



Human centenarian–associated SIRT6 mutants modulate hepatocyte metabolism and collagen deposition in multilineage hepatic 3D spheroids

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Abstract Non-alcoholic fatty liver disease (NAFLD), encompassing fatty liver and its progression into nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), is one of the rapidly rising health concerns worldwide. SIRT6 is an essential nuclear sirtuin that regulates numerous pathological processes including insulin resistance and inflammation, and recently it has been implicated in the amelioration of NAFLD progression. SIRT6 overexpression protects from formation of fibrotic lesions. However, the underlying molecular mechanisms are not fully delineated. Moreover,

new allelic variants of SIRT6 (N308K/A313S) were recently associated with the longevity in Ashkenazi Jews by improving genome maintenance and DNA repair, suppressing transposons and killing cancer cells. Whether these new SIRT6 variants play different or enhanced roles in liver diseases is currently unknown. In this study, we aimed to clarify how these new centenarian-associated SIRT6 genetic variants affect liver metabolism and associated diseases. We present evidence that overexpression of centenarian-associated SIRT6 variants dramatically altered the metabolomic and secretomic profiles of unchallenged immortalized human hepatocytes (IHH). Most amino acids were increased in the SIRT6 N308K/A313S overexpressing IHH when compared to IHH transfected with the SIRT6 wild-type sequence. Several unsaturated fatty acids and glycerophospholipids

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were increased, and ceramide tended to be decreased upon SIRT6 N308K/A313S overexpression. Furthermore, we found that overexpression of SIRT6 N308K/A313S in a 3D hepatic spheroid model formed by the co-culture of human immortalized hepatocytes (IHH) and hepatic stellate cells (LX2) inhibited collagen deposition and fibrotic gene expression in absence of metabolic or dietary challenges. Hence, our findings suggest that novel longevity associated SIRT6 N308K/A313S variants could favor the prevention of NASH by altering hepatocyte proteome and lipidome.

Keywords SIRT6 · Liver · Spheroids · Aging · Hepatocytes · Hepatic stellate cells · Metabolomics

Abbreviations

ER	Endoplasmic reticulum
HSC	Hepatic stellate cells
IHH	Immortalized human hepatocytes
LV	Lentiviruses
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
SIRT6	Sirtuin 6
SNP	Single-nucleotide polymorphism
XBP1	X-box binding protein 1

Introduction

Non-alcoholic fatty liver disease (NAFLD) became a global healthcare challenge reaching epidemic proportions, affecting up to 30% of the general adult population [1–4] and is projected to increase further in some developed countries by 2030 [5, 6]. NAFLD is characterized by excessive fat accumulation in the liver tissue and increased level of inflammatory markers [7]. The disease can progress towards non-alcoholic steatohepatitis (NASH), with persisting stimuli to fibrosis, and worsening to irreversible condition as cirrhosis, and hepatocellular carcinoma (HCC), a very aggressive tumor with poor outcome—<6% 5-year survival [8–11]. To date, there are no specific drugs approved for NAFLD-NASH therapy, making the identification of molecular targets to avoid fibrosis progression of primary importance for public health.

Sirtuins are NAD⁺-dependent class III histone deacetylases, which are involved in regulating numerous cellular processes including stress, insulin resistance, inflammation, chromatin silencing, cell cycle

regulation, transcription, and apoptosis [12, 13]. Of the seven sirtuins present in humans, SIRT6 is an essential nuclear one that has deacetylase, deacylase, and mono-ADP ribosyl-transferase activities. SIRT6 is a “longevity gene” with the potential to increase lifespan of a variety of organisms by improving genome stability and regulating nutrient metabolism [14–18]. During recent years, several studies have connected the activity of SIRT6 to various conditions including dyslipidemia, diabetes, heart diseases, neurodegenerative diseases, NAFLD, and HCC [18–23]. Accordingly, adipose tissue-specific knock-out (KO) of SIRT6 exacerbates obesity and insulin resistance in rodents [21, 24], while its overexpression showed a protective effect [25]. In the liver, hepatocyte-specific SIRT6 deletion increases the risk of developing NASH [26, 27], whereas its upregulation confers resistance to endoplasmic reticulum (ER) stress-induced hepatic steatosis by deacetylation of XBP1s [28].

Recently, novel genetic/allelic variants of SIRT6 gene (rs183444295, rs201141490, rs117385980) were described to be associated with human longevity and healthy aging by improving genome maintenance, stimulating DNA repair and mitigating its damage, suppressing transposons and killing cancer cells [15, 29, 30]. Especially variants rs183444295 (Ala313Ser—>A313S) and rs201141490 (Asn308Lys—>N308K) seem most promising target in prolongation of human lifespan as they are overrepresented in Ashkenazi Jews (AJ) centenarians [30]. AJ population derived from a small number (estimated to be in the several thousands) of founders. Historic factors resulted in the social isolation and inbreeding of the Ashkenazi Jews. This history resulted in both cultural and genetic homogeneity making this population useful for identification of genetic traits. Longevity in AJ population is strongly inherited and associated with lower rates of age-related diseases [31, 32]. These SIRT6 SNP variants are coding missense mutations that modify SIRT6 protein properties/activities, which could be responsible for the observed “longevity” phenotypes. Centenarian-associated SIRT6 variants have enhanced ADP-ribosylation activity, with effects on DNA double-strand break (DSB) repair, LINE1 elements, and tumor cell suppression. Moreover, centenarian SIRT6 improved the interaction with the nuclear scaffold protein LMNA [30]. However, other studies described no correlation between SIRT6

variants and longevity, and some of them suggested even to shorten lifespan [33, 34]. Whether these centenarian-associated SIRT6 genetic variants are associated with the decreased incidence of NAFLD and NASH in the very old is currently unknown [1, 35]. Hence, in this study, we aimed to clarify how these new centenarian-associated SIRT6 genetic variants affect liver metabolism and associated diseases.

Here, we present evidence that overexpression of centenarian-associated SIRT6 variants dramatically altered the metabolomic and secretomic profiles of unchallenged immortalized human hepatocytes. Furthermore, we adopted a 3D hepatic spheroid model formed by the co-culture of IHH and LX2, to model in vitro complex cellular interactions observed in the liver [36, 37]; we found that overexpression of centenarian SIRT6 variants in IHH inhibited collagen deposition and fibrotic gene expression in absence of metabolic or dietary challenges. Our findings suggest that Ashkenazi Jews centenarian-associated genetic/allelic variants of SIRT6 gene could favor the prevention of NASH.

Materials and methods

Cell lines and 3D spheroids

Cell culture

HepG2, Huh-7, and LX2 cell lines were obtained from CLS-GmbH (Eppelheim, Germany). The human hepatoma cancer cell lines HepG2 and HUH7, and the hepatic stellate cell line LX2, were cultured in high glucose (4.5 g/l) DMEM (1×) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Czech Republic), 15 mM Hepes buffer (Biowest, France), glutamine, 1% penicillin/streptomycin solution, and 100 µg/ml Normocin (InvivoGen, CA, USA) at 37 °C and 5% CO₂. Human hepatocyte cell line (IHH), isolated and immortalized by lentiviral transduction with the SV40T antigen and hTERT as previously described [38], were maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM/F-12) containing 1×10^{−6} M dexamethasone, 1×10^{−12} M human insulin (Humalog, Lilly) 10% FBS, and 1% penicillin/streptomycin. The cell culture medium was changed every 2 days and the

cells were sub-cultured using TrypLE Express when reaching 90% confluence.

3D spheroids

For the generation of the cell spheroids, cells were seeded into 96-well round-bottom ultra-low attachment plates (BIOFLOAT, faCellilite) at 10,000 viable cells per well. Each IHH LV transfect cell line (EMPTY, WT, N308K, and N308K/A313S) and normal IHH (control CTL) was co-cultured with LX2 cells with 20:1 ratio, to reproduce the physiological proportion in the liver parenchyma, where hepatocytes are major cell type with only ~5% hepatic stellate cells. The spheroids were grown in DMEM media supplemented as described above. The plates were incubated for 5 days at 37 °C in a humidified atmosphere of 5% CO₂.

Lentivirus production and transduction

Production Self-inactivating lentiviral vectors expressing the wild-type and the mutated variants of SIRT6 (kindly provided by Dr Laura Sturla) were constructed by PCR and restriction enzyme cloning, using the vector LV2-EF1a_mCER3; UbC_Hygro-WPRE [39] as a backbone. SIRT6 cDNAs adjoined with the IRES-driven Katushka2S reporter cassette were placed under the control of the constitutive human EF1α promoter (LV2-EF1a_SIRT6_IRES_Kat2S). Serving as a control, an empty vector without the primary ORF (LV2-EF1a_IRES_Kat2S) was constructed. To enable selection of positive cells following transduction, all vectors contained a separate Hygromycin B resistance cassette, driven by UBC promoter (Supplementary Fig. 1). Integrity of constructs was verified by sequencing and restriction analysis.

