LETTER TO THE EDITOR

Clonal hematopoiesis of indeterminate potential-related epigenetic age acceleration correlates with clonal hematopoiesis of indeterminate potential clone size in patients with high morbidity

Clonal hematopoiesis of indeterminate potential (CHIP) is defined as the presence of myeloid cancer-associated, somatic mutations with a variant allele frequency (VAF) of ≥2% in the hematopoietic cells of individuals without hematologic malignancy.[1] Its prevalence steeply increases with age, affecting about 15-20% of individuals of ≥60 years, indicating an association with aging processes. *DNMT3A*, *TET2*, and *ASXL1* are the most frequently mutated genes and can all be classified as epigenetic modifiers.[2-4] CHIP is associated with various age-related, adverse health conditions, e.g., cardiovascular diseases and stroke.[2] Additionally, CHIP bares the inherent risk of progression to hematologic malignancy.[1-3] Together, these adverse effects translate into an increased overall mortality.[2,3]

There are nine well-accepted hallmarks of aging, which comprise genomic instability and epigenetic alterations.[5] As CHIP can be interpreted as genomic instability, matching CHIP state with other established markers of aging is of high interest. DNA methylation patterns change over an individual's life, allowing the establishment of "epigenetic clocks" that estimate epigenetic age through regression of methylation ratios at predefined CpG sites on chronological age.[6-8] Epigenetic age acceleration has been shown to correlate with various age-associated conditions and overall mortality (reviewed by Horvath and Raj[6]).

Due to its interrelatedness with older age and aging-associated conditions, we asked whether the presence of CHIP itself is a surrogate marker of an enhanced biological aging process. We therefore determined epigenetic age measures using an established adaption of the epigenetic clock[9] originally published by Vidal-Bralo and colleagues[8] in a cohort of 381 individuals with previously determined CHIP status.[10]

Blood samples were collected from 417 inpatients from the Departments of Cardiology, Nephrology, Musculoskeletal Surgery, and Oncology at Charité – Universitätsmedizin Berlin as reported previously.[10] Tumor patients were included before starting (radio-)chemotherapy. Individuals with hematologic malignancy were excluded. The study was conducted in accordance with the Declaration of Helsinki and patients gave informed consent.

Whole blood DNA of the study participants was screened for CHIP (VAF ≥2%) as performed and published previously.[10] Methylation status of CpG sites of interest was measured according to a protocol for the methylation-sensitive single-nucleotide-primer-extension method (ms-SNuPE) developed by Vidal-Bralo and colleagues[8] and adapted by Vetter and colleagues.[9] DNA methylation (DNAm) age was calculated with the 7-CpG clock using a regression model from multiple linear regression of chronological age on methylation fractions of the Berlin Aging Study II cohort. Age acceleration (AA) was determined as the residuals from regressing DNAm age on chronological age. Intrinsic epigenetic age acceleration (IEAA) was determined as the residuals from regressing DNAm age on chronological age and blood cell counts.[9] Alpha-level was set at 0.05, and IBM SPSS Statistics software (IBM, USA) was used.

Methylation ratios were successfully determined for 381 of 417 individuals (=91%) (*Online Supplementary Figure S1A*). Mean chronological age was 74.7 years (standard deviation [SD]= ±7.9 years, range, 55-98 years) and 45% of individuals were female. Our cohort was characterized by a high prevalence of clinically relevant morbidities as detailed in Table 1. Of the 381 individuals, 106 (=28%) had evidence of CHIP. Median VAF was 6% (Figure 1A), and an overview of all mutations is presented in Figure 1B and the *Online Supplementary Table S2*. The mean DNAm age was 76.2 years (±9.7 years), mean DNAm age acceleration was ±8.4 years, and mean IEAA was ±8.1 years.

AA (n=381) and IEAA (n=297) were analyzed with regard to baseline and clinical characteristics. In line with existing data[6], AA and IEAA were significantly higher in males than in females (AA: P=0.006; IEAA: P=0.001) (Figure 1C). No statistically relevant differences in AA could be noticed with regard to medical conditions, except for hyperuricaemia (AA: P=0.001; IEAA: P<0.001) and kidney failure requiring hemodialysis (AA: P<0.001; IEAA: P=0.018) (*Online Supplementary Table S1*).

