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Septic Shock Non-Survivors Have Persistently Elevated Acylcarnitines Following Carnitine Supplementation

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Abstract

Introduction—Sepsis-induced metabolic disturbances include hyperlactatemia, disruption of glycolysis, protein catabolism, and altered fatty acid metabolism. It may also lower serum L-carnitine which supports the use of L-carnitine supplementation as a treatment to ameliorate several of these metabolic consequences.

Methods—To further understand the association between L-carnitine-induced changes in serum acylcarnitines, fatty acid metabolism and survival, serum samples from (T0), 12 hours following completion (T24) of L-carnitine (n=16) or placebo (n=15) administration, and 48 hours (T48) after enrollment from patients with septic shock enrolled in a randomized control trial were assayed for acylcarnitines, free fatty acids and insulin. Data were analyzed comparing 1-year survivors and non-survivors within treatment groups.

Results—Mortality was 8/16 (50%) and 12/15 (80%) at one-year, for L-carnitine and placebo-treated patients, respectively. Free carnitine, C2, C3, and C8 acylcarnitines were higher among non-survivors at enrollment. L-carnitine treatment increased levels of all measured acylcarnitines; an effect that was sustained for at least 36 hours following completion of the infusion and was more prominent among non-survivors. Several fatty acids followed a similar, though less consistent pattern. Glucose, lactate, and insulin levels did not differ based on survival or treatment arm.

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Conflicts of interest:

The authors have no conflicts of interest to disclose.

Conclusions—In human patients with septic shock, L-carnitine supplementation increases a broad range of acylcarnitine concentrations that persist after cessation of infusion, demonstrating both immediate and sustained effects on the serum metabolome. Non-survivors demonstrate a distinct metabolic response to L-carnitine compared to survivors, which may indicate pre-existing or more profound metabolic derangement that constrains any beneficial response to treatment.

Keywords

sepsis; pharmacometabolomics; metabolomics; free fatty acids; liquid chromatography-mass spectroscopy; gas chromatography

Introduction

Despite advances in early recognition and care of patients with severe sepsis, patients with septic shock continue to demonstrate a mortality rate of approximately 40% (1). Clinical trials targeting the condition have continued to yield disappointing results, and specific pharmacologic therapies for the condition are lacking. Traditionally, many of these trials target the well-known pathophysiologic manifestations of the sepsis syndrome, namely the inflammatory and coagulation cascades. In addition to an inflammatory disease, however, sepsis might be considered and approached as an acute metabolic illness. Sepsis induces a series of physiologic changes including increased glycolysis, protein catabolism, lipolysis, and neuroendocrine activation resulting in a number of metabolic changes including hyperglycemia, hyperlactatemia, and alternations in circulating amino acids, fatty acids (FA), and lipoproteins (2). In addition to these effects, several lines of evidence suggest specific changes in metabolic pathways involving L-carnitine. Sepsis induces a relative L-carnitine deficiency, with decreased muscle and plasma levels and increased urinary excretion (3). In addition, the activities of carnitine-dependent enzymes such as carnitine acyl and palmitoyltransferases are altered following lipopolysaccharide injection (4). Carnitine is also necessary for the conjugation and transfer of long-chain acyl CoA into the mitochondria for beta-oxidation of FA. Together, these data suggest a potentially important role for carnitine in the metabolic response in sepsis.

The advent of new, high-throughput metabolomics platforms has reinvigorated interest in the metabolic response of patients to sepsis. Previous studies have demonstrated systematic shifts in energy metabolism using such platforms and raised interesting hypotheses regarding novel therapeutic targets to improve energy metabolism and mitigate organ dysfunction in patients with sepsis (5–9). More recently, a large observational cohort study of patients found a broad dysregulation of acylcarnitine levels among sepsis non-survivors using untargeted metabolomics, suggesting potential disruptions in FA-related metabolic pathways (9). We previously completed a phase I pilot randomized control trial testing the hypothesis that a single bolus and 12-hour infusion of L-carnitine is safe and demonstrates preliminary efficacy (10). While not powered to detect a mortality benefit, a statistically significant reduction in 28-day mortality was observed among patients treated with L-carnitine and a multi-center phase II clinical trial is currently nearing completion (NC01665092).