Lentiviruses were produced by transient transfection of packaging constructs and helper plasmids in HEK293T cells essentially as described [40]. Supernatant containing viral particles was cleared from debris by brief centrifugation, filtered through 0.45-µm PVDF filter and concentrated by low-speed centrifugation (7000 rpm 16 h).

Transduction The cells, after reaching confluence, were split and seeded into 24-well plate with growth surface area of 2 cm², where 50×10⁴ cells per

well were seeded. Immediately after seeding, cells were infected with lentivirus containing SIRT6 constructs: LV2-EMPTY-IresKat2S, LV2-SIRT6(WT)-IresKat2S, LV2-SIRT6(N308K)-IresKat2S, and LV2-SIRT6(N308K/A313S)-IresKat2S (Supplementary Fig. 1; Table 1), in basal DMEM media supplemented with 4 µg/mL polybrene transfection reagent (TR-1003, Sigma-Aldrich) with MOI of 1–2. Cells were cultivated with lentiviral constructs for the period of 24 h, then the fresh medium was added and cells were cultivated for another 48 h. Then, the cells were treated with selection media comprising basal DMEM media with 500 µg/mL of hygromycin (H3274, Merck) for the period of 5 days (every other day the media was changed for the fresh one). After selection, remaining cells were expanded and checked for the presence of fluorescent Kat2S signal by fluorescence microscope ex/em 588/635. SIRT6 overexpression was confirmed by Western blot. All used transduced cell lines with appropriate LV and their abbreviations are summarized in Table 1.

Cell viability

HepG2 and Huh7 control and transduced cell line were maintained in cultivation in T75 flasks for expansion. After 2 weeks, cells were harvested, washed in PBS, resuspended and cell viability was evaluated mixing them 1:1 with Trypan blue and analyzed by Luna-FL Fluorescence Cell Counter (Logos Biosystems).

Insulin sensitivity test

Insulin sensitivity was tested by measuring PI3K-AKT signaling pathway activity by immunoblotting detection of AKT/phosphoAKT^(ser473) in cell

lysates. Briefly, IHH cell were seeded and let to grow until reach at least 70% of confluency, then they were serum starved (0.5% FBS) in low-glucose (1 g/l) DMEM media overnight (12 h) and treated or not with insulin solution (100 nM, I9278—Sigma-Aldrich) for 30 min. Cells were then harvested using 1xRIPA lysis buffer (20–188, Millipore, USA) and isolation of proteins and immunoblotting were done as described in the “Immunoblotting analyses” section.

Microscopy and fluorescence imaging

Immunofluorescence imaging was performed as previously described [41]. The spheroids were fixed with 4% PFA for 10 min directly on the cultivation plates and then transferred in mini-tubes. After being washed in PBS, the spheroids were kept in sucrose 15% for 1 h, embedded in tissue freezing media (OCT), and then cut to 7 µm at −20 °C with a cryotome (Leica Microsystems) and stored at −80 °C for further use. To evaluate the effect of SIRT6 variants and their overexpression on liver tissue fibrosis, spheroid histological sections were immunolabeled to detect collagen 1A. Slides were washed once in 1xPBS to dissolve the OCT and blocked in 1xPBS supplemented with 0.2% Tween-20 and 5% BSA. Primary antibody rabbit anti-collagen I (1:500, ab34710, Abcam) was diluted in DAKO antibody diluent (S202230-2, Agilent technologies) and incubated O.N. in humid chamber at room temperature. After three washes with 1xPBS, mix of secondary antibody (1:500) donkey anti-rabbit IgG coupled with Alexa Fluor™ 647 was applied and incubated for at least 1 h. After three washes in 1xPBS, slides were counterstained with DAPI (1 µg/ml) solution for 15 min and mounted in water-based hardening media (Mowiol). After hardening (overnight at 4 °C), images were captured using an AxioScan Z.1 (ZEISS) equipped with a Hamamatsu ORCA-Flash 4.0 camera, and ImageJ software (NIH, USA) analysis program was used to evaluate all immunofluorescence images. Fibrosis, determined as collagen 1A abundance in spheroid samples, was evaluated as the % of the total spheroid area delineated by DAPI fluorescence at 100× magnification, when at least 5 spheroids per each condition/cell line were used in three consecutive and independent experiments.

Table 1 Lentivirus construct used

Cell line group	Lentivirus construct	SIRT6 overexpression
CTL	-	-
EMPTY	LV2-EMPTY-IresKat2S	-
WT	LV2-SIRT6(WT)-IresKat2S	Yes
N308K	LV2-SIRT6(N308K)-IresKat2S	Yes
N308K/A313S	LV2-SIRT6(N308K/A313S)-IresKat2S	Yes

Soluble collagen measurement

The spheroids conditioned media (CM) was collected and centrifuged at $1000\times g$. The cell solution was homogenized on ice using a pre-chilled Dounce homogenizer. Following overnight incubation, the acidic solution was centrifuged at $10,000\times g$ for 15 min at 4 °C to pellet any debris and the clarified supernatant was transferred to a new microfuge tube. Collagen concentration was measured using the Soluble Collagen Assay Kit® (ab241015, Abcam, Cambridge, UK) according to manufacturer's instruction. The fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using an Agilent BioTek FLx800 microplate reader. The graphs were plotted using GraphPad Prism 8.

Immunoblotting analyses

Immunoblotting analyses were performed as previously described [42, 43]. Briefly, cells were harvested from using TrypLE Express and washed with 1xPBS and centrifuged at 300 g. Supernatant was discarded, and the obtained pellet was resuspended in 1xRIPA lysis buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (100×, ThermoFisher) and lysed on ice (4 °C) for 30 min with vigorous vortexing every 10 min. The samples were then centrifuged at 10000 g for 10 min at 4 °C, supernatant was transferred to new microfuge tube, and concentration of protein was measured by Pierce™ BCA Protein Assay Kit (23,225, ThermoFisher) according to manufacturer's instruction. Equal amount of protein samples (at least 20 µg) was mixed with 1×Laemmli Sample buffer (1,610,747, 4×, Bio-Rad), and after heating at 95 °C for 5 min and cooling on ice, equal volumes of proteins (40 µl) were loaded on 10% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (4,568,034, Bio-Rad) and separated by electrophoresis running at 120 V for 45 min. Protein transfer was performed on PVDF membranes using Trans-Blot Turbo RTA Mini 0.45 µm LF PVDF Transfer Kit (1,704,274, Bio-Rad) and Bio-Rad Trans-Blot Turbo Transfer System at 1.3A and 25 V for 10 min. Membranes were then blocked with 5% bovine serum albumin (BSA, P6154, BioWest) dissolved in TBST buffer (20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) for at least 30 min and incubated

with the specific primary antibodies (see below) diluted in TBST blocking solution, at appropriate dilutions. Following three washes in TBST buffer, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase diluted in TBST blocking buffer. After three further washes with TBST, protein levels were detected by Clarity Western ECL Substrate (1,705,061, Bio-Rad), and the signal detected on Bio-Rad ChemiDoc XRS+ imaging systems. For quantitative measurement, the scanned membranes were analyzed using the Image Lab™ Software (Bio-Rad).

In this study we used the following antibodies: Cell Signaling Technology (MA, USA), rabbit anti-Akt (1:1000), rabbit anti-Phospho-Akt (Ser473) (1:1000), and rabbit anti Histone H3 (D1H2, 1:1000); Abcam (UK), rabbit anti-collagen I (1:1000) and rabbit anti-SIRT6 antibody (1:1000, EPR18463); ThermoFisher Scientific (CA, USA), mouse IgG1 GAPDH monoclonal HRP conjugated antibody (1:2000), secondary goat anti-rabbit IgG HRP-linked (1:2000), and secondary goat anti-mouse IgG HRP-linked (1:2000);

Quantitative real-time PCR

qPCR was performed as previously described [44]. Briefly, column separation technique was used for mRNA isolation with a RNeasy mini-Kit (74,106, Qiagen, Germany), according to manufacturer's instructions. At least 4 biological replicates were prepared for each treatment group. Total RNA was quantified on NanoDrop 1000 spectrophotometer (ThermoFisher Scientific), and 1 µg of total isolated RNA was used to prepare cDNA using a high-capacity cDNA Reverse Transcription Kit (4,368,814, ThermoFisher Scientific). Real-time PCR was performed with at least two technical replicates using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and SYBR™ Select Master Mix (4,472,908, ThermoFisher Scientific). The PCR reaction was held in 10 µl volume and 250 ng of cDNA was added to each well. The primer sequences used in this study are listed in the Table 2.

