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The MicroRNA-based Liquid Biopsy Improves Early Assessment of Lethal Acetaminophen Poisoning: A Case Report

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Statistical Analysis C
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Manuscript Preparation E
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Patient: Female, 44-year-old
Final Diagnosis: Acute liver failure
Symptoms: APAP overdose
Medication: Acetaminophen
Clinical Procedure: —
Specialty: Molecular Biology

Objective: Unusual clinical course

Background: Acetaminophen overdose is the most common cause of acute liver failure. Nevertheless, new biomarker approaches enabling early prediction of the outcome of the acetaminophen overdose are needed. Recently, using next-generation sequencing analysis of serum from human study participants we uncovered injury-specific signatures of circulating microRNAs (miRNAs) that represented underlying molecular mechanisms of toxicity. This case study is first to show the application of miRNA profiling to assess prognosis of acetaminophen poisoning.

Case Report: The patient was admitted to the hospital following supra therapeutic acetaminophen ingestion. The patient showed elevated levels of biomarkers of hepatocellular injury alanine aminotransferase, aspartate transaminase, and glutamate dehydrogenase. Even though treatment with N-acetyl cysteine was initiated 24 hours post-ingestion, levels of alanine-aminotransferase and aspartate transaminase peaked at about 40 hours post ingestion of acetaminophen. We analyzed global circulating miRNA levels from 24 consecutive serum samples from this study participant covering the period from admission to time of death.

Conclusions: The resulting global miRNA profiles were compared with profiles from study participants with non-lethal acetaminophen poisoning and healthy controls. At the admission, the miRNA profiles of both lethal and non-lethal acetaminophen poisoning showed induction of cellular stress and oxidative damage. Later, the miRNA profiles of the lethal poisoning featured fibrosis and coagulation pathways while profiles of non-lethal cases resembled those of healthy study participants. Although additional confirmatory studies are needed, our case study is first to indicate that global miRNA profiles to be used as liquid biopsies have potential to facilitate the assessment of acetaminophen poisoning.

MeSH Keywords: Acetaminophen • Biological Markers • MicroRNAs

Abbreviations: **ALF** – acute liver failure; **ALT** – alanine-aminotransferase; **APAP** – acetaminophen; **AST** – aspartate transaminase; **GLDH** – glutamate dehydrogenase; **MELD** – Mayo End-Stage Liver Disease; **NAC** – N-acetyl cysteine; **NAPQI** – N-acetyl-p-benzoquinone imine; **NGS** – next-generation sequencing technology

Full-text PDF: https://www.amjcaserep.com/abstract/index/idArt/919289

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Background

Acetaminophen is the most commonly used analgesic and antipyretic medicine in the world. At therapeutic doses, acetaminophen is efficacious and relatively safe; however, its overdose is highly hepatotoxic. Since acetaminophen is widely used either alone or included in many over-the-counter remedies, accidental or intentional overdoses are very common. In fact, acetaminophen hepatotoxicity is classified as the most frequent cause of acute liver failure (ALF) in the developed world [1]. In the USA alone, it was responsible for 56 000 emergency department visits, 26 000 hospitalizations, and 458 deaths per year [2].

Recently, the molecular mechanism of acetaminophen hepatotoxicity has been extensively reviewed [3,4]. The hepatotoxicity of acetaminophen is triggered by its reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), produced by hepatic cytochrome P-450 namely CYP2E1 and CYP2A1. At therapeutic doses, the endogenous free radical scavenger, glutathione, efficiently eliminates the formed NAPQI. At toxic doses, the large amount of NAPQI overwhelms the glutathione reserves. In that case the free NAPQI produces mitochondrial protein adducts that provoke mitochondrial oxidative and nitrosative stress that is further amplified by activation of MAP kinases. This leads to activation of autophagy, as well as the mitochondrial permeability-inducing process known as "regulated necrosis" with subsequent activation of an inflammatory response. The only known treatment of acetaminophen poisoning is early administration of an exogenous reactive metabolite scavenger, N-acetyl cysteine (NAC), which increases the capacity of the hepatocytes to eliminate NAPQI.

Clinically, acetaminophen-induced hepatotoxicity is characterized as a hyperacute form of ALF that leads to progressive multi-organ failure [5]. Severe cases of acetaminophen poisoning feature highly elevated transaminases accompanied by gradual increases of total bilirubin, severe acidosis, coagulopathy and encephalopathy. If not treated by liver transplant, the condition is often lethal. Although severe acidosis and coagulopathy are associated with poor prognosis even before the onset of encephalopathy, the further development of prognostic biomarker approaches capable of identifying candidates for liver transplant is important [6]. Currently, the modified King's College [7–9] and Mayo End-Stage Liver Disease (MELD) scores are used for identifying patients at risk for potential liver transplantation [10]. However, neither the modified King's College Scores, MELD scores and addition of arterial blood lactate and INR can be optimally used under all circumstances leading to ALF, thus there is a pressing need for a more diversified and accurate predictor of ALF.

Recently, a panel of emerging biomarkers of drug-induced liver injury consisting of miR-122, high mobility group box1 and cytokeratin-18 has been shown to be able to stratify patients at the admission to the hospital based on risk of developing liver injury [11] which seemed promising. However, the biomarker panel did not substantiate the prognosis of the outcome of the injury.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post transcriptional level [12]. These molecules are released into the peripheral circulation upon cellular injury and consequently have shown a promise as a new class of tissue-specific biomarkers [13].

Previously, we have developed a non-invasive miRNA based "liquid biopsy" approach capable of investigating molecular mechanisms of toxicity and disease [14,15]. The approach relies on next-generation sequencing technology (NGS), an open analytical platform, which enables interrogating global profiles of circulating miRNAs and using bioinformatics approaches to decrypt underlying biological pathways. Initially, we examined global miRNA profiles in serum samples from 6 study participants with supra therapeutic acetaminophen-overdose collected during the period from admission to the release from hospital [14]. We identified 36 acetaminophen-inducible miRNAs that included several isomiRs and putative novel miRNAs. Importantly, the biological function of the identified miRNAs was consistent with established molecular mechanisms of acetaminophen-toxicity. In a subsequent study we used NGS to interrogate global serum miRNA profiles in groups of healthy study participants, study participants with supra therapeutic non-lethal acetaminophen-overdose, alcohol-induced liver cirrhosis, hepatitis B infection, and type 2 diabetes mellitus [15]. We identified signatures of circulating miRNAs that were specific for various phenotypes of liver injury and type 2 diabetes mellitus. The identified miRNA profiles again corresponded to mechanistically relevant biological pathways which were predominantly related to cellular stress response and apoptosis. For liver cirrhosis the miRNA profile displayed pathways associated with tissue remodeling, while hepatitis B patients showed inflammatory pathways and type 2 diabetes exhibited pathways associated with insulin signaling. The recovery from the acetaminophen overdose was clearly identifiable because of the increasing similarity of the acetaminophen patient miRNA profiles across the recovery period to the miRNA profile of healthy study participants [15].

