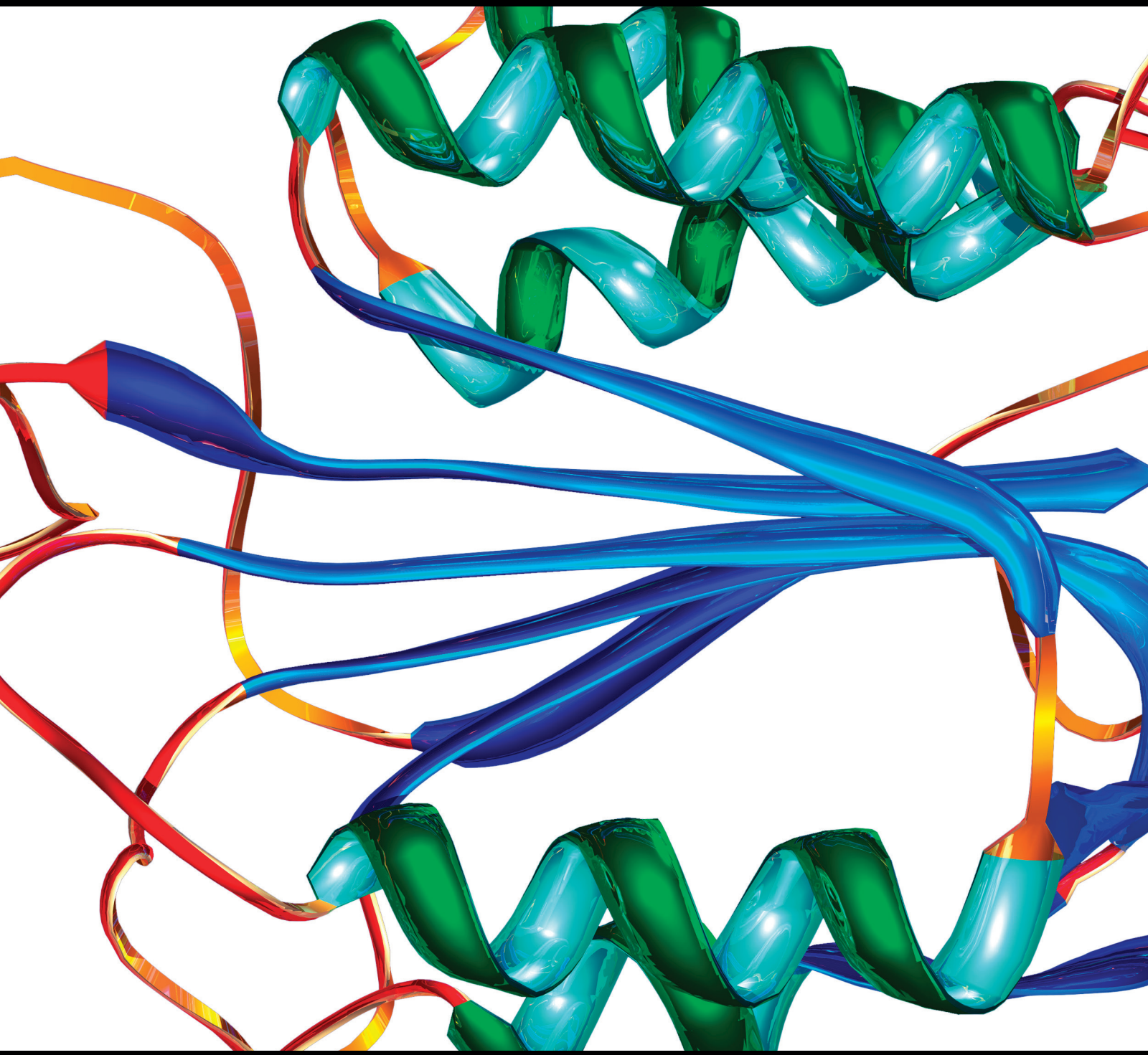
Disease Markers

Biomarkers in Emergency Medicine

Lead Guest Editor: Patrizia Cardelli
Guest Editors: Mina Hur and Salvatore Di Somma



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Editorial Board

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https://doi.org/10.1155/2018/4597489

Editorial
Biomarkers in Emergency Medicine

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Received 13 February 2018; Accepted 13 February 2018; Published 20 March 2018

