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ORIGINAL ARTICLE

U6 is unsuitable for normalization of serum miRNA levels in patients with sepsis or liver fibrosis

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MicroRNA (miRNA) levels in serum have recently emerged as potential novel biomarkers for various diseases. miRNAs are routinely measured by standard quantitative real-time PCR (qPCR); however, the high sensitivity of qPCR demands appropriate normalization to correct for nonbiological variation. Presently, RNU6B (U6) is used for data normalization of circulating miRNAs in many studies. However, it was suggested that serum levels of U6 themselves might differ between individuals. Therefore, no consensus has been reached on the best normalization strategy in 'circulating miRNA'. We analyzed U6 levels as well as levels of spiked-in SV40-RNA in sera of 44 healthy volunteers, 203 intensive care unit patients and 64 patients with liver fibrosis. Levels of U6 demonstrated a high variability in sera of healthy donors, patients with critical illness and liver fibrosis. This high variability could also be confirmed in sera of mice after the cecal ligation and puncture procedure. Most importantly, levels of circulating U6 were significantly upregulated in sera of patients with critical illness and sepsis compared with controls and correlated with established markers of inflammation. In patients with liver fibrosis, U6 levels were significantly downregulated. In contrast, levels of spiked-in SV40 displayed a significantly higher stability both in human cohorts (healthy, critical illness, liver fibrosis) and in mice. Thus, we conclude that U6 levels in the serum are dysregulated in a disease-specific manner. Therefore, U6 should not be used for data normalization of circulating miRNAs in inflammatory diseases and previous studies using this approach should be interpreted with caution. Further studies are warranted to identify specific regulatory processes of U6 levels in sepsis and liver fibrosis.

INTRODUCTION

MicroRNAs (miRNAs) represent a new class of RNAs that do not transcribe for proteins, but act as regulators of gene expression in multicellular and some unicellular eukaryotes.[1,2] Recently, the involvement of miRNAs was demonstrated in highly regulated processes such as inflammation, fibrosis and carcinogenesis.[3–5] Next to their role in the regulation of gene expression in various pathological and physiological processes, serum levels of some miRNAs emerged as biomarkers in different diseases such as inflammation/bacterial infection,[6–8] fibrosis[9–11] and cancer.[12,13]

Because of their low chemical complexity, lack of post-processing modifications, tissue-specific expression profiles and their extraordinary stability to storage and handling, circulating miRNAs represent potential biomarkers as well.

However, although the problem of proper detection of miRNAs was solved with the introduction of standard quantitative real-time PCR (qPCR) assays, the problem of proper data normalization is still unsolved and might explain the high variance between different studies analyzing alterations of circulating miRNAs.[11] In most available studies, the cell/tissue-miRNA normalizer 'RNU6B (U6)' is used for normalization of serum miRNAs.[14–19] However, it was suggested that serum levels of this RNA display high inter-individual variances[11,20,21] and different authors analyzed the suitability of other miRNAs such as miR-16,[22] miR-192,[8,23] miR-374a, miR-374b and let-7d[21] or combinations of miRNAs (such as miR-146a, miR-16, miR-195, miR-30e and miR-744) for normalization of circulating miRNAs levels.[24] However, the stable expression of these genes was not validated in larger

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human collectives with different disease collectives. For array analyses, Mestdagh et al.[25] just recently proposed a strategy based on a global expression mean to normalize miRNA levels; however, this approach cannot be easily adapted for single-gene PCR analysis. Thus, considering the lack of established endogenous 'normalizers', many authors recommend the use of external 'spiked-in' RNA for normalization of variations during RNA extraction and sample handling.[9,11,23,26–31]

In the present study, we analyze the suitability of circulating U6 for normalization of serum miRNAs in two large patient collectives with either critical illness or sepsis and liver fibrosis, thereby demonstrating that U6 is not suitable to serve as a reference gene in this setting.

MATERIALS AND METHODS

Study design and patient characteristics

Between March 2008 and July 2011, patients who were admitted to the internal intensive care unit (ICU; University Hospital RWTH Aachen) were included in this study (Table 1). According to previously published practice, those patients who were expected to have a only a short-term (<72 h) intensive care treatment were not included in this study.[32,33] Patient data, clinical information and blood samples were collected prospectively. The clinical course of patients was observed in a follow-up period by directly contacting the patients, the patients' relatives or their primary care physician. Patients who met the criteria proposed by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference Committee for sepsis, severe sepsis and septic shock were categorized as sepsis patients, and the others as non-sepsis patients.[34,35] Patients displaying a body mass index of >30 kg m^{-2} at ICU admission (before any treatment) were defined as obese. Patients with a medical history of diabetes mellitus and a concomitant intake of diabetes-related medication were defined as diabetics. As a control population, we analyzed 44 healthy blood donors with normal values for blood counts, C-reactive protein and liver enzymes. The study protocol was approved by the local ethics committee and conducted in accordance with the ethical standards laid down in the Declaration of Helsinki (ethics committee of the University Hospital Aachen, RWTH-University, Aachen, Germany, reference number EK 150/06). Written informed consent was obtained from the patient, his or her spouse or the appointed legal guardian.