Metabolomics

Metabolic profiling was performed by mass spectrometry coupled to ultra-high-performance liquid chromatography (UHPLC-MS) as previously

Table 2 Primer sequences used in this study

Gene		Sequence (5'-3')	Gene		Sequence (5'-3')
α SMA	F	AAAAGACAGCTACGTGGGTGA	CERS1	F	TACAGTGCCTACCTGCTGTTT
	R	GCCATGTTCTATCGGGTACTTC		R	TAGCGTAGCGTAGATGGAGTG
COL1A1	F	GTGCGATGACGTGATCTGTGA	DGAT1	F	GGTCCCCAATCACCTCATCTG
	R	CGGTGGTTTCTTGGTCGGT		R	TGCACAGGGATGTTCCAGTTC
TIMP1	F	ACCACCTTATACCAGCGTTATGA	FABP5	F	TGAAGGAGCTAGGAGTGGGAA
	R	GGTGTAGACGAACCGGATGTC		R	TGCACCATCTGTAAAGTTGCAG
VIMENTIN	F	AGTCCACTGAGTACCGGAGAC	FADS1	F	CTACCCCGCGCTACTTCAC
	R	CATTTACGCATCTGGCGTTC		R	CGGTGATCACTAGCCACC
MMP2	F	TACAGGATCATTGGCTACACACC	FASN	F	GGAGGTGGTGATAGCCGGTAT
	R	GGTCACATCGCTCCAGACT		R	TGGGTAATCCATAGAGCCCAG
ACC	F	ATGTCGTGGCTTGCACCTAGTA	SCD	F	TCTAGTCCTATACCACCACCA
	R	CCCCAAAGCGAGTAACAAATTCT		R	TCGTCTCCAACCTATCTCCTCC
CD36	F	GGCTGTGACCGGAACCTGTG	SGMS1	F	TGTGCCGAGTCTCCTCTGA
	R	AGGTCTCCAACCTGGCATTAGAA		R	CCGTTCTTGTGTGCTTCCAAA
GAPDH	F	GGTGCGTGCCAGTTGA			
	R	TACTTTCTCCCGCTTTTT			

described [41, 45]. Cell pellets or cell culture media were resuspended/diluted in cold extraction solvents spiked with metabolites not detected in the unspiked cell extracts (internal standards) and incubated at $-20\text{ }^{\circ}\text{C}$ for 1 h. The samples were then vortexed and centrifuged at $18,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 5 min, and the supernatants were collected and incubated at $4\text{ }^{\circ}\text{C}$, while the cell pellets were again resuspended in cold extraction solvents and incubated for a further 1 h at $-20\text{ }^{\circ}\text{C}$. The samples were again vortexed and centrifuged at $18,000\times g$ at $4\text{ }^{\circ}\text{C}$ for 5 min, and the supernatants were collected and pooled with the previous supernatant samples. The supernatants were then dried under vacuum, reconstituted in water and resuspended with agitation for 15 min before being centrifuged at $18,000\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$ and transferred to vials for UHPLC-MS analysis. Two different types of quality control (QC) samples were used to assess the data quality: (i) a QC calibration sample to correct the different response factors between and within batches and (ii) a QC validation sample to assess how well the data pre-processing procedure improved data quality [46]. Randomized sample injections were performed, with each of the QC calibration and validation extracts uniformly interspersed throughout the entire batch run. A specific UHPLC-MS method was used. The chromatographic separation and

mass spectrometric conditions employed have been previously described.

Data normalization and quality control Normalization factors were calculated for each metabolite by dividing their intensities in each sample by the recorded intensity of an appropriate internal standard in that same sample, following the procedure described by Martínez-Arranz et al. [47]. The most appropriate internal standard for each variable was defined as that which resulted in a minimum relative standard deviation after correction, as calculated from the QC calibration samples over all the analysis batches. In general, as one would have expected, best internal standard trends followed chemical structural similarities between spiked compounds and endogenous variables. Robust linear regression (internal standard corrected response as a function of sample injection order) was used to estimate in the QC calibration samples any intra-batch drift not corrected for by internal standard correction. For all variables, internal standard corrected response in each batch was divided by its corresponding intra-batch drift trend, such that normalized abundance values of the study samples were expressed with respect to the batch averaged QC calibration serum samples (arbitrarily set to 1). Any remaining sample injection variable response zero values in the corrected dataset

were replaced with missing values before generating the final dataset that was used for study sample statistical analyses.

Univariate data analysis Univariate statistical analyses were also performed for each metabolite measured in the hepatocytes and culture medium samples, calculating group percentage changes and Student's t test p value (or Welch's t test where unequal variances were found) for the comparisons among groups: WT vs. Empty; N308K vs. Empty; N308K/A313S vs. Empty; N308K vs. WT; N308K/A313S vs. WT; and N308K/A313S vs. N308K. As mentioned, data per sample and per metabolite, as well as data per sample and metabolic class, are included in the Supplementary Files 1 and 2. Intensity data, average group intensities, fold-changes and Student's t test per individual metabolite and metabolic class are also included in the Supplementary Files 1 and 2. In order to help in the visualization of the results, a heatmap per type of sample was generated displaying the results of the comparisons mentioned above. These heatmaps display the $\log_2$ (fold-change) of the metabolites included in the analysis together with the Student's t test for the comparisons performed. For each metabolite, changes between subgroups were calculated as the base 2 logarithm of fold-change. Darker blue and red colors indicate higher drops and elevations of the metabolite levels, respectively. These values are accompanied by a significance level based on p values from Student's t test. Three levels of increasing significance are considered: $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Statistical analyses

Statistical analyses were performed using GraphPad Prism Software (version 8.00 for Windows; GraphPad Inc., CA, USA). Statistical comparisons between groups were made using the parametric Student's t test, if the data had normal distribution in all tested subgroups; otherwise, the non-parametric Mann–Whitney U test was used instead. To determine statistical significance between more than two groups, a parametric one-way ANOVA was used when the data had a normal distribution, or otherwise, a non-parametric Kruskal–Wallis test, as appropriate. The data are expressed as the means $\pm$ SEM (unless indicated otherwise). Differences were considered

statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) or indicated otherwise.

Data availability statement

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

Results

SIRT6 variants' overexpression induces cell death in hepatoma cells

In order to study the effects of SIRT6 overexpression (OE) on NAFLD, we created stably transfected hepatic cell lines using lentiviral integration in HepG2 and HuH7, two hepatocellular carcinoma (HCC) cell lines widely used in various *in vitro* models of hepatic steatosis and fibrosis [48, 49]. When wild-type (WT) SIRT6 or its allele variants N308K and N308K/A313S were overexpressed, we observed a strong reduction in cell viability of about 60–90% in HepG2, and more than 90% in HuH7, after 2 weeks from LV transduction (Fig. 1). These results are in agreement with other studies that showed that SIRT6 overexpression induces massive apoptosis in a variety of cancer cell lines but not in normal non-transformed cells [50].

SIRT6 variants' overexpression in immortalized human hepatocytes (IHH)

Since it was not possible to use hepatoma-derived cell lines for our experiments, we employed immortalized human hepatocytes (IHH). Figure 2A shows the signal of the far-red fluorescence protein Katushka2S contained in the LV cassette, alone in the empty group, or together with one of SIRT6 versions (WT, N308K or N308K/A313S), demonstrating the successful infection. No Katushka2S signal was detected in the IHH control cells. SIRT6 protein expression was measured by Western blot (Fig. 2B–C), confirming the strong increase in SIRT6 levels in the groups transfected with LV-SIRT6 compared to either empty or CTL cells. SIRT6 is actively recruited to target gene promoters and represses gene transcription by removing acetylation of H3K9 and H3K56

