  age showed stronger associations with improved attentiveness. Given observed  
674 sexual dimorphism in both developmental rates [90] and biological age measures through a variety  
675 of proposed mechanisms [91], it may be unsurprising that relationship between biological age and  
676 development also differs between the sexes.

677 Furthermore, we observed that immunometabolic  $\Delta$  age was associated with greater odds of  
678 puberty onset, driven by effects observed among girls only. We did not observe any further  
679 significant associations with onset of puberty, however the sample size in the subset of children was  
680 small compared to the other developmental measures. There was also suggestive evidence for  
681 associations between DNA methylation  $\Delta$  age with onset of puberty with associations close to the  
682 nominal significance threshold. Three previous studies have reported associations between DNA  
683 methylation age acceleration and puberty onset and stage [24-26], and one study has reported  
684 associations between shorter telomere length and puberty onset [92]. However, directions of effect  
685 for telomere length in our study were in the opposite direction. While earlier age at puberty is  
686 representative of more advanced physical maturation, it has been associated with metabolic  
687 diseases in later life, including cancers [93] and all-cause mortality [94].

688 We found transcriptome data to be highly accurate in predicting chronological age, including in a  
689 test set of children assessed six months later, demonstrating that gene expression tracks closely with  
690 age in children, even over this relatively short period. We analyzed biological pathways and  
691 processes enriched among transcript clusters contributing to the transcriptome clock, observing the  
692 integral role of ribosome and ribosome biogenesis pathways, central to protein synthesis, and  
693 biological processes including the immunity related processes leukocyte migration and activation,  
694 and cell movement, activation, and secretion. Strikingly, gene expression in adults is similarly  
695 characterized by downregulation of ribosomal genes and enrichment of expression in immune  
696 related genes [8]. This indicates that, similar to DNA methylation changes [21], there is some overlap  
697 in gene expression related to both development in children and ageing in adults. Although formal

698 testing of enrichment of genes contributing to transcriptome clock presented here among age-  
699 associated genes in adults showed enrichment at only borderline statistical significance, the  
700 transcriptome clock predictors are an underrepresentation of the full profile of gene expression  
701 associated with age in children, due to the sparsity enforced during the variable selection training  
702 process.

703 Despite associations with growth and adiposity measures, transcriptome age generally showed  
704 weaker associations with other developmental outcomes than for the other biological age markers.  
705 While this in part can be attributed to the slightly smaller sample size for children with  
706 transcriptome data, it is also likely due to the high accuracy in predicting chronological age of the  
707 transcriptome clock, resulting in lower variation in the portion of transcriptome age that is not  
708 explained by chronological age, further reducing statistical power. This makes it challenging to judge  
709 the relevance of transcriptome age, if any, to developmental endpoints, which may be mixed since,  
710 non-significant direction of effects were observed with both maturity in attention and lung function,  
711 yet relative immaturity in behaviour. In fact, training clocks using chronological age, which while  
712 providing an accessible route to understanding molecular changes associated with age, does pose  
713 limitations generally for inference regarding biological ageing. Particularly for high-dimensional data  
714 such as DNA methylation, it has been shown that it is possible to predict chronological age near-  
715 perfectly [95], thereby limiting information on biological age and its variation. For this reason, newer  
716 epigenetic clocks have included clinical and mortality data, to improve clinical relevance and  
717 sensitivity to risk factors [13, 14], which should be considered in future studies developing clocks in  
718 children.

719 Other limitations include the cross-sectional design of the main analysis, which limits inference  
720 regarding the directionality of associations and allows the possibility of age-associated  
721 environmental factors to influence the clock development. Furthermore, there were differences in  
722 age by study centre. Children from the EDEN cohort study centre were generally older, which likely

723 introduced a degree of cohort bias into the age modelling. For this reason, we adjusted all  
724 associations by study centre and additionally assessed age correlation within each study centre.  
725 Although cohorts were recruited from the general population, certain ethnicities or socio-  
726 economically disadvantaged groups may have been under-represented, limiting generalizability  
727 somewhat. A bias towards over-representation of White ethnic groups is an issue generally with the  
728 development of biological clocks, which means associations observed with ethnicity should be  
729 interpreted cautiously. While the DNA methylation and transcriptome data was representative of  
730 the full genome, our coverage of the metabolome and proteome was limited to targeted assays. For  
731 analysis of age at the gene expression and protein/metabolite levels we developed new clocks, and  
732 these remain to be validated as true biological age indicators, through testing association with age-  
733 related disease and mortality in adults. While biological age markers exist for these data types, they  
734 are not appropriate for applying in our dataset, since the same model predictors (i.e., metabolites  
735 and proteins) were not included in the assays used here and/or the markers were not trained in  
736 pediatric populations, which will drastically reduce their accuracy as molecular changes within in  
737 childhood cannot be assumed to follow the same relation with age as in adulthood: For instance,  
738 DNA methylation shows a logarithmic dependence with age during childhood and a linear  
739 dependence in adulthood [12].

740 However, the strengths of this study include the large population sample, drawing from six countries  
741 from around Europe, increasing generalizability, and the integration of rich molecular data and a  
742 broad range of developmental outcomes into a single systematic analysis. Although our age range  
743 was somewhat limited, missing the infancy and adolescent periods, the age range covered a key  
744 childhood period, where energy expenditure (an indicator of level of overall physiology) has entered  
745 a period of steady increase following more rapid increases during infancy and before stabilization  
746 during adolescence [96].

747 In conclusion, in this large Pan-European study we have found that four indicators of biological age,  
748 representing complimentary molecular processes, were all associated with BMI after controlling for  
749 chronological age, indicating that adiposity is an important correlate of accelerated biological ageing  
750 in children. We developed a highly accurate “transcriptome age” clock although it was found to be  
751 relatively insensitive to other development phenotypes. We found that immunometabolic  $\Delta$  age was  
752 associated with cognitive maturity fitting a buildup of biological capital model of ageing in children,  
753 while shorter telomere length and DNA methylation  $\Delta$  age was associated with greater behavioral  
754 problems suggesting a “wear and tear” model of ageing in children. Our findings contribute to the  
755 interpretation and understanding of biological age measures in children, crucial for clinical and  
756 epidemiological research into early life risk factors for adverse ageing trajectories. Future long-term  
757 studies should investigate associations between age acceleration in children and adults to further  
758 test the antagonistic pleiotropy hypothesis.

### 759 Data availability

760 Due to data protection regulations in each participating country and participant data use  
761 agreements, human subject data used in this project cannot be freely shared. The raw data  
762 supporting the current study are available on request subject to ethical and legislative review. The  
763 “HELIX Data External Data Request Procedures” are available with the data inventory in this website:  
764 <http://www.projecthelix.eu/data-inventory>. The document describes who can apply to the data and  
765 how, the timings for approval and the conditions to data access and publication. Researchers who  
766 have an interest in using data from this project for reproducibility or in using data held in general in  
767 the HELIX data warehouse for research purposes can apply for access to data. Interested researchers  
768 should fill in the application protocol found in ANNEX I at  
769 [https://www.projecthelix.eu/files/helix\\_external\\_data\\_request\\_procedures\\_final.pdf](https://www.projecthelix.eu/files/helix_external_data_request_procedures_final.pdf) and send this  
770 protocol to [helixdata@isglobal.org](mailto:helixdata@isglobal.org). The applications are received by the HELIX Coordinator, and are  
771 processed and approved by the HELIX Project Executive Committee. All code used for data analysis

772 has been provided as supplementary material. Deidentified dataset for generation of figures 1 and 2  
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## 1008 Tables and Figures

1009

1010 Table 1. Summary Statistics for study population.

	TELOMERE LENGTH	DNA METHYLATION AGE	TRANSCRIPT- OME AGE	IMMUNO- METABOLIC AGE
N	N (%) or Mean (SD)	N (%) or Mean (SD)	N (%) or Mean (SD)	N (%) or Mean (SD)
	1,162	1,173	1,007	1,152
<b>DEMOGRAPHIC FACTORS</b>				
<b>AGE (YEARS)</b>	7.84 (1.54)	7.84 (1.54)	7.90(1.50)	7.86 (1.55)
<b>SEX-MALE</b>	639 (55)	644 (54.9)	547 (54.3)	628 (54.5)
<b>SEX-FEMALE</b>	523 (45)	529 (45.1)	460 (45.7)	524 (45.5)
<b>ETHNICITY-WHITE</b>	1039 (89.4)	1048 (89.3)	905 (89.9)	1032 (89.6)
<b>ETHNICITY-PAKISTANI/ASIAN</b>	96 (8.3)	98 (8.4)	76 (7.5)	93 (8.1)
<b>ETHNICITY -OTHER</b>	27 (2.3)	27 (2.3)	26 (2.6)	27 (2.3)
<b>COHORT-BIB</b>	200 (17.2)	203 (17.3)	162 (16.1)	191 (16.6)
<b>COHORT-EDEN</b>	145 (12.5)	146 (12.4)	109 (10.8)	149 (12.9)
<b>COHORT-INMA</b>	212 (18.2)	215 (18.3)	184 (18.3)	201 (17.4)
<b>COHORT-KANC</b>	196 (16.9)	198 (16.9)	151 (15)	197 (17.1)
<b>COHORT-MOBA</b>	211 (18.2)	212 (18.1)	245 (24.3)	222 (19.3)
<b>COHORT-RHEA</b>	198 (17)	199 (17)	156 (15.5)	192 (16.7)
<b>PRENATAL FACTORS</b>				
<b>MATERNAL NON-ACTIVE SMOKER DURING PREGNANCY</b>	988 (85)	998 (85.1)	859 (85.3)	981 (85.2)
<b>MATERNAL ACTIVE SMOKER DURING PREGNANCY</b>	174 (15)	175 (14.9)	148 (14.7)	171 (14.8)
<b>BIRTHWEIGHT (KG)</b>	3.37 (0.5)	3.37 (0.5)	3.38 (0.52)	3.38 (0.5)
<b>GESTATIONAL AGE (WEEKS)</b>	39.57 (1.67)	39.58 (1.67)	39.59 (1.75)	39.59 (1.66)
<b>FAMILY CAPITAL</b>				
<b>MATERNAL EDUCATION (LOW)</b>	165 (14.7)	166 (14.7)	140 (14.4)	157 (14.1)
<b>MATERNAL EDUCATION (MEDIUM)</b>	391 (34.8)	394 (34.8)	328 (33.8)	391 (35.1)
<b>MATERNAL EDUCATION (HIGH)</b>	568 (50.5)	573 (50.6)	503 (51.8)	565 (50.8)
<b>FAMILY AFFLUENCE (LOW)</b>	133 (11.5)	135 (11.5)	112 (11.1)	128 (11.1)
<b>FAMILY AFFLUENCE (MEDIUM)</b>	462 (39.8)	466 (39.8)	394 (39.2)	450 (39.1)
<b>FAMILY AFFLUENCE (HIGH)</b>	565 (48.7)	570 (48.7)	499 (49.7)	572 (49.7)
<b>FAMILY SOCIAL CAPITAL (LOW)</b>	513 (47.7)	516 (47.5)	422 (45.8)	496 (46.7)
<b>FAMILY SOCIAL CAPITAL (MEDIUM)</b>	264 (24.6)	269 (24.8)	228 (24.7)	259 (24.4)
<b>FAMILY SOCIAL CAPITAL (HIGH)</b>	298 (27.7)	301 (27.7)	272 (29.5)	307 (28.9)

<b>CHILD FACTORS</b>				
<b>NO PASSIVE SMOKE EXPOSURE</b>	723 (63.8)	732 (63.9)	639 (64.5)	718 (63.8)
<b>PASSIVE SMOKE EXPOSURE</b>	411 (36.2)	413 (36.1)	351 (35.5)	407 (36.2)
<b>PHYSICAL ACTIVITY-LOW</b>	418 (36.9)	420 (36.8)	349 (35.3)	416 (37.1)
<b>PHYSICAL ACTIVITY-MEDIUM</b>	336 (29.7)	341 (29.9)	295 (29.9)	330 (29.4)
<b>PHYSICAL ACTIVITY-HIGH</b>	378 (33.4)	381 (33.4)	344 (34.8)	375 (33.5)
<b>KIDMED DIET SCORE</b>	2.81 (1.77)	2.82 (1.78)	2.88 (1.77)	2.84 (1.76)
<b>DEVELOPMENTAL MEASURES</b>				
<b>HEIGHT Z-SCORE</b>	0.4 (0.97)	0.39 (0.98)	0.39 (0.96)	0.4 (0.98)
<b>BMI Z-SCORE</b>	0.43 (1.2)	0.43 (1.2)	0.4 (1.15)	0.42 (1.18)
<b>ADIPOSITY (BIA FAT-MASS %)</b>	6.76 (4.01)	6.77 (4.01)	6.52 (3.9)	6.72 (3.95)
<b>WORKING MEMORY (3-BACK D')</b>	1.1 (1.01)	1.1 (1.01)	1.13 (1)	1.1 (1.01)
<b>INATTENTIVENESS (ANT-HRT)</b>	301.97 (90.38)	301.93 (90.46)	297.69 (89.36)	301.35 (89.84)
<b>FLUID INTELLIGENCE (CPM)</b>	25.87 (6.33)	25.86 (6.32)	26.12 (6.26)	25.95 (6.3)
<b>INTERNALIZING BEHAVIOURS (CBCL)</b>	6.49 (5.9)	6.48 (5.9)	6.36 (5.89)	6.52 (5.87)
<b>EXTERNALIZING BEHAVIOURS (CBCL)</b>	6.81 (6.5)	6.82 (6.51)	6.67 (6.49)	6.74 (6.42)
<b>LUNG FUNCTION (FEV1)</b>	99.26 (13.46)	99.25 (13.47)	99.16 (13.02)	99.17 (13.47)
<b>PUBERTY NOT STARTED</b>	250 (46.6)	252 (46.5)	254 (49.7)	260 (48)
<b>PUBERTY STARTED (PDS &gt;1)</b>	287 (53.4)	290 (53.5)	257 (50.3)	282 (52)

1011

1012 **Table2: Associations between health risk factors and biological age measures.** Estimates calculated using linear regression, adjusted for  
 1013 chronological age, sex, ethnicity, and study centre. Bold indicates p<0.05 and \*indicates FDR <5%. Telomere length is expressed as standard  
 1014 deviation (SD) decrease in length (multiplied by -1) to provide estimates indicative of accelerated biological age, as the other biological age  
 1015 indicators. Telomere Length N= 1,162, DNA methylation age N = 1173, Transcriptome age N= 1,007, Immunometabolic age N = 1152  
 1016

	TELOMERE LENGTH		DNA METHYLATION AGE		TRANSCRIPTOME AGE		IMMUNOMETABOLIC AGE	
	SD Decrease (95%CI)	P value	Increase in years ΔAge (95%CI)	P value	Increase in years ΔAge (95%CI)	P value	Increase in years ΔAge (95%CI)	P value
<b>SEX-MALE</b>	-	-	-	-	-	-	-	-
<b>SEX-FEMALE</b>	-0.27 (-0.39, -0.16)	<b>3.30E-06*</b>	0.07 (-0.01, 0.16)	0.1	0 (-0.01, 0.02)	0.73	0.06 (-0.01, 0.13)	0.086
<b>PRENATAL FACTORS</b>								
<b>MATERNAL NON-ACTIVE SMOKER DURING PREGNANCY</b>	-	-	-	-	-	-	-	-
<b>MATERNAL ACTIVE SMOKER DURING PREGNANCY</b>	0.07 (-0.1, 0.23)	0.41	0.15 (0.03, 0.28)	<b>0.018</b>	0 (-0.02, 0.02)	0.88	-0.04 (-0.14, 0.06)	0.43
<b>BIRTHWEIGHT (KG)</b>	-0.098 (-0.218, 0.023)	0.11	-0.021 (-0.114, 0.072)	0.66	0.005 (-0.01, 0.02)	0.51	0.102 (0.027, 0.177)	<b>0.0075</b>
<b>GESTATIONAL AGE (WEEKS)</b>	-0.012 (-0.048, 0.024)	0.52	0.013 (-0.015, 0.041)	0.35	0 (-0.005, 0.004)	0.89	0.018 (-0.005, 0.04)	0.12
<b>FAMILY CAPITAL</b>								
<b>MATERNAL EDUCATION (LOW)</b>	-	-	-	-	-	-	-	-
<b>MATERNAL EDUCATION (MEDIUM)</b>	-0.06 (-0.26, 0.13)	0.53	0.02 (-0.14, 0.17)	0.84	0.01 (-0.02, 0.03)	0.61	0.08 (-0.04, 0.2)	0.21
<b>MATERNAL EDUCATION (HIGH)</b>	-0.1 (-0.29, 0.1)	0.32	-0.07 (-0.22, 0.08)	0.37	0 (-0.02, 0.03)	0.85	0.12 (0, 0.24)	0.051
<b>FAMILY AFFLUENCE (LOW)</b>	-	-	-	-	-	-	-	-
<b>FAMILY AFFLUENCE (MEDIUM)</b>	-0.15 (-0.34, 0.05)	0.13	-0.11 (-0.26, 0.03)	0.13	0 (-0.03, 0.02)	0.85	0.02 (-0.1, 0.14)	0.8
<b>FAMILY AFFLUENCE (HIGH)</b>	-0.27 (-0.47, -0.07)	<b>0.0081</b>	-0.14 (-0.29, 0.02)	0.083	0.01 (-0.01, 0.04)	0.35	0.09 (-0.04, 0.21)	0.17
<b>FAMILY SOCIAL CAPITAL (LOW)</b>	-	-	-	-	-	-	-	-
<b>FAMILY SOCIAL CAPITAL (MEDIUM)</b>	-0.06 (-0.21, 0.09)	0.45	-0.03 (-0.14, 0.08)	0.62	0.02 (0.01, 0.04)	<b>0.012</b>	-0.04 (-0.14, 0.05)	0.36

			0.09)					
<b>FAMILY SOCIAL CAPITAL (HIGH)</b>	-0.15 (-0.3, 0)	0.054	-0.12 (-0.23, 0)	<b>0.048</b>	0.02 (0.01, 0.04)	<b>0.011</b>	-0.06 (-0.15, 0.04)	0.25
<b>CHILD FACTORS</b>								
<b>NO PASSIVE SMOKE EXPOSURE</b>	-	-	-	-	-	-	-	-
<b>PASSIVE SMOKE EXPOSURE</b>	0.05 (-0.08, 0.18)	0.42	0.11 (0.02, 0.21)	<b>0.023</b>	0.01 (0, 0.03)	0.16	-0.01 (-0.09, 0.07)	0.76
<b>PHYSICAL ACTIVITY-LOW</b>	-	-	-	-	-	-	-	-
<b>PHYSICAL ACTIVITY-MEDIUM</b>	0.09 (-0.06, 0.23)	0.25	-0.08 (-0.2, 0.03)	0.15	-0.01 (-0.03, 0.01)	0.17	0.03 (-0.06, 0.12)	0.56
<b>PHYSICAL ACTIVITY-HIGH</b>	0.14 (-0.01, 0.29)	0.067	-0.1 (-0.22, 0.01)	0.08	0 (-0.02, 0.01)	0.69	-0.06 (-0.15, 0.04)	0.24
<b>KIDMED DIET SCORE</b>	-0.03 (-0.064, 0.005)	0.092	0.005 (-0.022, 0.031)	0.74	0.004 (-0.001, 0.008)	0.10	-0.005 (-0.027, 0.016)	0.64

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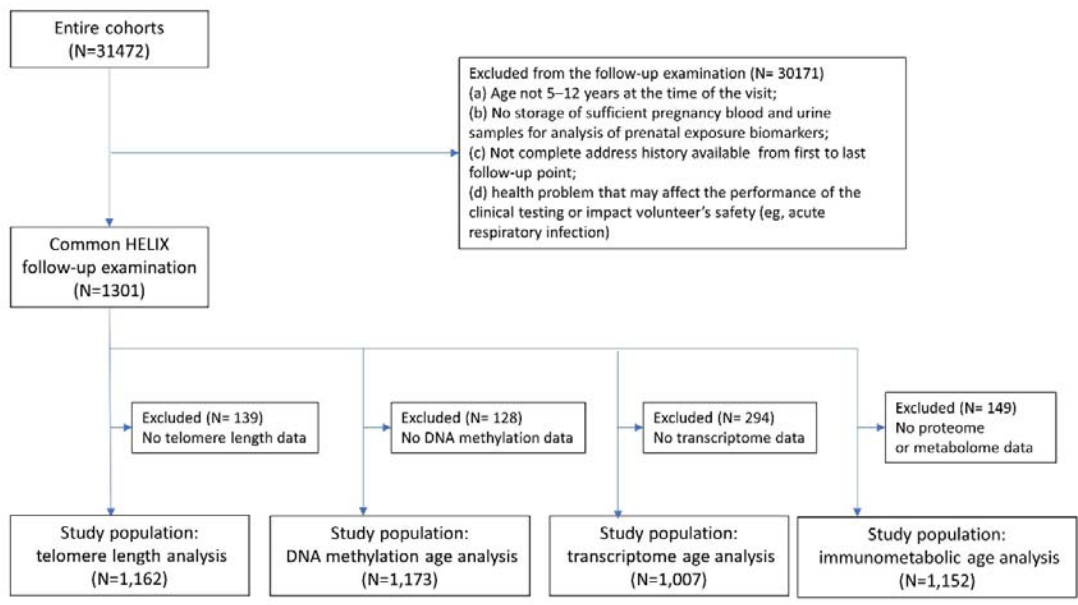


1019 **Table 3: Associations between biological age measures and developmental measures.** Estimates calculated using linear regression, adjusted for  
 1020 chronological age, sex, ethnicity, and study centre.

