Research Article
Viral Hepatitis

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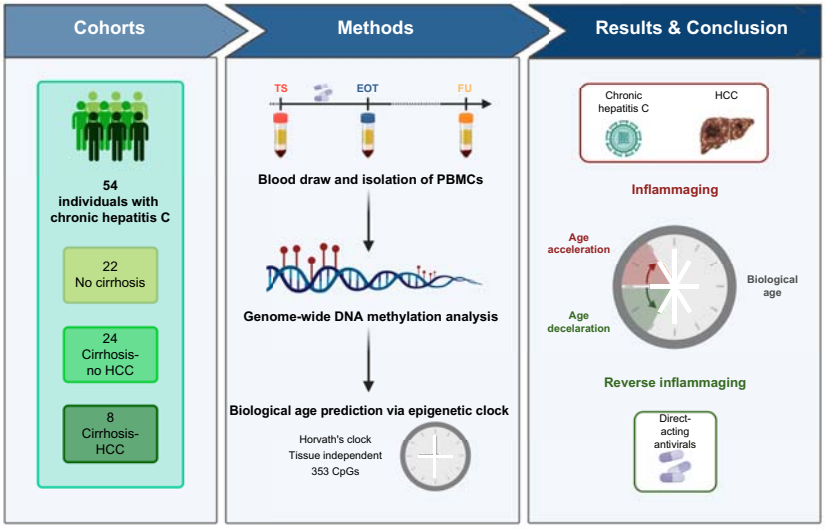
Graphical abstract



Highlights

- Individuals with chronic hepatitis C have accelerated epigenetic age compared to healthy controls.
- DAA treatment and HCV elimination partially reverse the accelerated epigenetic age during long-term follow-up.
- Accelerated epigenetic aging was not reversed during follow-up in those who developed HCC after HCV elimination.

Impact and implications

Chronic hepatitis C virus infection is now curable with direct-acting antivirals, but it remains unclear whether hepatitis C sequelae are fully reversible after viral elimination. Our results suggest that epigenetic changes or acceleration of biological age are reversible in principle, but this requires time, while a lack of reversibility appears to be associated with the development of hepatocellular carcinoma. While most clinical risk scores now take chronological age into account, it may be worthwhile to explore how biological age might improve these scores in the future. Biological age may be a cornerstone for the individualized clinical assessment of patients in the future, as it better reflects patients' lifestyle and environmental exposures over decades.

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Reverse inflammaging: Long-term effects of HCV cure on biological age

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Background & Aims: Chronic hepatitis C virus (HCV) infection can be cured with direct-acting antivirals (DAAs). However, not all sequelae of chronic hepatitis C appear to be completely reversible after sustained virologic response (SVR). Recently, chronic viral infections have been shown to be associated with biological age acceleration defined by the epigenetic clock. The aim of this study was to investigate whether chronic HCV infection is associated with epigenetic changes and biological age acceleration and whether this is reversible after SVR.

Methods: We included 54 well-characterized individuals with chronic hepatitis C who achieved SVR after DAA therapy at three time points: DAA treatment initiation, end of treatment, and long-term follow-up (median 96 weeks after end of treatment). Genome-wide DNA methylation status was determined in peripheral blood mononuclear cells (PBMCs) and used to calculate epigenetic age acceleration (EAA) using Horvath's clock.

Results: Individuals with HCV had an overall significant EAA of 3.12 years at baseline compared with -2.61 years in the age- and sex-matched reference group (p <0.00003). HCV elimination resulted in a significant long-term increase in DNA methylation dominated by hypermethylated CpGs in all patient groups. Accordingly, EAA decreased to 1.37 years at long-term follow-up. The decrease in EAA was significant only between the end of treatment and follow-up (p = 0.01). Interestingly, eight individuals who developed hepatocellular carcinoma after SVR had the highest EAA and showed no evidence of reversal after SVR.

Conclusions: Our data contribute to the understanding of the biological impact of HCV elimination after DAA therapy and demonstrate that HCV elimination can lead to "reverse inflammaging". In addition, our data support the potential use of biological age as a biomarker for HCV sequelae after SVR.

Introduction

Hepatitis C virus (HCV) infection continues to be a major global health burden. According to the World Health Organization (WHO), 1.5 million new HCV infections occur worldwide each year, with 58 million individuals living with chronic HCV infection.[1] The main long-term consequence of chronic hepatitis C is the development of cirrhosis, which is associated with a significant risk of hepatocellular carcinoma (HCC).[2] Furthermore, HCV infection can lead to extrahepatic manifestations such as chronic fatigue, diabetes mellitus or vasculitis.[3] As a result, approximately 290,000 people die each year as a result of HCV infection.[1]

Meanwhile, direct-acting antivirals (DAAs) are available, well tolerated and result in sustained virological response (SVR) rates of more than 95%, leading to a significant reduction of liver morbidity and mortality.[4] However, not all sequelae of chronic hepatitis C seem to be completely reversible after SVR. Patients with advanced fibrosis or cirrhosis have a residual risk for HCC.[5] Impaired quality of life[6] or extrahepatic manifestations such as cryoglobulinemic vasculitis[7] are only partially improved or not reversible in all patients. Interestingly, recently published studies have shown that HCV infection can leave an immunological imprint or scar after SVR, and the impaired immune response characteristic of chronic HCV infection is only partially restored.[8–10]

One mechanism that may explain this is that HCV infection can profoundly affect the epigenome, and it has been shown that many of the epigenetic changes caused by HCV remain as "scars" in different cell types, *i.e.* CD8 T cells and hepatocytes, after viral elimination.[11,12] Of note, HCV-induced epigenetic changes in hepatocytes have been associated with HCC risk which persisted after SVR.[13] DNA methylation (DNAm), defined as the covalent addition of a methyl group to a DNA nucleotide (usually the cytosine of a cytosine-guanine dinucleotide [CpG]), is the most well-studied epigenetic modification that affects transcription factor binding and controls accessibility to regulatory regions in the DNA, modulating gene expression.[14]

Keywords: Hepatitis C virus; epigenetic age; direct-acting antiviral; sustained virological response; cirrhosis; Inflammaging; DNA methylation.
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Numerous studies have shown that epigenetic changes, particularly DNAm, are affected by aging,[15,16] so DNAm status enables us to estimate an individual's biological age. One of the most widely used and validated methods for estimating biological age is "Horvath's clock," which is based on the methylation status of 193 CpGs that gain methylation and 160 CpGs that lose methylation over time.[17]

Thus, the aim of this study was to evaluate if chronic HCV infection is associated with biological age acceleration and if this is reversible after DAA therapy and HCV elimination. For this, we analyzed DNAm in peripheral blood cells isolated from a well-characterized cohort of 54 individuals with chronic hepatitis C before treatment, at the end of treatment and after long-term follow-up.