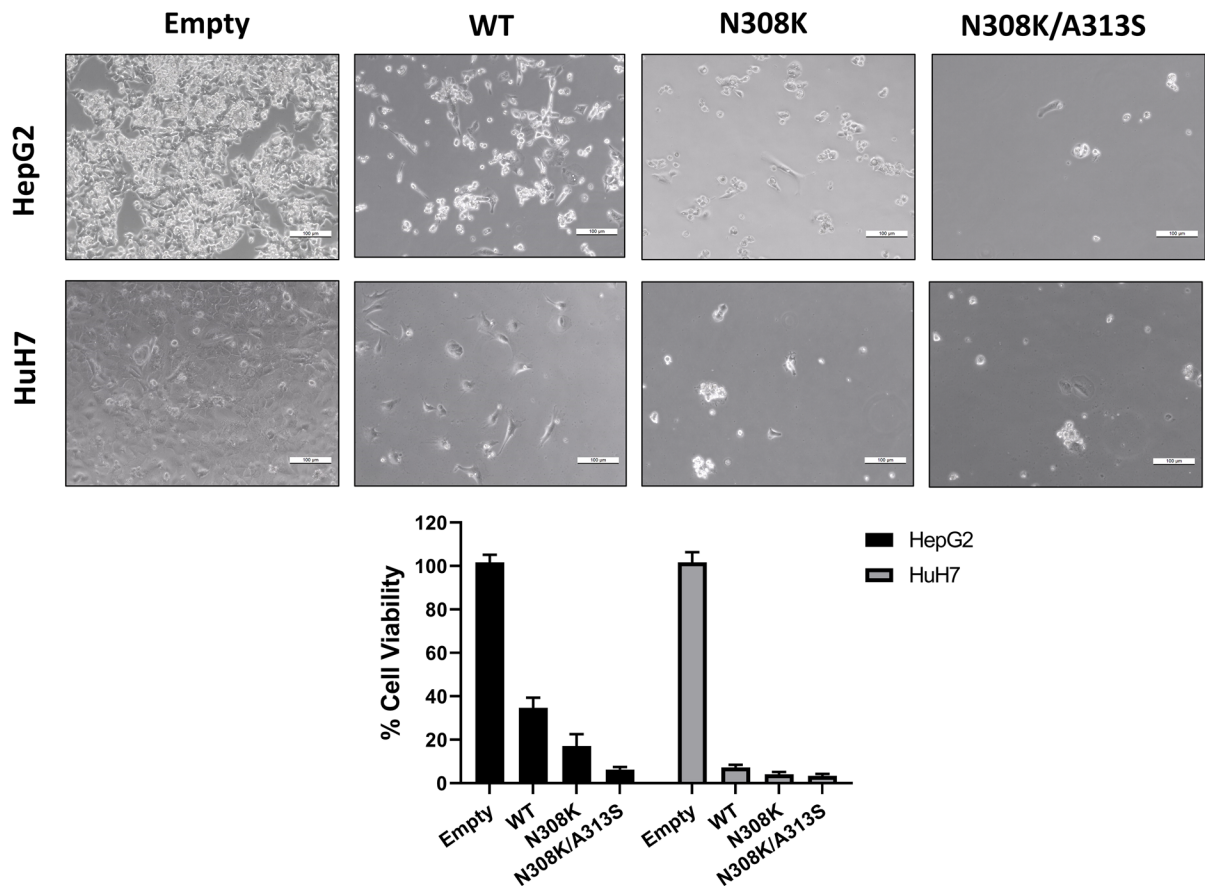


Fig. 1 Hepatoma cell lines viability decreases after SIRT6 overexpression. Representative figures from optimal microscope of HepG2 and HuH7 cell culture after 2 weeks from LV transfection, showing a markedly decrease in number of cells in SIRT6 groups. The quantification shows a decrease of

about 60–90% in HepG2 and ~90% in Huh7 in cell viability of the group overexpressing one of the SIRT6 variants compared to the empty control group ($N=3$). Data are presented as mean $\pm$ SEM

sites [51]. Accordingly, in the SIRT6 overexpression (OE) groups the levels of acetylated histone H3K9 were significantly reduced, while H3K56Ac showed a decreased trend (Fig. 2B–C), supporting the fact that, along with the increased SIRT6 expression, there was a concomitant increase in its deacetylase activity.

Overexpression of centenarian variants of SIRT6 does not affect hepatocyte insulin sensitivity

Because alterations of insulin receptor substrate PI3K (phosphoinositide 3-kinase) and AKT signaling pathways are well known to be closely associated with metabolic disorders, liver steatosis, and insulin resistance [52], we sought to determine by immunoblotting whether the overexpression of SIRT6 and its

longevity variants in IHH cells might lead to higher insulin sensitivity/activation of PI3K/AKT pathway. After overnight serum and glucose starvation of IHH cells, overexpressing or not SIRT6 and its allelic variants, we stimulated them with human insulin solution (100 nM) for 30 min and then we measured the pAKT^(Ser473) protein levels. We did not observe any significant difference of pAKT^(Ser473) levels among the conditions, either with or without insulin administration (Fig. 3A–B).

Overexpression of centenarian variants of SIRT6 profoundly alters the metabolomic profiles of IHH

SIRT6 overexpression in mice preserves glucose homeostasis through an improvement in gluconeogenesis

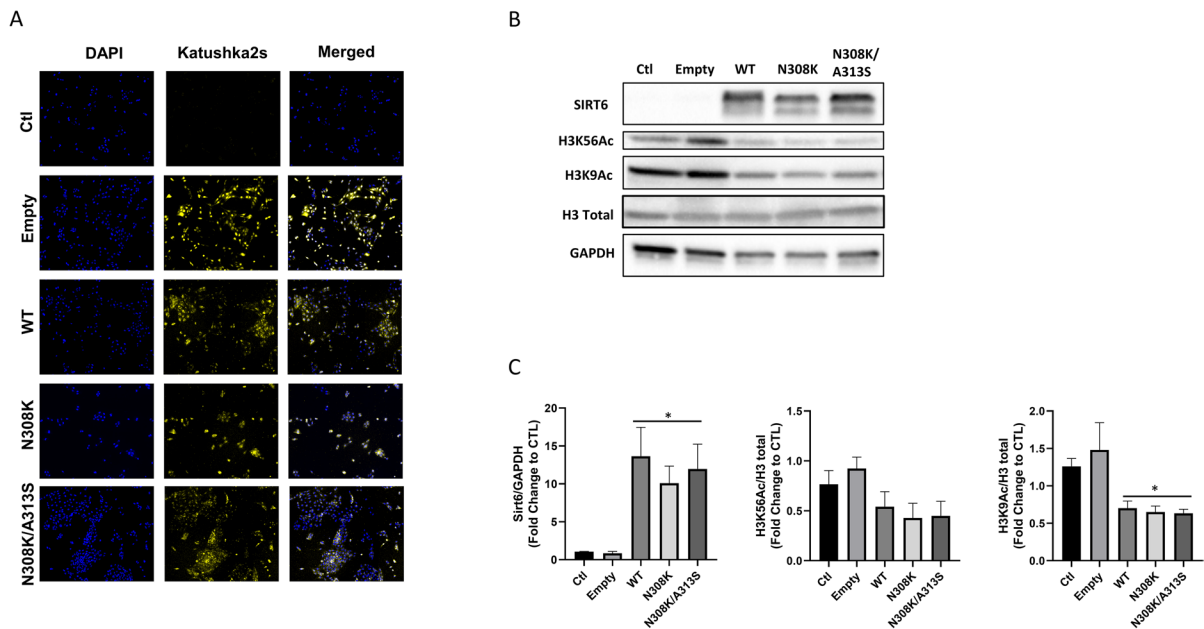


Fig. 2 Model of stable transfected IHH cells overexpressing SIRT6 allele variants. **A** Representative immunofluorescence images of Katushka2S staining showing the occurred lentivirus transfection for the empty vector and all of three SIRT6 variants (WT, N308K, N308K/A313S) in IHH cells. **B** Representative

immunoblotting of SIRT6, H3K56Ac, H3K9Ac, Histone 3, and GAPDH proteins in IHH transfected cells. **C** Quantification of SIRT6 and acetylated histones H3K9Ac and H3K56Ac protein levels in IHH cells ($N=5-7$). Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs empty group

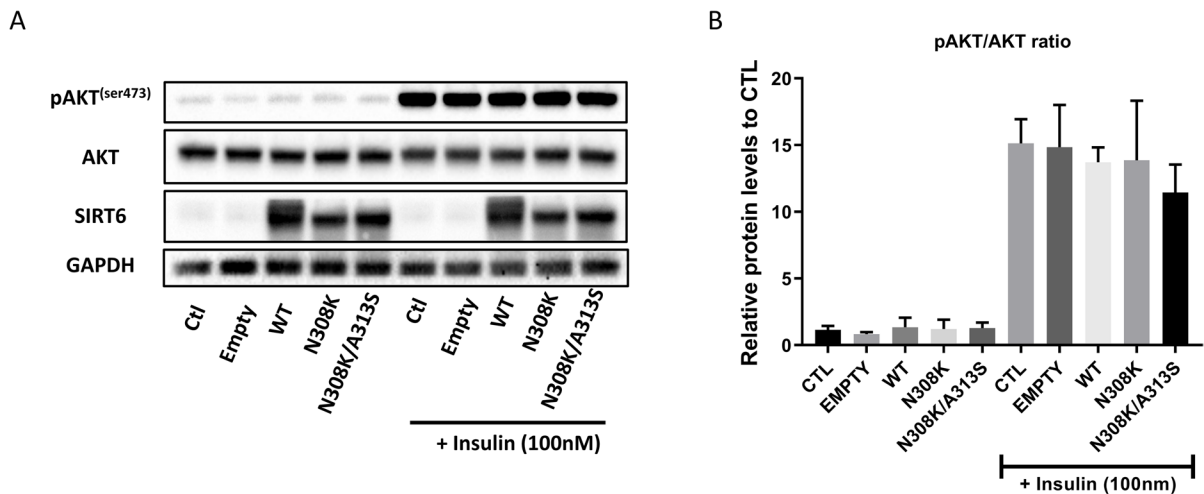


Fig. 3 SIRT6 overexpression did not affect pAKT/AKT ratio in IHH. **A** Representative immunoblotting of pAKT^(ser473), AKT, SIRT6, and GAPDH proteins with or without insulin treatment of 100 nM for 30 min. **B** Quantification of the

protein expression ratio pAKT^(ser473)/AKT relative to control group, showing the increased protein levels in all groups after insulin stimulation, but with no differences among the groups themselves ($N=6$). Data are presented as mean $\pm$ SEM