	Telomere Length			DNA methylation age			Transcriptome age			Immunometabolic age		
	N	SD increase / odds ratio <sup>a</sup> per SD shortening (95%CI)	P value	N	SD increase / odds ratio per year increase in Δ age (95%CI)	P value	N	SD increase / odds ratio per year increase in Δ age (95%CI)	P value	N	SD increase/ odds ratio per year increase in Δ age (95%CI)	P value
Height z-score	1162	0 (-0.05, 0.06)	0.89	1173	0.17 (0.09, 0.24)	<b>6.20E-06*</b>	1007	0.66 (0.13, 1.18)	<b>0.014</b>	1152	0.31 (0.22, 0.4)	<b>4.30E-11*</b>
BMI z-score	1162	0.12 (0.05, 0.19)	<b>0.00082*</b>	1173	0.18 (0.09, 0.27)	<b>7.70E-05*</b>	1007	0.9 (0.27, 1.53)	<b>0.005*</b>	1152	0.5 (0.4, 0.61)	<b>3.80E-19*</b>
Adiposity (BIA fat-mass %)	1153	0.07 (0.02, 0.12)	<b>0.0093*</b>	1164	0.12 (0.05, 0.19)	<b>0.0004*</b>	999	0.45 (-0.05, 0.94)	0.079	1144	0.2 (0.11, 0.28)	<b>5.00E-06*</b>
Working memory (3-back d') <sup>b</sup>	882	-0.03 (-0.09, 0.03)	0.37	890	0.05 (-0.03, 0.13)	0.19	784	0.06 (-0.51, 0.63)	0.84	876	0.15 (0.05, 0.26)	<b>0.0036*</b>
Inattentiveness (ANT-HRT)	1142	-0.01 (-0.05, 0.04)	0.78	1153	0.07 (0.01, 0.13)	<b>0.03</b>	997	-0.39 (-0.85, 0.08)	0.11	1135	-0.14 (-0.22, -0.06)	<b>5.00E-04*</b>
Fluid Intelligence (CPM)	1156	-0.03 (-0.07, 0.01)	0.21	1167	-0.03 (-0.08, 0.02)	0.21	1001	0.08 (-0.3, 0.47)	0.67	1147	0.06 (-0.01, 0.12)	0.08
Internalizing Behaviours (CBCL)	1156	0.03 (-0.03, 0.08)	0.33	1166	0.06 (-0.01, 0.13)	0.094	1002	0.4 (-0.12, 0.93)	0.13	1146	-0.09 (-0.18, 0)	0.053
Externalizing behaviours (CBCL)	1156	0.06 (0, 0.11)	<b>0.032</b>	1166	0.09 (0.02, 0.16)	<b>0.01</b>	1002	0.45 (-0.06, 0.97)	0.083	1146	-0.01 (-0.1, 0.08)	0.82
Lung Function (FEV1)	911	-0.01 (-0.07, 0.05)	0.75	921	-0.07 (-0.15, 0.01)	0.085	795	0.47 (-0.06, 1.01)	0.08	907	0.07 (-0.03, 0.17)	0.16
Puberty onset <sup>c</sup>	537	0.92 (0.76, 1.11)	0.36	542	1.25 (0.99, 1.57)	0.058	511	1.84 (0.41, 8.44)	0.42	542	1.41 (1.01, 1.97)	<b>0.046</b>

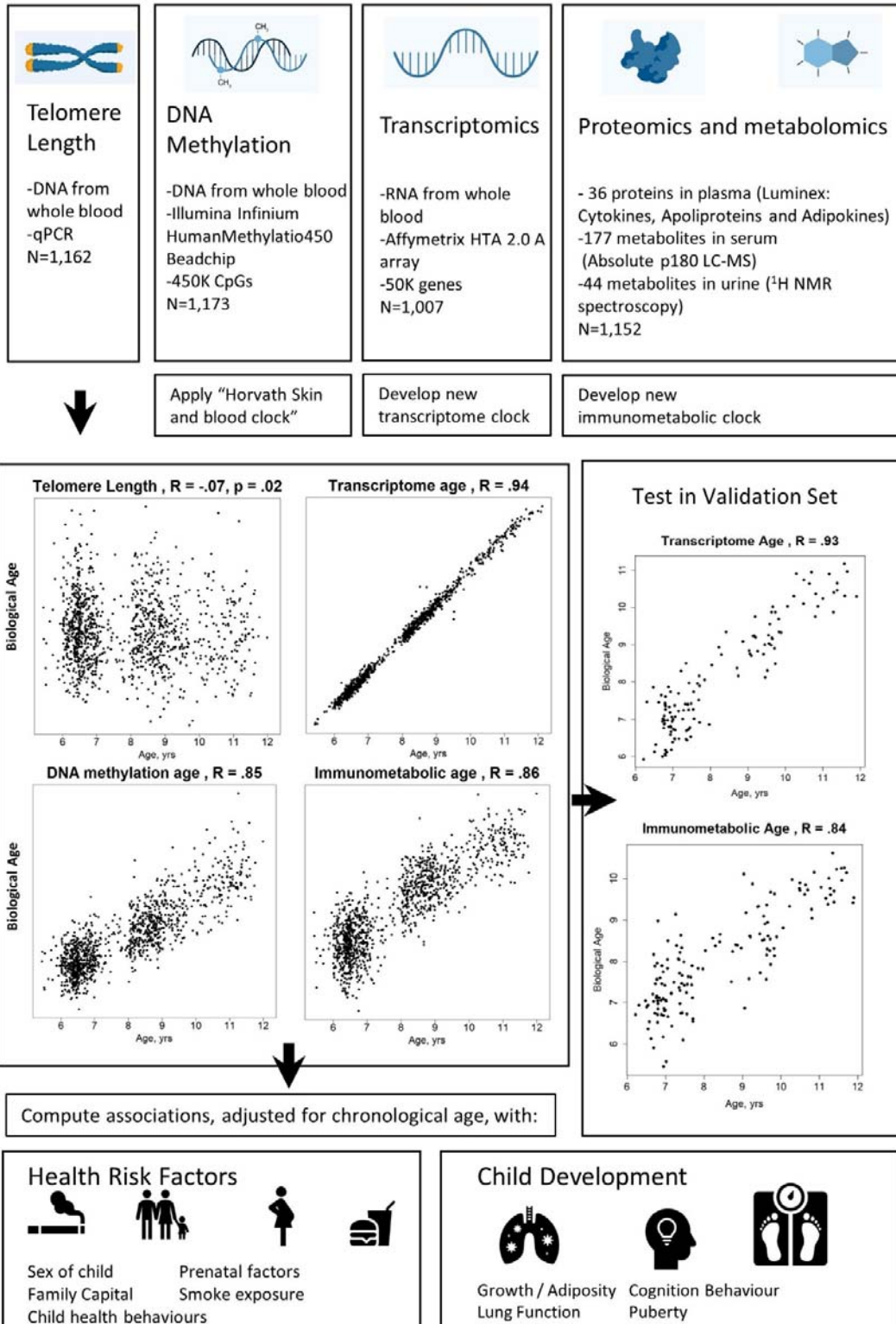
1021 Bold indicates p<0.05 and \*indicates FDR <5%. <sup>a</sup>Odds ratio provided for puberty onset only. <sup>b</sup>Not available in the Lithuanian KANC cohort. <sup>c</sup>Only assessed in  
 1022 children over 8 years old.

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1024

1025 Figure 1: Participant flowchart. See Supplementary file 1 for details of quality control of molecular data at sample and feature level.



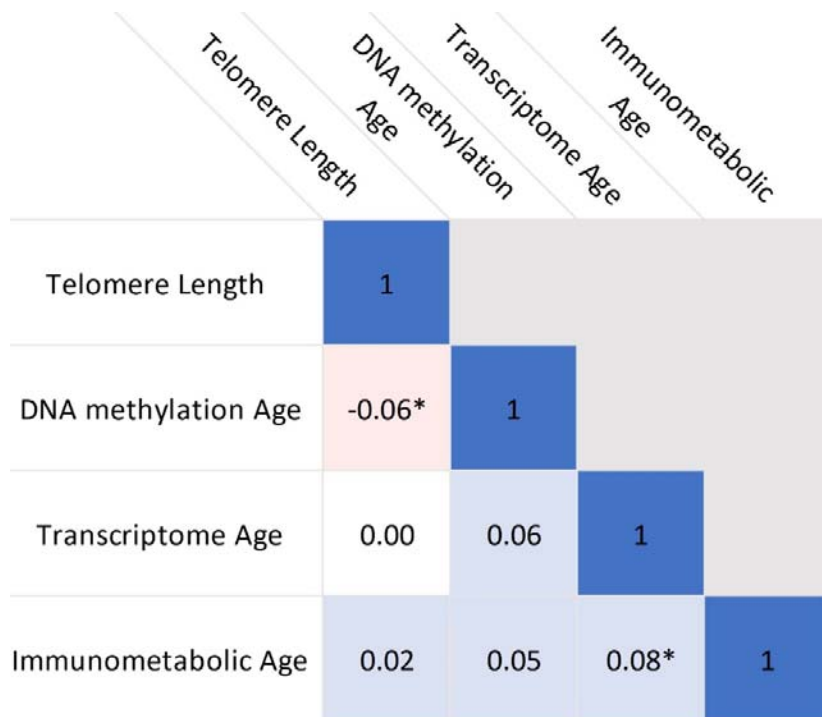
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1027 Figure 2: Study design schematic. Source data for reproducing correlation plots is provided in

1028 Figure 2-source data 1.

1029

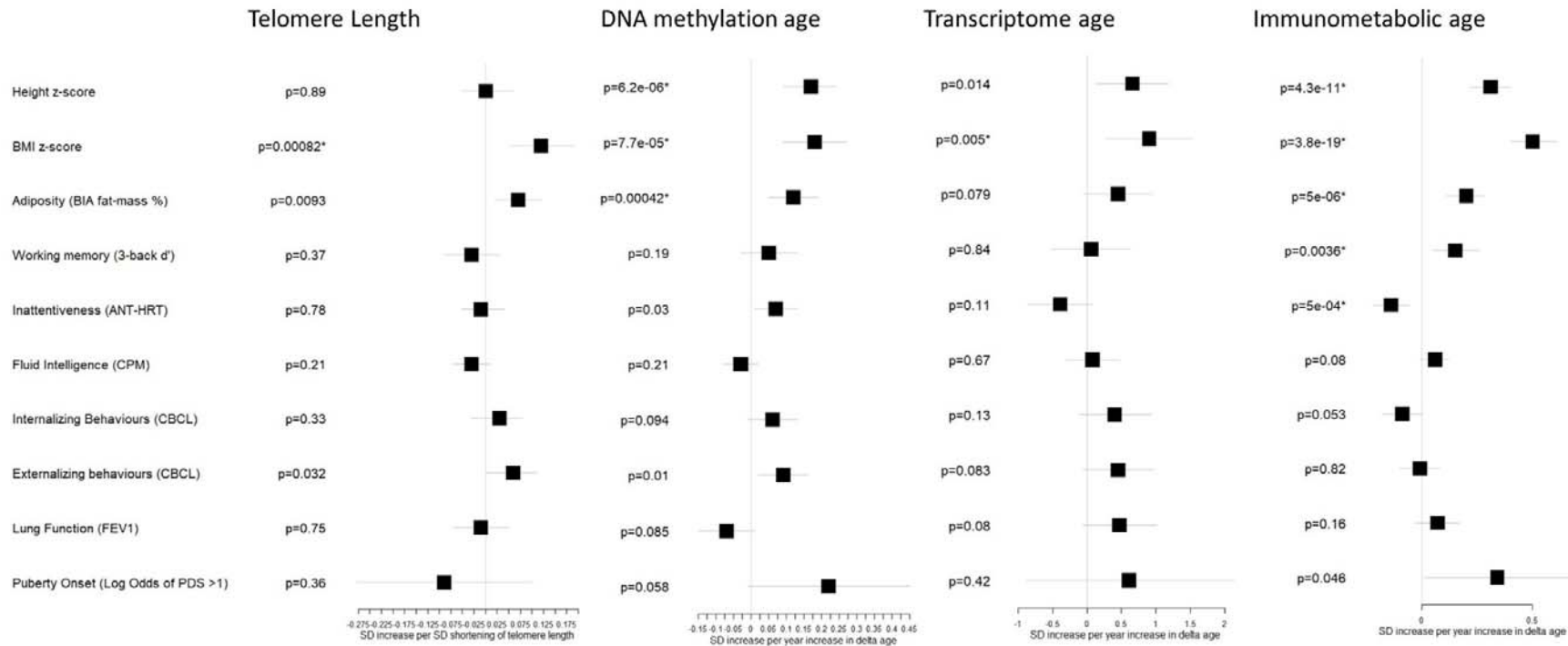
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1032

1033 **Figure 3: Correlations between biological age indicators.** Heatmap shows partial Pearson's  
1034 correlations, adjusted for chronological age and study centre. \* indicates  $p < 0.05$ . Source  
1035 data for reproducing plots is provided in Figure 3-source data 1.



1036

1037 Figure 4: Associations between biological age measures and developmental measures. Estimates calculated using linear regression, adjusted for  
 1038 chronological age, sex, ethnicity, and study centre. \*indicates FDR <5%. Telomere length is expressed as standard deviation (SD) decrease in  
 1039 length (multiplied by -1) to provide estimates indicative of accelerated biological age, as the other biological age indicators. . See Table 3 for  
 1040 numbers included in each analysis and exact point estimates and confidence intervals.

1041 Legend for Source Code File 1:

1042 R software source code for all data analyses. Datasets and variables are named within the code as  
1043 provided at <http://www.projecthelix.eu/data-inventory>.

1044

1045 Legends for Supplementary Figures:

1046 Figure 2-figure supplement 1: Comparison between immunometabolic and transcriptome age  
1047 between first and second study visits. Box plots (showing minimum, maximum, median, first quartile  
1048 and third quartile) of biological age measures at each panel study visit (approximately 6 months  
1049 apart). Panel clinic 1 was part of the main Helix subcohort examination. P values calculated from  
1050 paired t-tests.

1051 Figure 2-figure supplement 2: Age Prediction by study centre of transcriptome age. MAE = mean  
1052 absolute error. R and p values from Pearson's correlation.

1053 Figure 2-figure supplement 3: Age Prediction by study centre of immunometabolic age. MAE = mean  
1054 absolute error. R and p values from Pearson's correlation.

1055 Figure 4-figure supplement 1: Associations between biological age measures and developmental  
1056 measures, stratified by sex. Estimates calculated using linear regression, adjusted for chronological  
1057 age, sex, ethnicity, and study centre. Telomere length is expressed as % decrease in length  
1058 (multiplied by -1) to provide estimates indicative of accelerated biological age, as for the other  
1059 biological age indicators.

1060 Figure 4-figure supplement 2: Associations between telomere length and developmental measures  
1061 adjusted for A: chronological age, sex, ethnicity, and study centre; B: as for A plus estimated cell  
1062 counts; C: as for A plus family affluence and social capital, birthweight, maternal active smoking, and  
1063 child passive smoking; D as for C plus estimated cell counts. Telomere length is expressed as  
1064 standard deviation decrease in length (multiplied by -1) to provide estimates indicative of  
1065 accelerated biological age, as for the other biological age indicators.

1066 Figure 4-figure supplement 3: Associations between DNA methylation  $\Delta$  age and developmental  
1067 measures adjusted for A: chronological age, sex, ethnicity, and study centre; B: as for A plus  
1068 estimated cell counts; C: as for A plus family affluence and social capital, birthweight, maternal  
1069 active smoking, and child passive smoking; D as for C plus estimated cell counts.

1070 Figure 4-figure supplement 4: Associations between transcriptome  $\Delta$  age and developmental  
1071 measures adjusted for A: chronological age, sex, ethnicity, and study centre; B: as for A plus  
1072 estimated cell counts; C: as for A plus family affluence and social capital, birthweight, maternal  
1073 active smoking, and child passive smoking; D as for C plus estimated cell counts.

1074 Figure 4-figure supplement 5: Associations between immunometabolic  $\Delta$  age and developmental  
1075 measures adjusted for A: chronological age, sex, ethnicity, and study centre; B: as for A plus  
1076 estimated cell counts; C: as for A plus family affluence and social capital, birthweight, maternal  
1077 active smoking, and child passive smoking; D as for C plus estimated cell counts.

1078 Figure 4-figure supplement 6: Associations between biological age measures and developmental  
1079 measures, stratified by study centre (adjusted for chronological age, sex, and ethnicity). Associations  
1080 at least at  $p < 0.05$  in the pooled analysis are shown for A) telomere length (TL), B) DNA methylation  
1081 (DNAm) age, and C) Immunometabolic (IM) age.