Researchers navigate the ocean of biomarkers searching for proper targets and optimal utilization of them. Emergency medicine builds up the front line to maximize the utility of clinically validated biomarkers and is the cutting edge field to test the applicability of promising biomarkers emerging from thorough translational researches. The role of biomarkers in clinical decision making would be of greater significance for identification, risk stratification, monitoring, and prognostication of the patients in the critical- and acute-care settings. No doubt basic research to explore novel biomarkers in relation to the pathogenesis is as important as its clinical counterpart. This special issue includes five selected research papers that cover a variety of biomarker- and disease-related topics.

The paper by N. Shi et al. demonstrated that the plasma microRNA-127 (miR-127) level was significantly downregulated in acute pancreatitis (AP) patients with respiratory failure compared with the healthy volunteers and those without respiratory failure. It was the first study that integrated miR-127 and the inflammatory injuries of the pancreas and the lung. miR-127 might serve as a potential marker for the identification of AP with lung injury.

The paper by L.-J. Chen et al. explored the association between plasma miRNA-24-3p (miR-24) expression and coagulation factor X (FX) and XII (FXII) levels in major trauma and trauma-induced coagulopathy (TIC) patients. In their study, miR-24 was overexpressed in major trauma and TIC patients, and miR-24 expression correlated with FX level negatively, suggesting the possibility that miR-24 might inhibit the synthesis of FX during TIC.

P. Lochner et al. measured osteopontin (OPN: biomarker for inflammation) and neurofilament heavy chain (NfH: biomarker for axonal injury) in patients with acute optic neuritis and sex- and age-matched healthy controls. They demonstrated that OPN and NfH are elevated in these patients, supporting the presence of underlying inflammation and axonal injury as well as the prognostic utility of examining biomarkers in optic neuritis.

The paper by B. Morawiec et al. addressed the prognostic value of copeptin in patients admitted with chest pain and suspected acute coronary syndrome (ACS). They concluded that copeptin appears to be an independent predictor of long-term mortality in patients with suspected ACS. Copeptin may be also considered as an early marker for identifying high-risk patients who would develop heart failure.

The last paper by C. Spiekermann et al. explored S100 proteins A8 and A9 (S100A8/A9) as a promising diagnostic marker for peritonsillar abscess (PTA). Using a combination of S100A8/A9 levels and characteristic symptoms of PTA, they also developed a PTA score as an objective and appropriate tool to differentiate between peritonsillitis and PTA.

We hope that this special issue would bridge basic research and clinical practice on biomarkers, facilitate the scientific development in this research field, and eventually contribute to the unrivaled value of disease markers.

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Hindawi
Disease Markers
Volume 2018, Article ID 6597387, 8 pages
https://doi.org/10.1155/2018/6597387

Research Article

Copeptin as a Prognostic Marker in Acute Chest Pain and Suspected Acute Coronary Syndrome

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Received 7 June 2017; Revised 25 August 2017; Accepted 18 October 2017; Published 24 January 2018

Academic Editor: Salvatore Di Somma

Background. In patients admitted with chest pain and suspected acute coronary syndrome (ACS), it is crucial to early identify those who are at higher risk of adverse events. The study aim was to assess the predictive value of copeptin in patients admitted to the emergency department with chest pain and nonconclusive ECG. *Methods*. Consecutive patients suspected for an ACS were enrolled prospectively. Copeptin and high-sensitive troponin T (hs-TnT) were measured at admission. Patients were followed up at six and 12 months for the occurrence of death and major adverse cardiac and cerebrovascular events (MACCE). *Results*. Among 154 patients, 11 patients died and 26 experienced MACCE. Mortality was higher in copeptin-positive than copeptin-negative patients with no difference in the rate of MACCE. Copeptin reached the AUC 0.86 (0.75–0.97) for prognosis of mortality at six and 0.77 (0.65–0.88) at 12 months. It was higher than for hs-TnT and their combination at both time points. Copeptin was a strong predictor of mortality in the Cox analysis (HR14.1 at six and HR4.3 at 12 months). *Conclusions*. Copeptin appears to be an independent predictor of long-term mortality in a selected population of patients suspected for an ACS. The study registration number is ISRCTN14112941.