Characteristics of patients with liver fibrosis

We studied 64 patients with chronic liver diseases.[36] Characteristics of the study cohort are summarized in Table 4. Importantly, patients were excluded in case of systemic inflammatory response syndrome or sepsis, HIV-infection, systemic steroid medication (prednisolone >7.5 mg per day or equivalent doses) and malignant tumor(s) except for hepatocellular carcinoma. The study protocol was approved by the local ethics committee and conducted in accordance with the ethical standards laid down in the Declaration of Helsinki (ethics committee of the University Hospital Aachen, RWTH-University, Aachen, Germany, reference number EK 150/06). Written informed consent was obtained from the patient, his or her spouse or the appointed legal guardian.

Mouse model of polymicrobial sepsis

Male C57Bl/6 mice (6–8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Sex- and age-matched mice were subjected to the cecal ligation and puncture surgery, as described previously.[37] Blood was taken before and 24 h after surgery for analysis. Animals received human care according to European, national and institutional regulations.

miRNA isolation from serum

Four hundred microliters of serum were spiked with miScript miRNA mimic SV40 (Qiagen, Hilden, Germany, 2 μM, 1 μl per 100 μl serum). Eight hundred microliters of phenol (Qiazol, Qiagen) and 200 μl of chloroform were added to the sample and mixed vigorously for 15 s, followed by an incubation at room temperature for 10 min. Samples were centrifuged for 15 min at 12 000 g until complete phase separation. The aqueous phase, containing total RNA, was precipitated with 500 μl of 100% isopropanol and 2 μl of glycogen (Fermentas, St Leonroth, Germany) overnight at -20 °C. After centrifugation at 4 °C for 30 min (12 000 g), the pellets were washed once with 70% ethanol. Precipitated RNA was resuspended in 30 μl of RNase-free water (Ambion, Austin, TX, USA). To assess the quality of RNA, the samples were measured with a NanoDrop spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) and a small RNA assay using Agilent's Bioanalyzer was performed (Agilent Technologies, Böblingen, Germany).[9,38]

Quantitative real-time PCR

qPCR was performed as recently described.[9,38] In detail, 5 μl of the final RNA eluate was used to synthesize cDNA utilizing miScript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's protocol. cDNA samples (2 μl) were used for qPCR in a total volume of 25 μl using the miScript SYBR Green PCR Kit (Qiagen) and miRNA-specific primers (Qiagen) on a qPCR machine (Applied Biosystems 7300 Sequence Detection System, Applied Biosystems, Foster City, CA, USA). All real-time PCR reactions were performed in duplicates. Data were generated and analyzed using the SDS 2.3 and RQ manager 1.2 software packages (Applied Biosystems).

Table 1 Baseline characteristics of the ICU patients

Parameter	All patients
Number	203
Sex (male/female)	126/77
Age median (range) (years)	63 (18–89)
APACHE-II score median (range)	17 (2–40)
ICU days median (range)	7 (1–83)
Death during ICU (%)	21.6%
Death during ICU or follow-up (%)	41.9%
Ventilation time median (range) (h)	139 (0–1363)
Pre-existing diabetes, n (%)	78 (38.4%)
Body mass index	26.1 (16.6–86.5)
WBC median (range) ($\times 10^3$ per μl)	12.5 (0.1–67.4)
D-Dimers	2167 (0–9000)
CRP median (range) (mg dl^{-1})	94 (<5–230)
Procalcitonin median (range) (μg l^{-1})	0.7 (0–180.6)
Interleukin-6 median (range) (pg ml^{-1})	100 (0–83 000)
TNF (range) (pg ml^{-1})	49 (4.9–140)

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; CRP, C-reactive protein; ICU, intensive care unit; SAPS, Simplified Acute Physiology Score; TNF, tumor necrosis factor; WBC, white blood cell count.