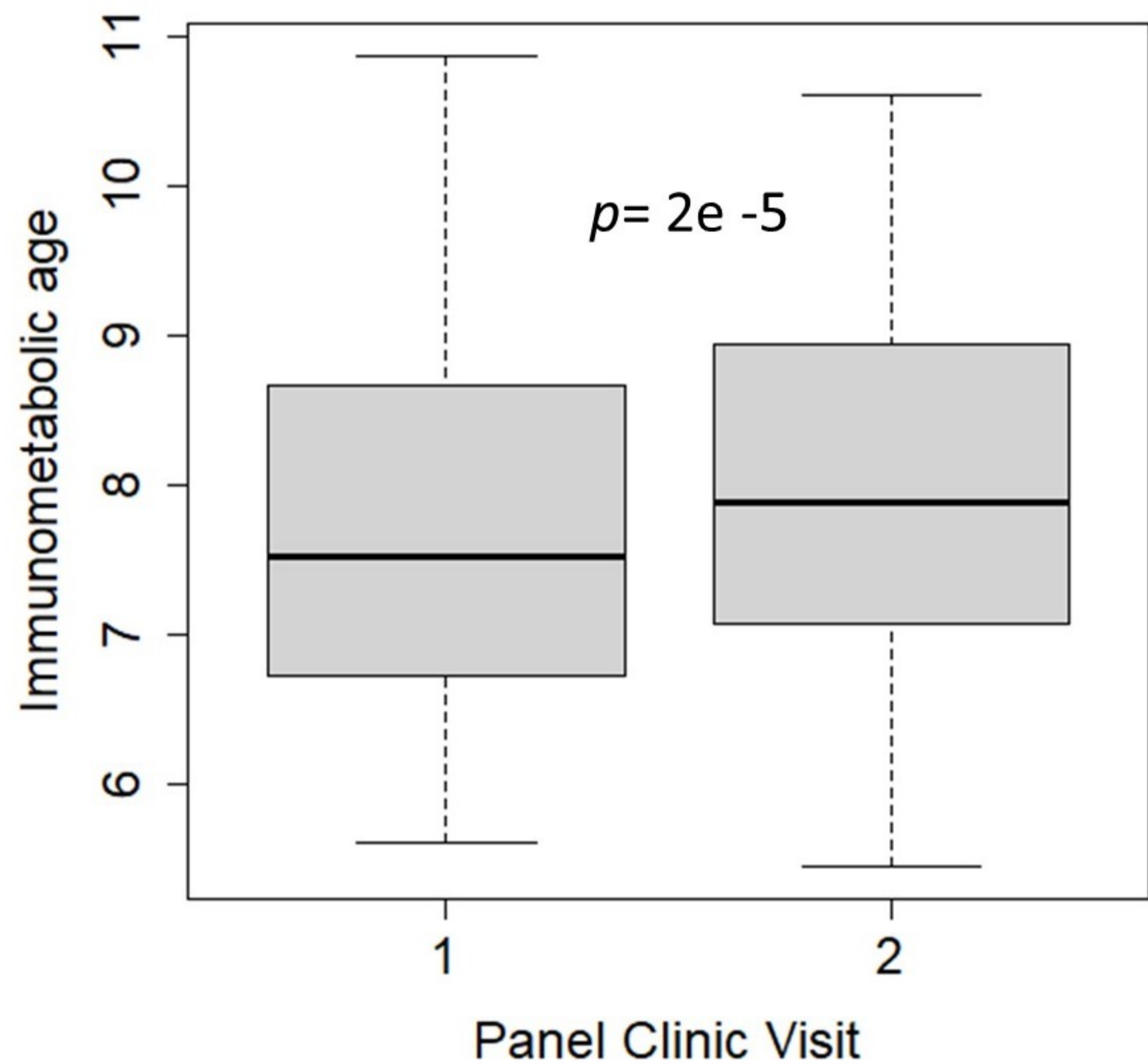
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1083 Figure 2 – source data 1

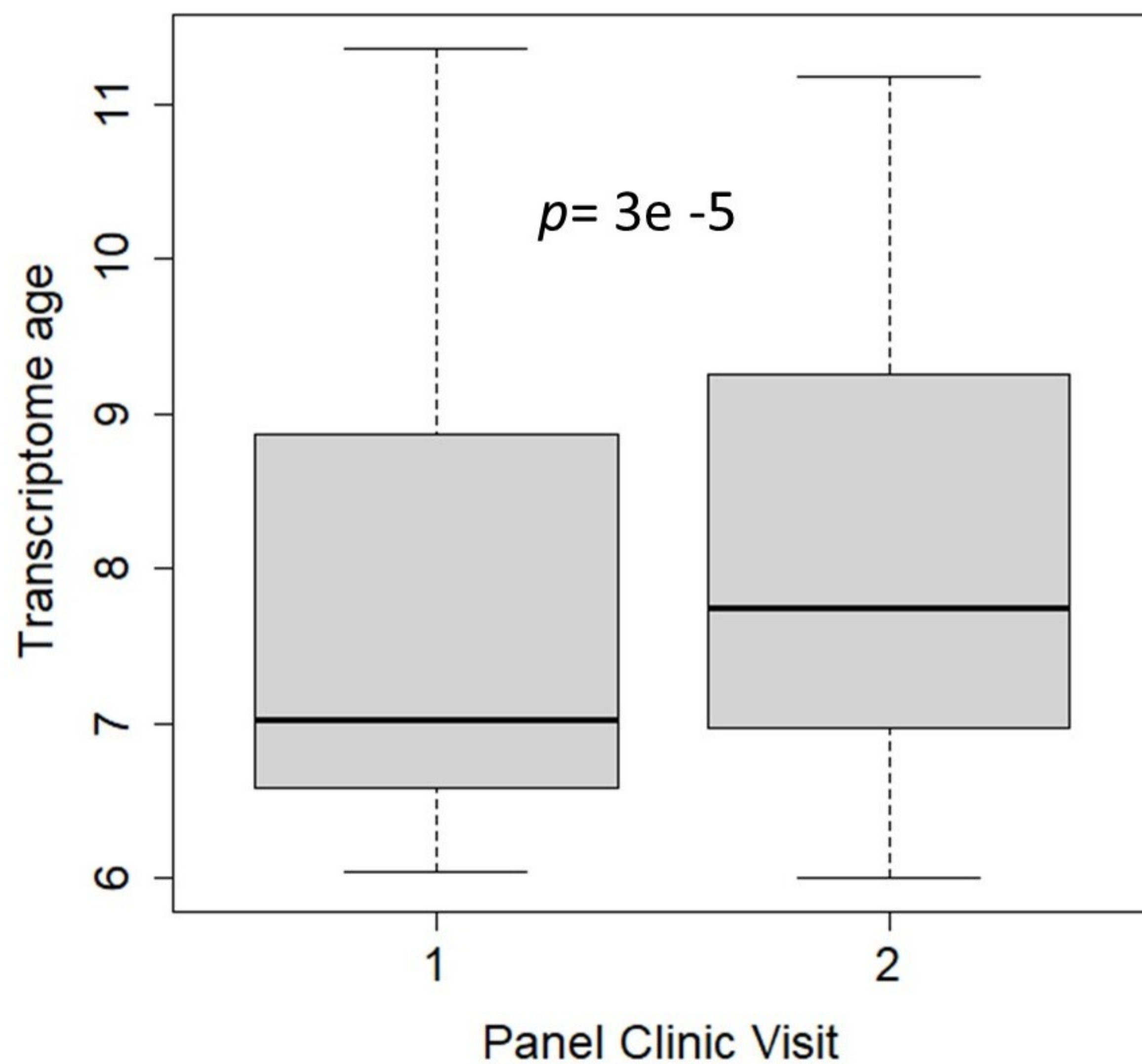
1084 Figure 3- source data 1

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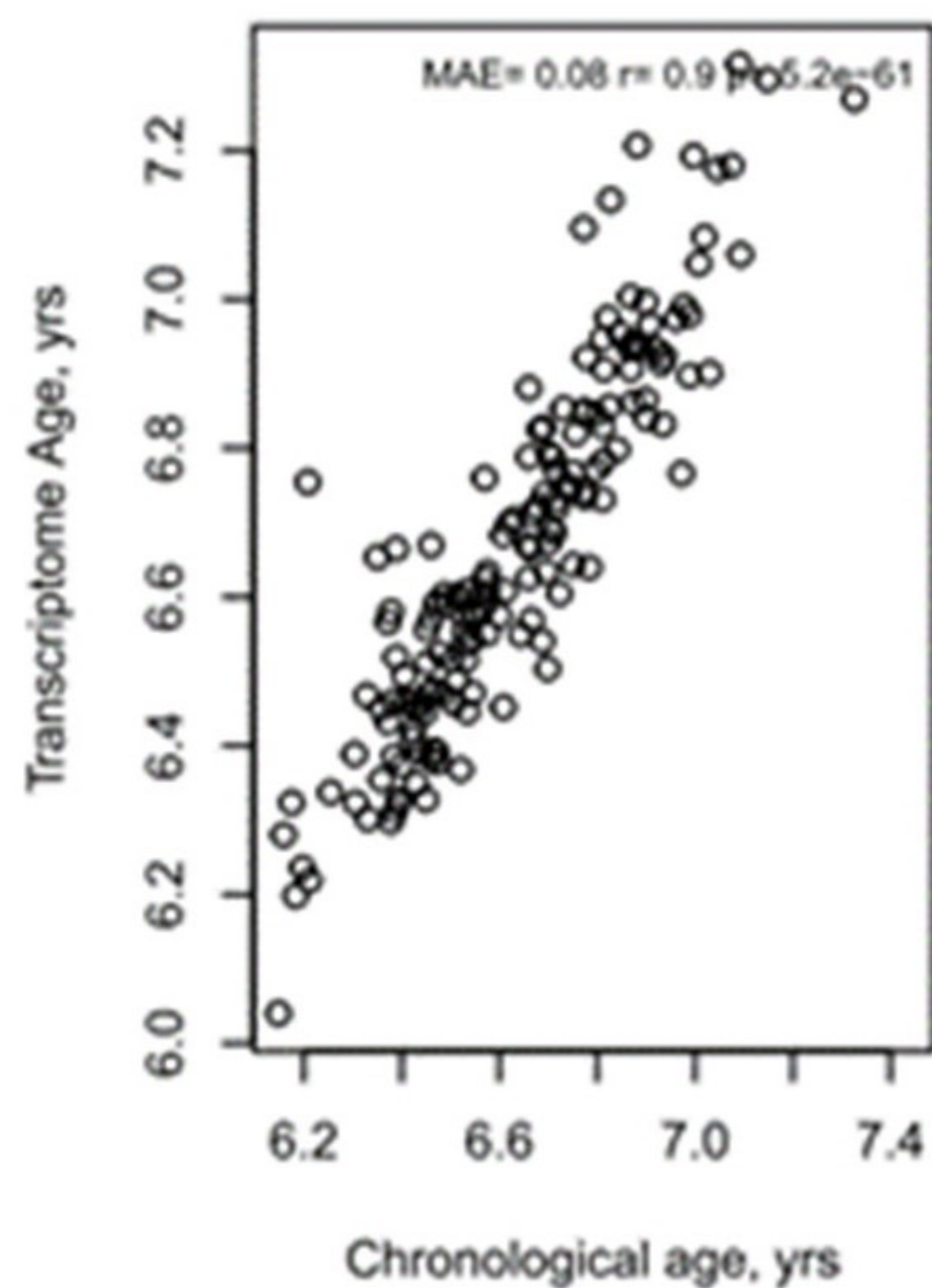
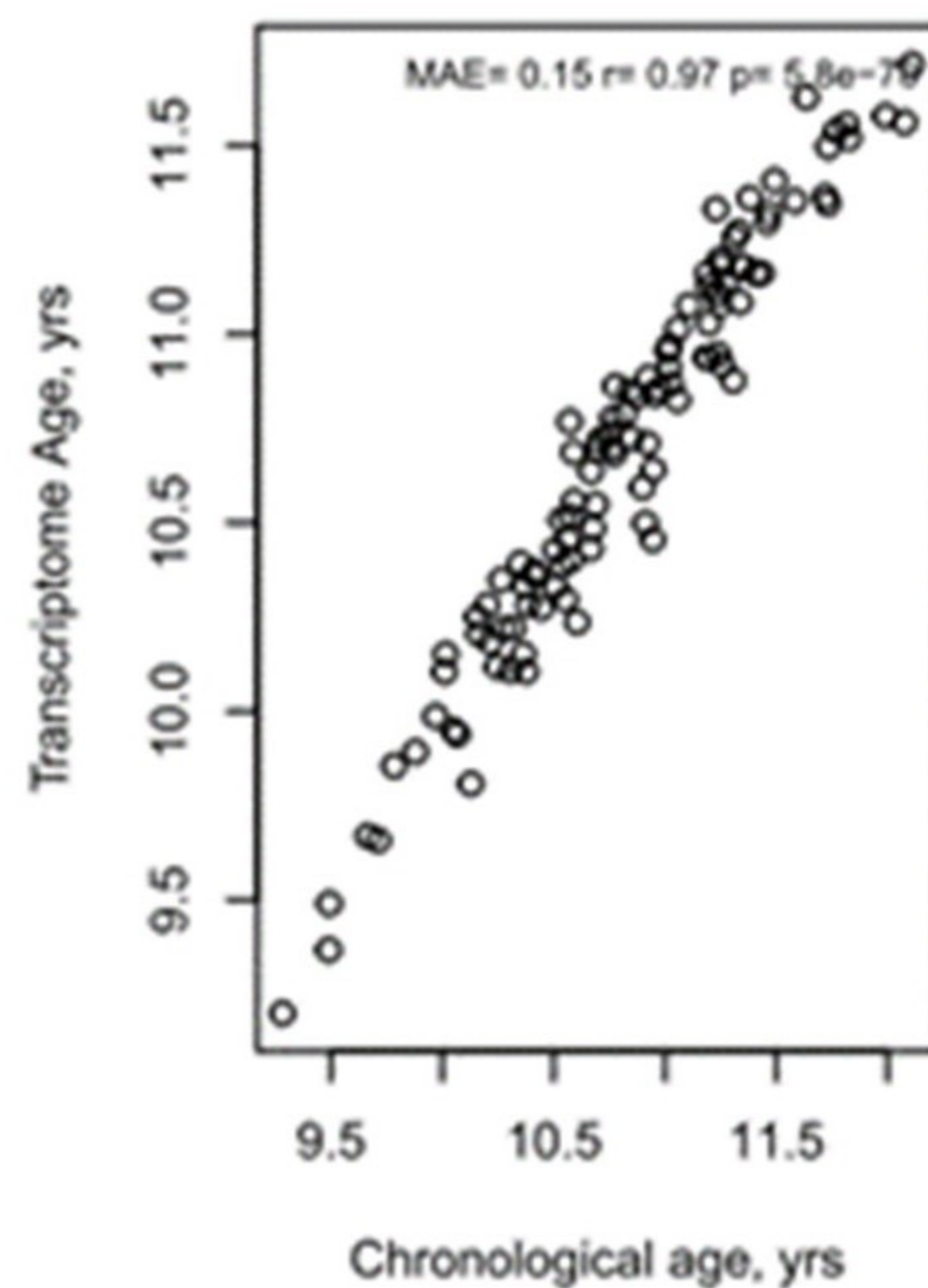
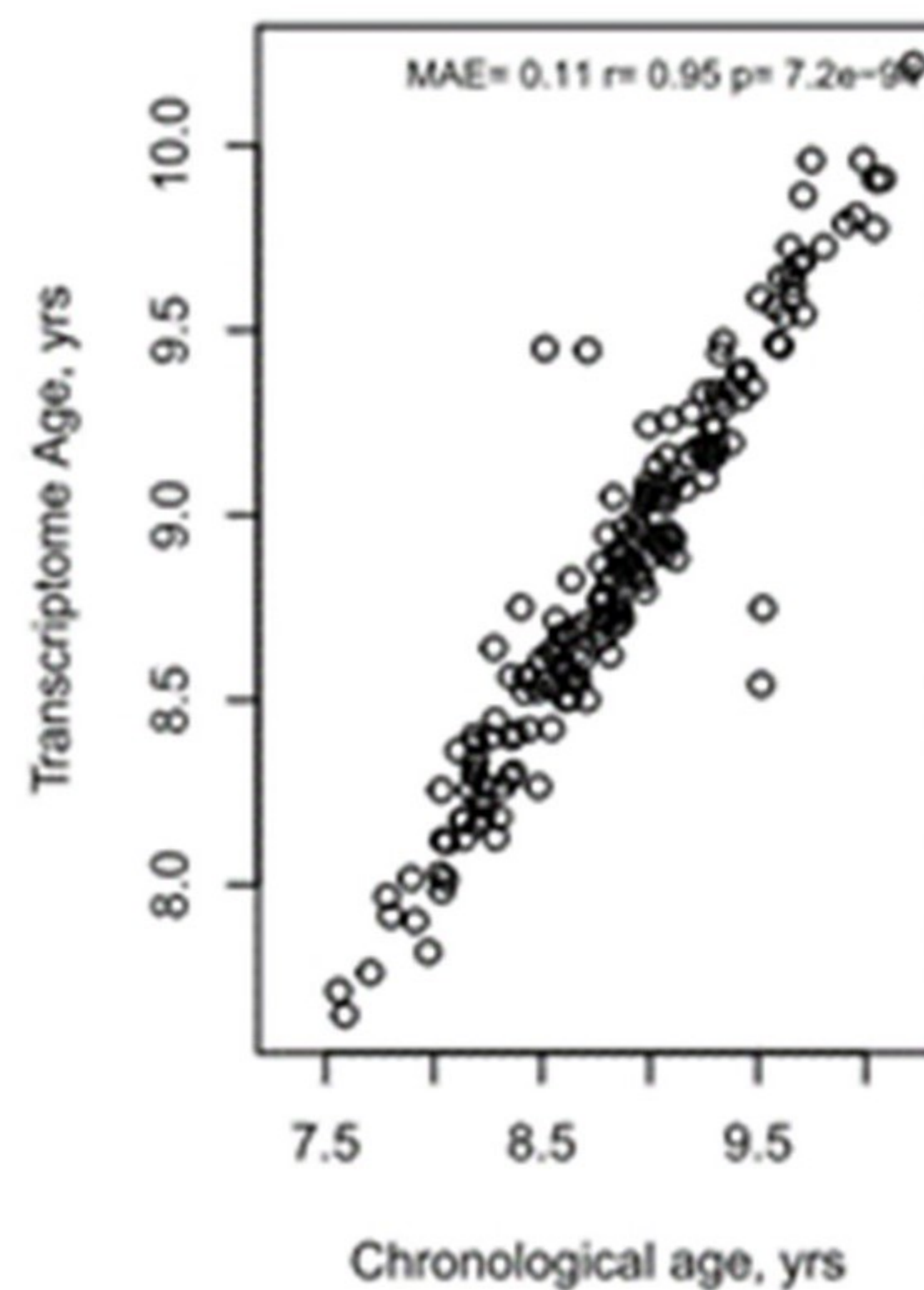
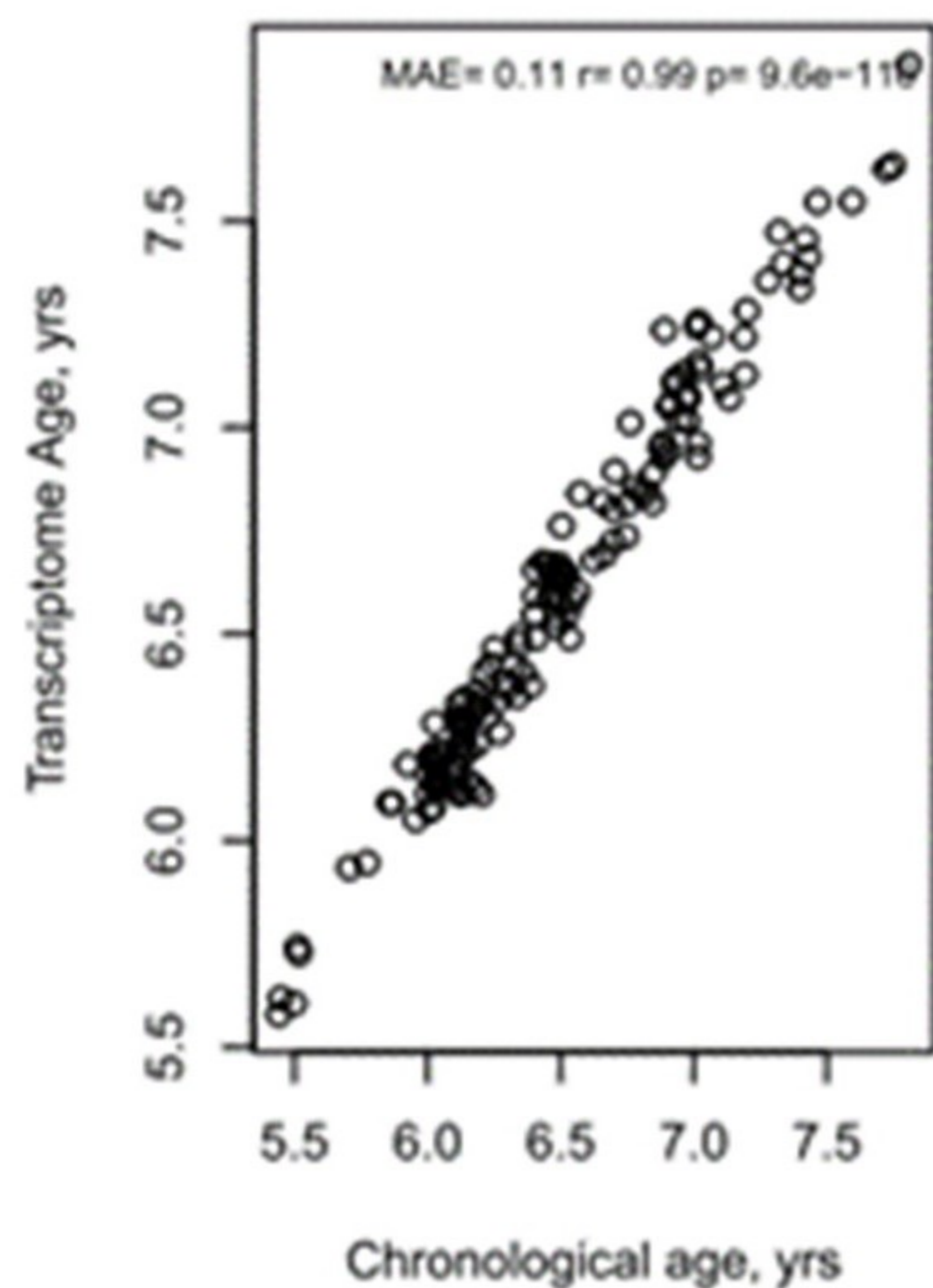
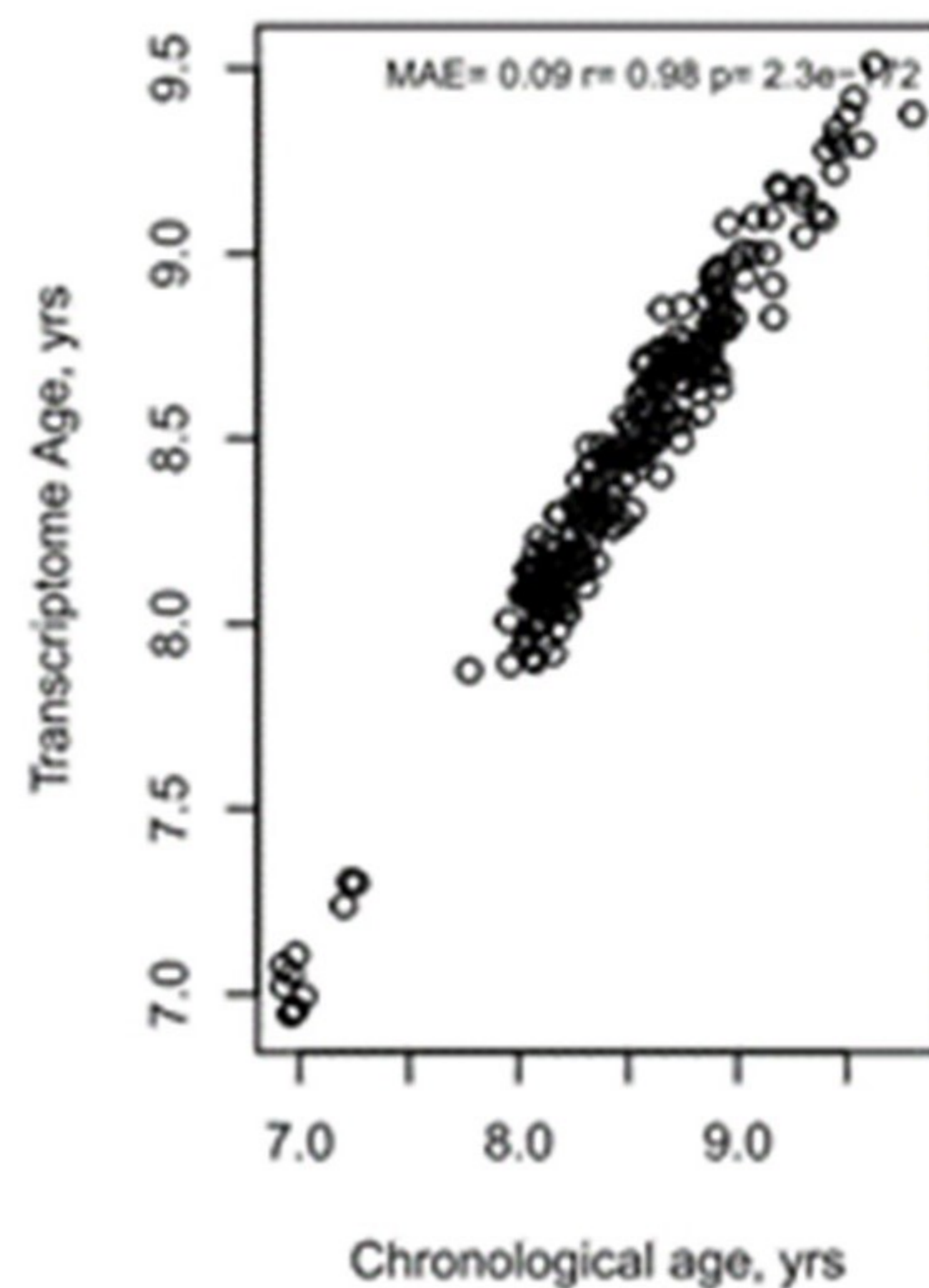
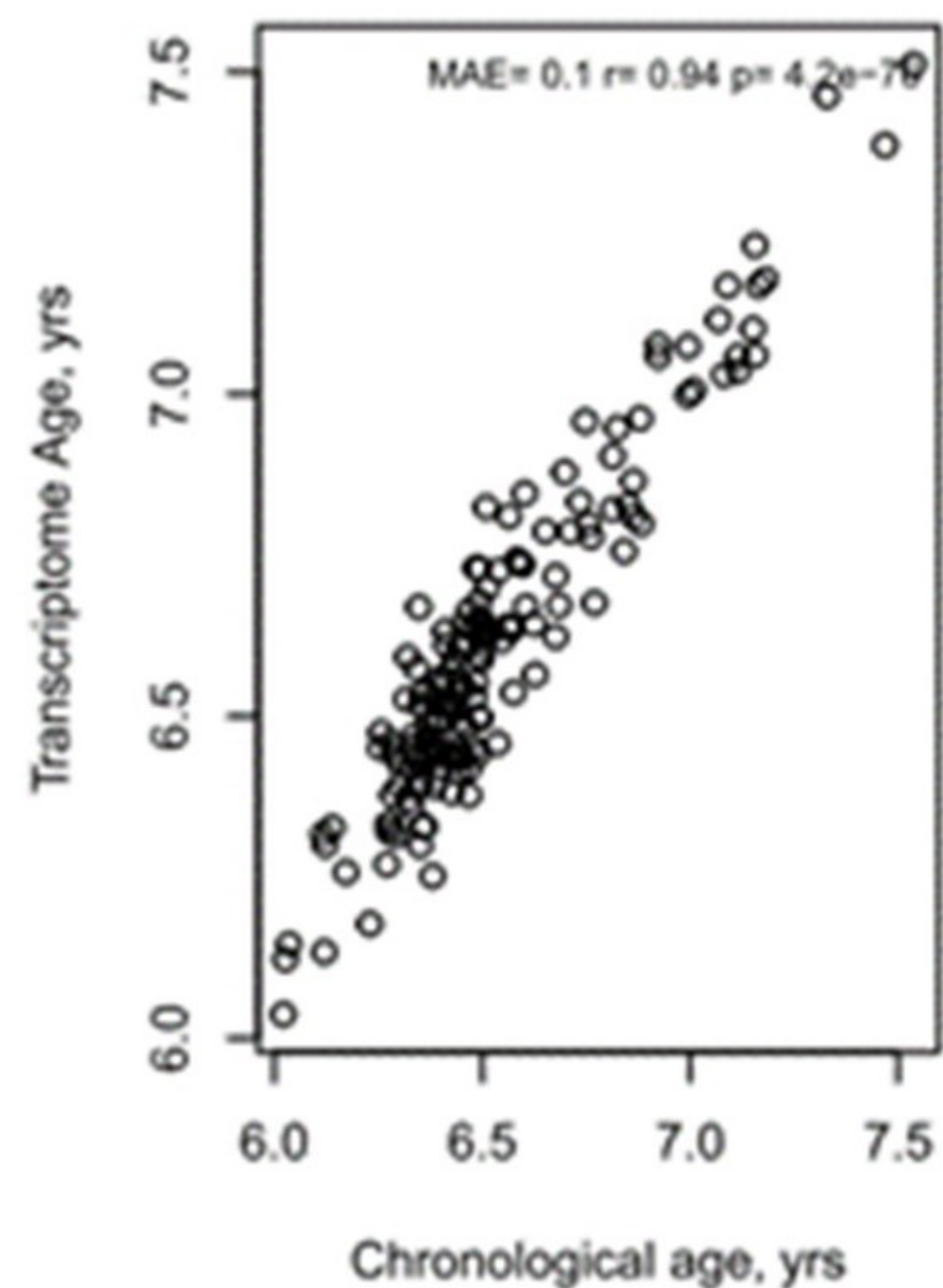
## Immunometabolic age

