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DNA methylation age and perceived age in elderly Danish twins

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Abstract

Perceived age is an easily accessible biomarker of aging. Here, we studied its relation to DNA methylation age (DNAm age) as introduced in (Horvath, 2013) in 180 elderly Danish twins. We found perceived age and DNAm age to be associated with chronological age ($P = 0.04$ resp. $P = 2.2e-10$) when correcting for gender, but did not see an association between perceived age and DNAm age ($P = 0.44$). Intrapair-analysis showed that the proportion of pairs where the twin with the highest perceived age also had the highest DNAm age was not different from 0.5 ($P = 1$), and we did not see a trend when dividing pairs according to their difference in perceived age ($P = 0.36$). Hence, intrapair analysis did not reveal links between perceived age and DNAm age. Moreover, none of the 353 CpGs underlying DNAm age was individually associated with perceived age after correction for multiple-testing ($P > 6e-4$, $FDR > 0.21$). Finally, when constructing an epigenetic signature based on these CpGs to predict perceived age, we only found a correlation of 0.18 (95%CI: -0.06 to 0.40) and a mean square error of 13.6 years² between observed and predicted values in the test dataset, indicating poor predictive strength. Altogether, our results suggest that perceived age and DNAm age capture different aging aspects.

1. Introduction

Aging related changes are manifold, span from molecules to organisms and include the loss of structural intactness and functional performance of tissues. Vice versa, these changes can be considered indicators of aging and can be used to predict aging phenotypes or mortality. Perceived age (how old one looks for one's age) is a simple estimate of the age of a person. In (Christensen et al., 2009), the correlation with chronological age was estimated to be 0.52 in an elderly sample. When controlling for chronological age, perceived age was also significantly associated with physical and cognitive functioning as well as leucocyte telomere length and survival. Further associations to bone status were found by (Nielsen et

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al., 2015). Overall, the major sources of variation in perceived age were identified to be genetic and environmental factors to equal parts (Gunn et al., 2009), and recently (Liu et al., 2016) performed the first GWAS of perceived age and found variants in *MC1R*, a pigmentation gene, to be associated.

Aging related changes are also reflected in epigenetic phenomena, such as DNA methylation. Recently, genome-wide DNA methylation levels of CG dinucleotides (CpGs) have become accessible, and studies have shown that methylation profiles change with age and are influenced by both genetic background and environmental factors (Huidobro et al., 2011). Links between epigenetic mechanisms and the development of diseases were uncovered in (Maunakea et al., 2010) and (Murphy and Mill, 2014). Age-related changes in methylation profiles have been used to construct DNA methylation signatures known as “epigenetic clocks” or DNA methylation age (DNAm age), which show considerably high correlations of 0.7 or greater with chronological age, (Horvath, 2013), (Hannum et al., 2013), and were shown to be associated with mortality, see (Marioni et al., 2015a, 2015b), (Christiansen et al., 2015) and (Chen et al., 2016). Moreover, DNAm age acceleration (residual of DNAm age regressed onto chronological age) was associated with, among other phenotypes, physical and mental fitness in elderly (Marioni et al., 2015a, 2015b) and frailty (Breitling et al., 2016).

Altogether, both DNAm age as well as perceived age have been suggested to be measures of biological age. The concept of biological age loosely aims at quantifying the true global state of aging individuals and resolving the discrepancy between the average life expectancy and individual life expectancies, cp. (Jackson et al., 2003) and (Klemera and Doubal, 2006). However, it is still largely unclear what induces individual differences in DNAm age and perceived age and a better understanding of their possible interplay would be desirable. It is the aim of this study to investigate, whether DNAm age and perceived age capture different aspects of aging, or whether they can be considered correlated estimates of a person’s biological age.

Our study sample consisted of 180 elderly Danish twins. DNA methylation age was estimated using the frequently applied Horvath prediction model. Perceived age had been assessed based on ratings from facial photographs.

2. Materials and methods

2.1. Study samples

The study samples were drawn from the Longitudinal Study of Aging Danish Twins (LSADT), which includes all Danish twins born between 1907 and 1923. The study was initiated in 1995, and surviving twins were followed-up every second year through 2007 (Christensen et al., 1999) and (McGue and Christensen, 2007).