1. Introduction

The optimal risk stratification in patients with acute coronary syndrome (ACS) without persistent ST segment elevation is a priority for clinicians. Patients after an ACS remain on higher risk of the development of heart failure, further ischemic events, and death. It is crucial to identify possibly early those subjects who present with chest pain and are at higher risk, in order to enable the introduction of adequate strategy in optimal time frames. The guidelines for diagnosis and treatment of non-ST segment elevation ACS focus attention on early stratification of short- and long-term risk [1]. However, cardiac troponin (cTn), despite being the gold standard marker for diagnosis of myocardial injury, remains suboptimal in terms of early risk assessment. Due to the character of the release profile, repetitive blood sampling for cTn is needed to achieve satisfactory prognostic accuracy [2]. The introduction of high-sensitive assays allowed earlier stratification of patients but did not justify single measurement or risk stratification based solely on these assays. While looking for complemental markers that would increase the accuracy of cTn, we face a large variety of biomarkers [3]. It is of utmost importance from a practical point of view to indicate those that could be used for diagnostic and prognostic purposes simultaneously as early as possible after symptom onset. Copeptin, the C-terminal part of the prohormone for vasopressin, is a marker of acute endogenous stress, with rapid release pattern [4]. Several previous studies showed

its good diagnostic accuracy in patients with an ACS [5–7]. Its potential prognostic role was described in patients with the history of an acute myocardial infarction (AMI) who developed heart failure [6, 8]. The strategy of combined use of copeptin and cTn, besides serving as a diagnostic tool, could identify patients with chest pain at higher risk for adverse events and the development of heart failure (HF) very early after symptom onset, thus enabling early triage decision. Despite the combination of copeptin and high-sensitive Tn (hs-Tn) was extensively described in terms of diagnosis of an ACS, there is insufficient data regarding its prognostic accuracy, especially in patients without persistent ST segment elevation. We therefore aimed to investigate if copeptin provides information on the prediction of outcome in patients admitted to tertiary cardiac centers with symptoms of ACS and nonconclusive ECG.

2. Methods

The Copeptin for Acute Coronary Syndrome (COPACS) is a prospective, investigator-initiated, observational study with the aim to evaluate the role of copeptin in the diagnostic and prognostic process in patients with acute chest pain to enhance rapid evaluation in the emergency department.

Details on the design and the chart of the study were widely described previously [9]. In brief, consecutive patients presenting to the emergency department of the 2nd Department of Cardiology in Zabrze, Medical University of Silesia, Katowice, Poland, were screened in 24/7 manner. Patients with chest pain lasting 5 minutes or more during the last 6 hours and the absence of persistent ST segment elevation in admission ECG were prospectively enrolled to the study. Major conditions with proved influence on copeptin elevation were regarded as exclusion criteria (e.g., end-stage renal disease, sepsis, anaemia, and hyponatremia). According to the tertiary character of the enrolling center, patients admitted to the emergency department conform a highly preselected population, referred mainly via regional hospital. In the structure of our health care system, high rate of patients with chest pain and without persistent ST segment elevation on ECG undergoes initial stratification in regional hospitals. Therefore, patients are referred and admitted to tertiary cardiac centers after initial qualification to invasive coronary angiography with longer delays from chest pain onset and high suspicion of an ACS. As an effect, first, vast majority of patients present later than six hours after chest pain onset and, second, high rate of patients is finally diagnosed with acute coronary syndrome.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Medical University of Silesia (decision number KNW/0022/KB1/187/11). All patients gave their written informed consent before inclusion to the study. The study registration number is ISRCTN14112941.

The design of the study, data gathering, and analysis were conducted according to the STARD guidelines for studies of diagnostic/prognostic accuracy. All authors contributed to the work by participating in the design, collecting and analyzing the data, and writing the paper, and all accepted the final draft of the manuscript.

After inclusion, each patient underwent initial clinical examination which included physical examination, 12-lead electrocardiogram (ECG), echocardiographic examination, and standard laboratory tests (blood count, sodium, potassium, creatinine, GFR, C-reactive protein [CRP], and N-terminal pro-B type natriuretic peptide [NT-proBNP]). Hs-TnT, creatine kinase myocardial bound (CK-MB), and copeptine were measured at admission (T0). Copeptin was double-blinded until final adjudication of the diagnosis. Hs-TnT and CK-MB were afterwards measured at six hours (T6) and repeated according to clinical indications.