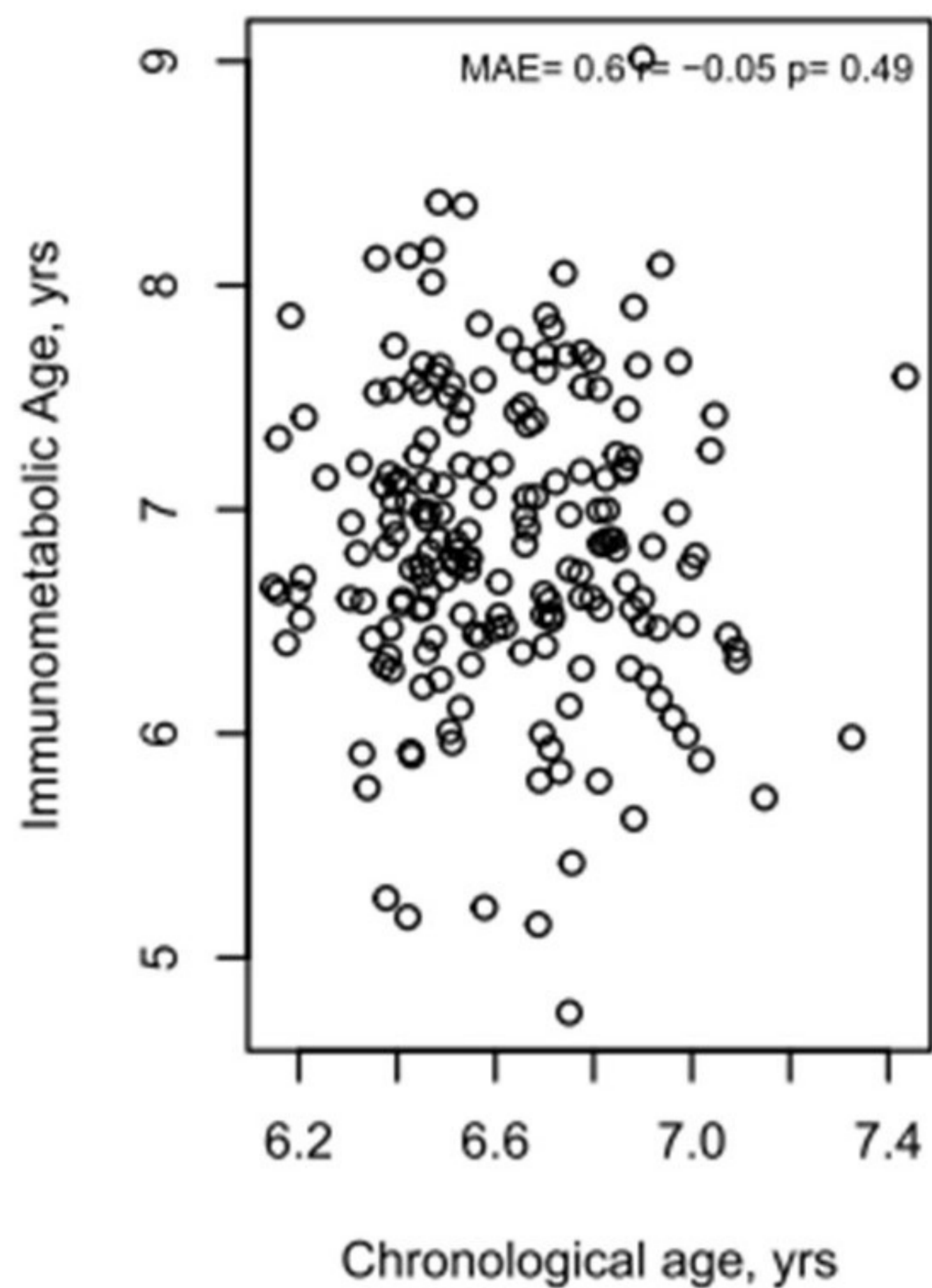
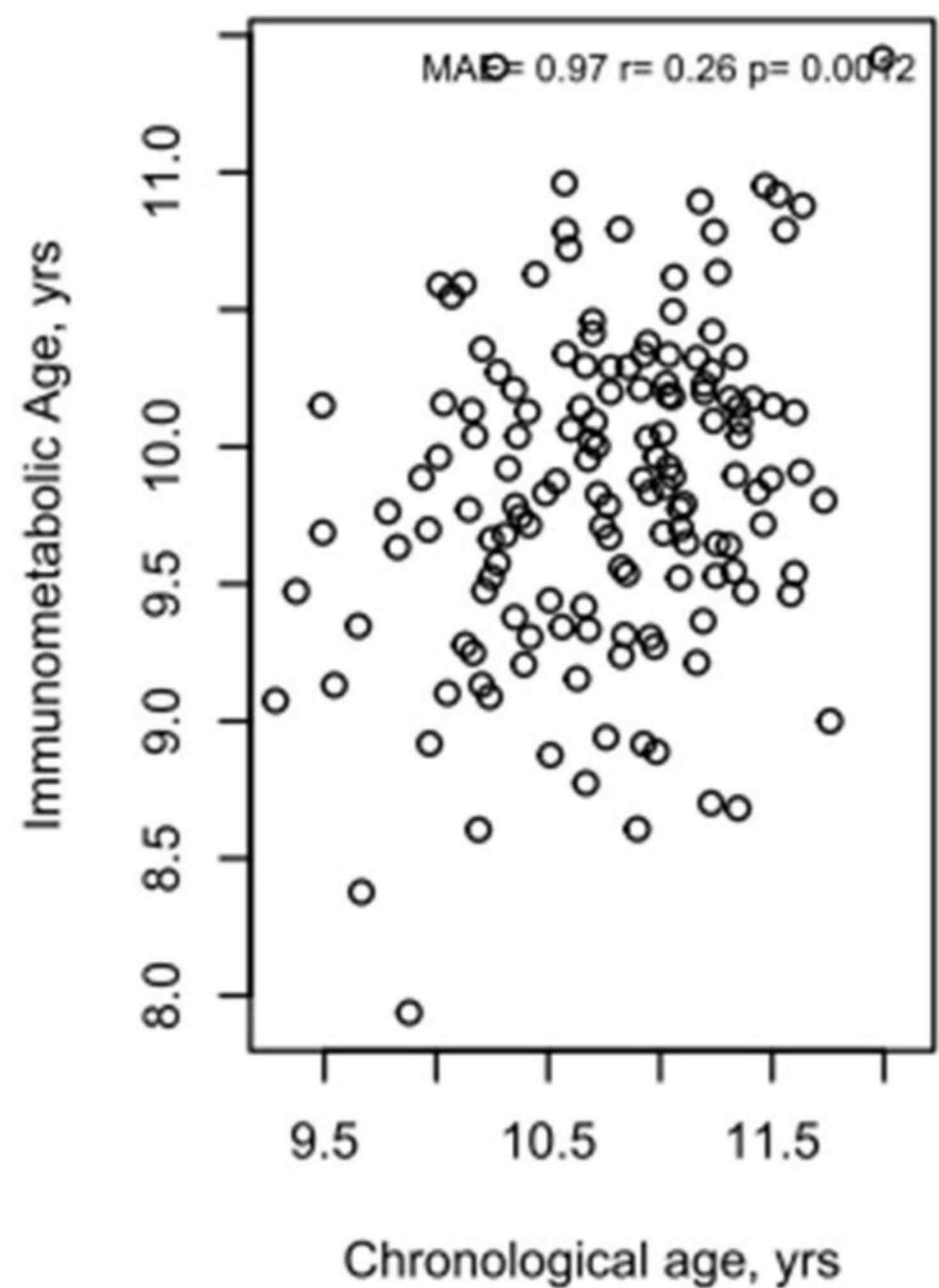
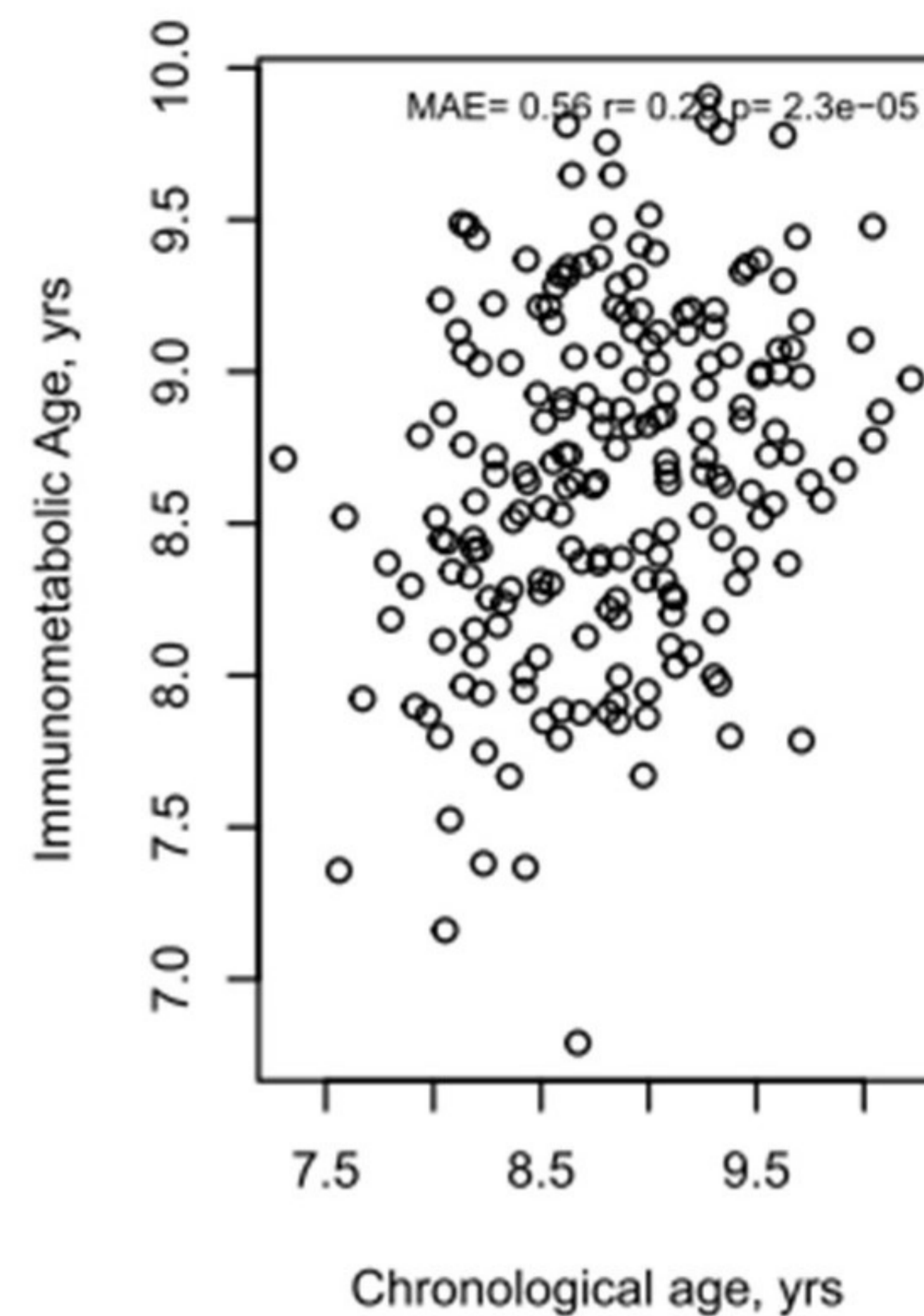
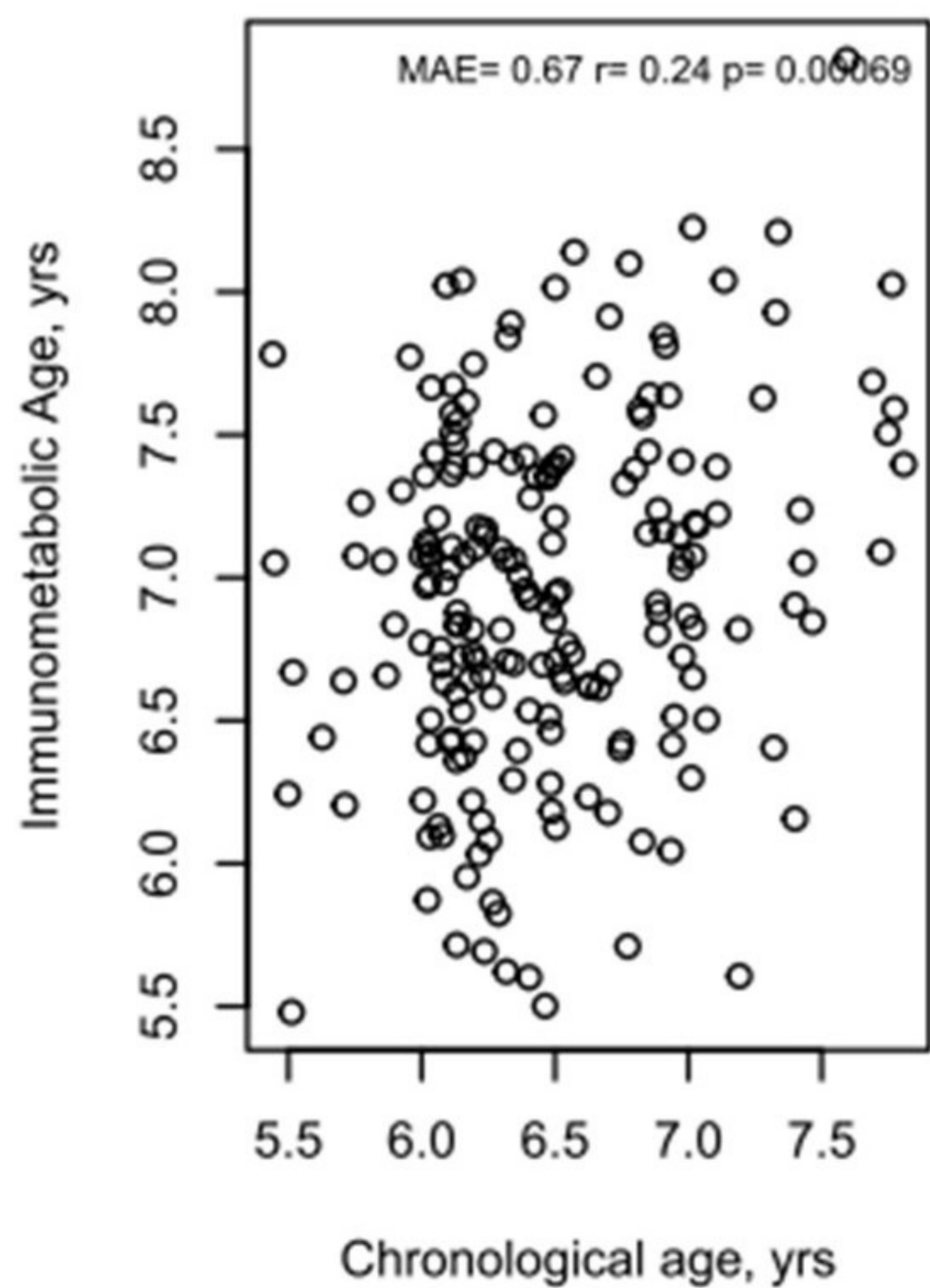
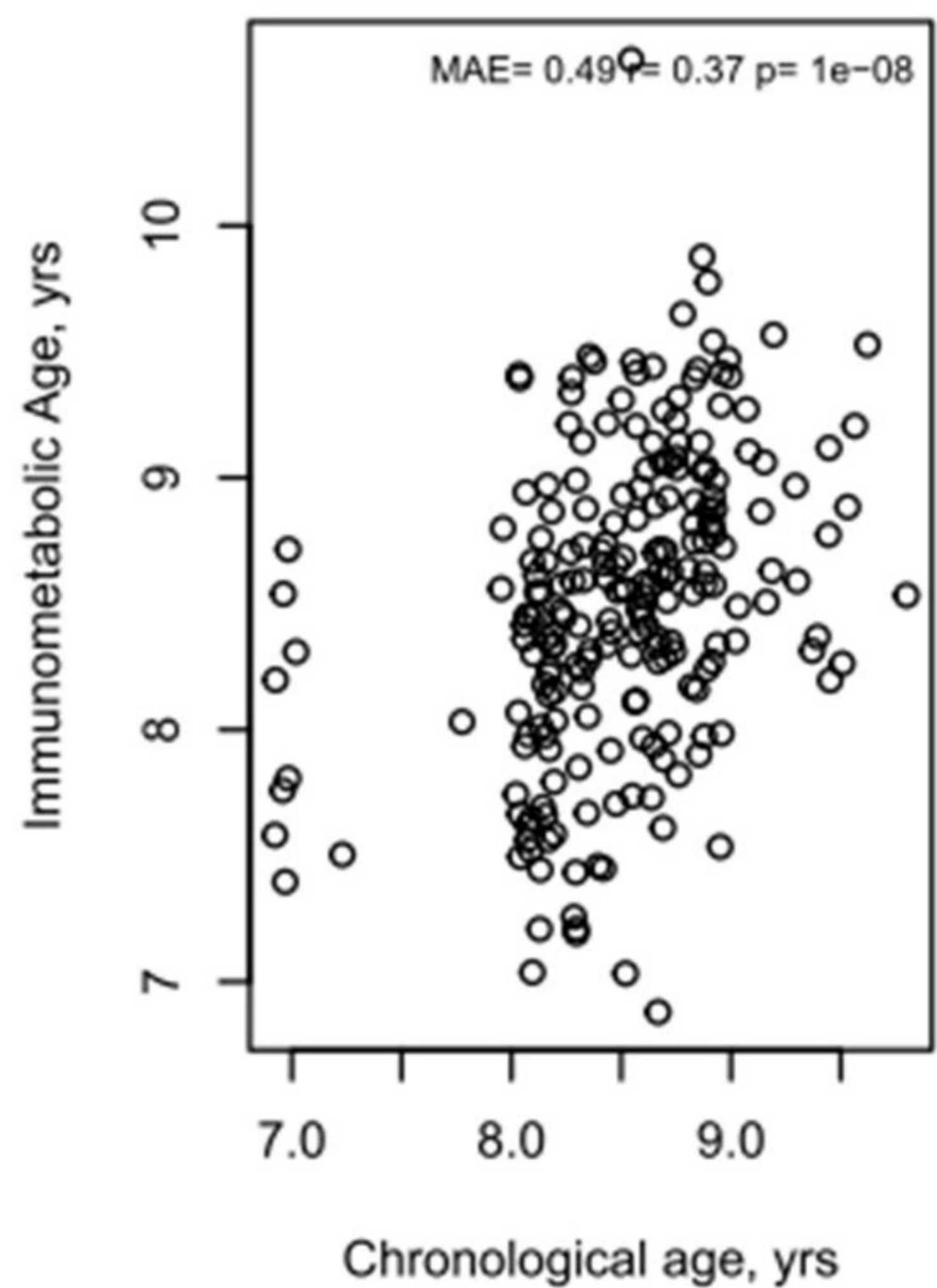
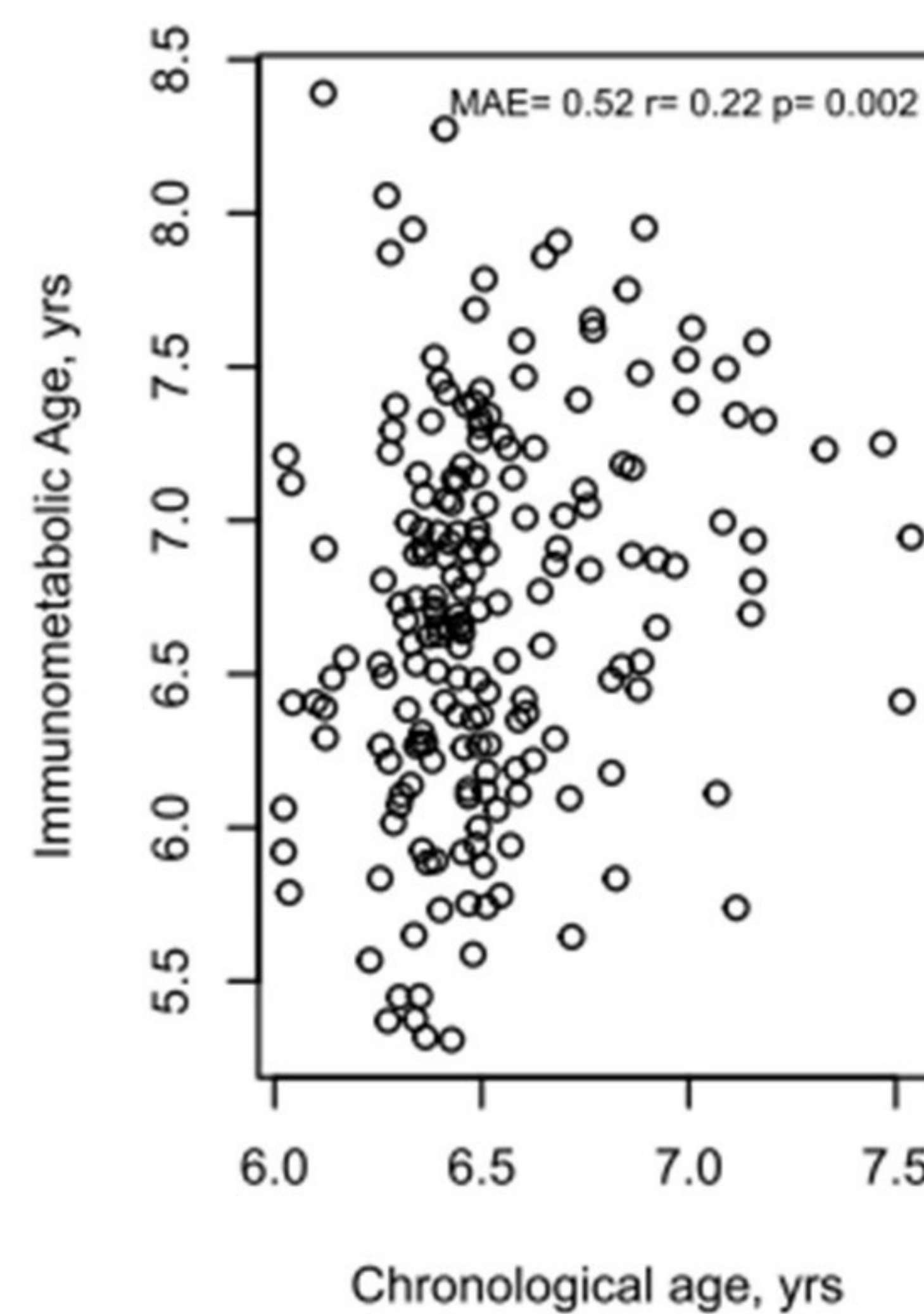