Perceived age was assessed by 10 nurses based on digital photographs from 2001 for all twins giving their consent (Christensen et al., 2009).

In 1997 and 2007, whole blood samples were collected. DNA methylation was first measured for all pairs in which both twins participated in both waves, and later, for all further monozygotic pairs in which both twins participated in 1997. All of these pairs were same sex pairs.

For this study, we only used the methylation data from the 1997 blood sample, and those 180 subjects were included who had both a blood sample from 1997 and an assessment of perceived age from 2001. Perceived age and DNAm age were thus measured 3.3–4.4 years apart in our study. Whenever we considered either perceived age or DNAm age together with chronological age in this study, we used the age when the picture was taken for the former and the age at blood sampling for the latter.

Our sample consisted of 180 subjects (119 females and 61 males). Among those were 67 twin pairs (45 monozygotic and 22 dizygotic). The co-twins of the remaining 46 twins were not part of this study, as they did not have measurements on perceived age. All included twin pairs were same sex pairs.

2.2. DNA methylation

DNA was isolated from buffy coats applying the salt precipitation method (Miller et al., 1988). Bisulfite treatment of 500 ng genomic DNA was performed with the EZ Methylation Gold kits (Zymo Research, Orange County, CA, USA). DNA methylation was measured using the Infinium HumanMethylation450 K BeadChip (Illumina, San Diego, CA, USA) following the manufacturer's protocols, and BeadChip images scanned using the iScan system. Data preprocessing was carried out using the free R packages MethylAid (van Iterson et al., 2014 and Tobi et al., 2015) and Minfi (Aryee et al., 2014). The following criteria were used for sample exclusion: firstly, samples where less than 95% of the probes had a detection P-value < 0.01 , and secondly, samples which failed inspection of the internal quality control probes of the bead chip, which are used by the MethylAid package for identifying low quality samples (see van Iterson et al. for details). Probes were excluded if they had a detection P-value > 0.01 , a raw intensity value of zero, had a low bead count (< 3 beads), were identified as being cross reactive (Chen et al., 2013) and/or had a measurement success rate below 95%. After probe filtering a sample success rate of 0.95 was applied; no samples were excluded at this step. Normalization was carried out by Functional normalization (Fortin et al., 2014) with 4 principal components.

Zygosity of the twin pairs was inspected considering values for the 65 SNPs of the bead chip, see (Wang et al., 2015). For 110 samples, this zygosity was confirmed by a previous ascertainment using microsatellite markers (Christiansen et al., 2003).

Blood cell counts were not available for the present study population. However, such data were available for 695 Danish twin individuals without perceived age data (Starnawska et al., 2017). Hence, blood leukocyte subtypes (monocytes, lymphocytes, basophils, neutrophils, eosinophils) for these individuals were used for imputation of the cell composition for the present study population. Imputation was performed by a modified version of the method supplied by PredictCellComposition (www.github.com/mvaniterson/predictcellcomposition) employing partial least squares regression after setting values of 0 to

0.0001, i.e. inflating percentages below the minimum value observed (0.0099), and transforming the cell composition using an isometric log ratio transform. As predictors we used the CpGs' beta values, age, sex and technical variables indicating array position and at which occasion DNA methylation had been measured (first or later; see Study Samples section).

For each twin, we calculated DNAm age following the protocol of (Horvath, 2013). Thereby, missing beta values were estimated using k-nearest neighbour averaging. All 353 CpGs underlying DNAm age were available in our dataset. Out of these, 284 CpGs had no missing values in any of the samples.

2.3. Statistical analysis

In this study we considered the following age measures: chronological age, perceived age, DNAm age as well as delta perceived age and delta DNAm age. For the calculation of delta perceived age and delta DNAm age (given by perceived age-chronological age and DNAm age-chronological age, respectively), we used the corresponding chronological age, see Study Samples section. When perceived age, DNAm age, and chronological age were included into an analysis at the same time, we used the average of the corresponding chronological ages (age at picture and age at blood sampling).

Overall we performed the following analyses: We tested for associations between either DNAm age or perceived age with chronological age. Then we tested for associations between (delta) perceived age and (delta) DNAm age. Intrapair analyses were used to investigate to what extent twins with the highest perceived age also have the highest DNAm age. Finally, we used individual CpGs to predict perceived age.