In our study, the general presence of CHIP was not associated with age acceleration (AA: P= 0.183; IEAA: P=0.513), nor were individual CHIP mutations (e.g., *DNMT3A*, *TET2*, *ASXL1*), functional CHIP groups or the presence of more than one mutation when compared to individuals without CHIP (Figure 2A to C; *Online Supplementary Figure S1A to C*). In order to obtain a more differentiated view, we investigated the relationship between CHIP clone size and epigenetic age acceleration. First, patients with CHIP were subdivided into three groups according to clone size.

Table 1. Baseline characteristics of 381 individuals analyzed in our study. Cases are reported as N (%) unless otherwise specified.

Variable	Missing data	Cases	CHIP+
Demographics			
Female	0	172 (45%)	46 (27%)
Male	0	209 (55%)	60 (29%)
Age, years (mean [SD])	0	74.7 (±7.9)	
Age, <61		3 (0.8%)	1 (33%)
Age, 61-70		129 (33.9%)	18 (14%)
Age, 71-80		171 (44.9%)	57 (33%)
Age, >80		78 (20.5%)	30 (38%)
Clinical data			
Malignant tumor	0	69 (18%)	22 (32%)
Coronary heart disease	4	183 (49%)	47 (26%)
Cardiac insufficiency	4	150 (40%)	42 (28%)
Cardiac arrhythmia (including atrial fibrillation)	4	193 (51%)	54 (28%)
Cerebrovascular disease	4	67 (18%)	17 (25%)
Peripheral artery disease	4	69 (18%)	24 (35%)
Arterial hypertension	4	307 (81%)	83 (27%)
Type 2 diabetes mellitus	4	113 (30%)	32 (28%)
Hyperlipidemia	4	207 (55%)	62 (30%)
Obesity (BMI ≥ 30)	4	87 (23%)	16 (18%)
Hyperuricemia	4	45 (12%)	15 (33%)
Kidney failure (all)	4	149 (40%)	44 (30%)
ESRD	4	39 (10%)	8 (21%)
Chronic obstructive pulmonary disease	4	50 (13%)	12 (24%)

SD: standard deviation; BMI: body mass index; ESRD: end stage renal disease. CHIP: clonal hematopoiesis of indeterminate potential.

Clones with a VAF <10% were considered "small to intermediate", while clones with a VAF of ≥10% were considered "large". A third group was defined by the absence of CHIP. Using ANOVA, significant differences between the individual groups were detected (*P*=0.023). This finding remained significant after correction for sex and age using ANCOVA (*P*=0.022). Differences in AA and IEAA were most pronounced when comparing individuals without CHIP to those with large clones (contrast analysis: *P*=0.01) (Figure 2D; *Online Supplementary Figure S2D*). The difference in mean AA amounted to 4.5 years. A comparison of AA between individuals without CHIP and individuals with clones of small to intermediate size showed no differences (*P*=0.718). Next, we sought to identify to which extent smaller clones still affect AA/IEAA. The lowest VAF with significant differences in AA/IEAA compared to AA/IEAA in individuals without CHIP was ≥5% (Kruskal-Wallis-Test: *P*=0.008; Mann-Whitney-Test: *P*=0.008). This effect was likewise significant for IEAA (Kruskal-Wallis-Test: *P*=0.016; Mann-Whitney-Test: *P*=0.044).

In order to further explore the relation between CHIP clone size and DNAm AA while accounting for the continuous character of VAF, we performed Spearman's correlations for DNAm age acceleration measures and VAF of individuals with CHIP. For individuals with more than one CHIP mutation, VAF of the largest clone was used. Our analysis revealed a statistically significant positive correlation between VAF and AA/IEAA (AA: R_S=0.328, *P*<0.001, IEAA: R_S=0.420, *P*<0.001) (Figure 2E and F). This association remained significant after correction for sex and age (AA: R_S=0.323, *P*<0.001, IEAA: R_S=0.411, *P*<0.001), further supporting a connection between CHIP clone size and accelerated aging processes.