Patients and methods

Study population and design

Out of 799 individuals with chronic HCV infection treated with DAAs at Hannover Medical School between January 2014 and March 2021, a total of 54 well-characterized patients were selected for this study (Fig. S1). All patients achieved SVR after the end of treatment. The patients are part of a biobank registry and all patients gave written informed consent. Blood samples were collected and stored according to established standard operating procedures.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and the local ethics committee approved this study *a priori* (Nr. 9474_BO_K_2020).

The study comprises three different groups of patients. Cohort A contains 22 individuals without cirrhosis. Exclusion criteria for this group are depicted in Fig. S1. The sampling time points for this group included the start of therapy, the end of treatment, and a follow-up period of 96 weeks. Cohort C includes eight individuals with cirrhosis who developed HCC after SVR. These eight individuals were matched with 24 other individuals with cirrhosis (cohort B) using a propensity score approach.[18] Thus, the total cohort included 32 individuals with cirrhosis. Sampling time points included initiation of therapy, end of treatment, and last available sampling time point (before the development of HCC in the HCC group). The detailed baseline characteristics of patients are shown in Table 1.

Finally, an age- and sex-matched healthy cohort from a publicly available dataset (GSE40279)[19] was used to compare epigenetic age acceleration (EAA) with that observed in individuals with chronic HCV. The datasets generated and analyzed during the current study are available in the Synapse repository (www.synapse.org/#!Synapse:syn32854048).

Sample preparation

Peripheral blood was drawn from patients at the Hannover Medical School outpatient clinic at the indicated time points, and peripheral blood mononuclear cells (PBMCs) were isolated according to a standard Ficoll Hypaque density centrifugation protocol (BioColl separating solution; Biochrom AG, Berlin, Germany). After isolation, cells were transferred to a freezing medium and stored in liquid nitrogen. For analysis purposes, the cells were thawed, counted and then further processed by us using the Monarch Genomic DNA Purification Kit T3010L (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions. We normalized the DNA concentration of all samples after elution to 60 ng/µl, randomized the samples on a 96-well plate and stored the plate at -20 °C.

Cohort matching

Cohorts B and C were matched using R Statistical Software (Version 4.0.5.) and the MatchIt package.[18] Nearest neighbor matching was used as the method of matching.[20] Matching variables included sex, age, transient elastography and follow-up length. Following the matching process, we checked for significant differences between the HCC and control group. There were no significant differences detectable in the matching variables.

Table 1. Baseline characteristics of analyzed HCV cohorts.

	Cohort A (HCV without cirrhosis)	Cohort B (HCV with cirrhosis not developing HCC after SVR)	Cohort C (HCV with cirrhosis developing HCC)	Reference
n	22	24	8	
Age	55.0 ± 5.4	55.8 ± 3.6	56.5 ± 4.0	
Sex	12 f 10 m	24 m	8 m	
BMI	25.6 ± 1.9	28.4 ± 1.7	28.7 ± 1.7	18.5-24.9
HCV genotype	1: 15 (68%) 2: 1 (4.5%) 3: 5 (23%) 4: 1 (4.5%)	1: 20 (83%) 2: - (0.0%) 3: 3 (13%) 4: 1 (4%)	1: 6 (75%) 2: - (0.0%) 3: 2 (25%) 4: - (0.0%)	
Hemoglobin (g/dl)	14.3 ± 0.7	14.1 ± 0.6	14.2 ± 1.4	13.5-17.2 (m) 12-15.6 (f)
Platelets (10^3/µl)	218 ± 24	109 ± 16	114 ± 34	160-370
Leukocytes (10^3/µl)	6.7 ± 0.6	5.2 ± 0.5	5.9 ± 1.0	3.9-10.2
INR	1.02 ± 0.04	1.2 ± 0.1	1.4 ± 0.5	0.9-1.25
AST (U/L)	53.5 ± 10.7	114 ± 26	145 ± 51	0-35 (m) 0-31 (f)
ALT (U/L)	68.1 ± 15.4	116 ± 29.1	146 ± 59	0-45 (m) 0-34 (f)
Albumin (g/L)	41.5 ± 1.3	36.3 ± 2.1	33.8 ± 3.0	35-52
Fibroscan (kPa)	7.4 ± 0.7	32.4 ± 8.0	36.9 ± 7.9	0-14.5
HCV-RNA (IU/ml)	2,673,526 ± 1,172,974	1,472,619 ± 797,720	701,000 ± 432,626	0

Clinical characteristics of different subgroups including: HCV individuals without cirrhosis (cohort A), HCV individuals with cirrhosis not developing HCC after SVR (cohort B) and HCV individuals with cirrhosis developing HCC (cohort C). Mean values and 95% confidence interval are shown.

DNA methylation measurements and quality control

DNAm measurement was performed at the Human Genomics Facility of Erasmus MC, Rotterdam, the Netherlands. 500 ng of DNA was bisulfite converted using the EZ-96 DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA) with the KingFisher Flex robot (Thermo Fisher Scientific, Breda, the Netherlands). The methylation status of 54 individuals was assessed in 8 μl bisulfite-treated DNA using the Infinium MethylationEPIC BeadChip, following Illumina's protocol.