## Transcriptome age





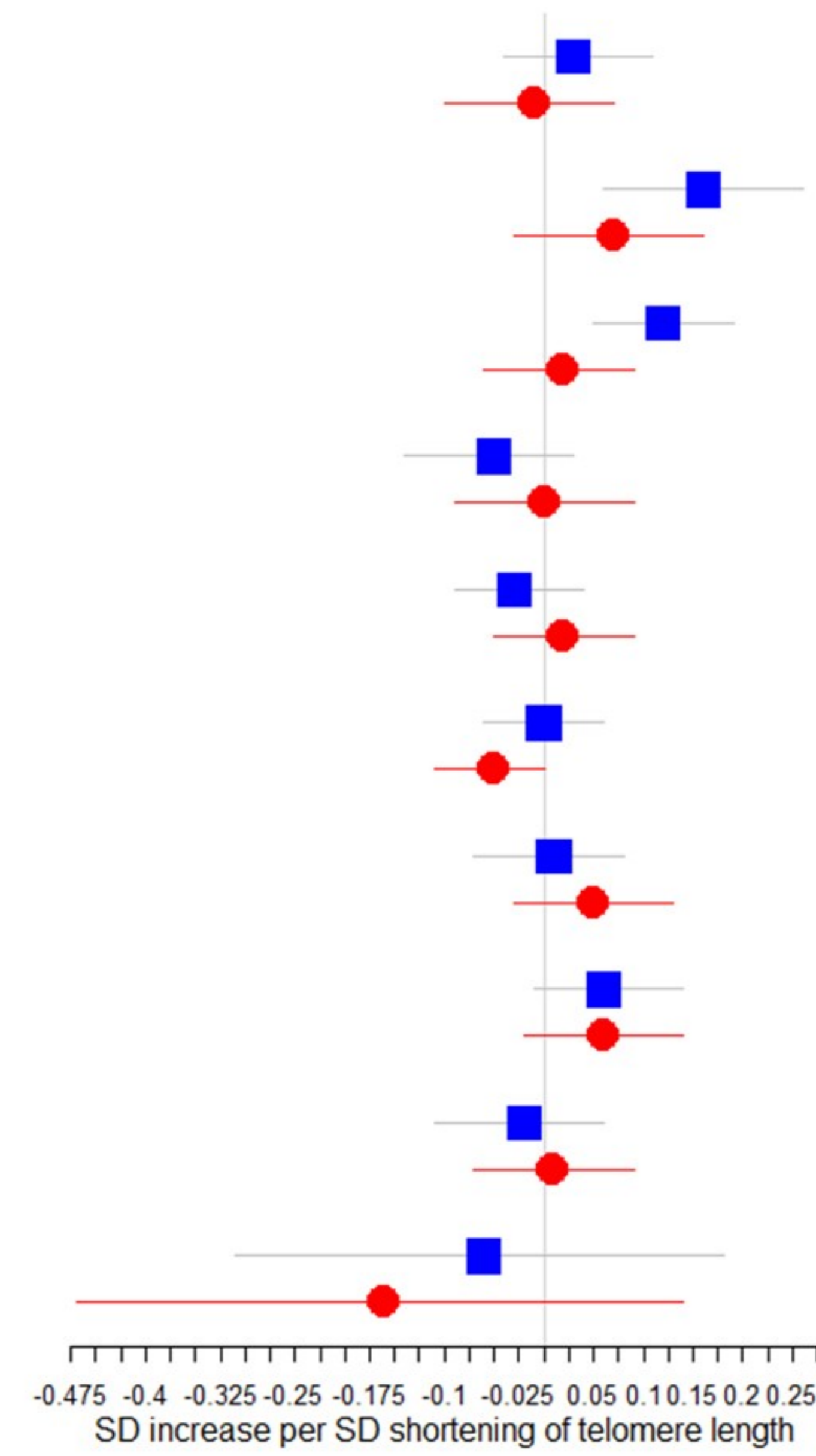
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### Telomere Length

■ Boys ● Girls

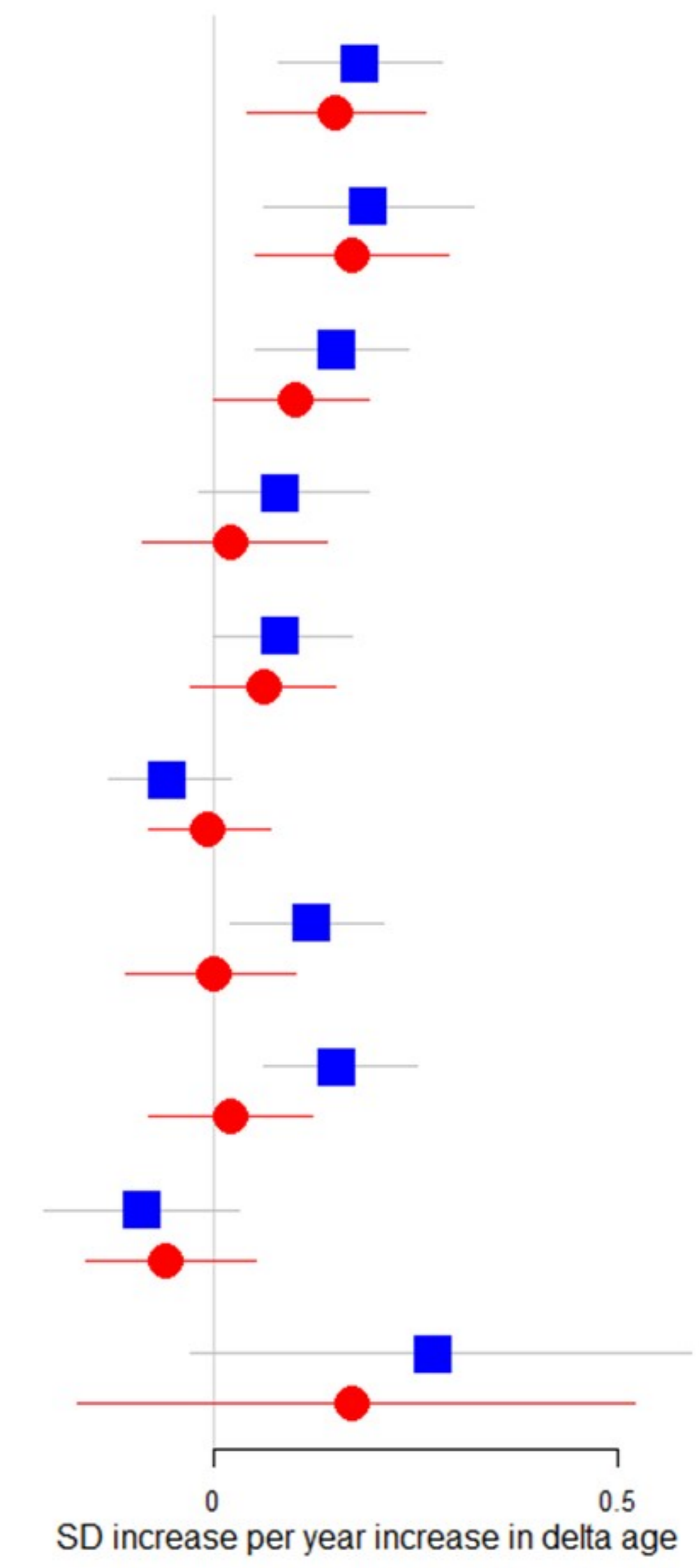
Variable	Boys p-value	Girls p-value
Height z-score	p=0.41	p=0.76
BMI z-score	p=0.0016	p=0.18
Adiposity (BIA fat-mass %)	p=0.0014	p=0.68
Working memory (3-back d')	p=0.19	p=0.96
Inattentiveness (ANT-HRT)	p=0.42	p=0.61
Fluid Intelligence (CPM)	p=0.98	p=0.07
Internalizing Behaviours (CBCL)	p=0.82	p=0.19
Externalizing behaviours (CBCL)	p=0.1	p=0.13
Lung Function (FEV1)	p=0.57	p=0.78
Puberty Onset (Log Odds of PDS >1)	p=0.62	p=0.29



### DNA Methylation Age

■ Boys ● Girls

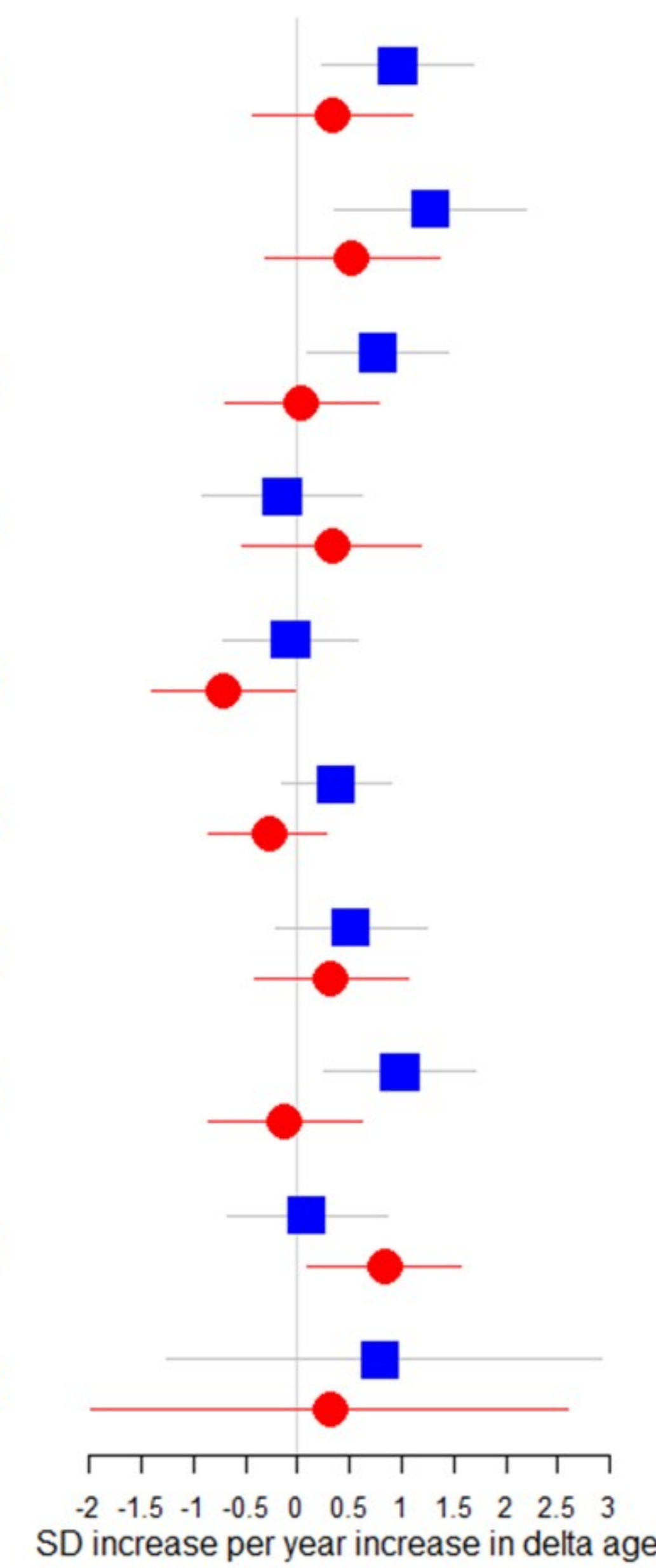
Variable	Boys p-value	Girls p-value
Height z-score	p=0.00029	p=0.0056
BMI z-score	p=0.004	p=0.0051
Adiposity (BIA fat-mass %)	p=0.002	p=0.056
Working memory (3-back d')	p=0.12	p=0.69
Inattentiveness (ANT-HRT)	p=0.064	p=0.19
Fluid Intelligence (CPM)	p=0.13	p=0.89
Internalizing Behaviours (CBCL)	p=0.019	p=0.93
Externalizing behaviours (CBCL)	p=0.0016	p=0.7
Lung Function (FEV1)	p=0.12	p=0.31
Puberty Onset (Log Odds of PDS >1)	p=0.081	p=0.34