(GCN), and it profoundly affected serum metabolomics during fasting [53]. Furthermore, we decided to conduct in-depth metabolic profiling in IHH cells and their

supernatants, upon overexpression of SIRT6 and its allelic variants. Ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) platforms

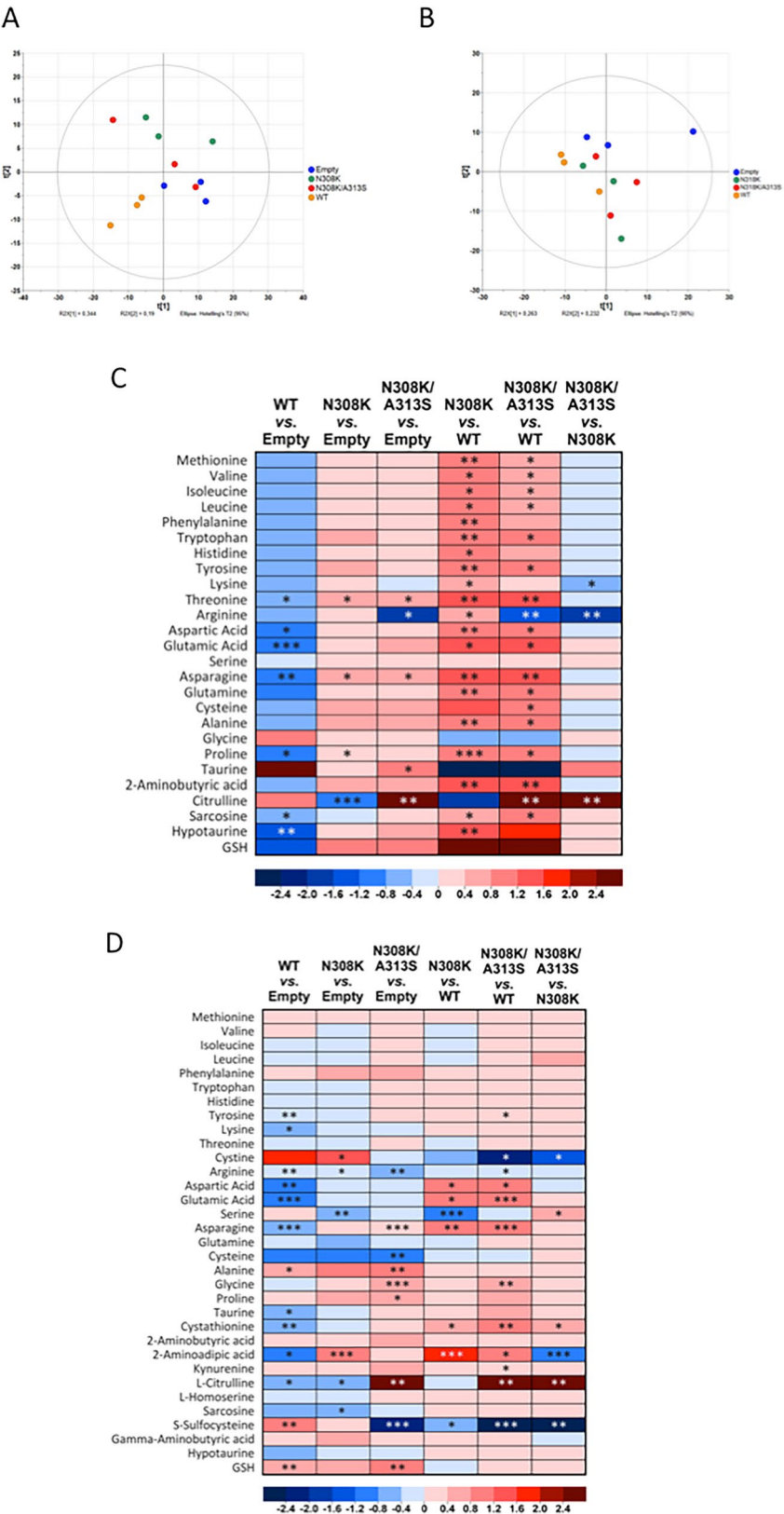
optimized for extensive coverage of the metabolome were used [54]. A total of 296 and 282 metabolic features were detected in the analyzed cell pellets and the culture media samples, respectively, and provided in Supplementary Files 1 and 2. Nomenclature and metabolic pathway descriptions for the metabolites covered by the methods are also detailed in Supplementary Files 1 and 2. First, a principal component analysis (PCA) of hepatocyte extracts was performed for the four IHH cell lines: empty, WT, N308K, or N308K/A313S. The score scatter plot of this PCA shows a separation of WT, N308K, and N308K/A313S samples when the first $t[1]$ and second $t[2]$ components are depicted, with the WT being more segregated compared to the other groups (Fig. 4A). The $t[1]$ and $t[2]$ components explain the 34.4% and 19.0%, respectively, of the variability among samples. Similarly, a PCA analysis of IHH culture media samples was performed. However, the separation between groups (Fig. 4B) was not as clear as in the case of the hepatocyte extracts (Fig. 4A). A higher number of changes in the metabolites' levels were found in the analysis of human hepatocytes (Supplementary Fig. 2) than in the comparisons performed between culture media groups (Supplementary Fig. 3). Most of the changes were observed between the WT samples and the other groups, being a fewer number of metabolites altered between both mutant groups and when they were compared to the hepatocytes transfected with the empty vector (Supplementary Fig. 2). The reduction of the levels of most glycerophospholipids in the cells transfected with the WT SIRT6 sequence when compared to the empty vector (Supplementary Fig. 2) is remarkable, although this reduction was not observed in the culture media (Supplementary Fig. 3). It is also relevant that almost the complete profile of amino acids (AA) was increased in the mutant groups when compared to the hepatocytes transfected with the WT SIRT6 sequence (Supplementary Fig. 2). However, fewer changes were observed in the levels of amino acids in the case of the culture media (Supplementary Fig. 3). Increments in several fatty acid (FA) and glycerophospholipids (especially lysophosphatidylethanolamines, LPE) were also detected for the comparisons between the hepatocytes transfected with the SIRT6 mutant sequences and the WT one, although a higher number of species were altered in the group N308K than in the N308K/A313S (Supplementary Fig. 2). A reduction in ceramides was only detected in N308K group when compared to the WT group, but not for N308K/A313S group (Supplementary Fig. 2).

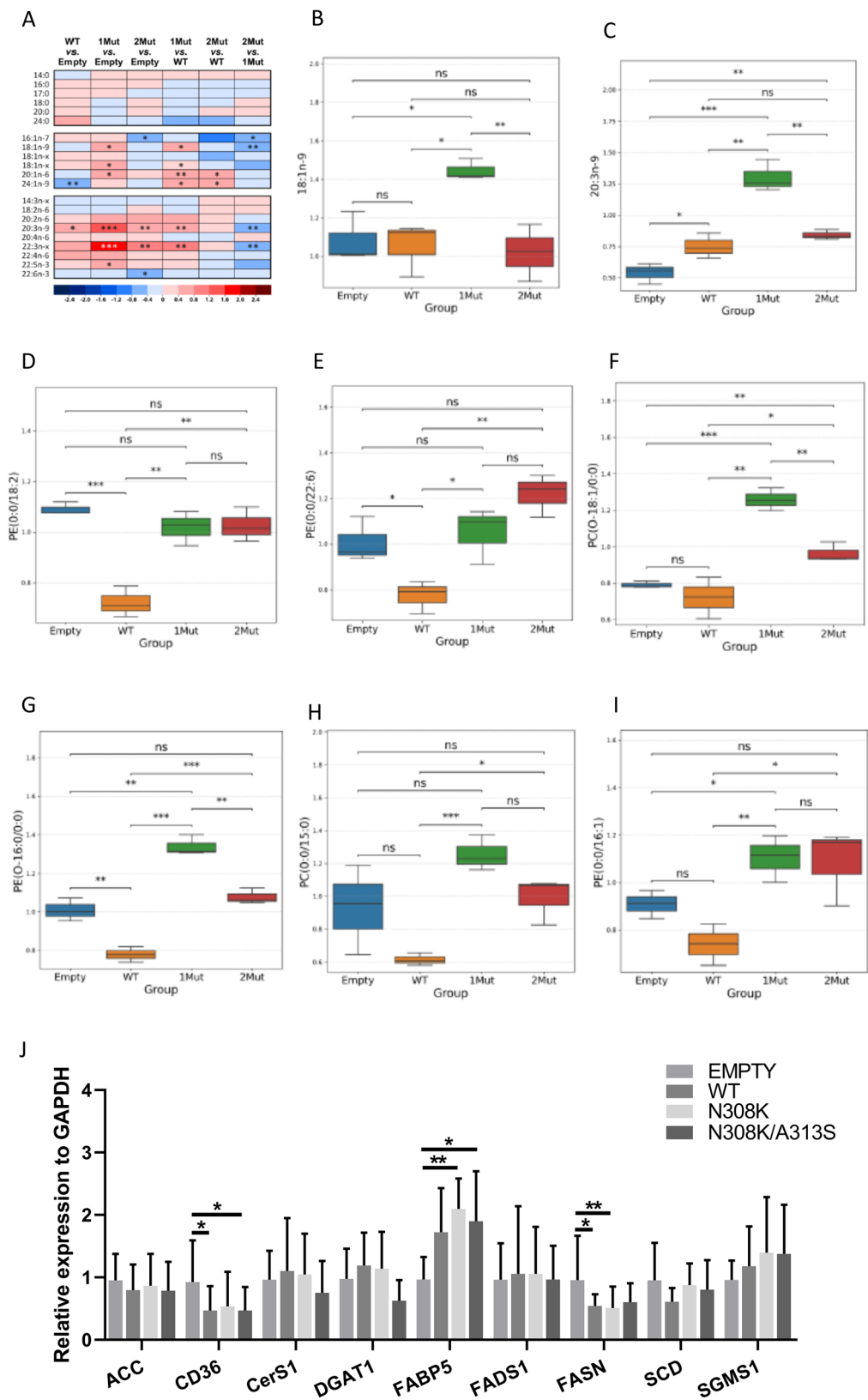
Several glycerophospholipids were also increased in the culture media of the mutant groups when compared to the WT group, especially ether-linked glycerophosphatidylcholines (ether-PC) in the comparison N308K vs. WT (Supplementary Fig. 3).