After receiving the raw IDAT files, we started our data preprocessing for each cohort in "R Statistical Software" using the Bioconductor package minfi.[21] We performed an extensive quality control by checking for sex concordance and removing low-quality probes (detection *p* value >0.01 in more than 10% of all samples), single nucleotide polymorphism-containing probes, cross-reactive probes[22] and sex chromosomal probes. All samples were checked for 20 control metrics generated by the "BeadArray Controls Reporter Software".[23] From the 162 samples, we excluded three samples not passing the pre-defined cut-off values by Illumina, indicating a failed bisulfite conversion.

In the first and second batch of samples, we excluded 1,470 and 1,302 failed probes, respectively, 43,254 cross-reactive probes,[22] 19,627 sex chromosome probes and 11,681 probes with SNPs at the CpG interrogation or at the single nucleotide extension. Overall, we included 793,532 and 793,365 unique high-quality CpGs from two batches and 162 samples in our analysis.

For normalization of the data, we used "dasen" from the "wateRmelon" R package.[24] We used the "IlluminaHumanMethylationEPICanno.ilm10b4.hg19" package to annotate all CpGs passing our quality control. In our downstream analysis we used M values for all of our analysis.

Biological age prediction

We used "agep" function from "wateRmelon" R package[24] to predict the biological (epigenetic) age of samples using Horvath's coefficients. EAA was defined as the difference between epigenetic and chronological age. The age- and sex-matched healthy cohort from a publicly available dataset (GSE40279)[19] has been used to obtain the expected distribution of EAA in the healthy population. Cell proportion was obtained via https://dnamage.genetics.ucla.edu/new.

Global methylation trends

We aimed to understand the global methylation trends by taking a look at the most changed CpGs over the course of treatment and follow-up. To detect those differences we designed a linear mixed effects model (lmer(methylation ~ time point + aspartate aminotransferase [AST] + CD8 T cells + CD4 T cells + natural killer cells + B cells + (1|Patient ID)), which included inflammation as well as immune phenotype parameters as covariates. Finally, a two-sided proportion test was used to determine whether increases and decreases in methylation differed at different *p* value thresholds.

Statistical analysis

We used R Statistical Software (Version 4.0.5.) to conduct our analyses. Based on this, we created all graphics in either R with "ggplot" and "ggpubr" packages or in GraphPad Prism (Version 8.3.1.). For detection of differences between dependent sample groups, "Wilcoxon signed-rank test" was used. Correspondingly, we used "Mann-Whitney *U* test" for differences between independent sample groups. "Spearman" method was used to calculate the correlation between EAA and estimated cell proportions as well as clinical lab parameters. *p* values for correlations between EAA and clinical lab parameters were adjusted using false discovery rate.

Results

Characteristics of the study cohort

Patients in cohort A (no cirrhosis) included 12 females and 10 males with a mean age of 55.0 years. AST and alanine aminotransferase (ALT) levels at baseline were elevated and declined during treatment and all patients achieved SVR and had normal levels at follow-up (Fig. S2). All individuals in this cohort had a transient elastography (FibroScan) value of less than 11.0 kPa indicating that no cirrhosis was apparent at treatment start. Patients in cohorts B and C (HCC after SVR and matched controls without HCC) were all male and had significantly lower platelet counts and albumin levels and higher international normalized ratio (Table 1). In addition, patients in cohorts B and C had higher levels of AST, ALT and higher transient elastography (FibroScan) values, which also declined during therapy (Fig. S2). At follow-up, 26/32 patients in cohorts B and C had normal ALT levels.

Importantly, the propensity score-matched control cohort B (no HCC) showed no significant differences in all lab parameters compared to cohort C (HCC after SVR), allowing a solid comparison. HCC occurred between 12 and 51 months after treatment start in cohort C (Fig. S8). All patients were HCV-RNA positive at baseline and achieved SVR.

Individuals with chronic hepatitis C show an accelerated epigenetic age

To understand the impact of chronic HCV infection on biological aging, we calculated the epigenetic age of all individuals with HCV and their age- and sex-matched controls using Horvath's epigenetic clock. Horvath's clock showed strong correlations between biological and chronological age, independent of the analyzed time point ($p < 2.2\times10^{-16}$) (Fig. 1).

Individuals with HCV showed an EAA at treatment start (median EAA = 3.12 years) compared to the age- and sex-matched reference group (median EAA = -1.45 years) ($p = 2.24\times10^{-4}$) (Fig. 2A). Further analysis revealed that EAA differed among the three patient groups studied. While those with chronic HCV without cirrhosis tended to have the lowest age acceleration at baseline ($p = 0.009$, median EAA = 2.45 years), individuals who developed HCC after SVR tended to have the highest age accelerations ($p = 0.017$, median EAA = 4.85 years) (Fig. 2B).

We additionally analyzed whether known conditions such as smoking status, type 2 diabetes mellitus, alcohol consumption, cardiovascular disease, metabolic syndrome, renal disease, and BMI, which might be associated with increasing EAA, had an impact on our results. There is a trend that patients who are active smokers or have a BMI ≥25 kg/m^2 have higher EAA (Fig. S6), which is consistent with previous reports in this