Statistical analysis

Raw C_t values of the U6 and SV40 qPCR were shown as difference to its median. To quantify the variability of U6 and SV40 levels 'mean of D^2-value' were built. Differences in the mean of these received D^2-values were analyzed with a t-test. In addition, an F-test with raw C_t value was performed to compare the variances.

Gaussian distribution was checked using the Shapiro–Wilk test. Data are displayed as median and range considering the skewed distribution of most of the parameters. Differences between two groups were assessed using the Mann–Whitney U-test and multiple comparisons between more than two groups have been conducted using the Kruskal–Wallis–analysis of variance and Mann–Whitney U-test for post hoc analysis. Box plot graphics illustrate comparisons between subgroups and they display a statistical summary of the median, quartiles, range and extreme values. The whiskers extend from the minimum to the maximum value excluding outside and far-out values that are displayed as separate points. An outside value (indicated by an open circle) was defined as a value that is smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range. A far-out value was defined as a value that is smaller than the lower quartile minus three times the interquartile range, or larger than the upper quartile plus three times the interquartile range. All values,

including 'outliers', have been included for statistical analyses. Correlations between variables have been analyzed using the Spearman correlation tests, where values of $P < 0.05$ were considered statistically significant. Differences in mice between before and 24 h after surgery were assessed using the Wilcoxon-matched pairs test. Statistical analyses were performed with SPSS (SPSS, Chicago, IL, USA) and GraphPad Prism 5 (Graph-Pad, San Diego, CA, USA).[33]

RESULTS

U6 levels display high variability in murine serum

U6 serum levels are widely used for the normalization of miRNA-based biomarkers in inflammatory diseases.[14–18] We therefore tested first whether U6 serum levels are suitable normalization parameters in a typical inflammatory disease model. For this, we first examined U6 serum levels in the well-established murine cecal ligation and puncture model, representing a highly synchronized model for polymicrobial sepsis. Variability of U6 serum levels were high compared with levels of spiked-in SV40, representing an alternative, widely accepted method for normalization of miRNA in serum samples.[9,11,23,26–30] Interestingly, serum levels of U6 displayed
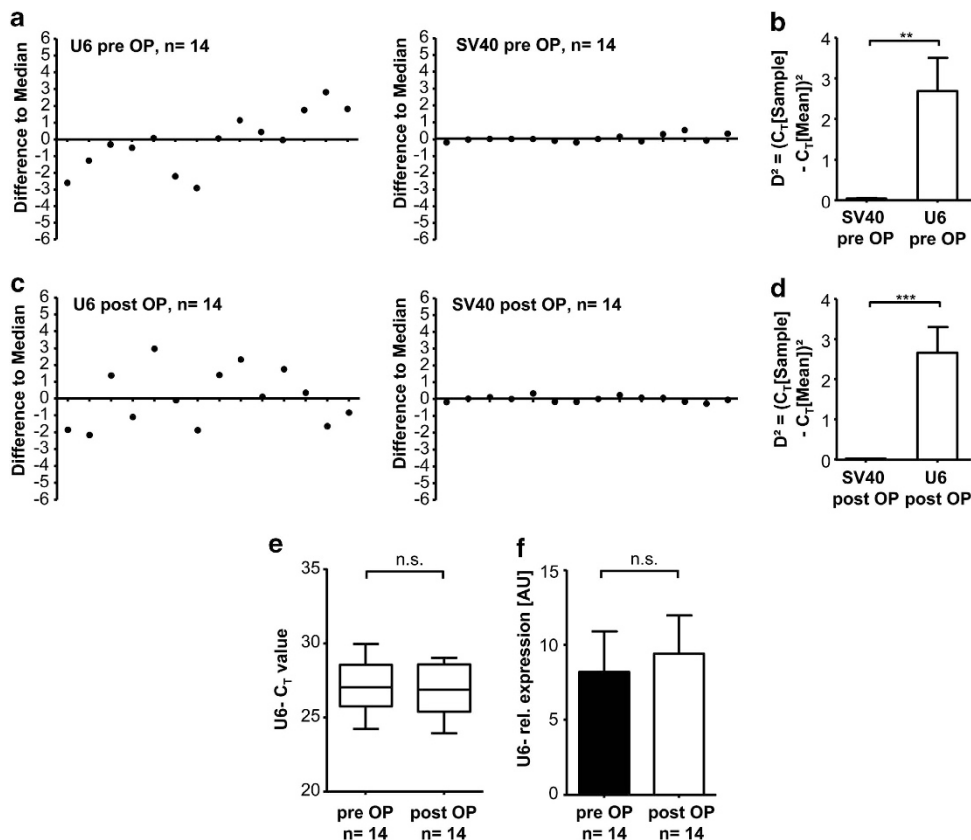