Using patient samples from the phase I pilot study, we previously demonstrated that carnitine-treated 1-year sepsis survivors had a distinct metabolic profile compared with non-survivors using quantitative proton nuclear magnetic resonance (^1H -NMR) metabolomics (11). Specifically, we observed multiple L-carnitine-induced metabolic changes, and most interestingly found nearly all of the clinical benefit in the intervention group could be attributed to a group of L-carnitine “utilizers” that could be identified based on their pretreatment metabolome. One of the most prominent findings of this study was that L-carnitine levels following supplementation was highly predictive of 28-day and 1-year survival, leading us to question what impact L-carnitine accumulation might have on acylcarnitines, FA, and glycolysis. We hypothesized that L-carnitine treatment would differentially affect systemic acylcarnitines and provide additional insights into the impact of L-carnitine treatment on the metabolic response to sepsis.

Materials and Methods

Description of the clinical trial

This is a secondary analysis of serum samples from patients enrolled in a FDA regulated (IND #107,086), IRB approved, clinical trial of L-carnitine administration registered on clinicaltrials.gov (NCT01193777), which has previously been published (10). All patients or their surrogates gave informed consent prior to enrollment. Inclusion criteria included 2 or more systemic inflammatory response syndrome (SIRS) criteria, confirmed or suspected infection demonstrated by administration of intravenous antibiotics, a sequential organ failure assessment (SOFA) (12) score of at least 5, continuous administration of vasopressors for at least 4 hours with a cumulative vasopressor index (13) of at least 3 following volume resuscitation, and enrollment within 16 hours of the recognition of sepsis. Abbreviated exclusion criteria included age <18 years, any primary diagnosis other than sepsis, a preexisting do not resuscitate order, a history of seizures (due to the risk of L-carnitine in lowering the seizure threshold), or known inborn error of metabolism. Patients were randomized in a double-blind fashion to receive either L-carnitine (single dose of 12 g delivered as a 4 g bolus followed by an 8 g infusion diluted in 1 L of normal saline and delivered over the subsequent 12 hours) or an equivalent volume of normal saline. Clinical variables were recorded prospectively at the bedside and were used in conjunction with prospectively ordered clinical laboratory tests at the same time points to calculate SOFA scores.

Blood draws and processing

Blood samples were collected via an existing intravenous or arterial catheter prior to dosing (T0) and 12h after the completion of the infusion (T24; $\pm$ 4h) and then again 24h later (T48; $\pm$ 4h) as previously described (11). Resulting serum was aliquoted into 500 μL samples and stored at (-80°C) until the time of assay.

^1H -NMR metabolomics

We utilized existing ^1H -NMR metabolomics data, specifically the concentration values of lactate and glucose, that were generated from technical replicates of the samples used for this study. We elected to use these rather than clinical measurements to ensure consistency in

interpreting the data, as different sample collection techniques, blood processing, use of whole blood versus serum, and use of point-of-care devices could introduce bias. These methods and results have been previously reported (11) and these data can be found at <http://www.metabolomicsworkbench.org/>.

Measurement of acylcarnitines by liquid chromatography-mass spectroscopy

To prepare serum for acylcarnitine analysis, extraction solvent consisting of a 1:1:1 mixture of methanol:acetonitrile:acetone plus a 1:200 dilution of a stock mixture of stable-isotope labeled acylcarnitine standards dissolved according to manufacturer instructions (NSK-B, Cambridge Isotope Laboratories) was prepared. Serum was extracted by the addition of 200 μ L extraction solvent to 50 μ L serum in a microcentrifuge tube, followed by vortexing for 10 seconds. After 5 minutes on ice, the samples were centrifuged ($15,000 \times g$, 5 min) and 200 μ L of supernatant was transferred to a clean tube and dried with a gentle stream of nitrogen gas. Dried samples were reconstituted in 50 μ L of a 9:1 mixture of water:methanol, which was transferred to autosampler vials with low-volume inserts for analysis. Acylcarnitine species were then analyzed without derivatization by RPLC-MS/MS using an Agilent 1200 LC coupled to an Agilent 6410 tandem quadrupole mass spectrometer (Santa Clara, CA). Absolute quantitation was performed for acylcarnitine species with exact-matching stable isotope internal standards (L-carnitine, C2, C3, C4, C5, C8, C14, C16) by multiplying the ratio of the unlabeled / labeled peak area by the known concentration of the original plasma sample. Other acylcarnitine species were assayed using relative quantitation by peak area. Additional analytical details about the assay can be found in the online supplement.