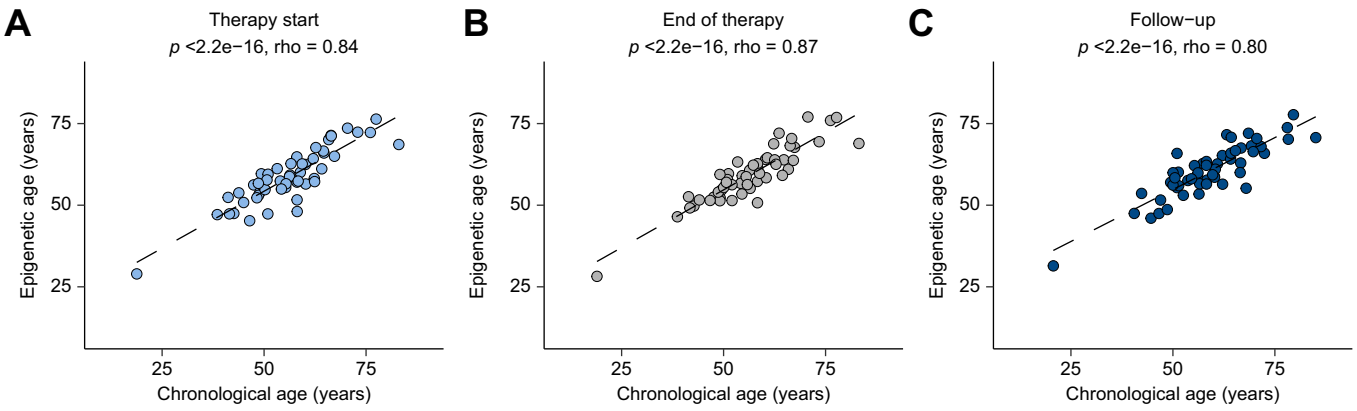

Fig. 1. Scatter plots showing the correlation between epigenetic age (Horvath's clock) and chronological age in individuals with chronic HCV. (A) At therapy start, (B) at end of therapy and (C) after long-term follow-up. The rank-based Spearman method was used for calculating the correlation.

area.[25] However, we did not find significant differences between the groups. BMI and smoking status were not part of the matching for the healthy cohort, but the rate of smoking and obesity was relatively low. Importantly, smoking status and BMI were very similar in those with cirrhosis (cohort B, 6/24 active smokers, BMI mean 28.4) and those with cirrhosis and HCC (cohort C, 3/8 active smokers, BMI mean 28.7). We further analyzed if the HCV genotype is associated with EAA. We observed a trend towards a higher EAA in individuals with HCV genotype 3 (p = 0.08) (Fig. S6).

Epigenetic age acceleration decreases after DAA therapy

Our longitudinal analysis over the course of DAA treatment and long-term follow-up showed that EAA is decreasing from baseline until the long-term follow-up (Fig. 3A). While the median age acceleration at baseline was 3.12 years, it decreased to only 1.37 years at long-term follow-up (n = 54, p = 0.07). The decrease in EAA was particularly significant between the end of therapy and the long-term follow-up (end of treatment – long-term follow-up: p = 0.01), whereas there was no significant difference in EAA between baseline and end of treatment (p = 0.56) (Fig. 3).

Interestingly, the patients who developed HCC after SVR showed not only the highest EAA but also did not show a significant decline of EAA after HCV elimination (therapy start – long-term follow-up: p = 0.51, median at therapy start: 4.85 years, median at long-term follow-up: 3.76 years). In contrast, propensity score-matched individuals without HCC after SVR showed a significant decline of EAA from baseline to the last follow-up (Fig. 4).

Significant increase in DNA methylation, dominated by hypermethylated CpGs, after DAA therapy

To understand what drives the changes in DNAm aging, we next investigated the effect of DAA therapy or HCV elimination on global methylation levels. It has been reported that overall DNAm levels are related to aging and in principle decrease with increasing age.[26] Interestingly, we observed that HCV

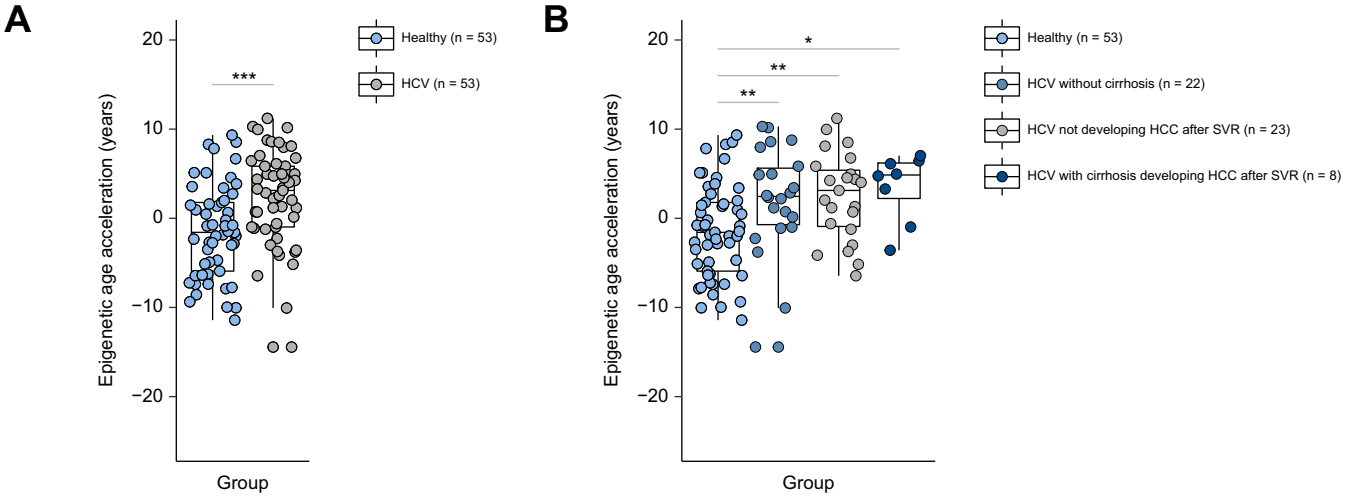

Fig. 2. Epigenetic age acceleration compared to the reference group and across different subgroups in individuals with chronic HCV at therapy start. (A) Comparison between healthy controls and all individuals with HCV, and (B) individuals without cirrhosis (cohort A), individuals with cirrhosis not developing HCC after SVR (cohort B) and individuals with cirrhosis developing HCC (cohort C) (B). Wilcoxon rank sum test was used to calculate the difference between groups. *p <0.05, **p <0.005, ***p <0.0005, ****p <0.00005. HCC, hepatocellular carcinoma; SVR, sustained virological response.