Figure 1 RNU6B (U6) levels display high variability in murine serum. (**a**, **c**) Serum U6 levels were analyzed by quantitative real-time PCR (qPCR) in C57Bl/6 mice before and 24 h after the cecal ligation and puncture (CLP) surgery and revealed a very high variability of U6 levels in the different mice ($n = 14$ per group). Levels of spiked-in SV40-RNA was determined by qPCR in samples from C57Bl/6 mice before and 24 h after CLP surgery, revealing a very low variability between the different samples. (**b**, **d**) The variability of U6 values and SV40 values was statistically compared, revealing a significantly higher variability in U6 values compared with that of SV40. (**e**) Raw U6 C_t values were compared between C57Bl/6 mice before and 24 h after CLP surgery, revealing no significant differences between both groups. (**f**) U6 serum levels were compared between C57Bl/6 mice before and 24 h after CLP surgery, revealing no significant differences between both groups also when data were normalized using SV40. **$P < 0.01$, ***$P < 0.001$.

high interindividual variability before and 24 h after the cecal ligation and puncture surgery despite an identical genetic background in inbred mice (Figures 1a and c), which was significantly higher compared with the spiked-in SV40-RNA method (Figure 1b (t-test: $P = 0.003$ F-test: $P < 0.001$) and Figure 3d (t-test: $P < 0.001$ F-test: $P < 0.001$)). Moreover, we observed a nonsignificant trend toward higher U6 levels in the cecal ligation and puncture-operated mice (Figures 1e and f). Of note, these results remained unchanged when data were normalized to spiked-in SV40-RNA (Figure 3f).

High variability of serum U6 levels in human serum

On the basis of our findings in mice, we next analyzed the variability of U6 levels in human serum and performed a systematic qPCR analysis on RNA extracts from serum samples of healthy donors. As shown in Figure 2a, serum levels of circulating U6 displayed high interindividual variability of up to eight cycles in qPCR analysis. In contrast, levels of spiked-in SV40-RNA showed a very low variability between the different samples (Figure 2b), and hence displayed a significantly lower variability than U6 (t-test: $P < 0.001$, F-test: $P < 0.001$; Figure 2c).

Levels of circulating U6 are elevated in critical illness and sepsis

On the basis of these findings, we next analyzed U6 serum levels in sera of 203 critically ill patients (126 men, 77 women with a median age of 63 years (range of 18–89 years) and a medium ICU-stay of 7 days (range of 1–83 days)) at admission to the ICU. Among these 203 critically ill patients, 126 patients conformed to the criteria of bacterial sepsis (Table 1). Most of these patients had pneumonia as underlying

disease leading to sepsis (Table 2). In non-septic patients, cardiopulmonary diseases (myocardial infarction, pulmonary embolism and cardiac pulmonary edema) and decompensated liver cirrhosis represented the predominant underlying disease etiologies (Table 2). Sepsis patients were more often in need of mechanical ventilation and for longer time periods than non-septic patients (Table 1). In sepsis patients, significantly higher levels of routinely used biomarkers of inflammation (C-reactive protein, procalcitonin, white blood cell count) were found (Table 1). Both groups did not differ in Acute Physiology and Chronic Health Evaluation II, and Simplified Acute Physiology Score 2 scores, vasopressor demand or laboratory parameters indicating liver or renal dysfunction (data not shown).

Similar to the results obtained in healthy volunteers, serum U6 levels demonstrated high variability also in sera of critically

Table 2 Disease etiology of the ICU-study population

	Sepsis n = 126	Non-sepsis n = 77
Etiology of sepsis critical illness		
Site of infection, *n* (%)		
Pulmonary	69 (54.8%)	
Abdominal	26 (20.6%)	
Urogenital	3 (2.4%)	
Other	28 (22.2%)	
Etiology of non-sepsis critical illness (%)		
Cardiopulmonary disease		26 (33.8%)
Decompensated liver cirrhosis		12 (15.6%)
Non-sepsis, other		39 (50.6%)