In this case study, we examined whether serum miRNA profiles have a potential to provide additional information for diagnosis and/or prognosis of lethal acetaminophen poisoning. To our knowledge, our study is the first to analyze global profiles of serum miRNAs using NGS in a patient with lethal acetaminophen overdose covering the time period from admission to time of death. The time course of miRNA profiles was then compared with profiles of 9 previously studied non-lethal

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cases of acetaminophen overdose. We show that, at the early time points, the miRNA profile of the lethal case was more prominent but consistent with profiles of non-lethal cases of acetaminophen poisoning. The underlying biological processes featured cellular stress and apoptosis as main components. As the acetaminophen poisoning progressed, the miRNA profiles of the lethal case started segregating from non-lethal cases of acetaminophen overdose. The miRNA profile of the lethal case, at the point of dissociation from non-lethal cases until the time of death, featured fibrosis and coagulation pathways. Although more confirmatory studies are clearly needed, this case study provides a proof of concept for the miRNA-based "liquid biopsy" analysis and lays foundation for further research. If confirmed, this approach has a potential to become a useful tool for diagnosis and prognosis of liver injury caused by acetaminophen poisoning and ultimately leading to better outcomes.

Case Report

The patient, who had a history of excessive alcohol and benzodiazepine consumption (with no known history of cirrhosis or primary liver cancers) and a past history of supra therapeutic acetaminophen consumption, was admitted to the hospital following supra therapeutic acetaminophen ingestion. acetaminophen ingestion (50 g) was in the form of an acute intake, of acetaminophen containing tablets about 24 hours before admission to the hospital. The study participant demonstrated elevated levels of serum biomarkers alanine aminotransferase (ALT), aspartate transaminase (AST) and glutamate dehydrogenase (GLDH) (Figure 1A). Treatment with NAC was initiated 24 hours' post-ingestion, administered as an initial bolus, then as a continuous intravenous drip for 24 hours. The patient tested negative for bacterial or viral causation of liver injury, and had no other extraordinary co-morbidities known to contribute to elevated liver enzymes. The levels of ALT and AST peaked at about 24 hours after admission to the hospital. In the case of GLDH, the maximum levels were reached 28 hours after admission and remained elevated until 86 hours post admission. At that time, the GLDH level started decreasing (Figure 1A). At the onset of hospital treatment, the patient had elevated arterial blood lactate values (Table 1) which fluctuated in response to intervention, but never fully stabilized over the duration of treatment. In contrast, biomarkers of hepatobiliary injury, alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT), were only slightly increased (Figure 1C). Interestingly, creatine kinase (CK), a biomarker of muscle impairment, showed a rapid increase only in samples taken immediately before the time of death (Figure 1A). The functional markers of liver injury, total bilirubin (TBIL), showed gradual increase throughout the entire period (Figure 1B). Initially, TBIL levels increased slowly, but accelerated 86 hours post admission reaching 35 mg/dL at the time of death. Since liver is the source of clotting factors, coagulopathy, measured as INR, is considered a hallmark of lost liver function. The patient developed coagulopathy at early time points reaching critical INR levels (5) at 31 hours post admission. The coagulopathy became severe at the later time points (Figure 1B). The serum levels of acetaminophen at the time of admission were 235 mcg/mL and showed a rapid decrease falling to less than 10 mcg/mL 136 hours post admission (Figure 1D). Values of ALT, Kings College Criteria, MELD Score, PT, INR, and lactic acid across all time points are presented in Table 1. The patient met the Kings College Criteria of needing a transplantation at approximately 48 hours after admission to the hospital.

To evaluate the potential of the miRNA-based liquid biopsy approach we analyzed global circulating miRNA levels from the 24 consecutive taken serum samples during the period from admission to time of death (199 hours). Subsequently, we compared the circulating miRNA profile of this lethal case to profiles from 9 previously analyzed cases of non-lethal acetaminophen overdose and 22 healthy study participants (patient characteristics in Supplementary Table 1)

The next generation sequencing yielded 76×10^6 quality-filtered and processed reads across all samples with a mean count of 4×10^6 (σ 4.99×10^6) per sample. The principal component analysis (PCA) revealed that 5 outliers were present in the data, samples that also showed very low sequencing reads, which were consequently removed from the analysis. In total, we identified 137 circulating miRNAs across all plasma samples with a minimum of 100 reads. Of these 137, our analysis identified 69 circulating miRNAs that showed a significant time-dependency over the entire time period (Table 2). The miRNA response showed 4 distinct patterns over the time course of the lethal acetaminophen poisoning. For 26 miRNAs the serum levels decreased relatively quickly and reached limit of detection within 116 hours after admission to the hospital (Figure 2A). The second set of 19 miRNAs (Figure 2B) showed a gradual decrease with many miRNAs never reaching the detection limit. Interestingly, we also identified a cluster of 10 circulating miRNAs that increased during the time course of the lethal acetaminophen poisoning (Figure 2C) and 14 circulating miRNAs with relatively high initial serum levels that showed only a slight increase or decrease (Figure 2D).