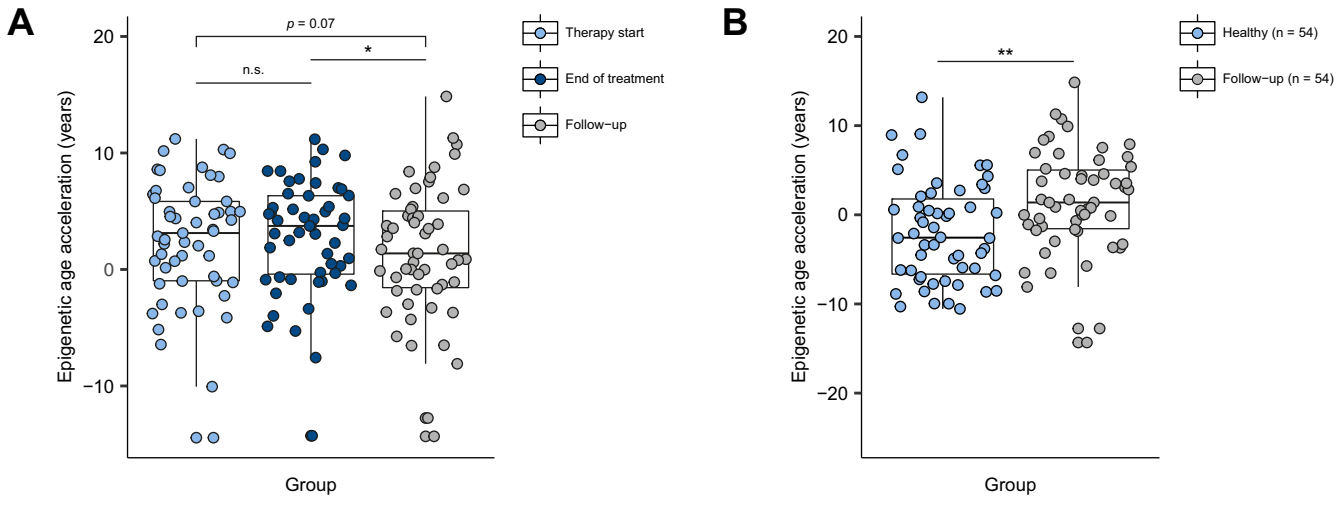

Fig. 3. Boxplots showing the age acceleration in individuals with chronic HCV (n = 54) at different time points over course of treatment and follow-up and age-matched healthy controls. (A) Comparison between different sampling points and (B) individuals with HCV at long-term follow-up and age-matched healthy controls. Two-sided Wilcoxon signed-rank test was used to calculate the difference between different sampling points and two-sided Mann-Whitney *U* test was used for comparison of follow-up with healthy controls. **p* <0.05.

elimination by DAA therapy leads to an increase in DNAm, which is inconsistent with the previous findings that methylation is inversely correlated with aging. This may suggest that DAA therapy has effects on epigenetic aging.

Specifically, in cohort A, treatment with DAAs did not result in an overall increase in DNAm between therapy start and end of treatment. In contrast, there was a significant increase in methylation dominated by hypermethylated CpGs between therapy start and 96-week follow-up. This increase was consistent at different chosen *p* value thresholds (*p* value threshold 10^{-4}: $p = 7.85\times10^{-5}$). In individuals with cirrhosis (cohorts B and C), a significant increase in methylation could be seen between therapy start and end of treatment (*p* value threshold 10^{-5}: $p = 0.003$) that remained consistent between therapy start and long-term follow-up (*p* value threshold 10^{-5}: $p = 0.006$). Similarly, compared to cohort A, this increase in methylation was mainly based on hypermethylated CpGs (Fig. 5, Table 2).

We compared the methylation change pattern of the CpG sites contained in Horvath's clock between therapy start and follow-up. Of the CpG sites that gain methylation over lifetime in Horvath's clock, 65.7% were more and 34.3% were less methylated. Of the CpG sites that lose methylation over lifetime in Horvath's clock, 43.8% were more and 56.2% were less methylated. When we compare those numbers directly, we see a trend that epigenetic aging was driven slightly more by the CpGs that gain methylation in the Horvath clock (65.7%) than by the CpGs that lose methylation (56.2%) (data not displayed).

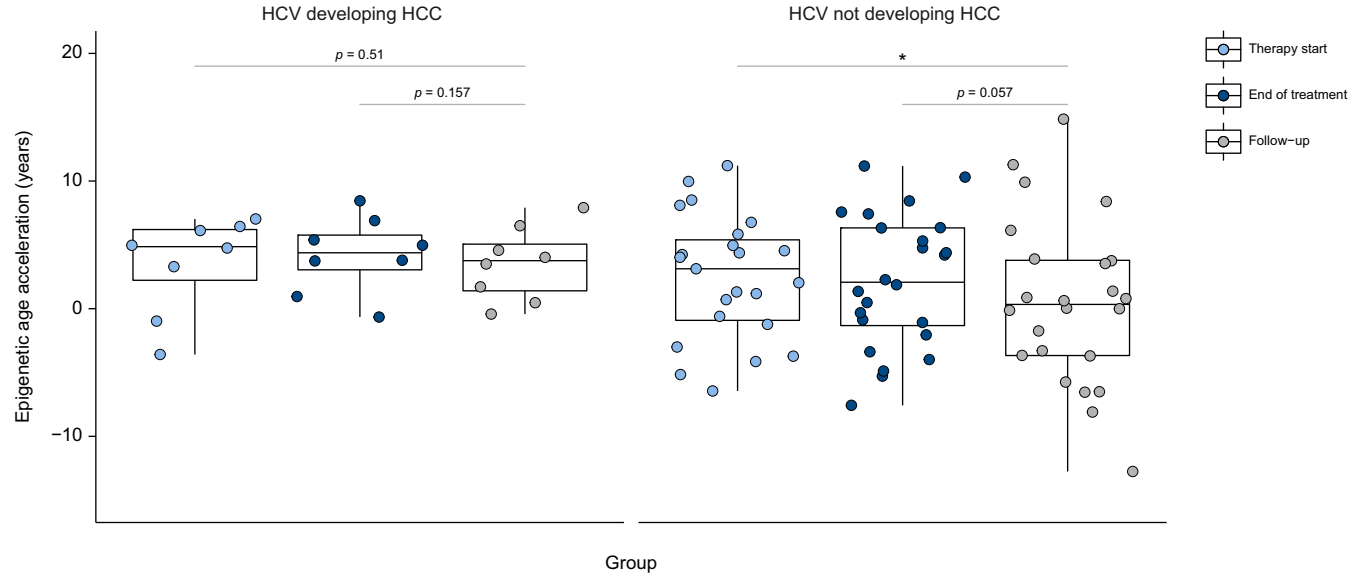

Fig. 4. Boxplots of epigenetic age acceleration in individuals developing hepatocellular carcinoma after SVR and matched control group over course of therapy. One-sided paired *t* test was used to calculate the difference between groups since EAA was normally distributed. **p* <0.05.