Levels of several amino acids were reduced in the hepatocytes transfected with the WT SIRT6 sequence when compared to the empty vector: threonine, aspartate, glutamate, asparagine, proline, sarcosine, and hypotaurine (Fig. 4C). Stunningly, however, most of these metabolites were increased in the mutant groups when compared to the empty-vector group (Fig. 4C). Some examples of these changes are included in the Supplementary Table 1. Although it is remarkable that almost the complete profile of amino acids and derivatives was increased in the comparisons of mutant groups vs. the WT group (Fig. 4C), the only exception was the reduction in the levels of arginine in the N308K/A313S group when compared to the other groups (Supplementary Table 1). In contrast, very few changes were detected between hepatocyte groups transfected with the mutations. Among them, a reduction of the levels of arginine and lysine and an increment of citrulline levels were found in the N308K/A313S group when compared to N308K group (Fig. 4C and Supplementary Table 1). Changes in amino acid levels were also observed between culture media groups (Fig. 4D). The most significant changes (lower p values) in the amino acid levels of WT-transfected hepatocytes' culture medium when compared to the empty vector group were the reduction of aspartate, glutamate, asparagine, and arginine (Fig. 4D and Supplementary Table 1). These reductions were also found in the cell pellets (Fig. 4C) except for arginine, for which the reduction did not reach a p value < 0.05 (Supplementary Table 1). Levels of asparagine, aspartic acid, or glutamic acid were increased in the culture media of mutant cells compared to WT samples (Supplementary Table 1), but arginine was reduced in N308K/A313S samples when compared to WT group, as also detected in the hepatocytes (Supplementary Table 1). Levels of several amino acids were also altered between both mutant groups: cystine, serine, cystathionine, aminoadipic acid, citrulline, and sulfocysteine (Supplementary Table 1).

No changes were detected in the levels of saturated fatty acids (SFA) among the groups of hepatocytes (Fig. 5A). However, several monounsaturated

Fig. 4 Metabolite profiling and changes in Amino acids levels. **A** core scatter plot of the PCA model of the human hepatocyte samples. First and second components are depicted. Model diagnostics ($A=3$; $R^2X=0.677$; $Q^2X=0.278$). The ellipse represents 95% confidence interval according to Hotelling's T2 test. **B** Score scatter plot of the PCA model of culture media extracts. Model diagnostics ($A=3$; $R^2X=0.662$; $Q^2X=0.115$). The ellipse represents 95% confidence interval according to Hotelling's T2 test. **C** Heatmap representation of the changes in amino acids and derivatives for the comparisons between groups of human hepatocytes. The color code represents the log2 (fold-change). Student's *t* test *p* values: **p* < 0.05, ***p* < 0.01; ****p* < 0.001. **D** Heatmap representation of the changes in amino acids and derivatives for the comparisons between culture media groups. The color code represents the log2 (fold-change). Student's *t* test *p* values: **p* < 0.05, ***p* < 0.01; ****p* < 0.001





◀**Fig. 5** Lipid profiling. **A** Heatmap representation of the changes in saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) for the comparisons between IHH lines. The color code represents the log₂ (fold-change). **B** Boxplots of 18:1n-9 in IHH. **C** Boxplots of 20:3n-9 in hepatocytes. **D–I** Boxplots of PE(0:0/18:2) (**D**), PE(0:0/22:6) (**E**), PE(18:1/0:0) (**F**), PE(O-16:0/0:0) (**G**), PE(0:0/15:0) (**H**) and PE(0:0/16:1) (**I**) in IHH. Student's *t* test *p* values: not significant (ns), **p*<0.05, ***p*<0.01; ****p*<0.001. **J** mRNA levels of ACC, CD36, CerS1, DGAT1, FABP5, FADS1, FASN, SCD, SGMS1 of the IHH groups. Data are presented as mean ± SEM

and polyunsaturated fatty acids such as the oleic acid (18:1n-9) and mead acid (20:3n-9) were markedly increased in hepatocytes of the N308K group, and to a lesser extent in hepatocytes of the N308K/A313S group when compared to CTL (empty) and SIRT6 WT (Fig. 5A–C).

Regarding glycerolipids, almost no changes were detected in diglyceride or triglyceride levels with the exception of a decrease of several unsaturated species in the WT-transfected hepatocytes when compared to the empty vector group, especially in species with longer acyl chains. Some of these triglycerides with longer acyl chains were also reduced in the N308K/A313S group when compared to control hepatocytes (Supplementary Fig. 4C). A reduction in the levels of most glycerophospholipids was found in the cells transfected with the WT SIRT6 sequence when compared to the empty vector (Supplementary Fig. 2), although almost no changes were detected in the culture media (Supplementary Fig. 3). However, an increment in lysophosphatidylethanolamines species (LPE) was detected for the comparisons between the mutant groups and the WT-transfected hepatocytes, although a higher number of species were altered in the group N308K than in the N308K/A313S (Fig. 5D–I).

Regarding sphingolipids (ceramides and sphingomyelins), several sphingomyelins were reduced in WT-transfected hepatocytes when compared to the empty-vector group, but there were not differences in their levels when mutants and empty groups were compared (Supplementary Table 1). Ceramides tended to be increased in WT-transfected hepatocytes when compared to the empty-vector group, but only the Cer(d18:1/22:0) reached a *p* value <0.05 (Supplementary Table 1). In addition, a reduction in ceramides was only detected in N308K group when compared to the WT group, but not for N308K/

A313S group (Supplementary Table 1). qPCR analysis of genes involved in lipid metabolism revealed significantly decreased levels of CD36 and FASN in IHH cells overexpressing WT SIRT6 or its allelic variants N308K and N308K/A313S, whereas levels of FABP5 were significantly increased in SIRT6 variants N308K and N308K/A313S when compared to SIRT6 Empty cells (Fig. 5J).

Altogether our data reveal profound metabolomics changes in IHH upon overexpression of SIRT6 WT and centenarian-associated mutants (N308K and N308K/A313S), compared to control cells. To summarize: (1) almost the complete profile of amino acids was increased in the mutant groups when compared to the hepatocytes transfected with the WT sequence. It was noteworthy the increment of citrulline levels in IHH from the N308K/A313S group; (2) an increment in several unsaturated fatty acid and glycerophospholipids was also detected for N308K and N308K/A313S, although a higher number of species were altered in the former; (3) ceramides tend to be increased in WT-transfected hepatocytes when compared to the control empty vector group. Also, a reduction in ceramides was detected in N308K group when compared to the WT hepatocytes, but not for N308K/A313S group; (4) almost no changes were found in the levels of diglyceride or triglyceride levels; (5) gene expression levels of CD36 and FASN were significantly decreased in all SIRT6 OE groups compared to the empty vector condition, while FABP5 showed an opposite trend.