Previously, we have shown that during recovery from acetaminophen overdose the miRNA profiles of non-lethal cases become gradually more similar to serum miRNA profiles detected in healthy study participants [15]. Therefore, we have compared serum miRNA profiles of the lethal case with previously measured cases of non-lethal acetaminophen overdose and with healthy study participants. The PCA of the global circulating miRNome revealed clear distinctions between the lethal and

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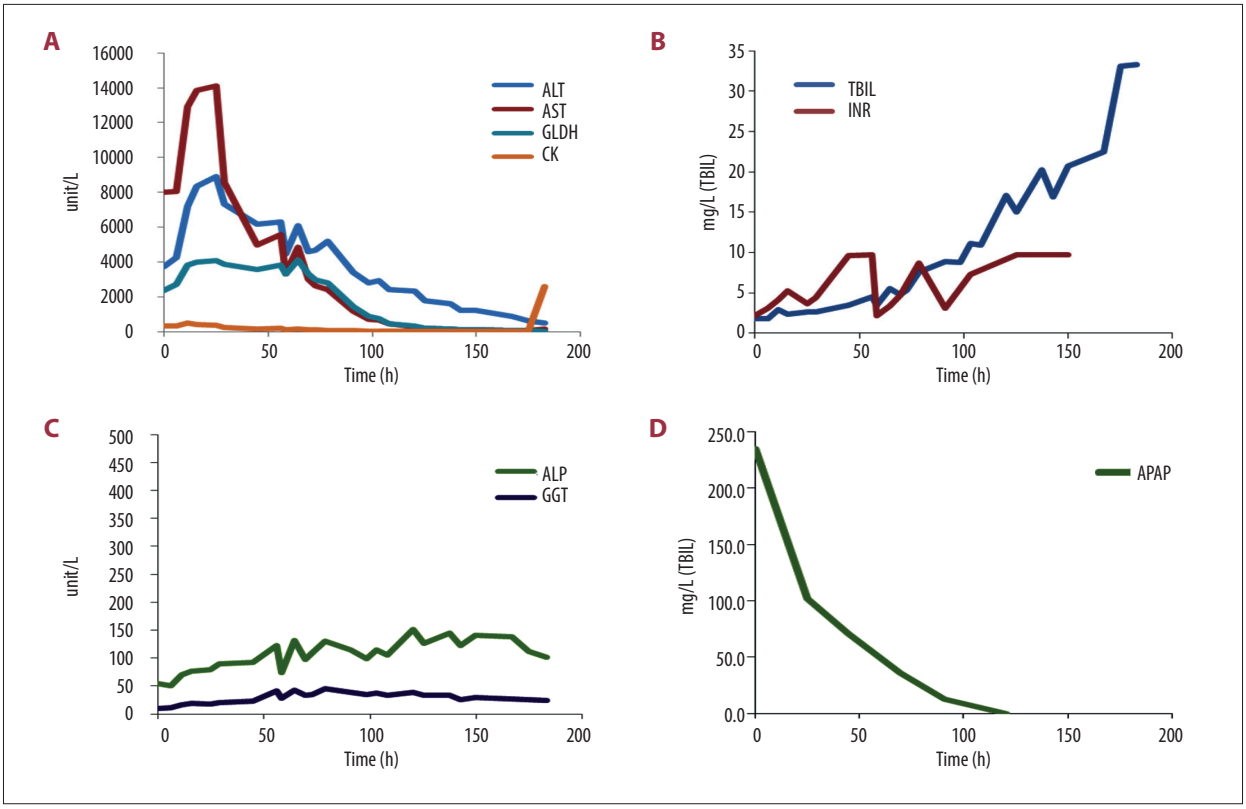

Figure 1. Serum Chemistry Values for subject with lethal APAP poisoning. Values for ALT (u/L), AST (u/L), GLDH (u/L), CK (u/L) (**A**); Values for TBIL (mg/dL) and INR (**B**); Values for ALP (u/L) and GGT (u/L) (**C**); Values for APAP (mcg/mL) (**D**).

non-lethal cases of the acetaminophen poisoning as well as healthy study participants (Figure 3). A PCA is used to visualize complex high-dimensional data by transposing the data to a new coordinate system using principal components (PC1 and PC2), which emphasize the variation in the underlying data. In the PCA visualization, the distance among individual points on the plot reflects a level of similarity among the underlying data. Thus, shorter distance among the points on the plot represents higher similarity between miRNA profiles. In this way, the high-dimensional data can be explored and visualized in a two-dimensional graph. In this analysis PC1 appeared to be associated with non-lethal outcome of acetaminophen poisoning. The right shift location of samples from acetaminophen-overdosed study participants is mainly driven by miR-122-5p, miR-4532, miR-193b-5p, miR-125b-2-3p, and miR-483-5p, and the distance from the cluster of healthy study participants is proportional to the degree of liver injury as corroborated by levels of ALT, a commonly used biomarker of liver injury. The samples collected at the early time point of the lethal poisoning showed the highest levels of ALT and AST and thus showed the largest PC1 right shift. As the acetaminophen-induced injury resolved, the subsequent miRNA profiles of samples of each individual non-lethal case of acetaminophen overdose moved toward the cluster of healthy study participants. One case of non-lethal acetaminophen overdose without elevated levels of ALT was indistinguishable from healthy study participants. In contrast, the PC2 differentiated the lethal case from non-lethal cases and healthy controls. The PC2 is mainly driven by miR-140-3p, miR-29c-3p, miR-22-5p, miR-4732-3p, and miR-128-3p and is visualized as a gradual downshift in PC2 values during the course of the supra therapeutic acetaminophen ingestion (Figure 3). Furthermore, the degree of PC2 downshift corresponds to the degree of liver injury as corroborated by increasing levels of TBIL. Figure 3A also displays ALT levels as bullet size per samples as well as TBIL levels as bullet color. ALT levels appear to be equally high among lethal and non-lethal study participants. TBIL levels are elevated at an early time-point for one non-lethal study participant, while the lethal study participant shows late elevation of TBIL relative to the early shift in miRNA levels. Figure 3B represents the MELD scores as bullet sizes. Utilization of MELD in combination with the modified King's College score, arterial blood lactate and INR would potentially provide better prognostic determinations. However, utilizing the limited predictive ability of the current MELD scores alone does not show a critical value for the lethal study participant at the early time point. Furthermore, 3 study participants from the non-lethal group show elevated MELD scores (Supplementary Table 1).

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Table 1. Clinical parameters across all time points. The table presents numeric values of ALT, Kings College Criteria, MELD Score, PT, INR and Lactic Acid of the lethal subject covering the period from admission to time of death.