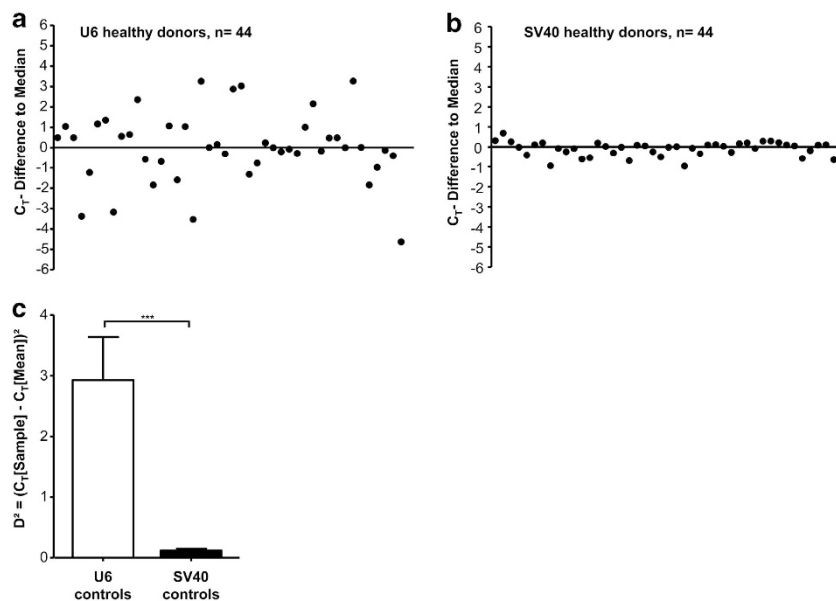Abbreviation: ICU, intensive care unit.

Figure 2 RNU6B (U6) levels display high variability in human serum. (**a**) Serum U6 levels were analyzed by quantitative real-time PCR (qPCR) in healthy donors and revealed a high interindividual variability ($n = 44$). (**b**) Levels of spiked-in SV40-RNA were determined by qPCR in samples from healthy donors, revealing a very low variability between the different samples. (**c**) The variability of U6 values and SV40 values was statistically compared, revealing a significantly higher variability in U6 values compared with that of SV40. ***$P < 0.001$.
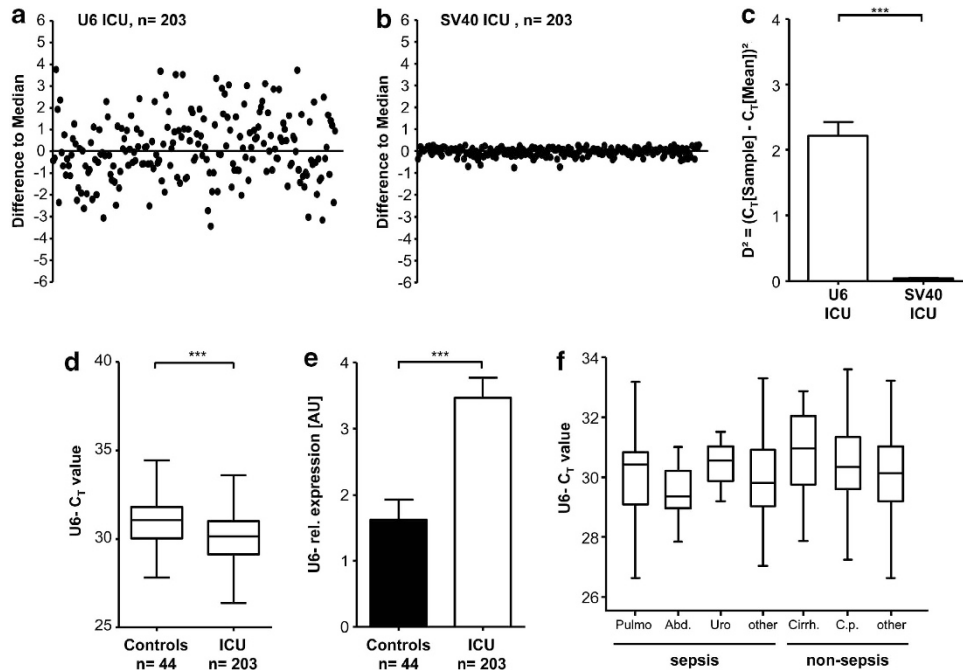
Figure 3 Levels of circulating RNU6B (U6) are elevated in critical illness. (**a**) Serum U6 levels at admission to the intensive care unit (ICU) were analyzed by quantitative real-time PCR (qPCR) and revealed a very high variability between different individuals ($n = 203$). (**b**) Levels of spiked-in SV40-RNA were determined by qPCR in samples from patients with critical illness, revealing a very low variability between the different samples. (**c**) The variability of U6 values and SV40 values was statistically compared, revealing a significantly higher variability in U6 values compared with that of SV40. (**d**) U6 serum levels were compared between critically ill patients and healthy donors, revealing significantly higher levels of U6 in the patients' group (corresponding to lower C_t values). (**e**) U6 serum levels were compared between critically ill patients and healthy donors, revealing significantly higher levels of U6 in the patients' group also when data were normalized using SV40. (**f**) U6 serum levels were analyzed with respect to the different etiologies of critical illness. Abd, abdominal; Cirrh, cirrhosis; C.p., cardiopulmonary; Uro, urological. ***$P < 0.001$.