### Transcriptome Age

■ Boys ● Girls

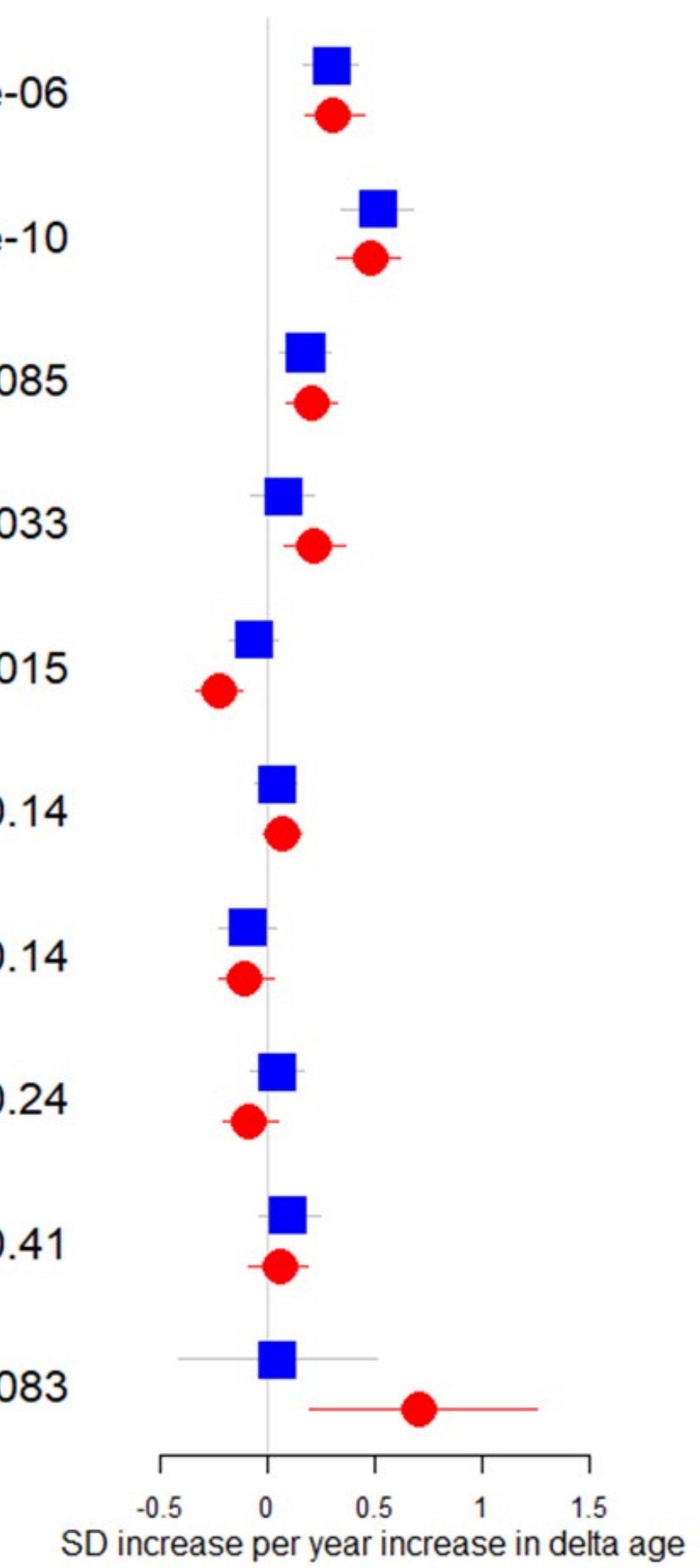
Variable	Boys p-value	Girls p-value
Height z-score	p=0.009	p=0.4
BMI z-score	p=0.0068	p=0.23
Adiposity (BIA fat-mass %)	p=0.027	p=0.91
Working memory (3-back d')	p=0.7	p=0.45
Inattentiveness (ANT-HRT)	p=0.83	p=0.04
Fluid Intelligence (CPM)	p=0.16	p=0.33
Internalizing Behaviours (CBCL)	p=0.17	p=0.4
Externalizing behaviours (CBCL)	p=0.0075	p=0.76
Lung Function (FEV1)	p=0.81	p=0.027
Puberty Onset (Log Odds of PDS >1)	p=0.46	p=0.78

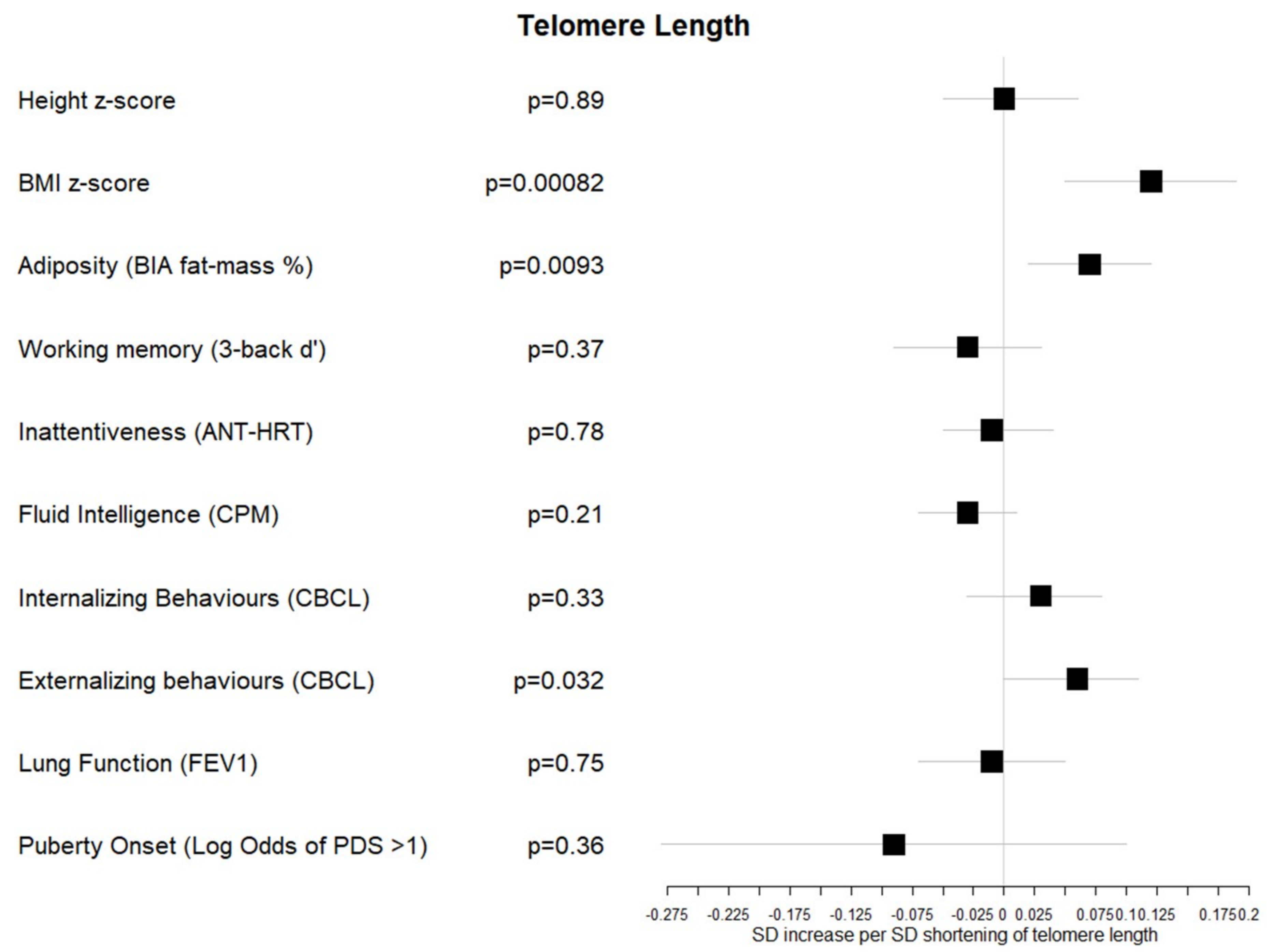
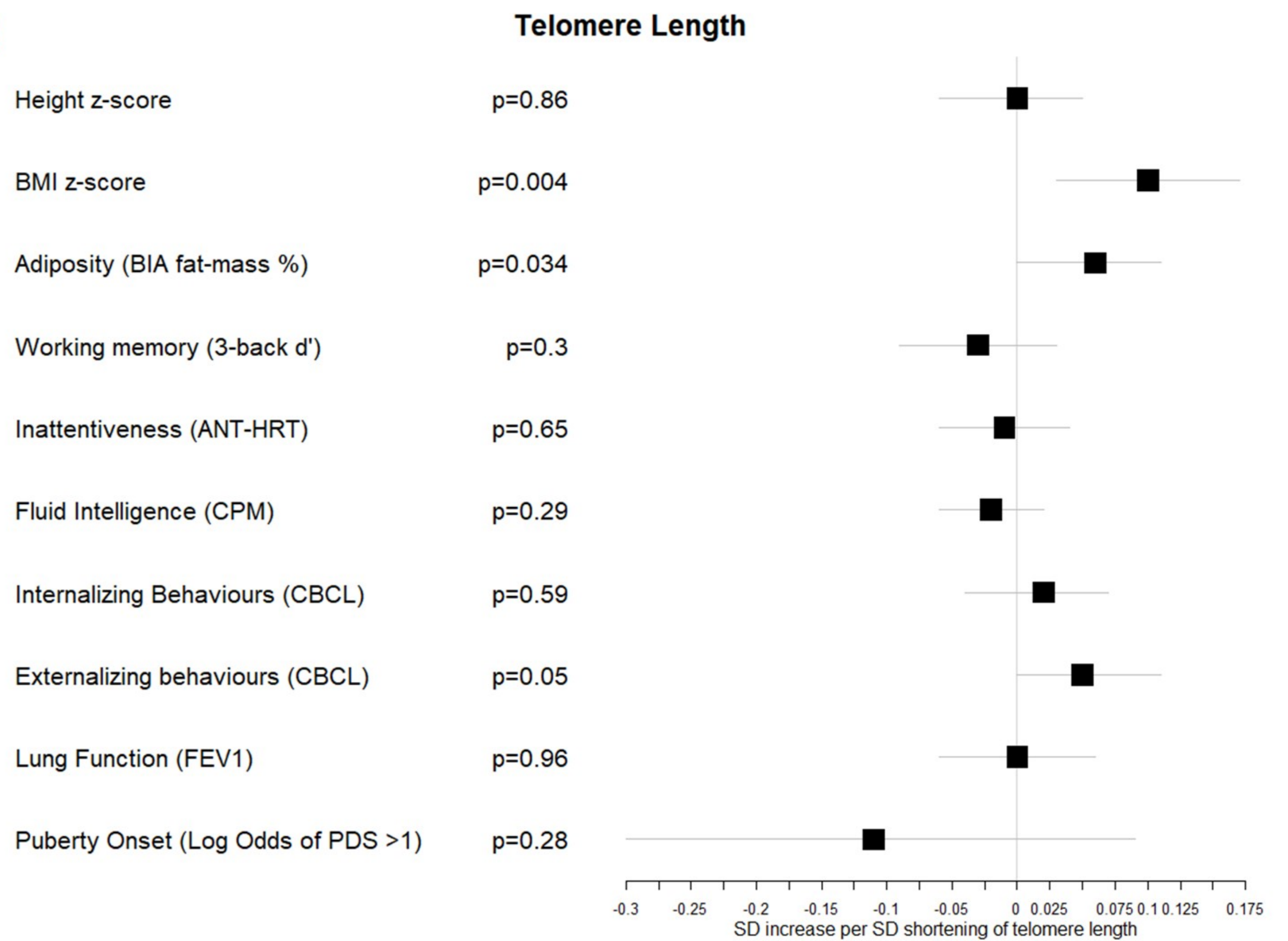
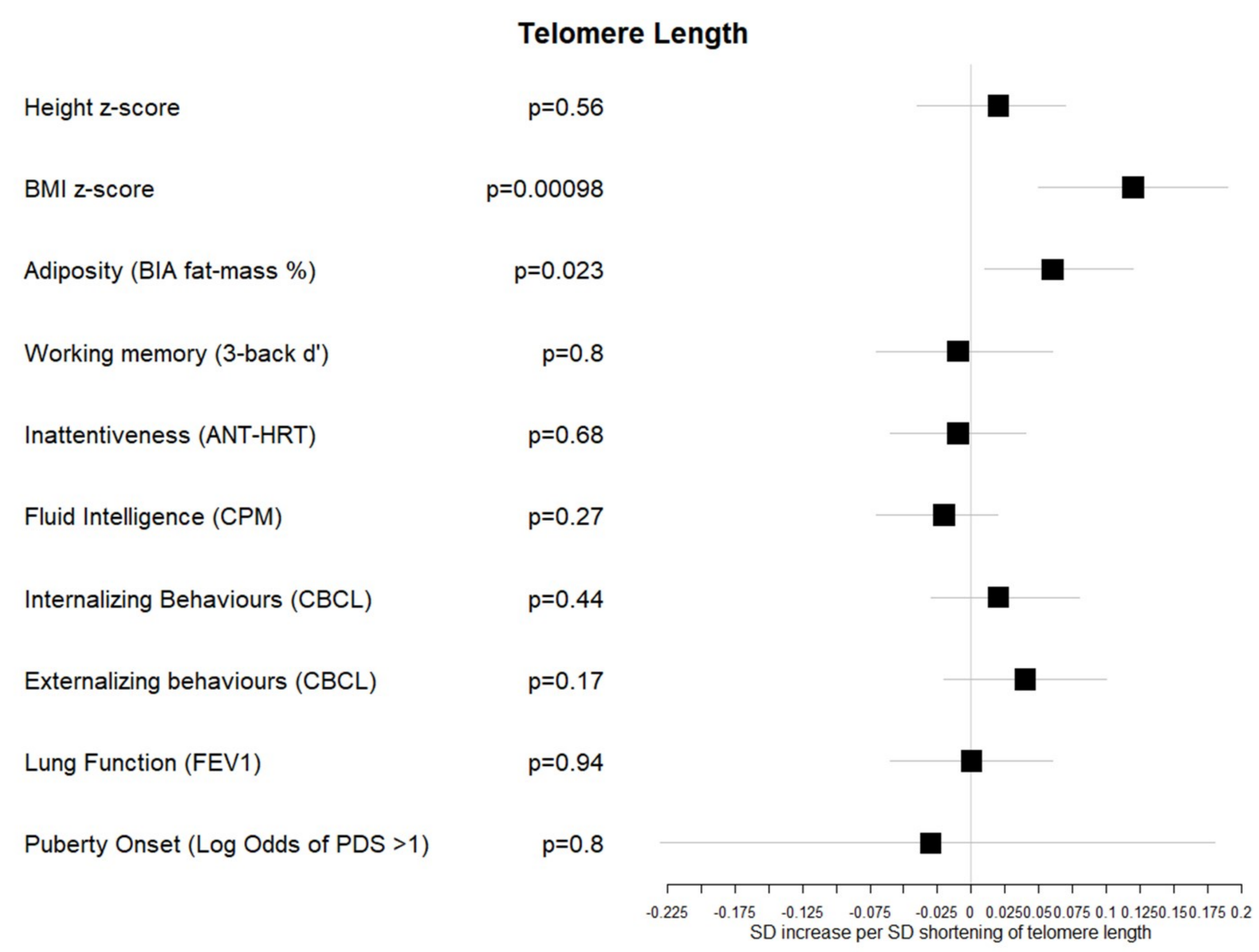
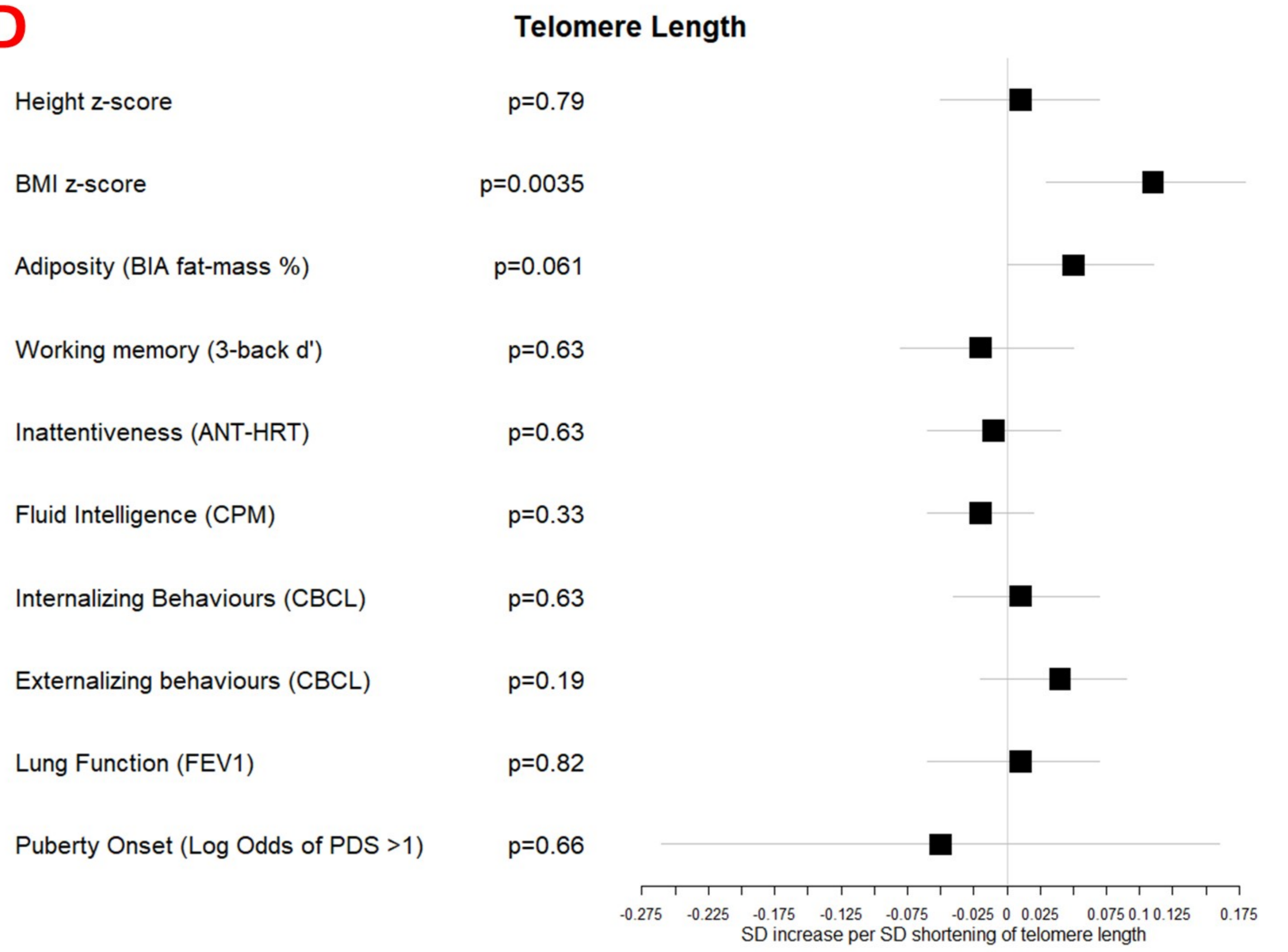