All regression analyses, that is regressing (delta) perceived age or (delta) DNAm age onto chronological age, (delta) perceived age onto (delta) DNAm age as well as perceived age onto individual CpGs, were carried out with mixed effect models using twin pairing as random factor in order to accommodate for possible correlations between twins from the same pair. Gender was considered a major confounder and was included in all regression analyses. When regressing (delta) perceived age onto (delta) DNAm age we also considered chronological age as optional covariate.

Moreover, for the analyses regressing perceived age onto a single CpG's beta value, Eosinophilo-, Mono- and Lymphocytter as well as a factor variable indicating the array were included as additional covariates besides gender in order to control for cell composition and technical confounders. To take multiple testing into account, we also calculated false discovery rates according to (Benjamini and Hochberg, 1995).

In connection with the intrapair analyses, twin pairs were divided into five groups according to their gap in perceived age based on quintiles. Finally, a trend test was applied to the proportions of pairs where the twin with the highest perceived age also had the highest DNAm age (depending on the size of the gap in perceived age).

In order to construct an epigenetic signature that predicts perceived age, we used a penalized regression approach implemented in the R package glmnet (Friedman et al., 2010). We

regressed perceived age onto gender and those CpGs underlying DNAm age that were available for all samples. The dataset was divided into a test and a training set, whereby the latter consisted of 113 randomly chosen, unrelated samples (i.e. one twin from each pair). The model was fitted in the training set with the alpha parameter of glmnet set to $\alpha = 0.5$ (elastic net regression) and choosing the lambda parameter, which controls the strength of the penalty, by leave-one-out cross-validation.

For the analyses, we used R version 3.3.1.

3. Results

Table 1 outlines the basic characteristics of the 180 twins from the Longitudinal Study of Aging Danish Twins (LSADT). The majority of the sample is female (66%). Mean perceived age was 78.62 (SD = 3.79) thus being approximately 3 years below the average chronological age when the picture was taken. This indicates that our study subjects are among the healthiest as the overall perceived age in (Christensen et al., 2009) fitted very well with the chronological age. (The sample of (Christensen et al., 2009) contained 1826 twins, which also contained our 180 samples, and mean perceived age and mean chronological age were 77.8 (SD = 4.2) and 77.7 (SD = 5.9).) DNAm age in our sample was on average 73.41 (SD = 6.29) years being approx. 4.5 years below the corresponding age at blood sampling.

3.1. DNAm age and perceived age versus chronological age

In Fig. 1a, DNAm age and perceived age were separately plotted against chronological age showing in both cases the expected correlation with chronological age. The overall correlation coefficient was 0.17 (95%CI: 0.02–0.31) for perceived age and 0.46 (95%CI: 0.34–0.57) for DNAm age. Additionally, it can be seen that both measures of biological age underestimate chronological age in this sample. Fig. 1a is further consistent with results from a regression of perceived age and DNAm age onto chronological age and gender, which showed an association to chronological age ($P < 0.05$) for both perceived age and DNAm age, cp. Table 2a. Gender was not significant in any of the two regressions ($P > 0.05$).

3.2. DNAm age versus perceived age

To investigate links between perceived age and DNAm age, Fig. 1b plots perceived age against DNAm age, and delta perceived age against delta DNAm age. No evident relation is found in either of these plots. Consistent with that, for perceived age and DNAm age, we see only a low correlation of -0.04 ; and for delta perceived age and delta DNAm age the correlation was -0.05 . We further explored the relation by regressing perceived age onto DNAm age at the same time controlling for gender and chronological age, cp. Table 2b. This analysis showed no significant association (Beta = -0.086 ; SE = 0.048; $P = 0.073$), which remained unchanged when removing chronological age from the included covariates. A regression of delta perceived age onto delta DNAm age when correcting for gender did not show an association either ($P > 0.05$). In none of the above regression analyses was gender significant ($P > 0.05$).