ill patients (Figure 3a), which was again significantly higher than that of spiked-in SV40-RNA (t-test: $P < 0.001$, F-test: $P < 0.001$; Figures 3b and c). Most importantly, absolute serum levels of U6 were significantly elevated in patients with critical illness and sepsis when compared with healthy volunteers (Figure 3d). Of note, these results remained unchanged when data were normalized to spiked-in SV40-RNA (Figure 3e). Interestingly, U6 serum levels did not significantly vary between septic and non-septic patients and were independent of the underlying disease entity (Figure 3f).

To further elucidate factors potentially affecting serum U6 levels, we performed correlation analyses with numerous laboratory parameters. Serum levels of U6 significantly correlated with established serum markers of inflammation, such as C-reactive protein ($r = 0.153$, $P < 0.030$), procalcitonin ($r = 0.207$, $P = 0.012$), tumor necrosis factor ($r = 0.331$, $P = 0.020$) and interleukin-10 ($r = 0.193$, $P < 0.039$; Table 3). In addition, we observed significant correlations of serum U6 levels with recently described markers of critical illness such as a proliferation-inducing ligand[39] ($r = 0.307$, $P < 0.001$), soluble urokinase-type plasminogen activator receptor[32] ($r = 0.281$, $P = 0.001$) and resistin[40] ($r = 0.285$, $P = 0.018$). Finally, U6 serum levels were significantly correlated with increased levels of D-dimers ($r = 0.391$, $P = 0.001$) and serum lactate ($r = 0.207$, $P = 0.003$), supporting a regulation of U6

serum levels in the setting of a systemic inflammatory response syndrome and critical illness. Interestingly, low U6 serum levels correlated with patients' insulin need during ICU treatment ($r = -0.268$; $P = 0.024$). In line, patients with type 2 diabetes displayed a trend toward lower U6 serum levels than patients without diabetes (Supplementary Figure 1). Finally, serum U6 levels were not correlated with the glomerular filtration rate, thus arguing against an unspecific retention of U6 in critically ill patients with an impaired renal function.

Levels of circulating U6 are downregulated in liver fibrosis
Although the previous results were gained in patients and mice with systemic disease and sepsis, we finally tested the regulation of U6 in liver fibrosis as a more organ-specific disease in which serum levels of miRNAs have been extensively studied.[11] We therefore measured serum U6 levels in a well-defined cohort of patients with liver fibrosis of different etiologies and disease stages (Table 4). In line with our previous findings, U6 levels demonstrated a high interindividual variability, especially when compared with that of spiked-in SV40 (t-test: $P < 0.001$, F-test: $P < 0.001$; Figures 4a and b). However, in contrast to patients with critical illness, U6 levels were significantly lower in sera of patients with liver fibrosis compared with healthy controls and correlated with serum interleukin-6 concentrations ($r = 0.426$, $P = 0.003$), a cytokine

Table 3 Correlations of U6 serum levels at admission day with other laboratory markers

Parameter	ICU patients	
	r	P
Markers of liver function		
AST	0.362	<0.001
ALT	0.273	<0.001
Markers of inflammation		
CRP	0.153	0.030
PCT	0.207	0.012
IL-10	0.193	0.039
TNF	0.331	0.020
D-Dimer	0.391	0.001
Markers of renal function		
GFR of cystatin C	0.011	0.901
Others variables		
APRIL	0.307	<0.001
suPAR	0.281	0.001
Resistin	0.295	0.018
Lactat	0.207	0.003
LDH	0.409	<0.001
Insulin	−0.268	0.024

Abbreviations: ALT, alanine aminotransferase; APRIL, a proliferation-inducing ligand; AST, aspartate aminotransferase; CRP, C-reactive protein; GFR, glomerular filtration rate; ICU, intensive care unit; IL-10, interleukin-10; LDH, lactate dehydrogenase; PCT, procalcitonin; r, correlation coefficient; suPAR, soluble urokinase-type plasminogen activator receptor; TNF, tumor necrosis factor; U6, RNU6B.
r- and P-values are determined by the Spearman rank correlation.

Table 4 Baseline characteristics of the patients with liver fibrosis.