Two previous studies consistently described a significant association of CHIP and epigenetic age acceleration based on methylation array data.[11,12] However, the whole genome sequencing (WGS) methodology applied for detection of CHIP in these investigations is characterized by low coverage, prohibiting the reliable detection of small to intermediate sized CHIP clones. This is reflected by comparably low CHIP frequencies of about 6% in both studies and no CHIP clones with a VAF <6% were included in Nachun and colleagues.[11] However, clones with a VAF of ≥2 and <6% account for a substantial part of CHIP.[13] In our cohort, 54% of CHIP carriers had CHIP mutations with VAF solely between 2-6%, which is within the expected frequency range.

Our key finding is the correlation of CHIP clone size with epigenetic age acceleration, showing that an increase in clone size is associated with increased epigenetic age acceleration. In contrast to the two previous studies, CHIP clones were identified by targeted sequencing with robust

coverage (average depth: 2,400x), allowing reliable detection of small- and intermediate-sized CHIP clones. We could therefore for the first time demonstrate that a significant epigenetic age acceleration can only be detected with CHIP clones of a VAF of 5% or greater, while smaller clones – contributing to a relevant proportion of CHIP in general – do not seem to be associated with acceleration of the epigenetic age. This association matches the results of multiple studies that report an increasing likelihood of CHIP-associated adverse conditions with ascending VAF.[2,13] To the best of our knowledge, the CpG of the clock implemented here are no major targets of the frequently mutated DNMT3A[14] and TET2.[15] Accelerated epigenetic age in CHIP carriers can therefore be interpreted as a reflection of actual accelerated biological aging rather than being an epiphenomenon of altered methylation processes caused by mutations. However, large clones might result from a predominant pool of HSC, possibly implicat-