Time (h)	ALT	Kings College Criteria	MELD Score	PT (sec)	INR	Lactic acid (mmol/L)
0.0	3737	No	18	21.6	2.2	7.4
6.0	4250	No	22	31.8	3.1	3.8
11.2	7178	No	25	42.6	4.1	4.8
15.7	8327	No	28	55.4	5.2	5.3
24.9	8900	No	28	38.7	3.7	6.4
28.9	7343	No	32	46.7	4.4	7.1
44.9	6178	No	40	>100	9.6	5.0
55.9	6313	Yes	40	>100	9.7	6.1
64.4	6098	Yes	34	35.3	3.4	4.4
69.4	4590	Yes	40	49.9	4.7	4.3
72.6	4660	Yes	40	ND	ND	4.3
78.6	5202	Yes	40	98.2	8.7	4.4
90.8	3383	Yes	32	31.3	3.1	2.8
98.1	2780	Yes	40	ND	ND	2.6
103.1	2918	Yes	40	80.8	7.3	2.4
108.3	2420	Yes	40	ND	ND	2.3
120.3	2345	Yes	40	ND	ND	1.4
125.3	1772	Yes	40	>100	9.7	1.5
137.5	1598	Yes	40	>100	9.7	1.2
142.7	1215	Yes	40	ND	ND	1.3
149.7	1232	Yes	40	>100	9.7	1.6
166.9	874	Yes	40	ND	ND	2.7
174.9	633	Yes	40	ND	ND	3.0
182.9	508	Yes	40	ND	ND	6.6

Since PC1 seemed to correspond with initial liver injury caused by acetaminophen overdose and PC2 was associated with lethal outcome, we set out to identify underlying molecular processes that drive each principal component. First, we identified miRNAs most important for PC1 and PC2. The gene targets for each miRNA set were then compared using gene set enrichment (GSE) analysis to the targets for all the miRNAs in the PCA and the significant pathways between PC1 and PC2 were contrasted. The GSE analysis of the gene targets for PC1 had a number of pathways related to autophagy, cell-cycle and notch signaling that includes response to cellular and oxidative stress which is consistent with the molecular mechanisms of action of acetaminophen hepatotoxicity (Table 3). In contrast, the GSE analysis for PC2 revealed pathways related to fibrosis and coagulation and thrombosis (Table 4). These results may relate to the breakdown of intercellular integrity and loss of liver function with resulting coagulopathies that have been observed in acetaminophen poisonings [16–18].

Discussion

Acetaminophen is the most commonly used analgesic and antipyretic in the world. Because of the wide availability of

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Table 2. Time-course dependent miRNAs. The table presents all miRNAs that showed a significant change over time. The beta coefficients (coef) and p-values of the linear model as well as the time course dependent clustering are presented in this table. Resulting p-values have been corrected for multiple testing according to the false discovery rats (FDR) <0.05. Samples have been clustered according to their time trend from admission to time of death (see supplementary methods).

Cluster	miRNA	Coef	FDR
1	hsa-miR-99a-5p	–0.06	9.88E-19
1	hsa-miR-122-5p	–0.08	4.79E-18
1	hsa-miR-193b-5p	–0.05	1.62E-16
1	hsa-miR-6087	–0.05	1.39E-15
1	hsa-miR-125b-2-3p	–0.06	1.06E-13
1	hsa-miR-4516	–0.04	1.71E-11
1	hsa-miR-1307-5p	–0.04	3.28E-09
1	hsa-miR-29a-3p	–0.04	9.41E-08
1	hsa-miR-194-5p	–0.05	2.67E-07
1	hsa-miR-340-5p	–0.03	2.98E-07
1	hsa-miR-885-5p	–0.05	4.24E-07
1	hsa-miR-483-5p	–0.04	2.27E-06
1	hsa-miR-23b-3p	–0.04	4.85E-06
1	hsa-miR-3615	–0.02	9.50E-06
1	hsa-miR-345-5p	–0.03	2.65E-04
1	hsa-miR-497-5p	–0.03	7.56E-04
1	hsa-let-7b-3p	–0.04	9.90E-04
1	hsa-miR-148b-3p	–0.04	3.52E-03
1	hsa-miR-425-5p	–0.02	4.13E-03
1	hsa-miR-24-3p	–0.03	5.71E-03
1	hsa-miR-1247-5p	–0.04	6.44E-03
1	hsa-miR-4492	–0.04	6.96E-03
1	hsa-miR-125b-1-3p	–0.03	8.08E-03
1	hsa-miR-1246	–0.06	8.87E-03
1	hsa-miR-1468-5p	–0.04	1.00E-02
1	hsa-miR-128-3p	–0.02	1.39E-02
2	hsa-miR-335-5p	–0.03	1.17E-15
2	hsa-miR-100-5p	–0.05	3.73E-14
2	hsa-miR-423-3p	–0.03	1.04E-11
2	hsa-miR-375	–0.05	2.68E-10
2	hsa-miR-215-5p/192-5p	–0.03	5.38E-08
2	hsa-miR-101-3p	–0.03	1.23E-07
2	hsa-miR-378a-3p/378c/378d/378e	–0.03	1.41E-07
2	hsa-miR-221-3p	–0.02	2.32E-07
2	hsa-miR-130b-3p	–0.02	1.38E-06
2	hsa-miR-26b-5p	–0.02	5.90E-06
2	hsa-let-7b-5p	–0.01	1.00E-05
2	hsa-miR-28-3p	–0.02	1.89E-05
2	hsa-miR-23a-3p	–0.02	9.17E-05
2	hsa-miR-125b-5p	–0.03	1.06E-04
2	hsa-miR-484	–0.02	3.68E-04
2	hsa-miR-660-5p	–0.02	3.80E-04
2	hsa-let-7d-3p	–0.02	5.07E-04
2	hsa-miR-584-5p	0.01	4.44E-03
2	hsa-miR-151b/151a-5p	–0.01	1.03E-02
3	hsa-miR-99b-5p	0.02	2.28E-13
3	hsa-miR-182-5p	0.02	1.33E-11
3	hsa-miR-191-5p	0.02	2.93E-10
3	hsa-miR-125a-5p	0.02	6.43E-06
3	hsa-miR-146b-5p	0.01	6.87E-06
3	hsa-miR-143-3p	0.01	1.21E-05
3	hsa-miR-451a	0.01	6.15E-04
3	hsa-miR-144-3p	0.01	1.79E-03
3	hsa-miR-142-5p	0.01	4.02E-03
3	hsa-miR-146a-5p	0.01	1.77E-02
4	hsa-miR-92a-3p	–0.02	8.74E-23
4	hsa-miR-320a/b/c/d	–0.02	1.27E-15
4	hsa-miR-130a-3p	–0.03	4.66E-14
4	hsa-miR-10b-5p	0.01	3.73E-12
4	hsa-miR-10a-5p	0.01	1.39E-10
4	hsa-miR-148a-3p	–0.04	1.54E-10
4	hsa-miR-27a-3p/27b-3p	–0.03	1.85E-10
4	hsa-miR-107/103a-3p/103b	–0.02	3.08E-09
4	hsa-miR-21-5p	–0.02	1.49E-08
4	hsa-miR-30d-5p	–0.03	5.75E-08
4	hsa-miR-30e-5p	–0.02	3.60E-07
4	hsa-miR-22-3p	–0.02	8.56E-07
4	hsa-miR-30a-5p	–0.01	1.18E-04
4	hsa-let-7a-5p/7c-5p	–0.01	2.43E-03