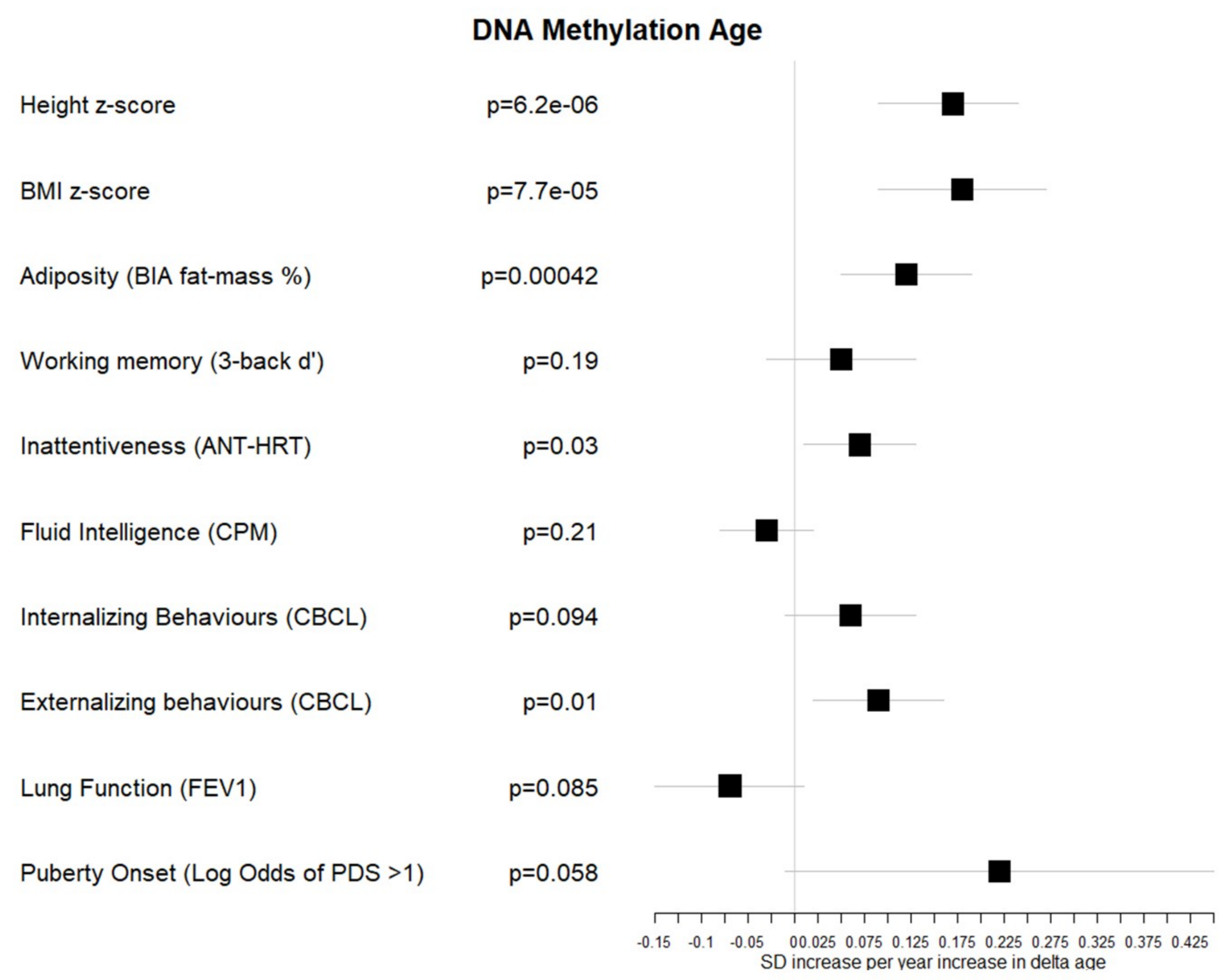
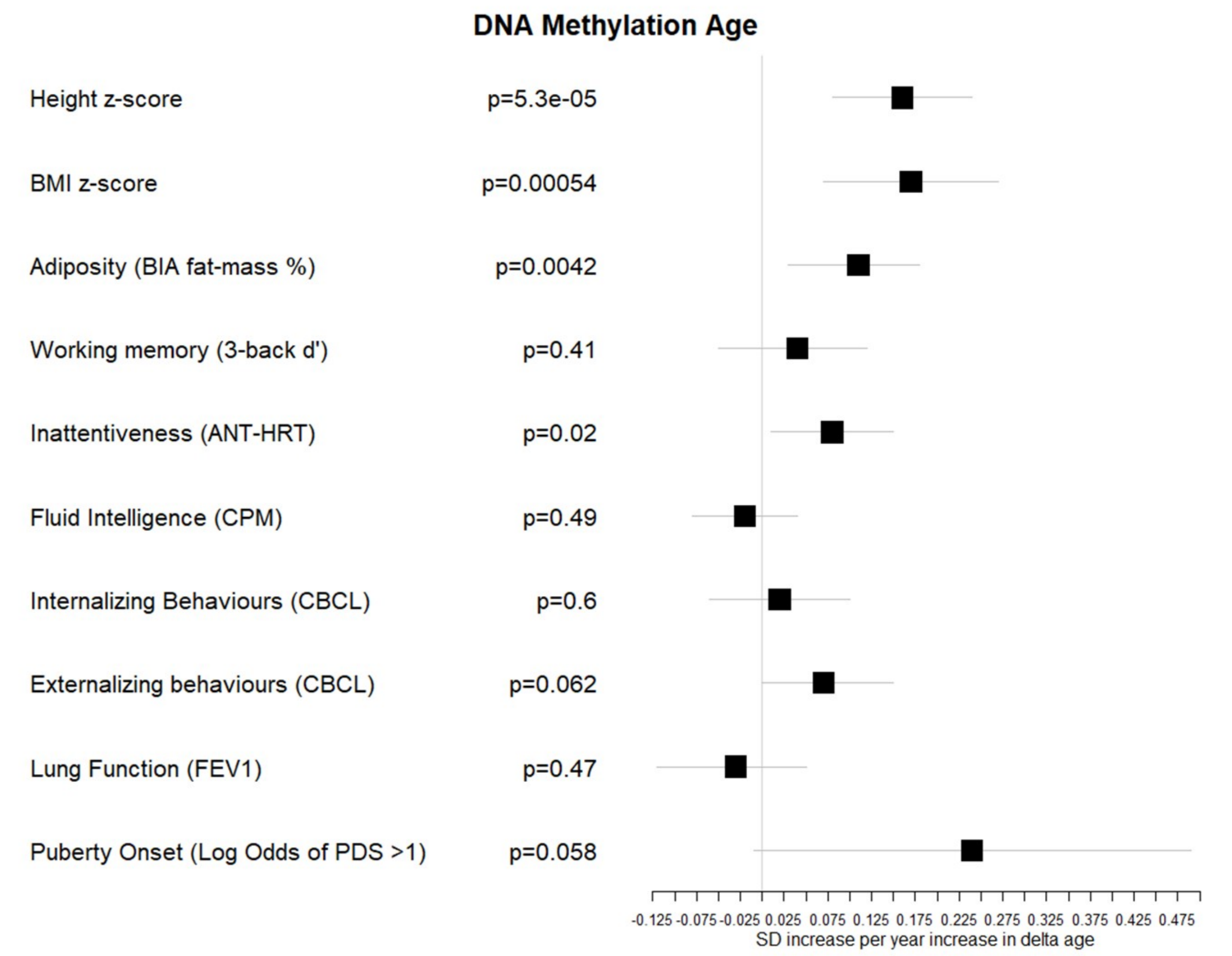
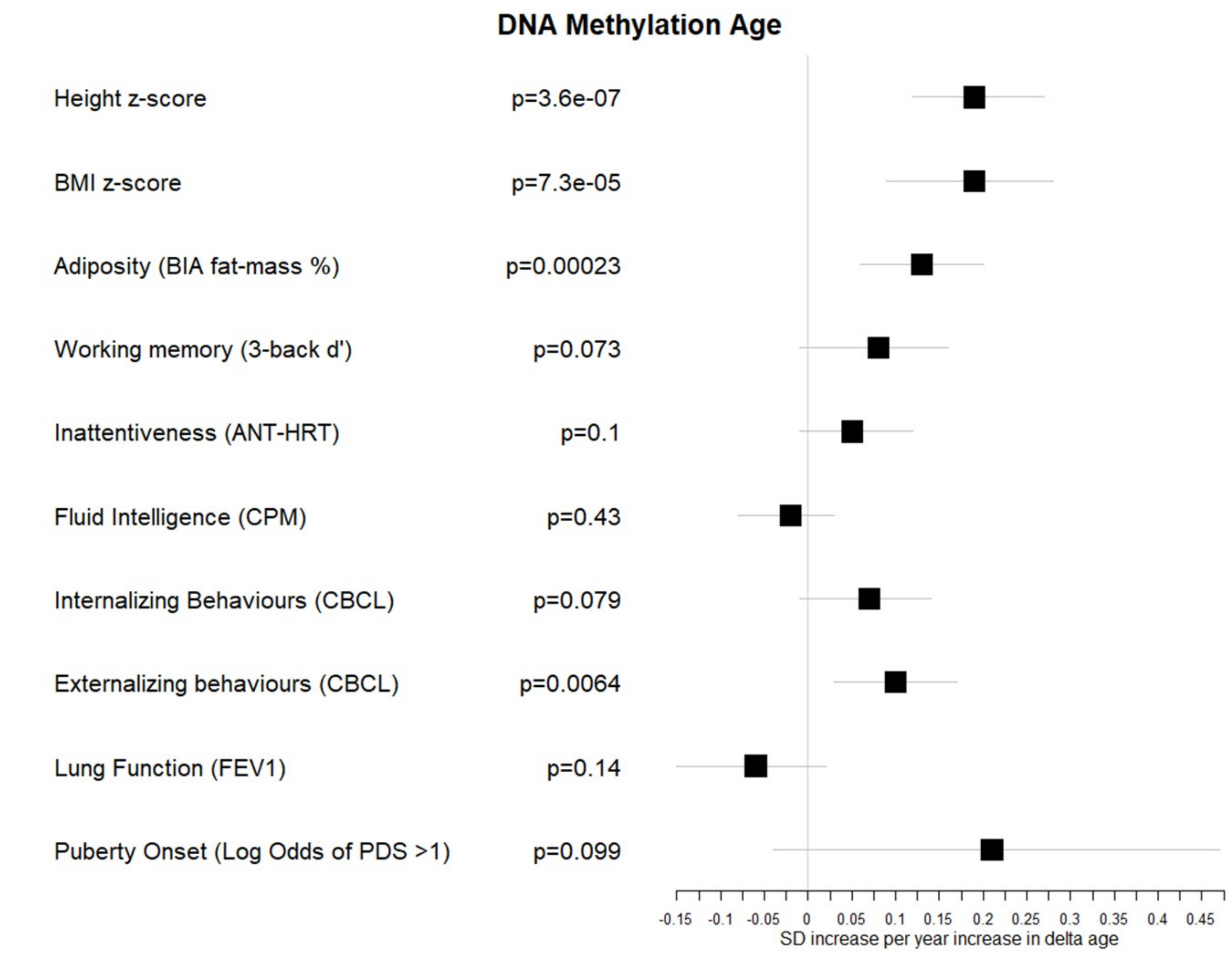
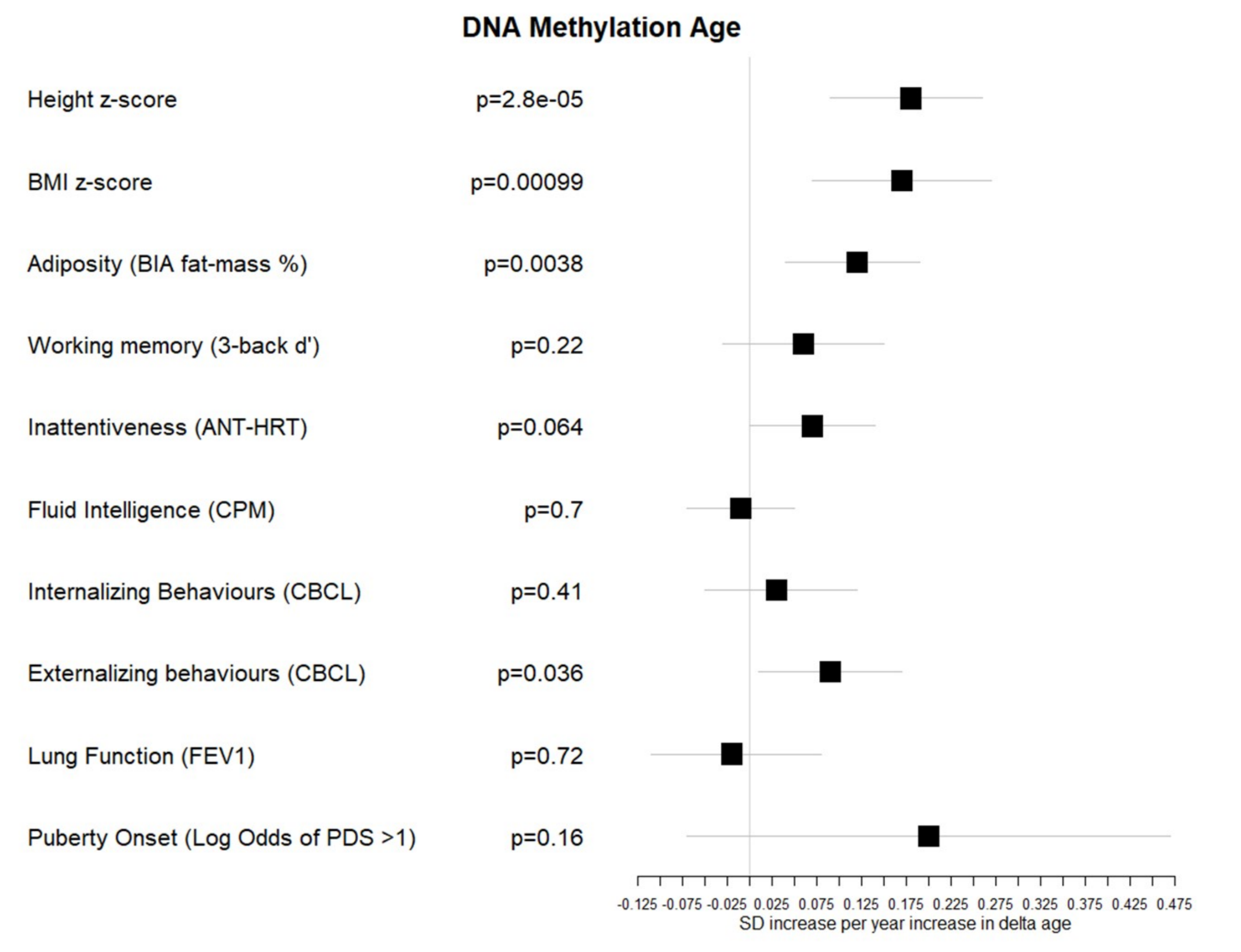
### Immunometabolic Age