Parameter	All patients
Number	64
Sex (male/female)	40/24
Age median (range) (years)	57 (29–82)
Stage of cirrhosis, n (%)	
No cirrhosis	7 (10.9%)
Child A	24 (37.5%)
Child B	11 (17.2%)
Child C	22 (34.4%)
Etiology, n (%)	
Alcohol	37 (57.8%)
HBV	2 (3.1%)
HCV	4 (6.3%)
PBC	19 (29.7%)
PSC	1 (1.6%)
Other	1 (1.6%)
HCC (yes/no)	53/11
MELD median (range)	12 (6–28)
Creatinine median (range) (mg dl^{-1})	0.6 (0.0–1.7)
Total bilirubin median (range) (mg dl^{-1})	2.1 (0.2–43.2)
IL-6 median (range) (pg ml^{-1})	15 (2–200)
AST median (range) (IU l^{-1})	81 (11–294)
ALT median (range) (IU l^{-1})	43 (12–568)
Quick median (range) (%)	69 (33–100)
Hyaluronan median (range)	280 (10–800)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL-6, interleukin-6; MELD, Model for End-stage Liver Disease Score; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

with important mechanistic functions in liver disease[41] (Figures 4c and d). These findings suggest that U6 serum level are differentially regulated between distinct disease entities, and therefore are not suitable for evaluation of serum biomarkers in liver disease.

DISCUSSION

The extraordinary potential of circulating miRNAs as 'next-generation' serum biomarkers is mainly based on their stability and resistance toward storage and handling. Serum miRNAs remain stable after being subjected to conditions that would normally degrade most RNAs, such as boiling, very low or high pH levels, extended storage and repetitive freezing-thawing cycles.[22] Previous studies demonstrated that distinct miRNAs were even preserved in archived 10-year-old serum samples.[28] Despite these excellent characteristics, different problems have prevented the integration of circulating miRNAs into clinical routine: on the one hand it was challenging for a long time to reliably detect miRNAs in body fluids and on the other hand until now no consensus on standardization and normalization of serum miRNA analysis could be established. Hence, previously published studies using differing methodologies or normalization strategies described

conflicting data and cross-comparison of miRNA-expression profiles failed to provide reliable results. As such, elevated levels of circulating miR-122 and mir-92a were described in patients with chronic hepatitis B virus infection after normalization to endogenous miR-181 values, whereas, in contrast, levels of these miRNAs were found to be decreased in the same setting after normalization with U6.[18,42]

In the last years, most authors proposed the use of U6 for normalization of circulating miRNAs. U6 has been shown to be consistently expressed in different tissues and cell types and is widely used for normalization of cell culture- or tissue-based miRNAs.[14–18] However, these findings from intracellular miRNAs cannot be unambiguously translated into the situation in body fluids such as plasma or serum, as the mechanisms regulating the levels of small RNAs in the blood are only poorly understood. miRNAs might be passively released from dying cells, but also be actively secreted. In both settings, it is highly unlikely that a constant intracellular level justifying its use as a 'house-keeping miRNA' in a tissue necessarily leads to constant serum levels. In line, we demonstrate that serum levels of U6 display an enormous interindividual variability in healthy volunteers as well as in untreated mice. Moreover, serum levels of U6 showed a
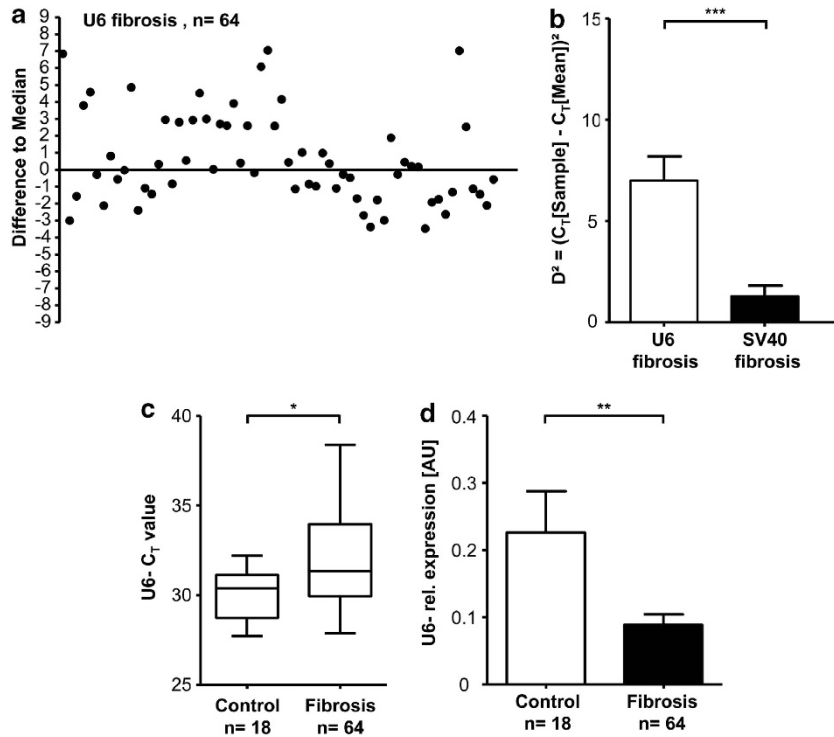
Figure 4 Levels of circulating RNU6B (U6) are downregulated in liver fibrosis. (a) Serum U6 levels were analyzed by quantitative real-time PCR (qPCR) in patients with liver fibrosis and revealed a very high variability between the different individuals ($n = 64$). (b) The variability of U6 values and SV40 values was statistically compared, revealing a significantly higher variability in U6 values compared with that of SV40. (c) U6 serum levels were compared between patients with hepatic fibrosis and healthy donors, revealing significantly lower levels of U6 in the patients' group. (d) U6 serum levels were compared between patients with hepatic fibrosis and healthy donors, revealing significantly lower levels of U6 in the patients' group also when data were normalized using SV40. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