Overexpression of centenarian variant (N308K/A313S) of SIRT6 inhibits collagen deposition and fibrotic gene expression in 3D spheroids formed by the co-culture of IHH and human hepatic stellate cells

Dietary supplementation of most amino acids, including branched-chain and essential ones, has therapeutic effects in liver diseases, including NAFLD and NASH [55, 56]. High levels of hepatic unsaturated fatty acids and lower levels of ceramides have been linked to lower fibrosis in NAFLD/NASH [57, 58]. The liver parenchyma is composed of various cell types: while hepatocytes make about 80% of total liver mass, the second most abundant hepatic cell type is represented by hepatic stellate cells (HSC), which account for 5–8%. The crosstalk

between these two major hepatic cell types and HSC-mediated collagen deposition largely controls the progression of fibrosis and inflammation in NAFLD/NASH [59]. First, we assessed the impact of SIRT6 protein overexpression in HSC cells alone. Figure 6A shows the signal of the far-red fluorescence protein Katushka2S contained in the LV cassette, alone in the empty group, or together with one of SIRT6 versions (WT, N308K or N308K/A313S), in LX2 cells, a HSC model, demonstrating the successful infection. This was supported by immunoblotting analysis (Fig. 6B). Overexpression of SIRT6 constructs in LX2 did not change the basal mRNA expression of fibrogenic markers (vimentin, TIMP1, COL1A1, and α SMA) (Fig. 6C). Therefore, LX2 basal phenotype in 2D was not affected by SIRT6. Next, driven by our metabolomics data on IHH, we investigated the in vitro interaction of hepatocytes

and HSC, adopting a 3D spheroid culture model of IHH overexpressing or not SIRT6 and its longevity-associated variants together with naïve HSC (LX2). The spheroid culture allows intercellular connections and communications, reproducing an environment closer to in vivo condition compared to monolayer cell culture, and to date, it has been used for many applications including the evaluation of liver function [36, 37, 60]. In our 3D hepatic spheroid model, IHH and LX2 were co-cultured for 5 days in ultra-low attachment 96 plates and then harvest and processed for the analyses. Figure 7A shows representative immunofluorescence images of spheroids sections stained with DAPI (nuclei) and COL1A1. The quantification analysis uncovered a significant decrease in collagen content in the spheroids with IHH overexpressing the N308K/A313S version of SIRT6 compared to all other groups (Fig. 7A). We

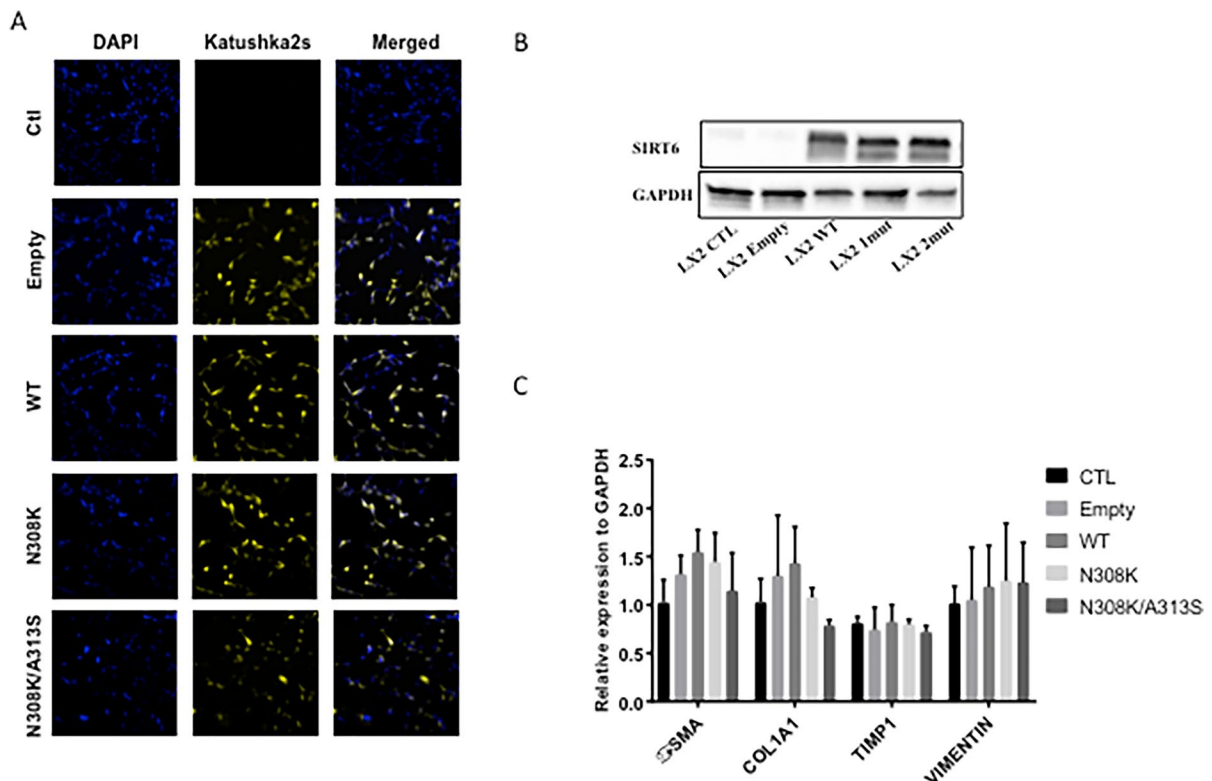


Fig. 6 Model of stable transfected LX2 cells overexpressing SIRT6 allele variants. **A** Representative immunofluorescence images of Katushka2S staining showing the occurred lentivirus transfection for the empty vector and all of three SIRT6 variants (WT, N308K, N308K/A313S) in LX2 cells. **B** Represent-

ative immunoblotting of SIRT6 and GAPDH proteins in LX2-transfected cells. **C** mRNA levels of α SMA, COL1A1, TIMP1, Vimentin, and MMP2 of the LX2 groups. Data are presented as mean $\pm$ SEM

then measured the soluble collagen released in the conditioned media by the spheroids, observing that in all groups overexpressing any of the SIRT6 variants the collagen levels were ~30% lower compared to the vector empty group (Fig. 7B). We also analyzed the mRNA expression of key fibrosis gene markers in the spheroids. The COL1A1 levels were significantly higher in the WT and N308K groups compared to either empty or N308K/A313S groups (Fig. 7C). Moreover, another important marker of fibrosis, MMP2, showed a decreasing trend in all SIRT6 overexpressing groups, with significant lower levels in N308K/A313S group, compared to empty group. Therefore, centenarian-associated SIRT6 variants confer basal anti-fibrotic effects in in vitro multilineage 3D hepatic spheroids.

Discussion

In this work, we have shown that LV-mediated overexpression of SIRT6 allele containing two linked substitutions (N308K/A313S) enriched in AJ centenarians [30], in immortalized human hepatocytes (IHH) altered dramatically their metabolomic profiles compared to WT SIRT6, without changes in insulin sensitivity. Almost all intracellular amino acids were increased by SIRT6 N308K/A313S, when compared to the hepatocytes transfected with the WT sequence. Moreover, several unsaturated fatty acid and glycerophospholipids were increased, and ceramide tended to be decreased upon SIRT6 mutants' overexpression. Also, overexpression of SIRT6 N308K/A313S inhibited collagen deposition and fibrotic gene expression in 3D spheroids composed of IHH and human hepatic

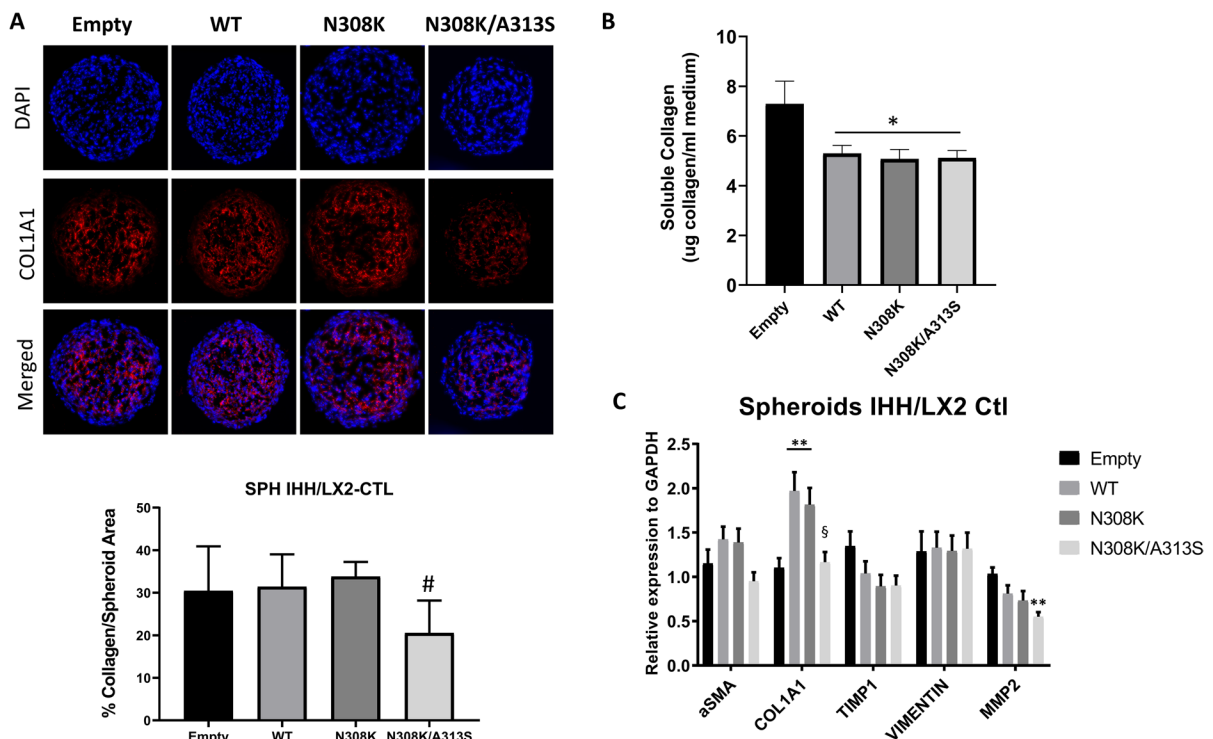


Fig. 7 SIRT6 overexpression lowered basal collagen levels in IHH/LX2 spheroids. **A** Representative immunofluorescence picture of spheroids sections stained for DAPI and/or COL1A1 protein, together with analysis quantification of percentage of collagen content in the spheroids structure. Collagen levels are significantly decreased in N308K/A313S group compared to empty, WT, and NK308K groups. **B** Quantification of soluble collagen content in the condition media of the dif-