Initial diagnosis was set by the emergency physician and was verified by a supervisor cardiologist based on available data and the ESC guidelines [1]. All patients were stratified according to the GRACE 1.0 risk score.

Further, all included patients underwent routine diagnostic and therapeutic procedures as indicated in the ESC guidelines for non-ST segment elevation ACS [1] and according to the study design [9]. Final diagnosis of non-ST elevation myocardial infarction (NSTEMI), unstable angina (UA), or other causes of chest pain was set based on independent opinions of two cardiologists, after analysis of all available data and tests gathered during the hospital stay. In case of incoherence of their diagnosis, a third cardiologist was asked for opinion.

Copeptin was measured once, at admission (T0) from the blood sample managed according to the instructions of the manufacturer of the test. The measurement was performed using the BRAHMS Copeptin KRYPTOR kit on BRAHMS KRYPTOR compact plus analyzer (BRAHMS GmbH, Hennigsdorf, Germany)—detection limit at 4.8–500 pmol/l, 20% coefficient of variation (CV) at 12 pmol/l, and the 97.5th percentile for healthy population at 17.4 pmol/l. According to the general rule for the optimal cutoff for a marker at the 99th percentile of healthy population, copeptin was regarded as positive when ≥17.4 pmol/l, following available information provided by the manufacturer on the most compliant value (97.5th percentile) to that recommended in the guidelines [1, 10]. For secondary analysis, the concentration of 10 pmol/l was used as a cutoff [10].

Troponin T was measured at admission (T0), after 6 hours from admission (T6), and at further time points according to the decision of treating physician. Troponin T was measured using an Elecsys Troponin T hs STAT kit on a cobas e 411 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) with a high-sensitive electrochemiluminescence method (limit of detection 3–10,000 ng/l, 99th percentile for healthy population 14 ng/l [95% CI 12.7–24.9 ng/l], and 10% CV 3 ng/l). Hs-TnT was regarded as positive when ≥14 ng/l, according to the manufacturer's indications and the guidelines [1].

The observational data of all patients were analyzed after six months and one year. Six-month follow-up was conducted in a phone call with the patient, relatives, or primary care physician and included the information on Canadian Cardiovascular Society (CCS) class, New York Heart Association (NYHA) class, and the occurrence of endpoints. At one

year, during a visit in the outpatient unit, following data were gathered: CCS and NYHA classes, the occurrence of endpoints, echocardiogram with the assessment of EF, and blood draw for NT-proBNP.

Primary endpoint was defined as death of cardiovascular origin. Secondary endpoints were major adverse cardiac and cerebrovascular events (MACCE) combined with death of cardiovascular origin, nonfatal AMI, UA, repeated cardiac revascularization, and stroke. Patients were also screened for the occurrence of major bleeding.

Maximal hs-TnT/CK-MB was the maximal concentration of the biomarker measured during the hospital stay. Smoker was regarded as past if one was free of smoking for at least one year before admission. Familial history of coronary artery disease was positive if AMI, stroke, or cardiac death occurred in at least one first-degree female relative at the age of <55 years or male relative at the age of <65 years. Coronary artery disease was diagnosed in coronary angiogram if the stenosis of coronary artery was >75% (or >50% for left main).

Data were checked for normality of distribution with the Shapiro-Wilk test. Continuous variables are presented as mean, standard deviation (SD), or median (interquartile range [IQR]) and were compared with the Student t-test or Mann–Whitney test and ANOVA or Kruskal-Wallis test, depending on their distribution. Categorical variables are presented as n (%) and were compared with chi-square test. The correlation between copeptin and other parameters was assessed with Spearman's method. To evaluate the prognostic accuracy, the receiver operating characteristic (ROC) curves with areas under the curve (AUC) were used and compared with the z test. Survival curves for copeptin and hs-TnT were performed with the Kaplan-Meier method. The influence of biomarkers and preselected baseline, clinical, and procedural parameters on the occurrence of endpoints was calculated in the Cox proportional hazard regression model. The taxonomic analysis (focus analysis) was used as a supplementary method to analyze prognostic accuracy in a multivariate manner and is described in online Supplementary Material 1. The p value of <0.05 was assumed significant throughout all analyses. All analyses were performed with Statistica software, version 10PL (StatSoft Inc., Tulsa, OK, USA); GraphPad Prism, version 6.00 (GraphPad, La Jolla, California, USA); and platform R, version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