3.3. Intrapair analyses

To control partly for genetic and environmental factors shared within a twin pair, we performed intrapair analyses using the 67 complete pairs. Considering in how many twin pairs the twin with the highest perceived age also had the highest DNAm age, we found 34 out of 67 pairs, which did not indicate the presence of a within-pair association between perceived age and DNAm age in our dataset. We further investigated whether this proportion varies with the size of the difference in perceived age, whereby we divided the twin pairs into five groups according to their gap in perceived age based on quintiles corresponding to [0,0.7], (0.7,1.7], (1.7,3.0], (3.0,5.4] and (5.4, 8.5] years, cp. Fig. 2. Although we observed proportions ranging from 0.3 to 0.7, none of these was significantly different from 0.5 and there was no clear trend ($P = 0.36$).

3.4. Prediction of perceived age using CpGs

Subsequently, we explored potential links between perceived age and DNAm age at the level of CpGs, considering the individual CpGs underlying DNAm age. We searched for an epigenetic signature, i.e. a subset of CpGs able to predict perceived age using the training set together with gender and those 284 CpGs as potential predictors that were available in all of our samples. The selected prediction model included 18 CpGs, but not gender. Fig. 3 shows predicted against observed perceived age in the training as well as the test dataset. In the training data, we found a correlation of 0.63 (95%CI: 0.51–0.73; p -value = 5.9×10^{-14}) and a mean square error of 10.4 years² between observed and predicted values. Evaluating this model in the test data, we found a correlation of 0.18 (95%CI: –0.06 to 0.40; P -value = 0.14) and a mean square error of 13.6 years², which is comparable to the variance of perceived age (Var = 13.3 years² in test data). Altogether, the predictive strength in the test data was very low, despite the fact that the test data consisted of co-twins of samples in the training data. Finally, all 353 CpGs underlying DNAm age were individually tested for an association with perceived age and delta perceived age. None of the CpGs obtained a p -value below 6.0×10^{-4} and 2.5×10^{-3} , respectively, and false discovery rates (FDRs) were found to be above 0.21 and 0.37, respectively. Hence we did not see a statistically significant association when taking multiple testing into account, referring to an adjusted p -value threshold of $0.05/353 = 1.4 \times 10^{-4}$.

4. Discussion

In this study, we investigated the relation between perceived age and DNAm age in a sample of elderly Danish twins. Firstly, we found both biomarkers to be significantly associated with chronological age. This has also been seen in previous works, cp. (Christensen et al., 2009) and (Horvath, 2013), although it was less pronounced in our study, which is likely due to a smaller sample size and smaller age range. However, we did not detect a significant association between perceived age and DNAm age. This was also the case when considering individual CpGs underlying DNAm age instead, and building a predictor for perceived age based on the same CpGs did not have considerable predictive strength either. Finally, an intrapair analysis did not reveal additional insights. Gender did not have a significant impact in any of our analyses. Altogether, our results suggest the hypothesis that perceived age and DNAm age either capture different aspects of the aging process or that these two biomarkers

are so weakly correlated that we were not able to capture the association with the current sample size.

The following speaks in favor of such a hypothesis: Perceived age can be considered a biological age, as it (if combined with chronological age) reflects a person's true health better than chronological age alone, see (Christensen et al., 2009). DNAm age, however, was originally constructed as a simple predictor of chronological age not aiming at predicting a person's health status (Horvath, 2013). The fact that the association between chronological age and DNAm age was statistically much stronger ($P\text{-value} = 2.2\text{e-}10$) than between chronological age and perceived age ($P\text{-value} = 0.04$) in our study potentially supports this point of view. Nonetheless, several studies have shown that DNAm age is independently of chronological age associated with aging phenotypes and mortality, e.g. (Breitling et al., 2016), (Christiansen et al., 2015) and (Marioni et al., 2015a, 2015b). This provides support for DNAm age being at the same time a measure of biological age.

On the other hand, the detection of an existing association between perceived age and DNAm age might be hampered by the following: Firstly, the fact that perceived age and DNAm age were measured 3.3–4.4 years apart in our study loosens potential correlations. Secondly, although the sample size of 180 subjects was moderately large, this could still result in a lack of power if the effect size is small. And thirdly, unaccounted confounding factors such as drug exposure might be involved (Mill and Heijmans, 2013).