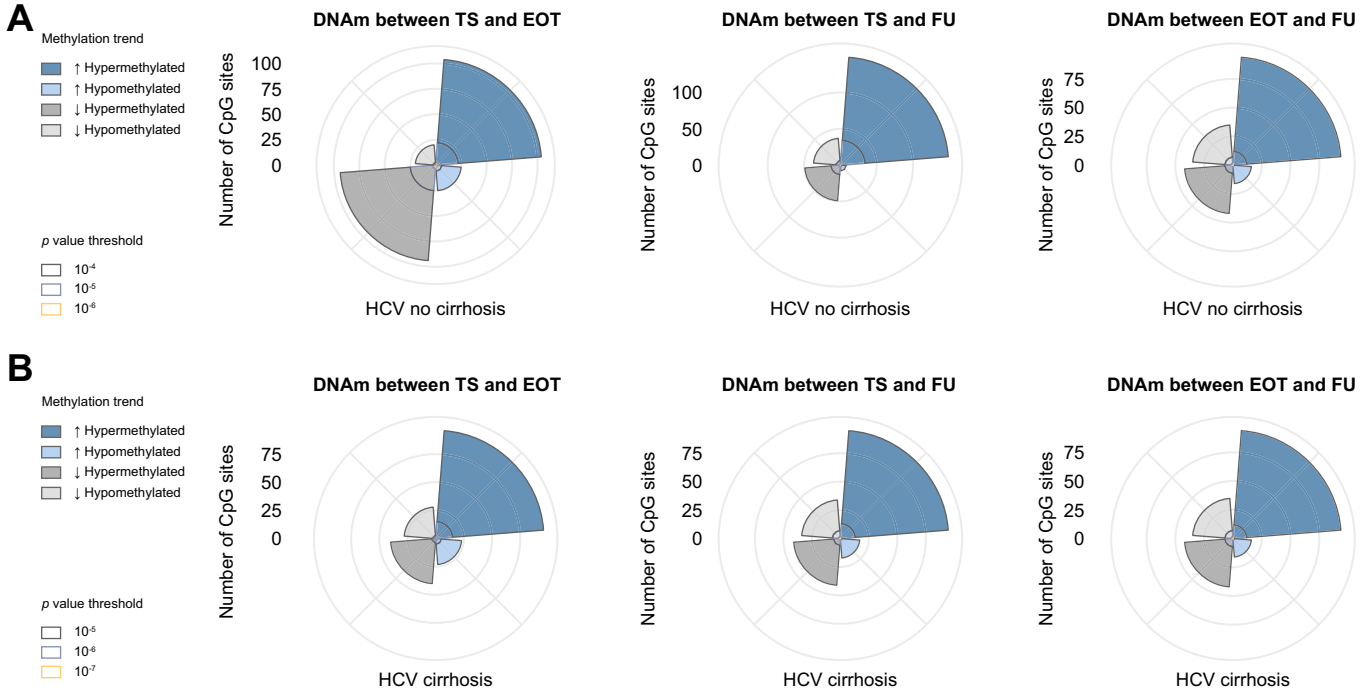

Fig. 5. Global methylation trends in individuals with HCV with and without cirrhosis. (A) No cirrhosis (cohort A) and (B) with cirrhosis (cohorts B and C). Color is indicating whether methylation increased (green) or decreased (red) and the overall methylation status of affected CpGs. Different sampling time points were compared to show short-term and long-term effects. DNAm, DNA methylation; EOT, end of treatment; FU, follow-up; TS, treatment start. (This figure appears in color on the web.)

Epigenetic age acceleration is not associated with estimated cell proportion but clinical phenotypes

As epigenetic changes can be driven by changes in cell composition, we next associated estimated cell counts with EAA in individuals with chronic HCV. In our approach, we focused on the main cell types with high overall frequencies. In conclusion, we were not able to detect any significant correlations between EAA and estimated cell counts before, during or after treatment.

The strongest association was between monocytes and EAA at follow-up. There was a consistent trend between CD8 T cells and EAA at all sampling points which was not significant after all. No consistent pattern was detectable for all other cell types. This stayed consistent in all subgroups of our analyses (Fig. S3-5).

We also aimed to understand if epigenetic age is driven by any specific clinical phenotype. We focused on clinical lab parameters that describe liver function such as ALT, albumin, platelets, transient elastography (FibroScan) values and gamma-glutamyltransferase, as well as leukocytes and creatinine. Liver stiffness, as indicated by transient elastography (FibroScan), was positively correlated with EAA (adj. $p = 0.03$, rho = 0.24) in individuals with cirrhosis (cohorts B+C), while platelet counts were negatively correlated (adj. $p = 0.03$, rho = -0.27) in the same cohorts. We did not observe any significant correlations in cohort A (Fig. 6).

The decline in liver stiffness after DAA therapy did correlate with the changes in EAA (Fig. S7) in those with cirrhosis (cohorts B + C) ($p = 0.048$, r = 0.37).

Discussion

The results of our study suggest that chronic HCV infection leads to a general acceleration of epigenetic aging predicted by Horvath's clock and that HCV elimination by DAA therapy can partially slow, halt, or reverse biological aging. The exact mechanism leading to this age acceleration and its reversal remains elusive. It has already been suggested that chronic infections such as HIV and HBV are associated with older or

Table 2. Global methylation trends in HCV cirrhosis (cohorts B and C) and non-cirrhosis (cohort A) patients.

