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

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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

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, 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 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.

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 years 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 increase 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.

2. MATERIALS AND METHODS

2.1. Cell lines and 3D spheroids

Cell culture

HepG2, Huh-7 and LX2 cell lines were obtained from CLS-GmbH (Eppenheim, 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 10000 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 five days at 37 °C in a humidified atmosphere of 5% CO₂.

2.2. 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 EF1 α 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 μ m PVDF filter and concentrated by low-speed centrifugation (7000 rpm 16h).

Transduction: The cells, after reaching confluence, were split and seeded into 24-well plate with growth surface area of 2cm², where 50x10⁴ 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 Figure 1; Table 1), in basal DMEM media supplemented with 4ug/mL polybrene transfection reagent (TR-1003, Sigma-Aldrich) with MOI of 1-2. Cells were cultivated with Lentiviral constructs for the period of 24 hours, then the fresh medium was added and cells were cultivated for another 48 hours. Then the cells were treated with selection media comprising of basal DMEM media with 500ug/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 below.

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

2.3. Cell viability

HepG2 and Huh7 control and transduced cell line were maintained in cultivation in T75 flasks for expansion. After two 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).

2.4. 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 till reach at least 70% of confluency, then they were serum starved (0.5% FBS) in low-Glucose (1g/l) DMEM media overnight (12h) and treated or not with insulin solution (100nM, I9278 - Sigma-Aldrich) for 30 minutes. Cells were then harvested using 1xRIPA lysis buffer (20-188, Millipore, USA) and isolation of proteins and immunoblotting were done as described in section 2.7 of Materials and Methods section.

2.5. Microscopy and fluorescence imaging

Immunofluorescence imaging was performed as previously described [41]. The spheroids were fixed with 4% PFA for 10 minutes directly on the cultivation plates and then transferred in mini-tubes. After wash in PBS the spheroids were kept in sucrose 15% for 1 hour, embedded in tissue freezing media (OCT) and then cut to 7 μ m 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 1h. After three washes in 1xPBS, slides were counterstained with DAPI (1 μ g/ml) solution for 15 minutes 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 100x magnification, when at least 5 spheroids per each condition/cell line were used in three consecutive and independent experiments.

2.6. 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.

2.7. Immunoblotting analyses

Immunoblotting analyses were performed as previously described [42, 43]. Briefly, cells were harvested from using TrypLE Express, washed with 1xPBS and centrifuged at 300g. Supernatant was discarded and the obtained pellet was resuspended in 1xRIPA lysis buffer supplemented with Halt[™] Protease and Phosphatase Inhibitor Cocktail (100X, ThermoFisher) and lysed on ice (4°C) for 30 minutes with vigorous vortexing every 10min. The samples were then centrifuged at 10000g 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 (23225, ThermoFisher) according to manufacturer's instruction. Equal amount of protein samples (at least 20 µg) was mixed with 1x Laemmli Sample buffer (1610747, 4x, Bio-Rad) and after heating at 95°C for 5 min and cooling on ice, equal volume of proteins (40 µl) were loaded on 10% Mini-PROTEAN[®] TGX Stain-Free[™] Protein Gels (4568034, Bio-Rad) and separated by electrophoresis running at 120 volts for 45 minutes. Protein transfer was performed on PVDF membranes using Trans-Blot Turbo RTA Mini 0.45 µm LF PVDF Transfer Kit (1704274, Bio-Rad) and Bio-Rad Trans-Blot Turbo Transfer System at 1.3A and 25V 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 minutes 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 (1705061, 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 follow antibodies: Cell Signaling Technology (MA, USA) - rabbit anti-Akt (1:1000), rabbit anti-Phospho-Akt (Ser473) (1:1000), rabbit anti Histone H3 (D1H2, 1:1000), Abcam (UK) - rabbit anti Collagen I (1:1000), rabbit anti SIRT6 antibody (1:1000, EPR18463), ThermoFischer 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);

2.8. 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 (74106, 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 1µg of total isolated RNA was used to prepare cDNA using a High-Capacity cDNA Reverse Transcription Kit (4368814, 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 (4472908, 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 1.

Table 2: Primer sequences used in this study.

Gene		Sequence (5'-3')	Gene		Sequence (5'-3')
αSMA	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	CGGTTCGATCACTAGCCACC
MMP2	F	TACAGGATCATTGGCTACACACC	FASN	F	GGAGGTGGTGATAGCCGGTAT
	R	GGTCACATCGCTCCAGACT		R	TGGGTAATCCATAGAGCCCAG
ACC	F	ATGTCTGGCTTGACCTAGTA	SCD	F	TCTAGCTCCTATAACCACCACCA
	R	CCCCAAAGCGAGTAACAAATTCT		R	TCGTCTCCAACCTTATCTCCTCC
CD36	F	GGCTGTGACCGGAACTGTG	SGMS1	F	TGTGCCGAGTCTCCTCTGA
	R	AGGTCTCCAACCTGGCATTAGAA		R	CCGTTCTTGTGTGCTTCCAAA
GAPDH	F	GGTGCCTGCCAGTTGA			
	R	TACTTTCTCCCCGCTTTTT			