Insulin measurements

Serum samples were assayed for insulin concentration using a double-antibody radioimmunoassay that employs a ^{125}I -Human insulin tracer (Linco Research), a guinea pig anti-porcine insulin first antibody (MDRTC, 68.5% cross-reaction to human proinsulin), and a goat anti-guinea pig gamma globulin (Antibodies Inc.)-PEG second antibody and standardized against the Human Insulin International Reference Preparation (NIBSC) as previously described (14).

Free fatty acid (FA) measurements

The protocol of lipid extraction was adapted from a published method (15) with slight modification as follows: Total lipids were extracted using a mixture of chloroform-methanol (1:2) containing 0.01% butylated hydroxytoluene with known amount of heptadecanoic acid (C17:0) as an internal standard, followed by further treatment with chloroform and NaCl (0.9%) for phase separation. The organic layer containing lipids was separated, FA were isolated using silica gel thin-layer chromatography, subjected to trans-methylation with BF_3 -methanol (16) and analyzed by gas chromatography (GC). Analysis was performed on an Agilent GC model 6890N using Chemstation software. The GC column was an Agilent HP 88, 30 meter, 0.25 mm I.D. and 0.20 μ m film thickness. Hydrogen was used as a carrier gas as well as for the FID detector and nitrogen was used as a makeup gas. Analyses were carried out with a temperature program of 125° C to 220° C. The coefficient of variation for GC analyses was within 2.5 to 3.6%.

Data analysis

Clinical data were summarized using simple descriptive statistics, and differences between groups were determined using chi-square, Fisher exact, Student's t-test, or a Mann-Whitney U test, as appropriate. For the purposes of this analysis, patients were categorized by 1-year mortality and treatment arm. Since the primary focus of the work was to determine differences in carnitine and acylcarnitine concentrations between carnitine or placebo-treated patients based on survival, we compared data at each time point using a Mann-Whitney U test. Resulting p values for carnitine, acylcarnitine and FA concentration comparisons were corrected for multiple comparisons using false discovery rate (17) and are reported as q values. All figures were constructed and statistical two-sided tests were performed in PRISM (version 7.0c for Mac; GraphPad Software, Inc., La Jolla, CA USA); p-values ≤ 0.05 were considered significant.

Results

We enrolled 31 patients in the trial, 16 in the L-carnitine arm and 15 in the placebo arm. Patient demographics and clinical characteristics stratified by treatment allocation and survival can be found in Table 1 and Table 2, respectively. Overall, patients enrolled in the study were critically ill with a median (IQR) SOFA score of 12 (6,14), all patients required vasopressor support, and the cohort had an intubation rate of 71%. Although there were disproportionately fewer females in the study (n=10 vs 21 males), there was only one female survivor at one year (Table 1). As noted previously (10), patients enrolled into the treatment arm had significantly higher SOFA scores (primarily related to the respiratory component and a higher rate of intubation, data not shown), though the treatment arms were otherwise well matched. One-year mortality was 80% and 50% in the placebo and treatment arms, respectively, with a trend towards improved survival ($p = 0.06$), suggesting potential clinical efficacy of L-carnitine that is undergoing further testing in an ongoing clinical trial (NC01665092) (10, 18).

At enrollment, there were no significant differences in the levels of free or any acylcarnitine levels when comparing treatment arms. Further visual inspection of free and acylcarnitine levels in placebo-treated patients demonstrated relative stability over 48h, regardless of patient survival (Figure 1A–H, right), and no measured difference was statistically significant. On the other hand, among patients treated with L-carnitine, levels of all measured carnitine species (C0, C2, C3, C4, C5, C6, and C8), with the exception of C16, were higher in carnitine-treated non-survivors compared to survivors at T0, T24 and T48 (Figure 1A–H, left). Non-survivors demonstrated a global, more profound increase in free carnitine and acylcarnitines in response to supplementation as compared to survivors, an effect that was most notable 12 hours following completion of the infusion (T24) but still observable 36 hours after cessation (T48). Other acylcarnitines followed similar temporal trends (see Figure E1 in the online supplement). Notably, these differences could not be explained by differences in renal function as assessed by serum creatinine, which neither differed significantly between non-survivors and survivors in the entire study [1.7 (1.5, 2.3) and 2.1 (1.4, 3.4) mg/dL; $p = 0.49$] nor in the subset of patients treated with L-carnitine [2.1

(1.6, 2.6) and 1.8 (1.5, 2.8) mg/dL; $p = 0.49$). A representative chromatogram of acylcarnitines is shown in Figure E2 in the online supplement.