During the study period, a total of 1665 were screened. After exclusion of 424 patients presented with STEMI and 1241 patients due to other exclusion criteria (40 patients with anaemia, 31 with hyponatremia, 13 with laboratory errors, 995 patients with chest pain out of inclusion criteria, and 8 patients due to withdrawal of the informed consent), a total of 154 patients were enrolled to the study. Mean age of the population was 63, SD was 12 years, and 65% were male. After adjudication of the final diagnosis, 105 patients (68%) were diagnosed with NSTEMI, 30 patients (20%) were diagnosed with UA, and 19 patients (12%) presented other causes of chest pain. Mean GRACE risk score was 124 (104–147) for the population. Copeptin was positive in 48 patients (31%) with the median of 11.56 (5.67–21.61) pmol/l.

Patients positive and negative for copeptin had a similar clinical profile. Groups differed significantly with age; patients were older in the copeptin-positive group. Regarding the risk profile, patients positive for copeptin had higher hs-TnT concentrations measured at every time point; higher six-hour and maximal concentration of CK-MB; higher admission NT-proBNP, CRP, and leukocytosis and lower GFR; and higher GRACE risk score. Patients with positive copeptin result had similar procedural characteristics and received equal medical therapy as copeptin-negative patients with significant differences with regard to in-hospital catecholamine administration, more frequent in copeptin-positive patients. Results are summarized in Table 1. Similar results were observed for patients positive and negative for copeptin with the cutoff 10 pmol/l and are presented in Supplemental Table S1.

Positive correlation of copeptin was found with age ($r = 0.36$ [0.18–0.52]), maximal hs-TnT ($r = 0.24$ [0.05–0.42]), six-hour and maximal CK-MB ($r = 0.34$ [0.16–0.5] and $r = 0.35$ [0.17–0.51], resp.), leukocytosis ($r = 0.36$ [0.18–0.52]), and the GRACE risk score ($r = 0.48$ [0.32–0.62]). Negative correlation reached statistical significance with GFR ($r = -0.27$ [−0.44; −0.08]) and EF ($r = -0.3$ [−0.46; −0.11]).

Follow-up was completed in 98% of patients at six months and 95% of patients at one year. Overall, 11 patients died (8%) and 26 (18%) experienced MACCE (11 deaths, 3 AMI, 10 PCI, and 2 strokes). No major bleeding was reported during the follow-up. Patients who died had significantly higher concentrations of copeptin than survivors (103 [21–168] pmol/l versus 11 [5.3–20] pmol/l, resp., $p = 0.001$), while there was no statistically significant difference in copeptin level between patients who experienced MACCE and other patients (17 [6.0–52] pmol/l versus 11 [5.4–20] pmol/l, resp., $p = 0.19$).

At one year, copeptin-positive patients had higher NT-proBNP concentrations than copeptin-negative patients (216 [140–457] pg/ml versus 147 [80–359] pg/ml, $p = 0.049$). They also had higher NYHA class with more patients in class III (20% versus 8%) and less in class I (50% versus 68%) than copeptin-negative patients ($p = 0.014$). Concentrations of NT-proBNP remained higher in copeptin-positive than in copeptin-negative patients when the cutoff 10 pmol/l was used (250 [118–567] pg/ml versus 135 [77–227] pg/ml, $p = 0.003$), and no statistically significant difference was observed in NYHA class between both groups ($p = 0.10$).

According to Kaplan-Meier analysis, patients with negative copeptin levels had significantly better survival than copeptin-positive patients at six months (103/104 versus 41/47 patients, $p = 0.001$) and one year (98/102 versus 38/45 patients, $p = 0.011$) (Figure 1(a)). After dichotomization with the cutoff 10 pmol/l, similar results were observed at six months (69/71 versus 67/76, $p = 0.033$) and one year (71/71 versus 73/80 patients, $p = 0.011$) (Supplemental Figure S1). No statistically significant difference in survival was observed regarding hs-TnT levels throughout the

Table 1: Baseline characteristics in patients positive and negative for copeptin.