	Therapy start – End of treatment		Therapy start – Long-term follow-up		End of treatment – Long-term follow-up	
Top CpGs (*p* value threshold)	*p* value	95% CI	*p* value	95% CI	*p* value	95% CI
Cohort A (HCV without cirrhosis)						
11 (10^{-6})	1	0.25–0.82	0.55	0.32–0.88	0.55	0.32–0.88
45 (10^{-5})	1	0.34–0.64	0.074	0.49–0.78	0.074	0.49–0.78
187 (10^{-4})	0.31	0.47–0.61	7.85×10^{-05}***	0.57–0.71	4.22×10^{-05}***	0.58–0.72
Cohort B + C (HCV with cirrhosis)						
5 (10^{-7})	0.074	0.46–1.00	1.00	0.07–0.83	1.00	0.07–0.83
22 (10^{-6})	0.14	0.45–0.85	0.83	0.33–0.75	1.00	0.31–0.69
160 (10^{-5})	0.0034**	0.54–0.69	0.0057**	0.53–0.69	0.0091**	0.53–0.68

Two-sided proportion test was used to determine whether in- and decrease in methylation differed at top *p* value thresholds.

accelerated biological age.[27,28] Thus, viral factors themselves may induce epigenetic modifications.[29] However, a recently published study did not observe this effect in individuals monoinfected with HCV. In this study, EAA was associated with advanced fibrosis and HIV coinfection in individuals with HCV. Of note, the individuals with HCV in the study by Gindin *et al.* were predominantly African American and were compared with the publicly available dataset consisting of Caucasian and Hispanic individuals.[30] Genetic background may have influenced the results as African Americans seem to have a lower extrinsic epigenetic aging rate than Caucasians and Hispanics.[31] In addition, longitudinal data were not available and there was no information on ALT levels as a marker for liver inflammation. Chronic inflammatory processes play a central role in the aging process, which is referred to as "inflammaging", because inflammation promotes, among other things, the formation of reactive oxygen species that can cause DNA damage and thus also contribute to epigenetic changes.[32]

Eliminating inflammation or the trigger of inflammation, *e.g.* with antiviral therapy, may slow, halt or even reverse biological aging. Recently, it was shown that individuals infected with HIV showed improvement in epigenetic age after 96 weeks of antiretroviral therapy.[33] Gindin *et al.* showed that one-year antiviral treatment of chronic hepatitis B was associated with a modest reduction in age acceleration.[30]

Our data also suggest that the process of EAA can in principle be halted or reversed, as patients showed a significant increase in overall DNAm and a decrease in EAA predicted by the Horvath clock after DAA therapy and HCV elimination. However, this process appears to take time and is not immediately evident with the end of treatment. Also, not all patients show a decline to values of healthy controls. This is consistent with previous studies showing that HCV elimination does not always lead to complete clinical resolution[34] and also immunological imprints of HCV infection remain after SVR.[8] This may be particularly important in individuals with advanced fibrosis or cirrhosis who have a residual risk of developing HCC after SVR.[35,36] It has been demonstrated that HCV-induced epigenetic changes in hepatocytes were associated with HCC and this persisted after SVR.[13] In addition, it has been shown that an accelerated age correlates with a higher risk of cancer-associated mortality and overall mortality.[37] Consistent with this concept, in our study, patients who developed HCC after SVR had the highest EAA and showed no significant age deceleration after DAA treatment. This is important data for understanding disease pathogenesis and highlighting the need for high-quality markers for HCC risk stratification after SVR. Early detection of HCC can lead to decreased overall mortality. Our results suggest that epigenetic age determination offers new insights and could be a tool to improve conventional risk scores such as GALAD (gender, age, AFP-L3, AFP and DCP) that comprise chronological age.[7,38] Instead of using chronological age, which is an imperfect surrogate measure of the aging process,[15] biological age may ultimately better reflect the risk of developing HCC. Thus, DNAm analysis of peripheral blood cells could serve as a liquid biomarker to improve the management of individuals with chronic hepatitis C or other individuals with chronic inflammatory conditions. For the development of a biomarker, it is important to consider that aging is accompanied by a change in blood cell type composition, *e.g.* the proportion of naïve or senescent cytotoxic T cells changes with age, which could create a bias. The age