Total free FA did not differ significantly between survivors and non-survivors prior to enrollment (50 (30,97) versus 65 (55, 102) nmol / 100 μ L, $p = 0.15$). Levels of the various measured FA also did not differ significantly between treatment groups prior to initiation of treatment with the exception of 22:0 which was higher in L-carnitine treated patients (0.22 (0.13, 0.28) versus 0.14 (0.09, 0.2)). Similar to the trend observed in the acylcarnitines, levels of the various FA tended to be higher in the L-carnitine treated survivors versus non-survivors (Figure 2), though only 20:0 and 22:0 met statistical significance (Figure 2K and Q) at T0. There were no significant differences in FA levels in placebo treated survivors compared to non-survivors prior to enrollment. Following treatment, the levels of 18:3(n-6), 20:5, 22:0, and 24:1 (Figure 2J, P and W) trended up in L-carnitine treated non-survivors compared with survivors. There were no significant differences in FA concentrations between survivors and non-survivors in the placebo groups although 22:1 trended higher (Figure 2R) in survivors compared with non-survivors. A representative chromatogram of FA can be found in the online supplement (Figure E3).

Pre-treatment concentrations of glucose and lactate (both as measured by NMR) and lactate:glucose ratios did not differ between non-survivors and survivors [3193 (2594, 4169) vs 2675 (2249, 5309) μ M, $p = 0.4$; 1775 (1193, 2271) vs 1482 (1193, 2271) μ M, $p = >0.99$; 0.48 (0.36, 0.86) vs 0.54 (0.5, 1.3), $p = 0.4$, respectively], but insulin concentration was significantly lower in non-survivors [8.9 (5.4, 17.2) vs 28.2 (13.6, 64.6) μ U/mL, $p = 0.01$]. Finally, in order to further evaluate the relationship between potential markers of altered glycolysis in response to L-carnitine supplementation, we analyzed glucose, lactate, lactate:glucose ratio, and insulin levels over the same time points. Carnitine-treated non-survivors had lower pre-treatment insulin levels than carnitine-treated survivors (7.8 (4.4, 19.5) vs 49.6 (11.8, 94.6) μ U/mL; $p=0.04$) but otherwise we found no significant differences in glucose, lactate, lactate:glucose ratio, or insulin levels when analyzing patients based on survival and treatment (data not shown). However, these data were limited by significant individual variability among patients and incomplete clinical data related to fasting status or feeding regimens and supplemental insulin administration.

Discussion

In this study, we report the impact of L-carnitine treatment on serum concentrations of acylcarnitines and FA in patients with septic shock, which we have previously reported to have potentially beneficial effects on patient outcomes. Here we show that intravenous L-carnitine supplementation to patients with septic shock leads to an immediate and sustained increase in short, medium, and long-chain acylcarnitines that persists for at least 36 hours following completion of intravenous infusion. These data demonstrate that this method of supplementation does indeed result in cellular uptake and substrate utilization through the formation of acylcarnitines supporting the biologic plausibility of the treatment. These increases in acylcarnitine concentrations are not unexpected as this has previously been reported with carnitine supplementation (19–21). Rather, the primary finding of this study is that carnitine-treated septic shock non-survivors had substantially higher concentrations of