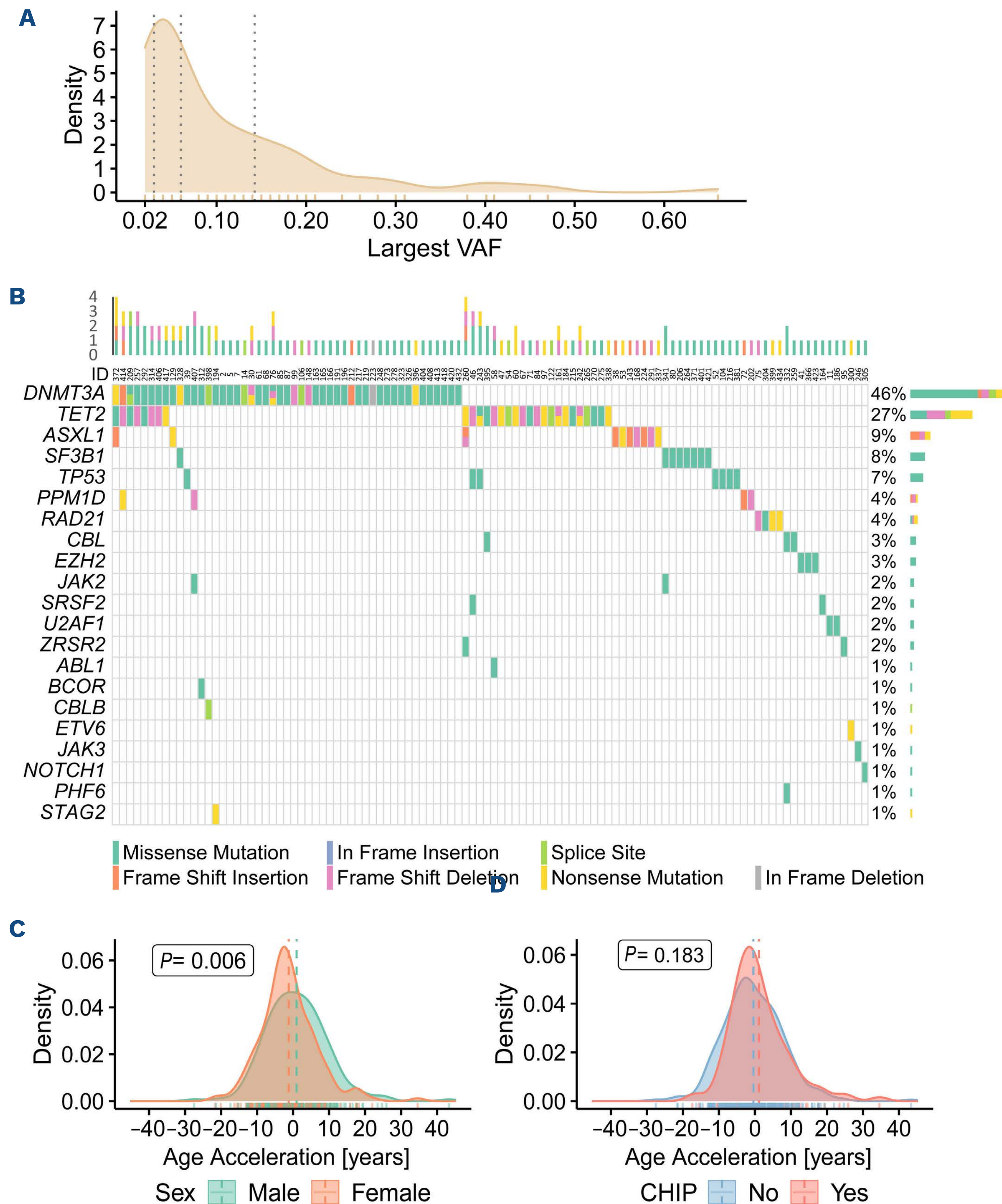

Figure 1. Clonal hematopoiesis of indeterminate potential (CHIP) characteristics and distribution of epigenetic age acceleration according to sex and CHIP status. (A) Density plot depicting the distribution of variant allele frequency (VAF) of largest CHIP mutation per individual. Dotted lines show 1st, 2nd and 3rd quartile. (B) Plot depicting number and type of mutations for the 106 individuals with present CHIP as determined by Arends *et al.*[10] (C and D) Density plots depicting the distribution of age acceleration according to sex (C) and CHIP status (D). Dashed lines show respective means. *P*-values were determined by Mann-Whitney-test.

ing higher cycle numbers and mitotic aging. Even though mitotic and epigenetic aging probably do not address the same aging processes,[9,16] we acknowledge that further research is needed to explore these relations.

Compared to the two previous studies that used methylation array based epigenetic clocks (e.g., by Horvath), our epigenetic age estimations relied on relatively few CpG sites. Nevertheless, our clock performs comparably well and high intercorrelation with Horvath clock was shown.[17] Unlike the previous studies, we could not detect an association of individual mutations with a particularly accelerated epigenetic age. However, due to rather small sample sizes