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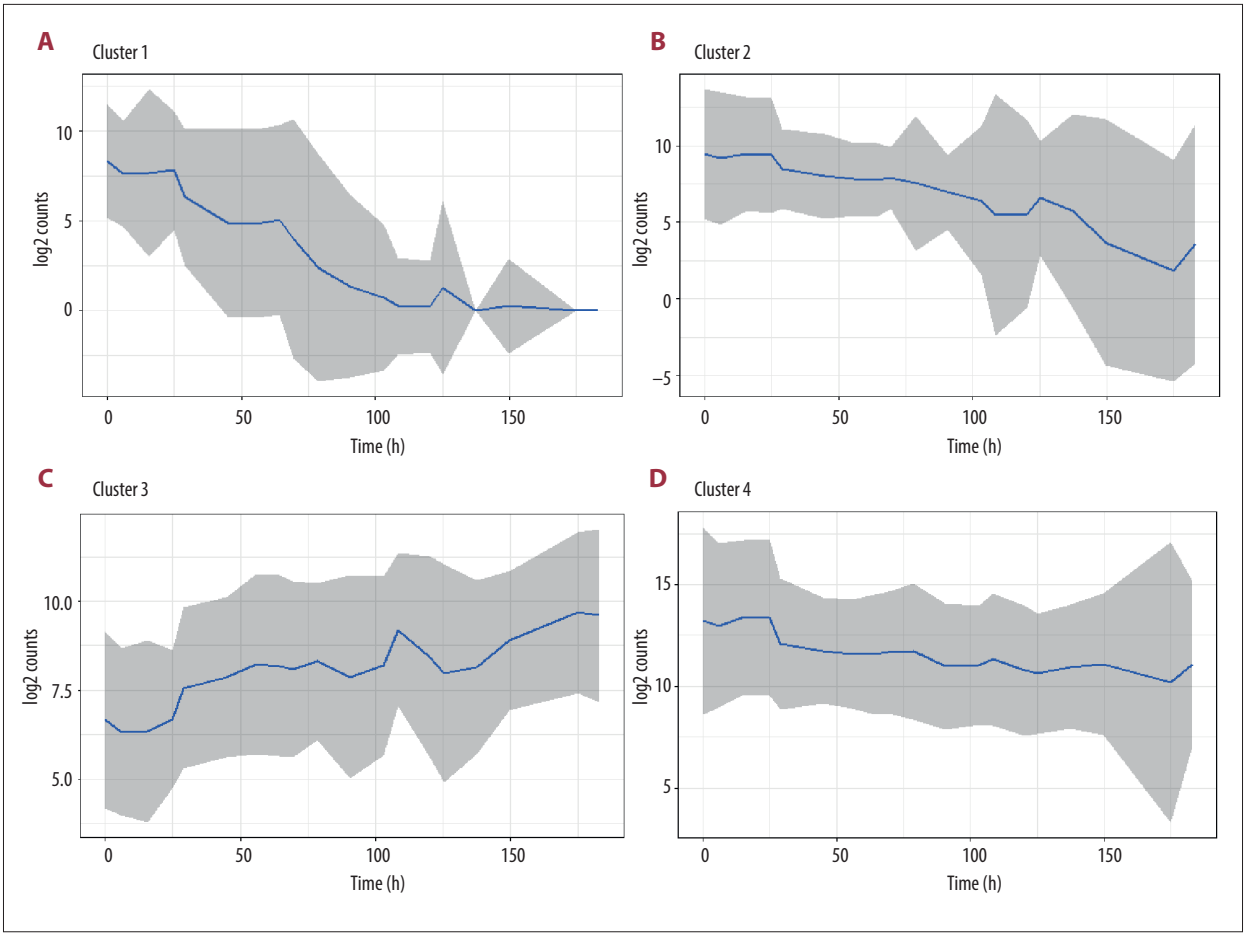

Figure 2. Clustering of time-course dependent circulating miRNAs. The figure shows the average abundance (in log2 Counts) and standard deviation of all circulating miRNAs per cluster over time after the supra therapeutic APAP ingestion. Cluster 1 represents all circulating miRNAs showing a quick decrease (**A**) and cluster 2 all circulating miRNAs with a retarded decrease (**B**) towards the detection limit. Cluster 3 represents circulating miRNAs that increase in time with the lethal outcome (**C**), while cluster 4 contains circulating miRNAs with a moderate decrease or increase over time (**D**).

acetaminophen, alone or as part of many over-the-counter remedies, supra therapeutic poisonings are quite common, which constitutes an important public health challenge. Therefore, the development of biomarker strategies that enable better diagnosis and importantly prognosis of the outcome of the acetaminophen induced acute liver injury is important. Here we evaluated the potential of non-invasive global profiling of circulating miRNA as "liquid biopsies" to differentiate a lethal case of acetaminophen poisoning from non-lethal cases and healthy study participants. Furthermore, we evaluated the ability of miRNA profiles to provide mechanistic information that could improve the assessment of acetaminophen poisoning.