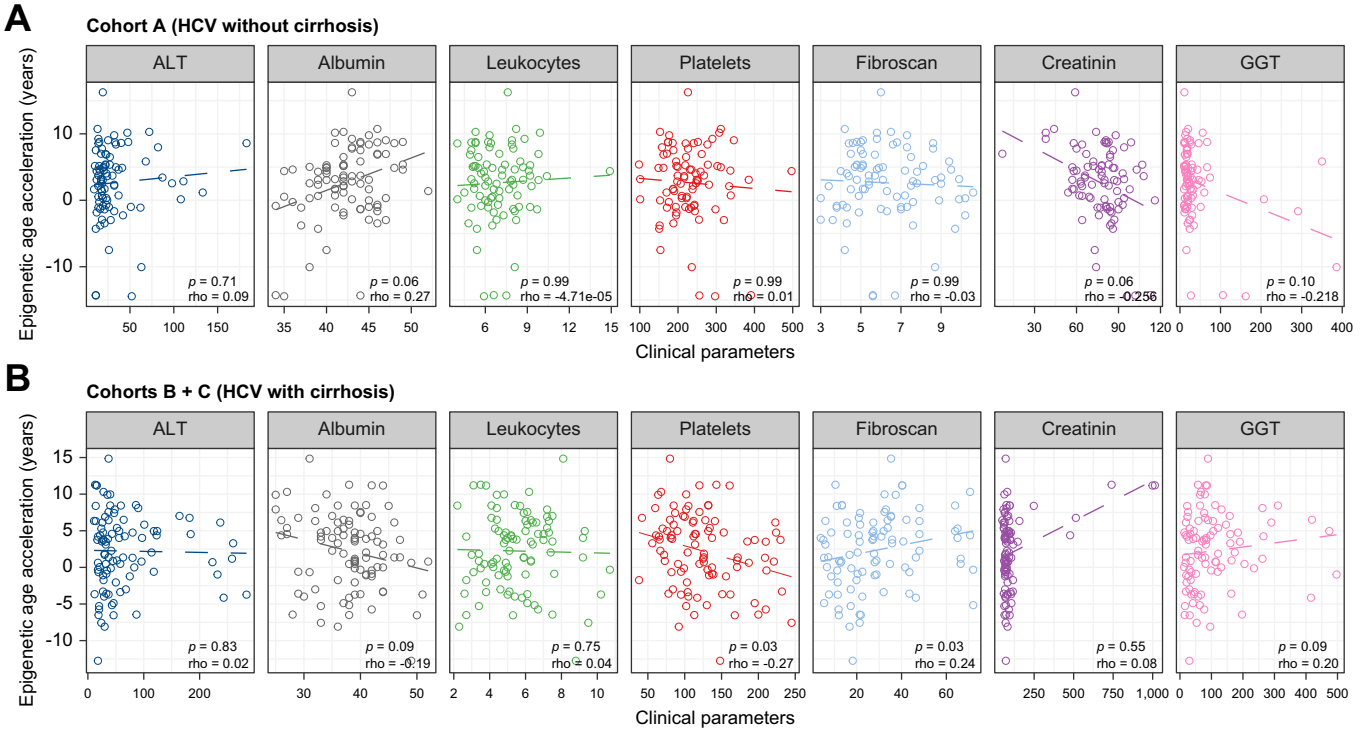

Fig. 6. Scatter plots showing the correlation between clinical phenotype and epigenetic age acceleration in individuals with chronic HCV with and without cirrhosis. (A) No cirrhosis and (B) with cirrhosis. The rank-based Spearman method was used for calculating the correlation. *p* values for correlations were adjusted using false discovery rate. (This figure appears in color on the web.)

calculated by the Horvath clock seems to be largely unaffected by these changes,[15] which is consistent with our data as the changes in DNAm were not significantly associated with altered cell type composition.

Our study certainly has strengths and limitations. The strengths lie in a very well-characterized and matched clinical cohort of 54 participants. The cohort of patients who developed HCC is very unique as we could analyze patient samples before the development of HCC. As the number of individuals with HCC was limited, larger studies are needed to evaluate whether the dynamics of age acceleration can support clinical decision making, with respect to HCC risk stratification, in individuals with chronic HCV. We compared our HCV patients with a healthy cohort, but this cohort did not contain longitudinal data, limiting our interpretation regarding the effect of HCV therapy in the long-term follow-up. Indeed, epigenetic age appears to increase more slowly over the life course than chronological age, but this is especially true for older cohorts, and any notable effect would take decades.[39] Overall, the age acceleration estimated by DNAm shows remarkable stability after childhood.[40]

Another limitation is that we analyzed PBMCs and not liver cells, the main target of HCV. Liver biopsies are not available because there is now no indication to obtain these biopsies in routine clinical practice. PBMCs are the only available living cell populations that can be obtained from blood. A recent study has shown epigenetic alterations in the liver to be associated with HCC risk[13] but little is known about the correlation between epigenetic changes in the liver and in PBMCs. As mentioned above, our main hypothesis is that this may not be a direct effect of the virus on epigenetic age, but rather an indirect effect due to the chronic inflammation in these patients that affects not only the infected hepatocyte. However, we observed a trend that HCV genotype 3 was associated with higher EAA, which is intriguing because HCV genotype 3 is associated with an increased risk of HCC.[35,41] This could indicate a viral effect, but indirect factors need to be discussed as well; for example, genotype 3 is associated with hepatic steatosis.[41] Whether viral factors affect epigenetic aging is certainly interesting and requires further investigation, as the number of individuals with different genotypes in our study was limited.

In conclusion, our study contributes to the understanding of the biological effects of HCV elimination after DAA therapy and offers new conceptual ideas for the use of DNAm in peripheral blood cells as a biomarker that supports the current effort for more individualized infectious disease medicine.

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Abbreviations

ALT, alanine aminotransferase, AST, aspartate aminotransferase; DAA, direct-acting antiviral; DNAm, DNA methylation; EAA, epigenetic age acceleration; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cell; SVR, sustained virological response.

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Conflict of interest

MC reports personal fees from Abbvie, personal fees from Falk Foundation, personal fees from Gilead, personal fees from GlaxoSmithKline, personal fees from Jansen-Cilag, personal fees from Merck/MSD, personal fees from Novartis, personal fees from Roche, personal fees from Spring Bank Pharmaceuticals, and personal fees from Swedish Orphan Biovitrum, outside the submitted work. BM reports personal fees from Abbott, personal fees from Abbvie, personal fees from Astellas, personal fees from Bristol-Myers Squibb, personal fees from Falk Foundation, personal fees from Fujirebio, personal fees from Gilead, personal fees from Jansen-Cilag, personal fees from Merck/MSD, personal fees from Norgine, personal fees from Roche, outside the submitted work. HW reports grants and personal fees from Abbvie, grants, personal fees and non-financial support from Abbott, grants, personal fees and non-financial support from Roche Diagnostics, personal fees from Siemens, grants and personal fees from BMS, grants and personal fees from Gilead, grants and personal fees from Novartis, grants and personal fees from Roche, personal fees from Janssen, grants and personal fees from Merck/MSD, grants and personal fees from Eiger, grants and personal fees from Falk and Falk Foundation, other from Transgene, non-financial support and other from Myr-GmbH, outside the submitted work. CX, YM, ZL, JM, JT, MU, JD, AK, CO have nothing to disclose.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

MC and CX conceived the project and coordinated the analyses. CO, AK, and MC were involved in designing of experiments. CO, ZL, AK, JD, MU, CX and MC drafted the manuscript. BM and HW were involved in recruitment of patients. JT was involved in creation of the clinical cohort, JM helped acquiring the data. CO acquired and analyzed the data with ZL and YM supervised by CX. All authors read and approved the manuscript.

Data availability statement

The datasets generated and analyzed during the current study are available in the Synapse repository (www.synapse.org/#!Synapse:syn32854048).

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Supplementary data

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