ferent groups. All the group overexpressing one of the SIRT6 variant showed a significant decrease of about 30% in soluble collagen levels compared to empty group. **C** mRNA levels of α SMA, COL1A1, TIMP1, vimentin, MMP2 of the five spheroids groups. Data are presented as mean $\pm$ SEM. * p < 0.05 vs empty group. ** p < 0.01 vs empty group, § p < 0.05 vs WT and N308K groups, # p < 0.05 vs all other groups

stellate cells. The robust link between SIRT6 enzymatic activity and increased longevity in mice has been previously shown. Whole-body SIRT6 overexpression in the mixed-CB6 mouse background leads to a significant extension of male lifespan and health span, associated with inhibition of IGF-1 signaling [18]. Non-coding genetic polymorphisms in the SIRT6 gene region were also associated with human longevity in candidate SNP analyses [29, 33, 61]. However, the centenarian-associated SIRT6 variants used in our study are the first ones known to enhance SIRT6 functions and potentially contributing to human longevity: SIRT6 N308K/A313S has enhanced mono-ADP ribosylase activity, but a reduced deacetylase activity. This confers enhancement in DNA repair, tumor suppressor activity, and a resistance to oxidative stress [30]. Consistently, we found that overexpression of SIRT6 variants in hepatoma cells (HepG2 and Huh-7), well-established *in vitro* models of hepatic metabolism but carrying pro-tumorigenic properties, led to even greater cell death compared to WT SIRT6. SIRT6 has been robustly reported to have protective roles against NAFLD/NASH in mice [26–28]. To our knowledge, there are no data available on centenarian AJ individuals (carrying or not SIRT6 mutations) and the incidence of liver diseases such as NAFLD or NASH. Regardless, epidemiological data suggest that the incidence of hepatocellular carcinoma (HCC) drops significantly in the very elderly (individuals aged more than 70), while the risk for NAFLD/NASH-related mortality does not increase in this age range [1, 62]. The peaks for these ailments are observed rather in the 6–7th decade of life. Moreover, a study conducted from 1985 to 2002 on 842 consecutive autopsies in centenarians did not identify liver disease as a cause of death [63]. The potential genetic causes, SIRT6-dependent or independent, for this advanced age-related protection from liver diseases remain unexplored. SIRT6-tg mice display increased health span and lifespan, and restoration of age-related deterioration in normoglycemia [53]. However, paradoxically, compared to young wild type mice, young SIRT6-tg attained higher blood glucose levels [53]: our data showed that SIRT6 variants do not impinge on the insulin-sensitive AKT activation in IHH, no difference in insulin sensitivity between SIRT6 WT and mutants, as assessed by activation of the AKT pathway. This may be consistent with the fact that

SIRT6 acts downstream of AKT phosphorylation and transcriptionally represses it at the level of chromatin [64]. The metabolic platform we used in this study allows the optimal profiling of: (1) fatty acyls, bile acids, steroids, and lysoglycerophospholipids; (2) glycerolipids, glycerophospholipids, sterol lipids, and sphingolipids; (3) amino acids and derivatives. SIRT6 WT overexpression induced metabolomics changes in IHH, including global amino acids' level downregulation, which appear consistent with the metabolomic changes induced by SIRT6 transgene overexpression in mice during fasting or fed state [53]. However, in this respect, SIRT6 variants' overexpression in IHH displayed an opposite phenotype, with a global cellular increase in most amino acids, notably of citrulline. Interestingly, dietary supplementation of amino acids, including citrulline, has preventive and therapeutic effects in liver diseases, including NAFLD and NASH [55, 56, 65]. Moreover, we detected increased levels of IHH LPE species and unsaturated fatty acids, such as oleic acid and mead acid, as well as lower levels of ceramides, modifications that have been linked to lower fibrosis and help stratifying NAFLD/NASH patients [57, 58, 66]. Interestingly, SIRT6 WT and centenarian-associated SIRT variants displayed mostly opposite effects also on LPE, unsaturated fatty acids and ceramides hepatocyte levels. All these changes were observed at a basal state, in absence of nutritional challenge such as incubation with free fatty acids (mimicking a high fat diet in individuals). Hepatocyte metabolomic changes were more prominent and consistent at the intracellular level compared to the extracellular media. Although several fatty acids and amino acid transporters have been identified and investigated with respect to substrate specificity, transport mechanism, and zonal distribution, the respective molecular machineries are not fully understood. Centenarian SIRT6 mutants could modulate hepatocyte intracellular lipid and amino acid homeostasis in part independent from their transport to/from the extracellular environment. The increased gene expression levels of FABP5 in concert with the decreased mRNA levels of CD36 and FASN let us speculate that FABP5 dependent import of fatty acids could be responsible for the elevated intracellular levels of fatty acids detected in SIRT6 overexpressing cells. However, no other significant deregulated genes involved in the cellular lipid metabolism were observed, indicating that other

mechanisms of SIRT6 metabolic control may be in effect. In particular post-translation modification (PTMs) of proteins could be one of the important mechanisms of SIRT6 to regulate intracellular lipid composition and amounts as PTMs at specific amino acid residues of proteins dramatically change the function of a protein through alteration of its properties, which could then mediate dynamic processes within cellular signaling and metabolism networks [67–69]. These modifications are often transient in nature and have great impact on cellular biology. Recently, it was shown that PTMs of various intracellular lipid metabolism protein may differ considerably between tumors and their normal tissue counterparts and thus serve as driving forces for tumorigenesis [70]. Despite ongoing efforts [71], to our knowledge, there are no longitudinal serum metabolomics studies on healthy individuals to uncover their predisposition or resistance to develop NAFLD/NASH. As centenarians tend to be exempt from liver diseases [1, 62, 63]), we chose to study the basal in vitro interaction of the two major hepatic cell types (hepatocytes and hepatic stellate cells), adopting a 3D spheroid culture model, which has been well established for the evaluation of liver function [36, 37, 60]. Our findings show that overexpression of SIRT6 N308K/A313S specifically inhibited the deposition of both basal cellular soluble collagen and fibrotic gene expression in this 3D model. As already mentioned, the AJ centenarian-associated SIRT6 mutations used in our study displayed weaker deacetylase activity, but stronger mADPr activity, over a range of NAD⁺ concentrations and substrates in vitro, compared to SIRT6 WT [30]. SIRT6 mADPr activity may translate into activation of DNA damage-protecting poly-ADP ribose polymerase 1 (PARP1) [72] and consequently reduce the higher mutational burden observed in fibrotic/cirrhotic livers compared to healthy ones [73]. Increases in mADPr and PARP1 activity may affect profoundly lipid species and unsaturated fatty acid membrane composition [74], and regulate mRNA translation rate through negative elongation factor (NELF)-dependent regulation of RNA Polymerase II (Pol II) [75]: the increased transcription rate might reflect the global increased amino acid levels upon SIRT6 mutants overexpression in IHH. This study presents several limitations, as we did not study nutritional/fibrogenic challenges on 3D spheroids (high free fatty acid exposure, TGF- β , etc.), as our study rationale led to

modelling the healthy baseline hepatic status of AJ centenarians' livers; moreover, we did not model the SIRT6 centenarian-associated mutations in established mice models of NAFLD/NASH [76, 77], due to the fact the human N308 and A313 amino acids are not conserved in mice (*data not shown*). Generation of “humanized” mice models carrying N308K/A313S would allow understanding at a mechanistic level how centenarian-associated SIRT6 variants display hepato-protective effects in vivo.

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Author contribution MV: Conceptualization. JF, MR: Data curation. JF, MR, HS, IP, PP, VG: Formal analysis. EL, MV: Funding acquisition. JF, MR: Investigation; EL, JF, MR, HS, IP, PP: Methodology; MV: Project administration; IP, PP: Resource; MV: Supervision; JF, MR, MV: Writing—original draft; EL, VG, JF, MR, IP, PP, MV: Writing—review & editing.

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Data availability All the data supporting the findings of this study are available within the article and its supplementary material files or from the corresponding author upon reasonable request.

Declarations

Competing interests EL is the founder of, and MV and VG are consultants for, GenFlow Biosciences. None of the other authors has conflict of interests.

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