We interrogated profiles of circulating serum miRNA using NGS from a single case with lethal acetaminophen poisoning from time of hospitalization till time of death and compared with previously established miRNA profiles of non-lethal cases of acetaminophen poisoning and healthy study participants [14,15]. The miRNA sequencing identified a total of 137 miRNAs across all samples. From those, the levels of 69 miRNAs responded in a time dependent manner (Figure 2). Although the samples from the lethal case had a 4-fold lower yield of miRNA that resulted in decreased sensitivity for miRNA detection, 46 time-dependent miRNAs identified in the lethal case were also present in our previously studied non-lethal cases of acetaminophen poisoning [14,15]. Furthermore, the miRNAs identified in this study and our previous studies [14,15] were consistent with recent reports that show direct involvement of these miRNAs in response to acetaminophen hepatotoxicity. In fact, a recent report on acetaminophen-overdosed children showed significant serum level elevations for miR-122-5p, miR-378a-5p, miR-125b-5p, and miR-27b-3p [19]. All 4 have been also found significantly changed in our study with hsa-miR-378a-5p being present as hsa-miR-378a-3p. Another study harvesting HepaRG cells and medium at multiple time points after acetaminophen exposure *in vitro* reported 29 miRNAs of which 9

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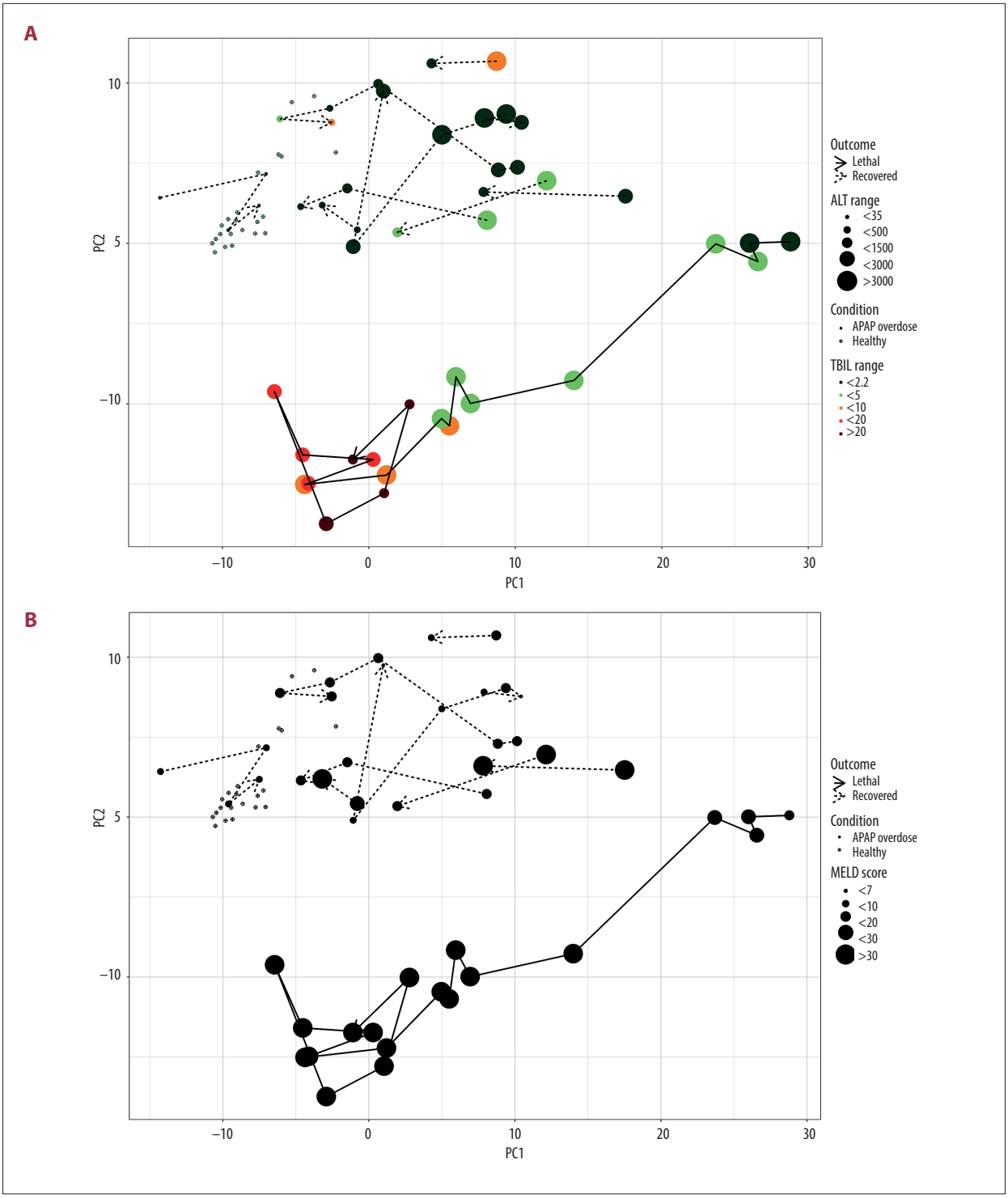

Figure 3. Principal component analysis of patients with recovered and fatal APAP overdose. The PCA presents the serum circulating miRNA levels and simultaneously the ALT and TBIL levels per subject and sample. The graph displays individual samples as bullets according to the principal components 1 and 2 values. Lines connect individual patients and the arrows indicate the relative order of sampling for all subjects. Time of each sample can be seen in Table 1 for the lethal case and in Supplemenatry Table 1 for the recovered subjects. The size of the dots is proportional to ALT levels (**A**) and MELD scores (**B**) while color indicates TBIL levels (**A**). The asterisk indicates the time when the patient met the Kings College Criteria of needing a transplant at approximately 48 hrs after admission to the hospital (**B**).

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Table 3. Top pathway predictions for PC1 miRNAs' target genes. Pathway analysis of miRNA target genes corresponding to PC1 (see supplementary methods).