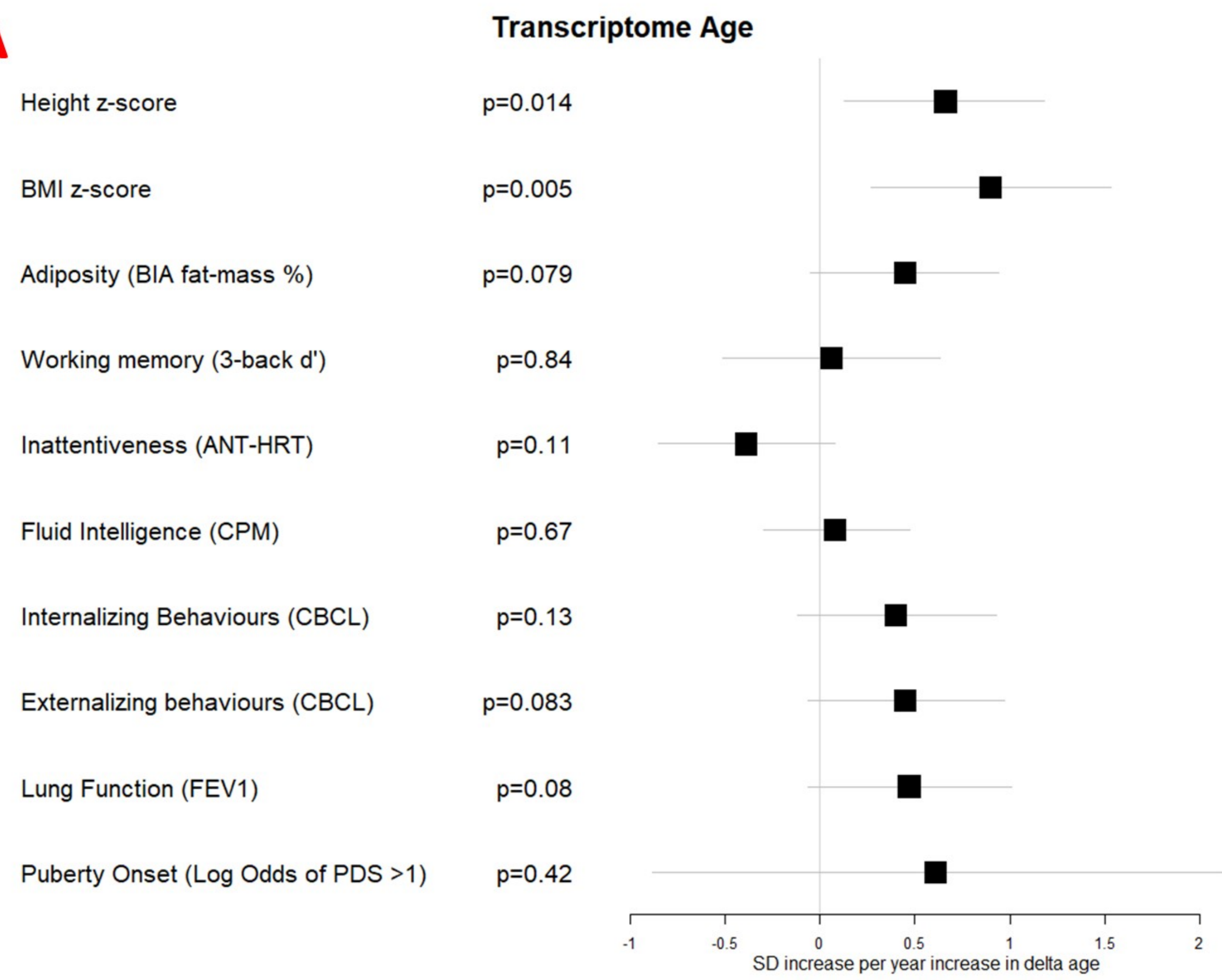
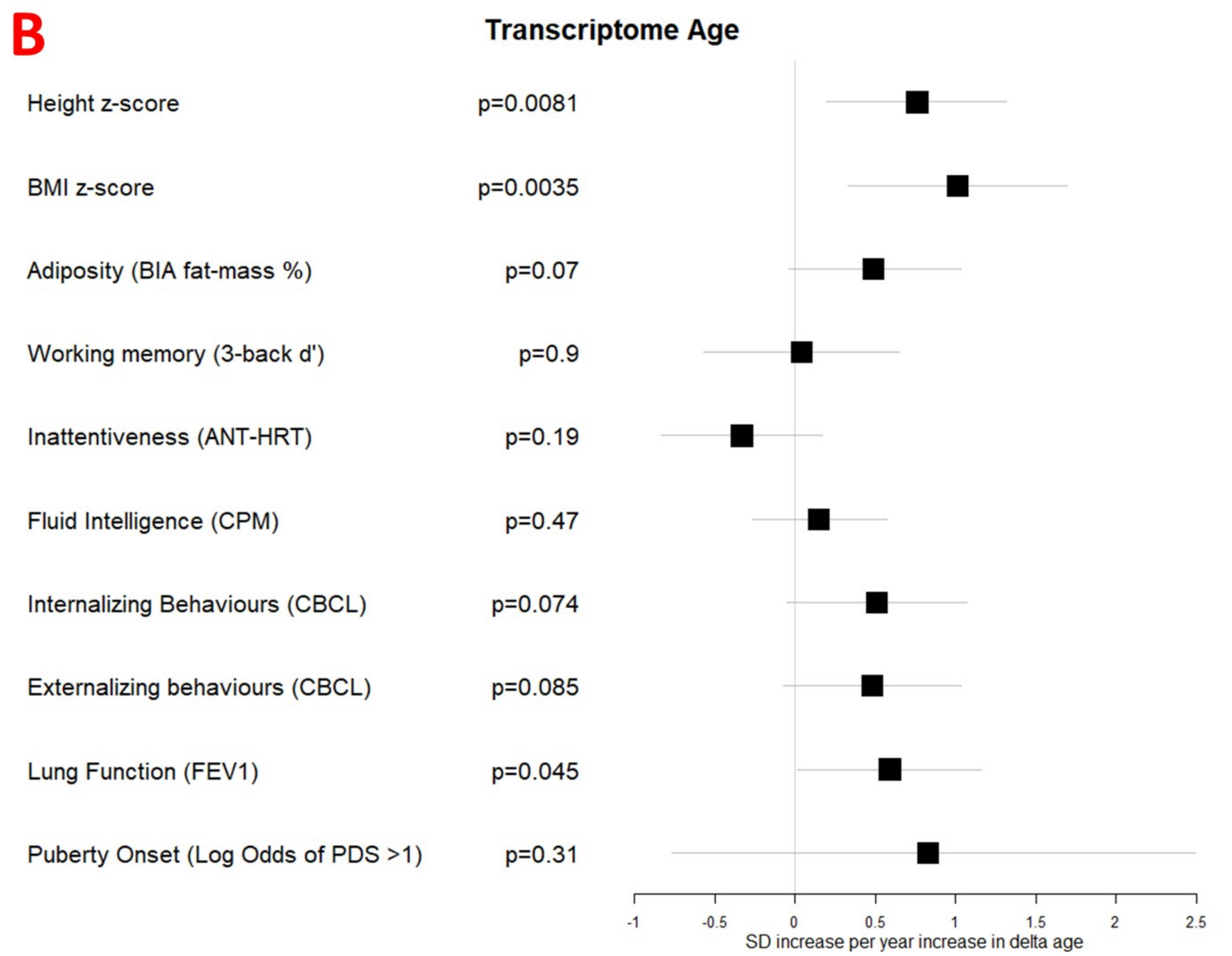
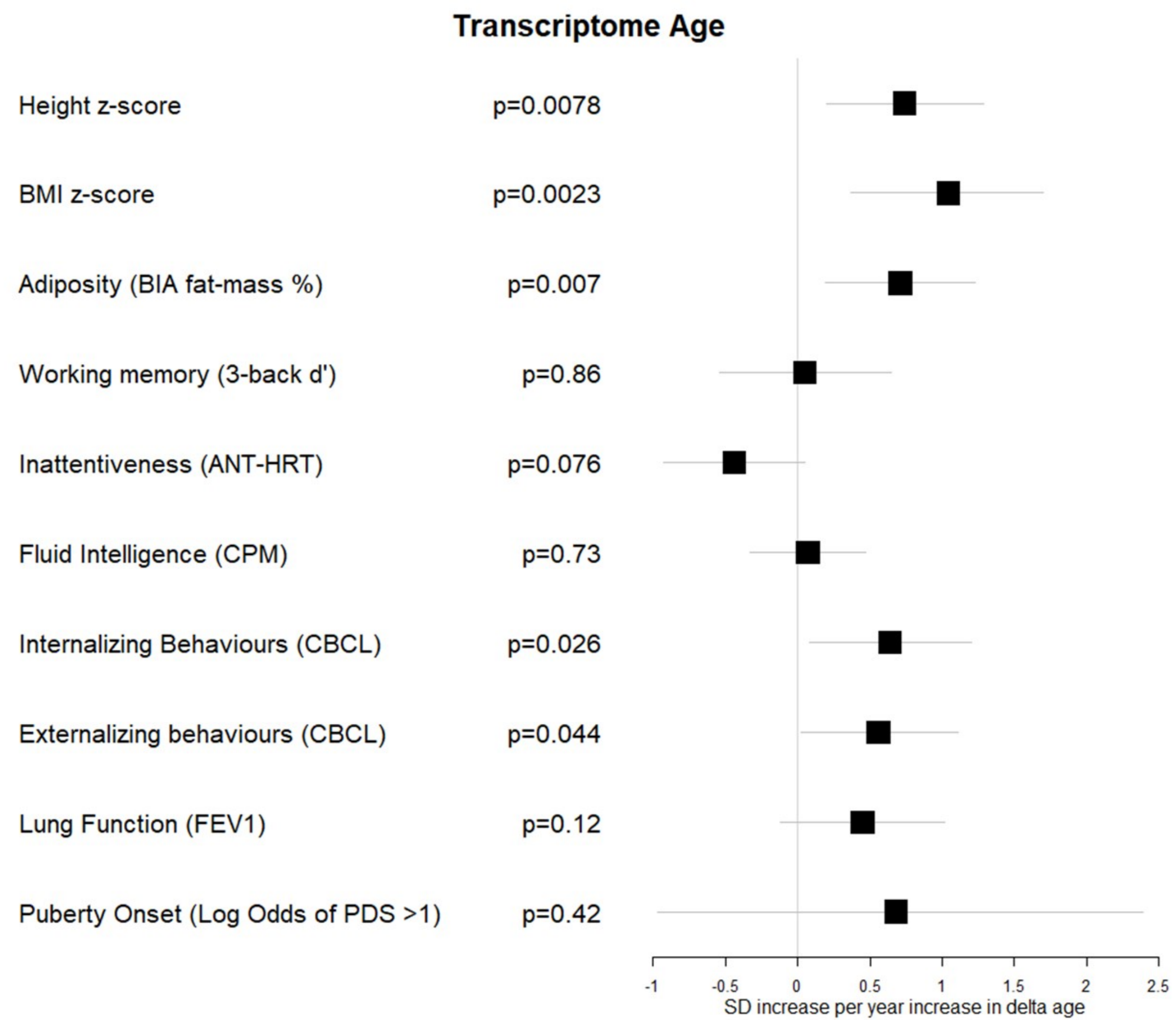
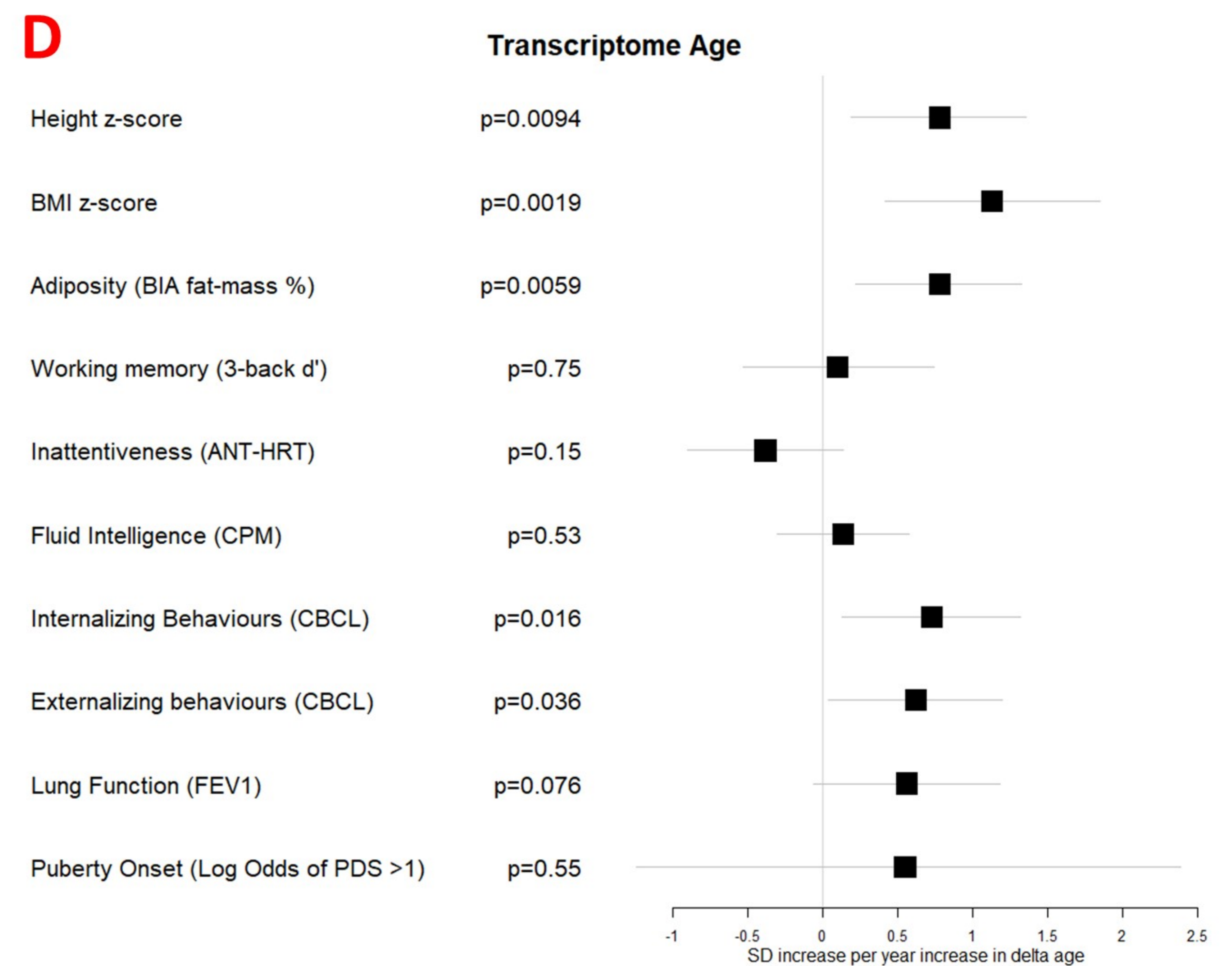
■ Boys ● Girls

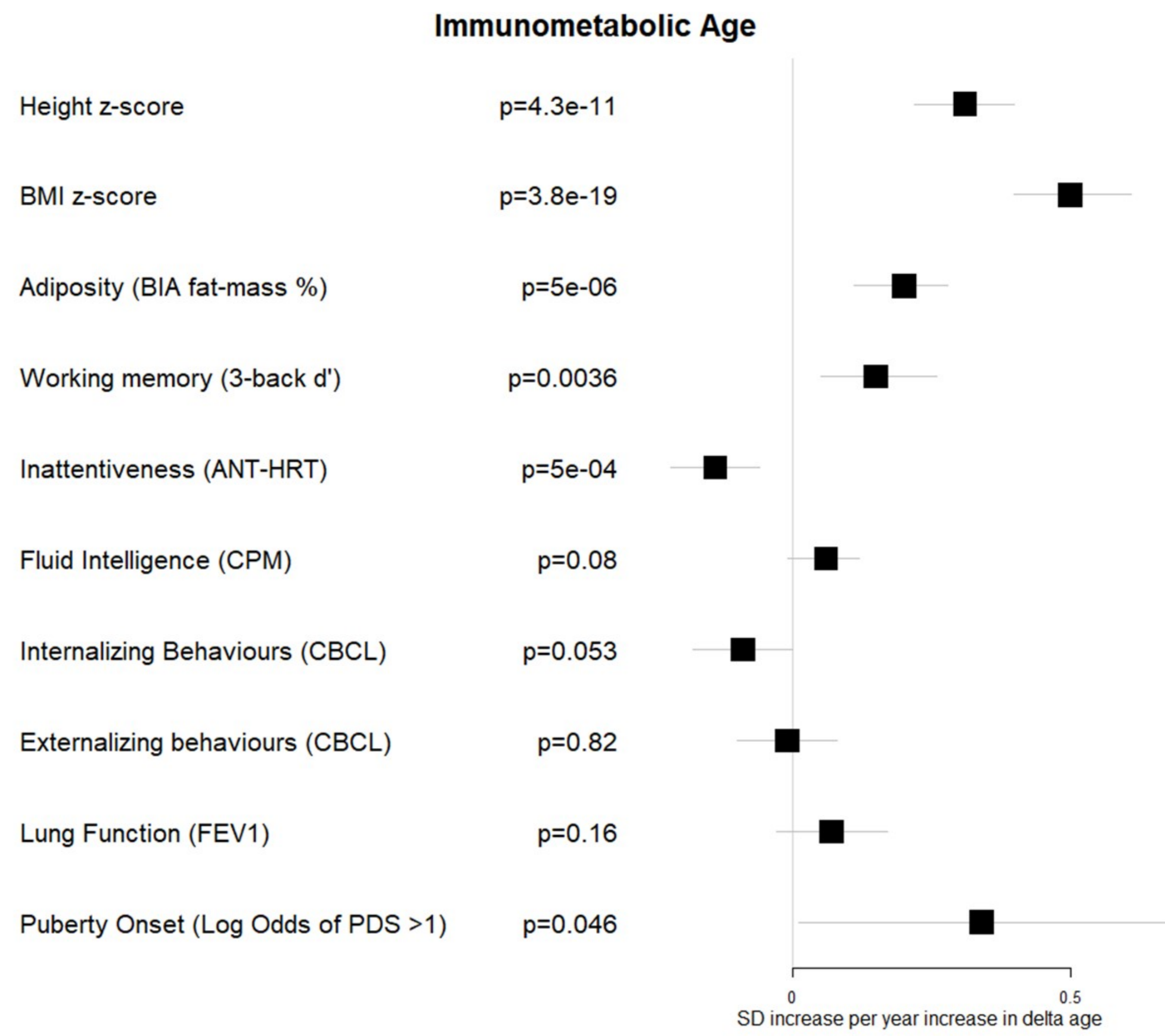
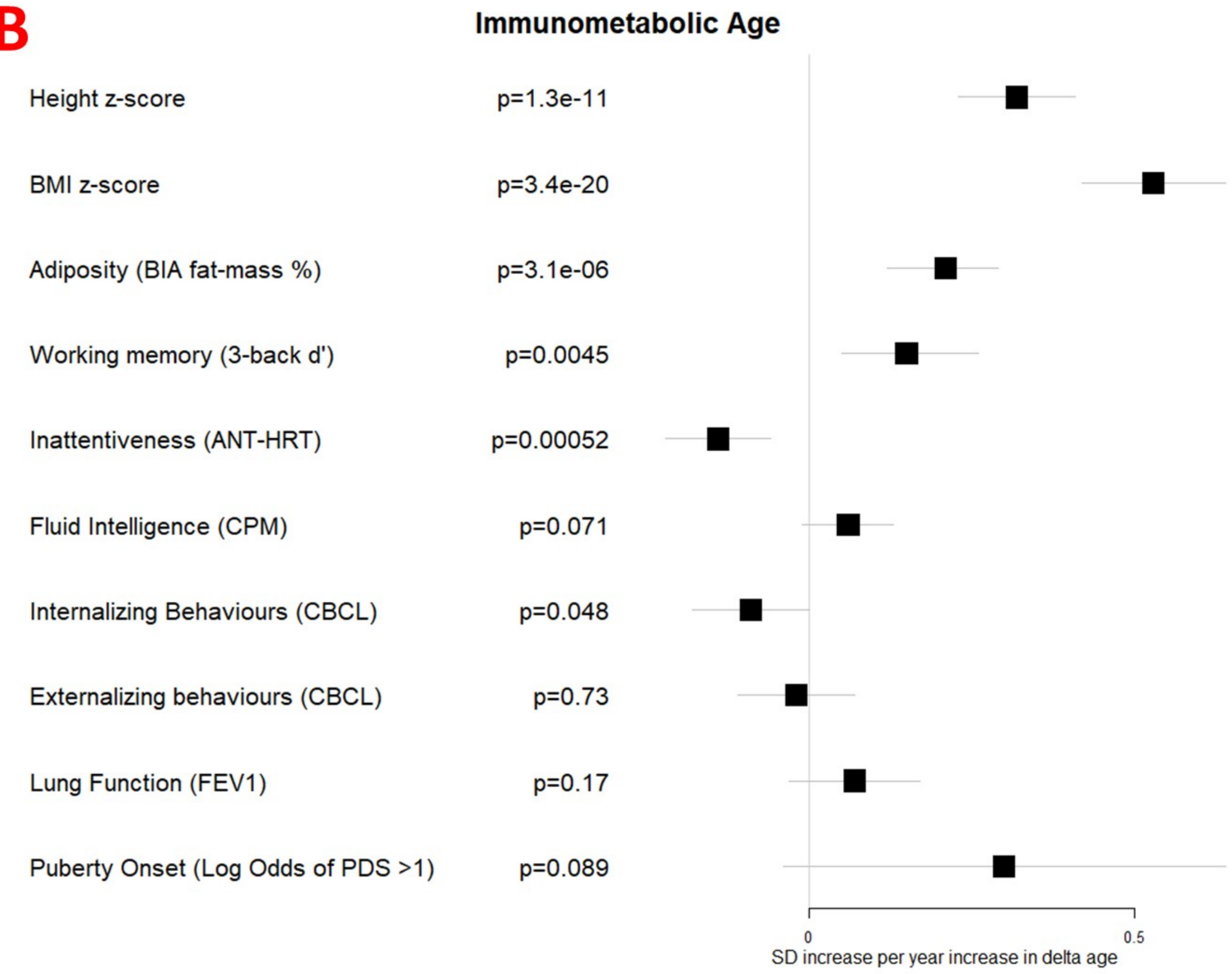
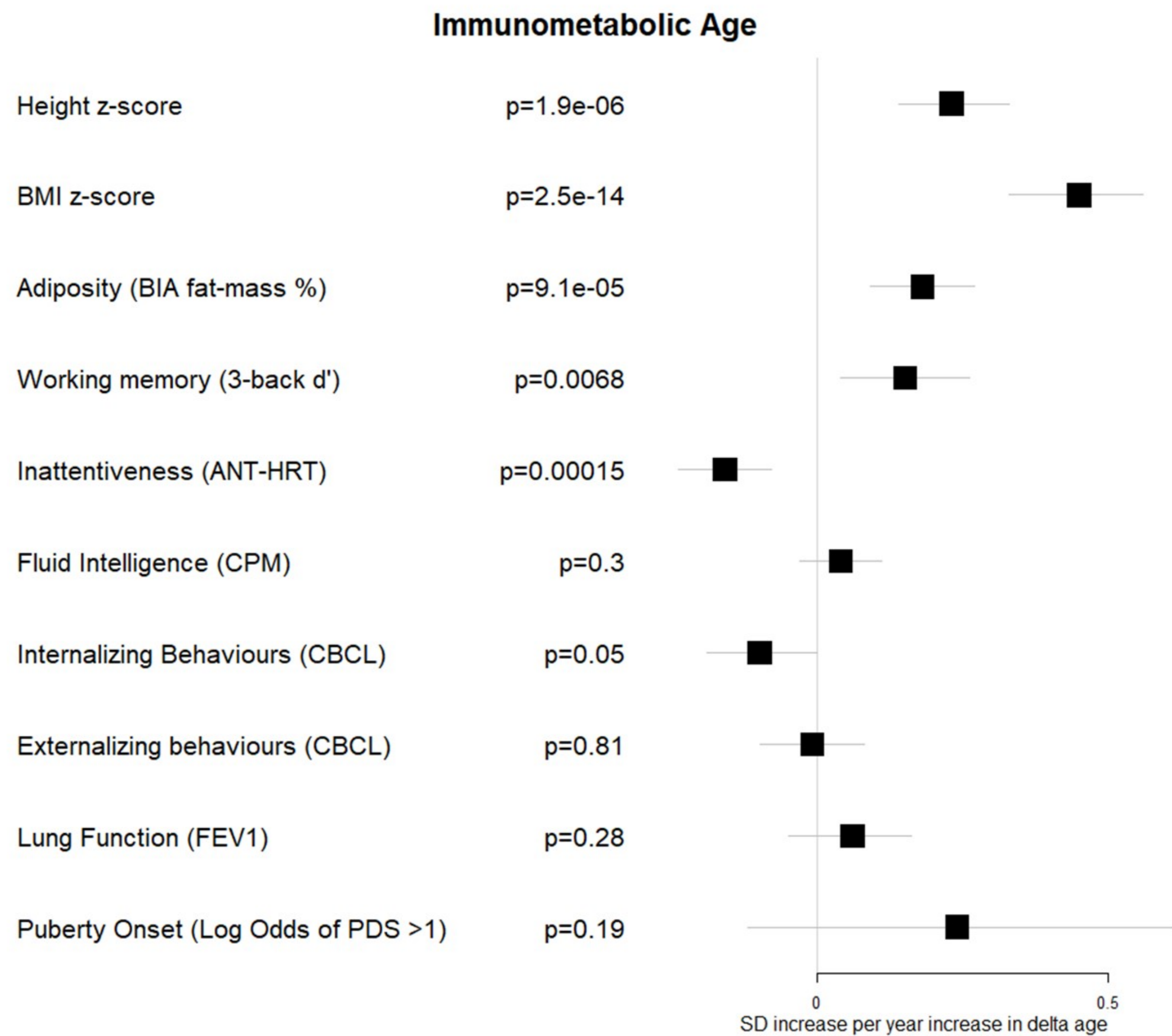
Variable	Boys p-value	Girls p-value
Height z-score	p=6.5e-06	p=3.2e-06
BMI z-score	p=7.7e-10	p=1.6e-10
Adiposity (BIA fat-mass %)	p=0.0035	p=0.00085
Working memory (3-back d')	p=0.3	p=0.0033
Inattentiveness (ANT-HRT)	p=0.28	p=0.00015
Fluid Intelligence (CPM)	p=0.35	p=0.14
Internalizing Behaviours (CBCL)	p=0.17	p=0.14
Externalizing behaviours (CBCL)	p=0.42	p=0.24
Lung Function (FEV1)	p=0.17	p=0.41
Puberty Onset (Log Odds of PDS >1)	p=0.83	p=0.0083



**A****B****C****D**

**A****B****C****D**

**A****B****C****D**

**A****B****C****D**