carnitine and acylcarnitines following L-carnitine administration compared with survivors. This finding cannot simply be explained by differences in endogenous serum carnitine at T0 because the median difference in carnitine concentration at T24 was much greater (i.e., 485 (IQR: 401, 998) μM ; see Figure 2A) than the difference in the median concentration at T0 (17 (IQR: 11, 25) μM ; see Figure 1A) between survivors and non-survivors (22). It also cannot be explained by differences in renal function between the two groups. As L-carnitine is renally cleared, we might have expected the differences between L-carnitine treated survivors and non-survivors to reflect differences in renal function, though similar creatinine levels at enrollment suggest against this explanation. Orally ingested L-carnitine is metabolized by gut flora which results in the generation of trimethylamine (23). However, IV administration bypasses this mechanism and to the best of our knowledge there are no other degradative or metabolic pathways of carnitine that could explain these differences; carnitine is primarily excreted unchanged in the urine (24). Alternatively, we contend that these data imply that there is a differential metabolic response to carnitine supplementation in septic shock that reflects the underlying the metabolic dysfunction of sepsis which could be used to inform subsequent experimental design, either clinical or preclinical. This has been substantiated by our previous work which showed that L-carnitine treated responders have a unique metabolic profile (11). Finally, the uptake and retention of acylcarnitines in tissues and organs, and its role in those compartments was not assessed given the limitations of clinical research, and remains an open area for future investigations.

Sepsis induces all manner of metabolic disturbances that have been long recognized, including evidence of alterations in glycolysis (most notably hyperglycemia and hyperlactatemia, but also including alterations in pyruvate), protein catabolism, and FA metabolism (25). Most recently, large scale unbiased metabolomics approaches have compared patients with sepsis of varying severity which identified acylcarnitines as predictive of patient outcome (5, 9). In these studies, patients did not receive supplemental carnitine. Here, we show that administration of L-carnitine provokes the observed metabolic phenotype since placebo treated patients had no differences in their levels of acylcarnitines at enrollment. It is worthwhile to note that we did not observe a significant effect of L-carnitine treatment on various metabolic markers related to glycolysis, namely glucose, lactate, and insulin. This is relevant, as it might be hypothesized that L-carnitine treatment affects these pathways via its indirect effects on pyruvate dehydrogenase activity through the shuttling of excess acetyl-coA out of the inner mitochondrial space. While we did not observe a significant effect on glycolysis based on our simple measurements, we likewise cannot completely rule-out such an effect due to the large inter-patient variability in the levels that we observed. Rather, this pathway would require a more tightly controlled clinical study in a larger cohort to definitely interrogate the potential effects of L-carnitine on glycolysis.

Given the absence of differences in long-chain FA (<C22) concentrations between carnitine-treated survivors and non-survivors, which serve as the substrates of acylcarnitines, we conclude that the variance in carnitine-induced increases in acylcarnitines between these patients is attributable to the disposition of carnitine (21). Although the mechanism of carnitine disposition was not specifically assessed in this study, it is possible that the lower plasma carnitine and acylcarnitine levels in survivors reflect improved uptake and

maintenance of carnitine in the tissues, which could include urinary excretion of carnitine and acylcarnitines, where it would have potential to enhance lipid metabolism and help ameliorate the metabolic crisis associated with sepsis. We acknowledge that there was greater variability in FA concentrations across patients which may reflect a range of fasting and fed states (26). Given this and our small sample size, it is difficult to detect a consistent pattern of FA levels that may distinguish survivors and non-survivors. Nevertheless, the explanation for the broad range of carnitine and acylcarnitine concentrations following a single intravenous dose of carnitine, which, to the best of our knowledge has not been previously reported, warrants further study. It is also worth mentioning that sex-related differences in carnitine pharmacokinetics have been described (27). Specifically, females had higher levels of carnitine and acetylcarnitine following a single intravenous dose of acetylcarnitine (500mg). In our study at T24, male carnitine treated non-survivors (n=5) had a median (IQR) carnitine concentration of 626 (428, 789) μM compared with females (n=4), who had a concentration of 1250 (102, 1416) μM . While the study of sex-related differences was not an objective of this work and it remains unclear whether sex influences sepsis survival (28), evaluation of this biological variable will be important for deciphering carnitine-induced changes in the metabolome in future studies.