Pathway	PC1 p-value	PC2 p-value
Pancreatic adenocarcinoma signaling	1.74E-06	1.76E-03
Notch signaling	9.32E-04	4.94E-01
Autophagy	1.85E-03	–
Cell cycle: G1/S checkpoint regulation	3.70E-04	5.48E-02
EGF signaling	3.02E-03	3.05E-01
IL-6 signaling	3.31E-03	2.62E-01
UVC-induced MAPK signaling	7.76E-03	–
STAT3 pathway	2.57E-03	1.58E-01
Type II diabetes mellitus signaling	9.49E-03	–
Ovarian cancer signaling	1.55E-04	7.17E-03

Table 4. Top pathway predictions for PC2 miRNAs' target genes. Pathway analysis of miRNA target genes corresponding to PC2 (see supplementary methods).

Pathway	PC1 p-value	PC2 p-value
Hepatic fibrosis/hepatic stellate cell activation	4.65E-02	1.03E-04
Intrinsic prothrombin activation pathway	–	1.66E-03
PI3K/AKT signaling	7.96E-02	2.01E-03
PTEN signaling	5.87E-02	1.55E-03
Reelin signaling in neurons	–	1.37E-02
Tight junction signaling	4.96E-01	1.44E-02
Role of tissue factor in cancer	6.28E-02	3.19E-03
FAK signaling	1.97E-01	1.09E-02
Hereditary breast cancer signaling	1.56E-01	1.09E-02
LXR/RXR activation	–	3.54E-02

overlapped with this study (miR-10b-5p, miR-122-5p, miR-1246, miR-143-3p, miR-192-5p, miR-320a, miR-320b, miR-320c, and miR-320d). These authors also reported hsa-miR-320a to be significantly increased in cells and medium, while miR-122-5p and miR-194-5p were reduced in cells but increased in medium. Since exogenous transfection of miR-320a effectively rescued HepaRG cells from acetaminophen-induced toxicity it was suggested that this miRNA is leaked upon hepatocyte damage, while miR-122 and miR-194-5p are eliminated from cells via exportation, interfering with the suppressive role of both miRNAs in hepatotoxicity [20]. In our study, miR-122 and miR-194-5p levels showed large elevations of their serum levels that quickly dropped below the detection limit within 86 hours after admission to the hospital (cluster 1), while miR-320a was present at high serum levels from admission till the time of death (cluster 4). In previously published studies the levels of miR-122, miR-21, and miR-221 were lower in lethal cases of acetaminophen-induced ALF than in non-lethal cases of acetaminophen overdose [21,22]. In our case study, the study participant with acetaminophen-induced lethal ALF had indeed lower levels of miR-21 and 221 when compared to the study participants that recovered (fold change: -4.6 and -1.2, *P*-value: 0.005 and 0.64 respectively). In contrast miR-122 was higher in our lethal case during the day of admission (fold change: 22.6, *P*-value: 0.001) followed with a sharp decrease over the time course. The inconsistency of miR-122 can be due to various times of miRNA assessment post ingestion and the lack of a time course in the previously published studies. Taken together, our data are consistent with published datasets indicating that NGS provides a robust method for studying circulating miRNA in human serum.

Using PCA we have shown a clear distinction in miRNA profiles among a lethal case, non-lethal cases and healthy study participants (Figure 3). In this visualization, the PC1 right shift was proportional to the degree of initial injury. As time progressed, miRNA profiles from study participants recovering from non-lethal acetaminophen poisoning, gained similar profiles to healthy study participants. This is apparent as the gradual left shift on PC1 scale indicating recovery (Figure 3). Receding levels of ALT also confirmed the recovery from liver injury. The miRNA profiles at early time points after lethal acetaminophen poisoning were in line with non-lethal cases albeit with a more pronounced right shift indicating a higher degree of initial acetaminophen-induced liver injury. In contrast to non-lethal cases, later time points showed a significant PC2 downshift. This was accompanied by a decrease in ALT and an increase of TBIL, thus clearly indicating loss of liver function. The PC2 downshift was detectable as early as 24 hours after admission to the hospital care. Clinically, severe acetaminophen poisoning features elevated levels of transaminases with a gradual increase of TBIL accompanied with severe acidosis, coagulopathy and encephalopathy progressing to multi-organ failure [5]. In this lethal case, the extent of acidosis was variable and did not provide suitable information for predicting the outcome of acetaminophen poisoning. On the other hand, our patient showed coagulopathy, determined as INR increases, reaching clinically critical levels of 5 within 40 hours of admission (Figure 1B) while both PT/INR were at maximal detection levels by 64 hours after admission. At later time points the PT/INR values fluctuated before reaching maximum levels at 141 hours after admission (Table 1) until

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the time-of-death. While the large fluctuations of PT/INR values may well complicate the prediction of the outcome of the poisoning, these fluctuations occurred in response to "life saving" interventional steps in the intensive care unit (ICU) by administration of blood products (among other interventions) at the times when PT/INR were elevated. In contrast, the PCA analysis showed a steady downshift of PC2 and consequently provided a more consistent prognosis of the acetaminophen overdose.

MELD scores are utilized as a model for end-stage liver disease, which predicts severity and 3-month survival of liver damage and assists in liver transplant planning. Current calculation utilizes sodium for those patients with MELD scores greater than 11, as outlined by the Health and Human Services "Organ Procurement and Transplantation Network". Although MELD scores are not routinely used of for prognostic evaluation of acetaminophen poisoning, we tested whether MELD scores would differentiate the lethal outcome in our case study (Table 1). In contrast to the miRNA profiles, the MELD scores were not indicative for the outcome of the lethal or recovered acetaminophen overdoses (Figure 3B). To our knowledge, this case study is the first to demonstrate the potential of miRNA-based liquid biopsies to differentiate the outcome of acetaminophen-induced liver injury.

To decrypt underlying biological pathways from miRNA profiles that drive PC1 and PC2, we identified sets of miRNAs and their gene targets that were the most important for each PC. The GSE analysis of the target genes for PC1 identified a number of pathways related to autophagy, cell-cycle, and notch signaling that are associated with response to cellular and oxidative stress (Table 3). These pathways are likely to represent the liver's attempt to compensate for the DNA and protein damage which occurs with acetaminophen toxicity [23–26]. Our analysis is fully in line with known molecular mechanisms of acetaminophen toxicity. In fact, acetaminophen is activated by cytochrome P450 to a reactive metabolite NAPQI that causes protein adducts leading to mitochondrial oxidative and nitrosative stress with resulting autophagy and activation of regulated necrosis [3,4]. We also observed cancer-related pathways; however, inspection of the underlying genes indicated that a number of cell-cycle related genes were changing. Presumably, cancer pathways were highlighted due to the overlap between cancer pathways and cell-cycle genes. In casea of PC2 that are associated with lethal outcome of acetaminophen poisoning, the most important pathways were hepatic fibrosis and intrinsic prothrombin activation pathways. This is in line with the acknowledged clinical manifestation of severe acetaminophen poisoning [5] that includes coagulopathy. Since the liver produces these coagulation factors, our data are consistent with loss of liver function that is also corroborated by increasing levels of total bilirubin.