disease-specific regulation pattern in the respective patient cohorts when compared with healthy volunteers: although elevated in critical illness, we found lower U6 levels in patients with liver fibrosis compared with healthy donors. These data clearly highlight that (i) serum levels of U6 are not stable between different individuals and (ii) serum levels of U6 are specifically upregulated or downregulated in different diseases, thus preventing a potential use of U6 for normalization of circulating miRNA analysis.

We demonstrate that U6 levels show a high variability not only in human patients but also in a mouse model of polymicrobial sepsis. This is especially interesting with regard to the fact that many miRNA biomarkers as well as functional experiments were first established in murine disease models.[6] Importantly, a recent study, comparing genomic profiles in response to different inflammatory stimuli between human patients and corresponding mouse models, showed hardly any correlation of expression levels of inflammatory genes between humans and mice, thus challenging the translational potential of mouse models in inflammatory disease in general.[43] However, studies on miRNAs in other inflammatory models suggested that regulatory patterns of miRNAs might be much more conserved between mice and men than that of other inflammatory ligands.[7,27,44] Therefore, it is of fundamental importance that proper normalization strategies are

used also in mouse experiments on miRNA regulation, as these findings might have a strong translational potential for human disease.

Interestingly, U6 levels were recently shown to be decreased during hepatocarcinogenesis and chronic liver injury.[20] Together with our findings in liver fibrosis, these data suggest a specific regulatory link between molecular processes in liver cells and serum U6 levels. Of note, in our cohort of patients, U6 serum levels correlated with serum levels of prominent proinflammatory cytokines such as tumor necrosis factor or interleukin-6, thus highlighting the involvement of inflammatory processes in controlling U6 serum levels. Various inflammatory cytokines were recently linked with alterations of miRNA expression in cells. In line, we and others recently demonstrated a significant downregulation of miR-150 in immune cells upon stimulation with LPS.[45] Remarkably, this intracellular regulation was reflected by lower miR-150 levels in supernatants of these cells,[7] highlighting that inflammatory stimuli might influence the equilibrium of intracellular/extracellular miRNAs. miRNAs were recently described to be packaged in exosomes or microvesicles. Moreover, it was recently demonstrated that the number and serum concentrations of such microvesicles are increased in a setting of systemic inflammation.[46] Thus, one might speculate that inflammatory cytokines might affect serum miRNA levels on

the one hand by regulating their intracellular expression and on the other hand by regulating their release/secretion into the blood.

In contrast to using endogenous genes for normalization of circulating miRNAs, the addition of synthetic versions of miRNAs from other organisms such as *Caenorhabditis elegans* in serum and/or plasma samples represents an interesting approach to circumventing normalization issues, and was therefore used in a variety of recently published studies. We demonstrate that the variability of SV40 levels were significantly lower compared with that of serum U6 levels, suggesting that the use of SV40 or other spiked-in RNAs might be more suitable for normalization of serum miRNA data than the use of endogenous U6 levels. Of note, such spiked-in RNAs could be easily integrated into standardized clinical assay systems, as it is already done in clinical routine for ELISA-based assays in which no absolute results but results relative to an integrated standard specimen are gained. Thus, a normalization of circulating miRNA analysis by spiked-in miRNAs might open the door for the translation of results from basic science to patients' care.

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