2.9. Metabolomics

Metabolic profiling was performed by mass spectrometry coupled to ultra-high performance liquid chromatography (UHPLC-MS) as previously 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°C for 1 h. The samples were then vortexed and centrifuged at $18,000 \times g$ at 4°C for 5 min, and the supernatants were collected and incubated at 4°C while the cell pellets were again resuspended in cold extraction solvents and incubated for a further 1 h at -20°C . The samples were again vortexed and centrifuged at $18,000 \times g$ at 4°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°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 $p < 0.001$.

2.10. 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.

2.11. Data availability statement

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

3. RESULTS

3.1. *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 two weeks from LV transduction (Figure 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].

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

3.2. 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 minutes 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 (Figure 3A-B).

3.3. *Overexpression of centenarian variants of SIRT6 profoundly alters the metabolomic profiles of IHH.*

SIRT6 overexpression in mice preserve glucose homeostasis through an improvement in gluconeogenesis (GCN), and it profoundly affected serum metabolomics during fasting [53]. Further, 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 File 1 and Supplementary File 2. Nomenclature and metabolic pathway descriptions for the metabolites covered by the methods are also detailed in Supplementary File 1 and Supplementary File 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 (Figure 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 (Figure 4B) was not as clear as in the case of the hepatocyte extracts (Figure 4A). A higher number of changes in the metabolites' levels were found in the analysis of human hepatocytes (Supplementary Figure 2) than in the comparisons performed between culture media groups (Supplementary Figure 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 Figure 2). It is remarkable the reduction of the levels of most glycerophospholipids in the cells transfected with the WT SIRT6 sequence when compared to the empty vector (Supplementary Figure 2), although this reduction was not observed in the culture media (Supplementary Figure 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 Figure 2). However, fewer changes were observed in the levels of amino acids in the case of the culture media (Supplementary Figure 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 Figure 2). A reduction in ceramides was only detected in N308K group when compared to the WT group, but not for N308K/A313S group (Supplementary Figure 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 Figure 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 (Figure 4C). Stunningly, however, most of these metabolites were increased

in the mutant groups when compared to the empty-vector group (Figure 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 (Figure 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 (Figure 4C and Supplementary Table 1). Changes in amino acids levels were also observed between culture media groups (Figure 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 (Figure 4D and Supplementary Table 1). These reductions were also found in the cell pellets (Figure 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 (Figure 5A). However, several monounsaturated 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 (Figure 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. This can be easily visualized in the carbon plots displayed in Supplementary Figure 4A and 4B, which represent the influence of the number of carbons and double bond content in the increment or decrement of diglyceride and triglycerides in the WT group compared to the empty vector control group. Some of these triglycerides with longer acyl chains were also reduced in the N308K/A313S group when compared to control hepatocytes (Supplementary Figure 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 Figure 2), although almost no changes were detected in the culture media (Supplementary Figure 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 (Figure 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 (Figure 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. As well, 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.

3.4. 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 (Figure 6B). Overexpression of SIRT6 constructs in LX2 did not change the basal mRNA expression of fibrogenic markers (vimentin, TIMP1, COL1A1 and α SMA) (Figure 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 (Figure 7A). We 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 (Figure 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 (Figure 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.

4. 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 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, as well as 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. Centenarians 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*.

Author contributions

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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Supplementary Information

Supplementary material associated with this article can be found, in the online version.

Declarations

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

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Data availability statement

All the data supporting the findings of this study are available within the article and its Supplementary materials files or from the corresponding author upon reasonable request.

Figure Legends

Figure 1: Hepatoma cell lines viability decreases after SIRT6 overexpression. Representative figures from optimal microscope of HepG2 and HuH7 cell culture after two 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.

Figure 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 proteins levels in IHH cells (N=5-7). Data are presented as mean \pm SEM. *p < 0.05 vs Empty group.

Figure 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 minutes. (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.

Figure 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; R2X=0.677; Q2X=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; R2X=0.662; Q2X=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.

Figure 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 \pm SEM.

Figure 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) Representative 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.

Figure 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 different 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.

Supplementary Table 1: Selection of metabolites with significant changed levels upon different SIRT6 OE variant overexpression.

Supplementary Figure 1: Schematic representation of the lentiviruses (LV) used in this study.

Supplementary Figure 2: Amino acids profiling. Heatmap representing binary comparisons between hepatocyte groups per metabolite. Heatmap color codes for log₂ (fold-change) and Student's t-test p-values are indicated at the bottom of the figure.

Supplementary Figure 3: Amino acids profiling. Heatmap representing binary comparisons between culture media groups per metabolite. Heatmap color codes for log₂ (fold-change) and Student's t-test p-values are indicated at the bottom of the figure.

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