There are several important limitations to consider in this study. Most importantly, the sample size was limited by the original clinical trial. Nevertheless, given the highly significant differences between groups, broad and consistent trend in acylcarnitine levels, and biologic plausibility, we feel confident in the findings related to the impact of carnitine supplementation on acylcarnitine concentrations, though secondary analyses should be viewed as hypothesis generating. Second, detailed assessments of metabolic function, insulin sensitivity, and metabolic flexibility using methods such as carbon flux studies were not performed, so any conclusions regarding metabolic pathways and flexibility must be very guarded and rather serve as the impetus and rationale for future studies. Third, while it is possible that the observed changes led to improvements in energy balance and contributed to the observed improvement in clinical outcomes, due to measurements at individual time points in serum rather than tissue, and without the benefit of a flux analysis, we cannot definitely conclude what if any effects on energy metabolism carnitine treatment has on patients with septic shock, which would require follow-up studies. Finally, the patients enrolled in this clinical trial were all critically ill with a 28-day mortality in the placebo group approaching 60% and 1 year mortality of 75%. Patients demonstrated a significant vasopressor and organ failure requirement based on the study design, and therefore this cohort should not be considered representative of all patients with sepsis or even all those with septic shock, and generalization of these findings should be undertaken with caution.

Conclusion

In human patients with septic shock, L-carnitine supplementation induces a broad concentration range of acylcarnitines that persists after cessation of infusion, demonstrating both immediate and sustained effects on the serum metabolome. Non-survivors demonstrate a distinct metabolic response to L-carnitine compared to survivors, which may indicate pre-existing or more profound metabolic derangement that constrains any beneficial response to treatment. If these findings are confirmed and subsequently tested in clinical trials, the use of

pretreatment metabolite levels to guide metabolic therapies would demonstrate a real-world application of pharmacometabolomics-driven precision medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 2.

Temporal changes in serum concentrations of 14:0–24:1 fatty acids in L-carnitine- and placebo-treated patients, stratified by survival (A–W). While L-carnitine treated, non-survivors demonstrated trends towards having higher levels of various long-chain (C14–C21) and very long-chain fatty acids than survivors [(C) 16:0, (G) 18:1(n-9), (K) 20:0 (Q) 22:0, (R) 22:1, and (V) 24:0], prior to and following [(J) 18:3(n-6), (P) 20:5, (Q) 22:0, and (W) 24:1] carnitine administration, no clear and consistent trend or single biochemical pathway appeared to be implicated as specifically impaired or altered. In placebo-treated

patients, the findings were less remarkable in that there were no differences or trends in FA profiles between survivors and non-survivors.

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Table 1

Baseline demographics and clinical characteristics of 1 year survivors versus non-survivors.

Variable	Survivors (N = 11)	Non-survivors (N = 20)	p-value
Age (IQR)	63 (50, 69)	67 (60, 75)	0.11
Race, n (%)			
Caucasian	7 (64)	18 (90)	0.08
African American	4 (36)	2 (10)	0.08
Male, n (%)	10 (90)	11 (55)	0.04
Comorbidities, n (%)			
Coronary artery disease	3 (27)	4 (20)	0.64
Cerebrovascular accident	3 (27)	1 (5)	0.08
Hypertension	8 (73)	14 (70)	0.87
Insulin dependent diabetes	2 (18)	3 (15)	0.82
Non-insulin dependent diabetes	2 (18)	4 (20)	0.90
Chronic Renal Insufficiency	3 (27)	3 (15)	0.41
Malignancy	1 (9)	4 (20)	0.43
SOFA score (IQR)	14 (8, 14)	11.5 (10, 14)	0.95

SOFA - Sequential Organ Failure Assessment Score; IQR – interquartile range

Table 2

Baseline demographics and clinical characteristics patients grouped by L-carnitine versus placebo treatment allocation.

Variable	L-carnitine (N = 16)	Placebo (N = 15)	p-value
Age (IQR)	63 (55, 72)	65 (56, 71)	0.66
Race, n (%)			
Caucasian	13 (81)	12 (80)	0.93
African American	3 (19)	3 (20)	0.93
Male, n (%)	11 (69)	10 (67)	0.90
Comorbidities, n (%)			
Coronary artery disease	4 (25)	3 (20)	0.74
Cerebrovascular accident	1 (6)	3 (20)	0.25
Hypertension	11(69)	11 (73)	0.78
Insulin dependent diabetes	1 (6)	4 (27)	0.12
Non-insulin dependent diabetes	3 (19)	3 (20)	0.93
Chronic Renal Insufficiency	4 (25)	2 (13)	0.41
Malignancy	2 (13)	3 (20)	0.57
SOFA score (IQR)	14 (11.5, 14)	10 (7, 10)	0.01

SOFA - Sequential Organ Failure Assessment Score; IQR – interquartile range