Finally, additional reasons might lie in the special nature of the study population. Samples have been selected based on the fact that both twins of a pair were alive in 1997, when they were 77.96 years of age (or for the included DZ pairs even alive in 2007), which likely leaves us with subjects relatively fit compared to the general population as also indicated in both biomarkers, which showed age estimates substantially lower than chronological age (on average 3.08 years for perceived age and 4.55 years for DNAm age). This kind of sampling bias could possibly distort an association between DNAm age and perceived age.

Only very limited knowledge is available about the genetic background of perceived age, which could relate to our findings. Recently it has been estimated that approximately half of the variation of perceived age is due to genetic factors, whereas the other half is due to environmental influences (Gunn et al., 2009). The first GWAS of perceived age (Liu et al., 2016) performed in a sample of elderly Dutch individuals identified multiple SNPs in the MC1R gene, a pigmentation gene, to be associated with perceived age, and hence pointed to specific genetic variants. As epigenetic mechanisms are ubiquitous in aging processes, it can be expected that epigenetic variation also contributes to both the genetic and non-genetic component of perceived age. Nonetheless, to our knowledge, no study has considered the epigenetics of perceived age up to now. Our article systematically explores the link between DNAm age and perceived age, and our results suggest that these two biomarkers capture different aspects of ageing.

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Fig. 1.

a: Horvath DNAm age and perceived age against chronological age. The relations are visualized by a loess curve. The dashed curve in each plot is the line of identity. b: Perceived age against DNAm age, and delta perceived age (perceived age-chronological age) against delta DNAm age (DNAm age-chronological age). The relations are visualized by a loess curve. The dashed curve in each plot is the line of identity.

Fig. 2.

Proportion of perceived-oldest twins also being DNAm-oldest according to intrapair difference in perceived age.

The plot shows the proportion of twin pairs where the twin with the highest perceived age also had the highest DNAm age against the respective intrapair difference in perceived age. Thereby, intrapair differences have been divided into quintiles Q1 = [0,0.7], Q2 = (0.7,1.7], Q3 = (1.7,3.0], Q4 = (3.0,5.4] and Q5 = (5.4, 8.5] (in years) and each quintile corresponds to 13 or 14 pairs.

Fig. 3. Predicted against observed perceived age in the training and test data containing 113 and 67 samples, respectively. The relations are visualized by a loess curves. The dashed curves are the corresponding lines of identity.

Table 1

Characteristics of the study population.

Sex	Age at picture (SD)	Perceived age (SD)	Age at blood sampling (SD)	DNAm age (SD)	N
Male	81.58 (3.47)	78.01 (3.42)	77.81 (3.39)	73.75 (5.99)	61
Female	81.76 (3.59)	78.93 (3.95)	78.04 (3.59)	73.23 (6.46)	119
Total	81.70 (3.54)	78.62 (3.79)	77.96 (3.51)	73.41 (6.29)	180

Table 2a
Perceived age and DNAm age vs. chronological age and gender; results of regression analyses; P-values 0.05 are shown in bold.

Perceived age				DNAm age			
Covariates	Beta	SE	P-value	Covariates	Beta	SE	P-value
Age (picture)	0.2	0.1	0.04	Age (blood)	0.8	0.1	2.2e-10
Gender	0.8	0.7	0.22	Gender	-0.7	1.0	0.50

Perceived age and delta perceived age vs. DNAm age and delta DNAm age, respectively; results of regression analyses correcting for gender (and chronological age); P-values 0.05 are shown in bold.

Table 2b

Perceived age				Delta perceived age			
Model including chronological age				Model Including chronological age			
Covariates	Beta	SE	P-value	Covariates	Beta	SE	P-value
DNAm age	-0.1	0.05	0.07	Delta DNAm age	-0.1	0.05	0.07
Age	0.3	0.1	8.1e-3	Age	-0.8	0.1	2.8e-21
Gender	0.8	0.7	0.25	Gender	0.8	0.7	0.24

Model without chronological age				Model without chronological age			
Covariates	Beta	SE	P-value	Covariates	Beta	SE	P-value
DNAm age	-0.03	0.04	0.44	Delta DNAm age	-0.1	0.1	0.35
Gender	0.9	0.7	0.20	Gender	0.5	0.9	0.60