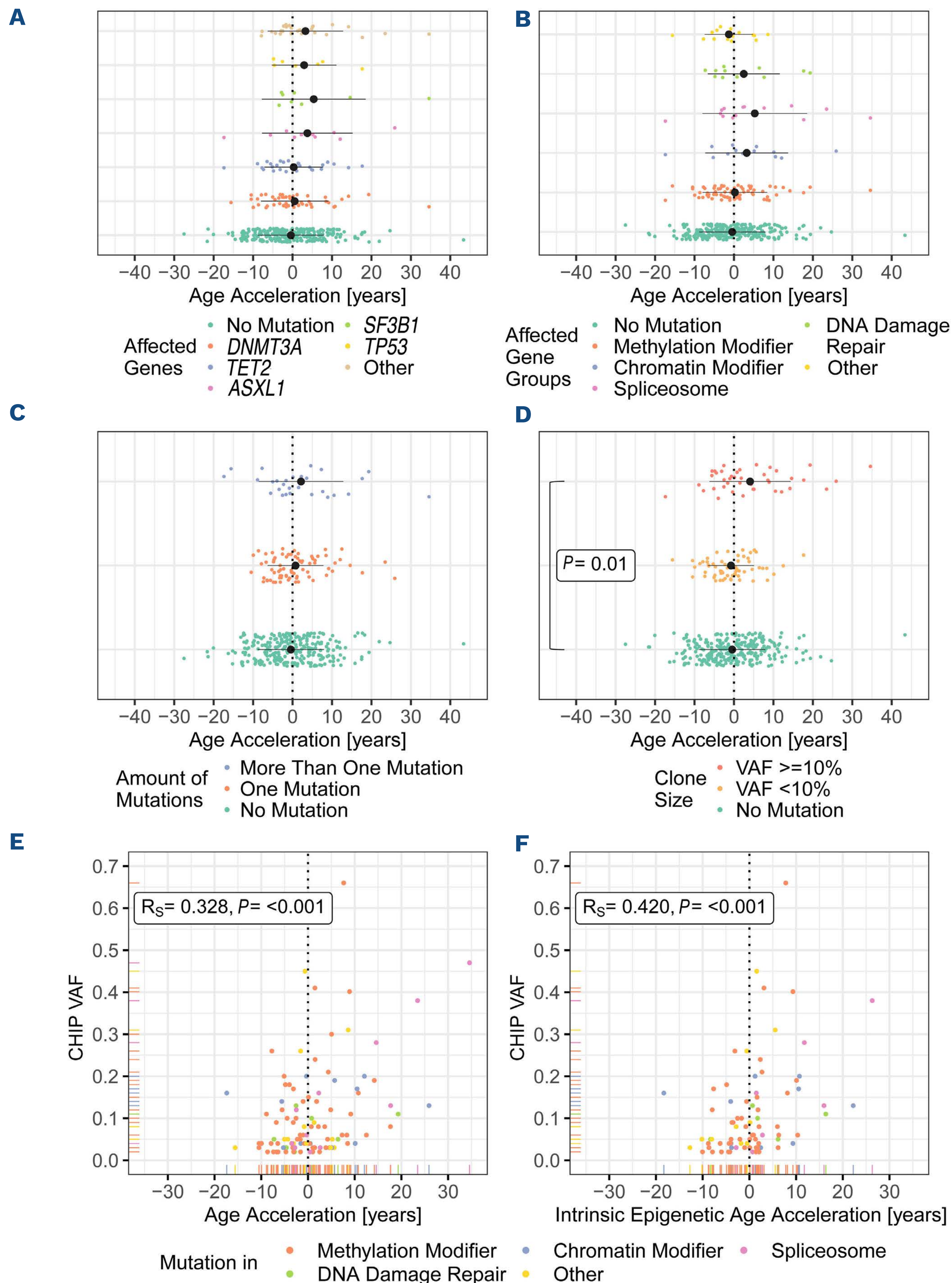

Figure 2. Clonal hematopoiesis of indeterminate potential characteristics and epigenetic age acceleration. (A) Jittered dot plot of epigenetic age acceleration (AA) by affected gene. (B) Jittered dot plot of AA by affected gene group. (C) Jittered dot plot of AA by number of clonal hematopoiesis of indeterminate potential (CHIP) mutations. (D) Jittered dot plot of AA by CHIP clone size. Mean and standard deviation are shown in black. (E and F) Dot plots of AA against CHIP variant allele frequency (VAF). Spearman's correlation of VAF against AA (E) or intrinsic epigenetic age acceleration (IEAA) (F). For patients with multiple CHIP mutations, largest VAF was used for calculation.

in our subgroups, we cannot fully exclude type II errors in this place.
While the study population is heterogeneous with respect to the underlying medical conditions, the overall disease burden of the study cohort is above the population average. Therefore, the study design is not suited to evaluate the impact of individual medical conditions on epigenetic aging. While a generally high level of comorbidities likely restores comparability with regard to CHIP as an independent factor, our study is not powered to investigate associations between medical conditions and combined CHIP/AA status.
In conclusion, our study revealed a correlation of CHIP clone size and accelerated epigenetic aging. This finding matches and extends our knowledge on the role of CHIP clone size and sets the foundation for future investigations exploring the interrelatedness of CHIP and aging.

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Contributions

JF and VV performed experiments, analyzed and interpreted data; MF, ID, and FD designed and supervised research and experiments, CMA collected samples and clinical data, performed experiments, analyzed and interpreted data; JF and MF wrote the manuscript; VV, CMA, TL, LB, FD and ID revised the manuscript. All authors approved the final version.

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Data sharing statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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