The hallmarks of lethal outcome of acetaminophen poisoning are severe acidosis and coagulopathy preceding the development of encephalopathy followed by multi-organ failure and death [5]. Recently, a panel of biomarkers consisting of miR-122, cytokeratin-18, and high mobility group box1 was shown capable of stratifying patients with acetaminophen overdose based on risk of developing liver injury [11]. However, the biomarker panel was not able to predict the outcome of acetaminophen-induced liver injury. Although acidosis and coagulopathy are main factors used in predicting outcome of the acetaminophen poisoning [5], developing more sensitive biomarkers capable of detecting and predicting the outcome of acetaminophen poisoning is important. In fact, in our case study, our patient with lethal acetaminophen poisoning demonstrated both metabolic acidosis and pulmonary alkalosis. In addition, with our liquid biopsy approach, the PC2 downshift provided earlier and more robust prediction of the lethal outcome of acetaminophen poisoning but also, since our liquid biopsy approach is anchored in relevant underlying molecular pathways, it might become useful for assessment of prognosis of acute liver failure in general.

We are aware that inter-individual variability in RNA levels may confound the miRNA response. However, since the healthy controls as well as non-lethal overdosed study participants cluster very closely together on the PCA within their group, we suggest that this would also be true for further lethal overdose cases. This was also supported by the observation that the earlier time points of the lethal case clustered close with the early time points of the non-lethal cases. Furthermore, miRNA profiles originated from the study participant with minimal injury clustered with the healthy controls (Figure 3). Since we evaluated 24 consecutive samples from the lethal case, 27 samples from the 9 non-lethal cases of acetaminophen poisoning, and 22 samples from healthy study participants, we were able to determine the statistically significant changes on the miRNA level. Obviously, lethal acetaminophen poisoning is very difficult to study in humans due to the lack of samples. More study participants with lethal outcome would be needed to confirm this observation; however, this is currently not feasible. Nevertheless, the results of this single case study are important for the scientific community in that it provides a foundation on which further research can be built.

Conclusions

In summary, to our knowledge this is the first case study interrogating a time course of global miRNA profiles of a lethal case of acetaminophen poisoning. Using bioinformatics, we identified relevant biological pathways associated with liver injury, recovery and progression to ALF. Our results showed that miRNA profile analysis differentiated the lethal outcome

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better than the standard biomarker panel including currently used clinical parameters for the assessment of the outcome of the acetaminophen-induced liver failure (persistent acidosis and coagulopathy). Although more confirmatory studies are needed, this case study indicates that miRNA profiles might help to improve the assessment of prognosis of acetaminophen-induced liver injury and ultimately to provide clinicians with more time to plan therapeutic interventions.

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Ethics approval and consent to participate

Serum samples were collected from healthy study participants and from study participants who had undergone supra therapeutic acetaminophen overdose and NAC treatment, from clinics within the University of Michigan health care system, under an approved IRB (HUM44422). This study was specifically approved and performed in accordance with the UMHS/Medical School Institutional Review Board (IRBMED) guidelines and regulations. All participants (or their authorized signers), provided consent to participate and all data were fully anonymized

Statement

The funder provided support in the form of salaries for authors SJS, MG, and JA, reviewed the final manuscript but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interests

The project was funded by Pfizer, Inc., Mark Gosink and Shelli Schomaker are employed by Pfizer, Inc. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all any policies on sharing data and materials.

Supplementary Data

Supplementary Table 1. Patient characteristics and clinical chemistry data.

Patient	Outcome	Time (h)	ALT	Kings College Criteria	MELD Score	PT (sec)	INR (sec)	Lactic Acid (mmol/L)
1	Recovered	0	2966	No	13	12.0	1.2	ND
1	Recovered	24	2056	No	12	10.0	0.9	ND
1	Recovered	72	800	No	12	9.8	0.9	ND
1	Recovered	96	473	No	10	10.1	0.9	ND
1	Recovered	144	206	No	10	ND	1.0	ND
1	Recovered	168	160	No	10	ND	0.9	ND
2	Recovered	0	4178	No	8	15.3	1.5	ND
2	Recovered	24	2853	No	6	12.4	1.2	ND
3	Recovered	0	2610	No	40	24.0	2.5	1.5
3	Recovered	48	1123	No	40	15.8	1.6	ND
4	Recovered	0	3347	No	12	17.1	1.7	ND
4	Recovered	72	1297	No	7	11.5	1.1	ND
5	Recovered	0	3541	No	40	67.3	6.7	5.0
5	Recovered	40	1321	No	17	14.6	1.0	9.2
6	Recovered	0	5064	No	12	21.1	1.4	2.1
6	Recovered	24	3515	No	9	20.0	1.1	ND
6	Recovered	48	2405	No	7	11.1	1.1	ND
6	Recovered	72	1832	No	6	10.6	1.0	ND

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Patient	Outcome	Time (h)	ALT	Kings College Criteria	MELD Score	PT (sec)	INR (sec)	Lactic Acid (mmol/L)
7	Recovered	0	386	No	36	43.3	4.3	1.9
7	Recovered	24	458	No	28	30.3	3.0	1.9
8	Recovered	0	3057	No	15	3.5	1.4	2.0
8	Recovered	96	616	No	12	13.4	1.5	1.2
8	Recovered	216	155	No	18	23.7	2.3	ND
9	Recovered	0	16	No	7	ND	1.1	1.4
9	Recovered	12	10	No	7	ND	1.1	1.4
9	Recovered	24	9	No	7	ND	1.1	ND
9	Recovered	48	13	No	7	ND	1.1	ND

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