	Overall cohort ($n = 154$)	Copeptin < 17.4 pmol/l ($n = 106$)	Copeptin ≥ 17.4 pmol/l ($n = 48$)	p value
Baseline parameters and medical history				
Age (years)	63 (57–73)	62 (56–69)	65 (57–78)	0.04
Male sex	100, 65%	72, 65.5%	28, 58.3%	0.25
BMI (kg/m^2)	28.7 (42.9–32.3)	28 (25–32)	29 (25–32)	0.93
CAD	67, 44%	50, 45.5%	17, 35.4%	0.17
Hypertension	114, 74%	75, 68.2%	39, 81.3%	0.17
Diabetes mellitus	42, 27%	28, 25.5%	14, 29.2%	0.72
PAD	4, 2.6%	3, 2.7%	1, 2.1%	0.79
Familial history of CAD	21, 14%	18, 16.4%	3, 6.3%	0.07
Current smoker	51, 33%	38, 34.5%	13, 27.1%	0.28
Past smoker	31, 20%	23, 20.9%	8, 16.7%	0.47
Dyslipidemia	67,44%	49, 44.5%	18, 37.5%	0.31
History of AMI	46, 30%	36, 32.7%	10, 20.8%	0.10
History of PCI	48, 31%	36, 32.7%	12, 25.0%	0.27
History of CABG	7, 4.5%	7, 6.4%	0, 0%	0.07
History of stroke	4, 2.6%	3, 2.7%	1, 2.1%	0.79
Baseline clinical status				
Heart rate (beats/min)	75 (66–88)	70 (65–80)	75 (70–85)	0.07
Systolic BP	140 (123–160)	140 (125–160)	140 (120–160)	0.54
EF (%)	55 (45–60)	55 (45–60)	55 (43–60)	0.56
NYHA class III or IV	4, 2.6%	1, 0.9%	3, 6.3%	0.06
Killip class				0.50
1	139, 90%	98, 89.1%	41, 85.4%	
2	14, 9.1%	7, 6.4%	7, 14.6%	
3	1, 0.6%	1, 0.9%	0, 0%	
4	0, 0%	0, 0%	0, 0%	
GRACE	124 (104–146)	120 (101–141)	131 (111–167)	0.03
Laboratory parameters				
Hs-TnT T0 (ng/l)	33 (13–143)	25 (11–125)	68 (30–177)	0.01
Hs-TnT T6 (ng/l)	75 (16–397)	32 (13–247)	234 (43–2284)	<0.001
Hs-TnT max (ng/l)	105 (23–530)	52 (17–274)	236 (74–2070)	<0.001
CK-MB T0 (IU/l)	20 (15–30)	19 (14–28)	22 (17–36)	0.06
CK-MB T6 (IU/l)	21 (14–45)	18 (13–32)	33 (18–107)	<0.001
CK-MB max (IU/l)	27 (18–53)	25 (17–42)	42 (25–111)	<0.001
NT-proBNP (pg/ml)	350 (163–1074)	289 (150–872)	491 (223–1979)	0.02
CRP (mg/l)	2.9 (1.3–5.5)	3 (1–5)	4 (2–7)	0.03
Leukocytosis ($10^3/\mu$l)	8.4 (6.9–10.3)	8 (6–9)	10 (8–12)	<0.001
Hemoglobin (g/dl)	14 (13–15)	14 (13–15)	14 (13–15)	0.34
GFR (ml/min/1.73m^2)	92 (76–110)	93 (77–115)	90 (67–99)	0.08
In-hospital parameters				
Diagnosis of CAD	116, 75%	83, 75.5%	33, 68.8%	0.11
Medical therapy	38, 25%	25, 22.7%	13, 27.1%	0.64
PCI	90, 58%	60, 54.5%	30, 62.5%	0.49
CABG	33, 21%	25, 22.7%	8, 16.7%	0.33
Catecholamines	4, 2.6%	0, 0%	4, 8.3%	0.003
ASA	141, 92%	97, 88.2%	44, 91.7%	0.79
DAPT	99, 64%	68, 61.8%	31, 64.6%	0.96
β-Blocker	134, 87%	93, 84.5%	41, 85.4%	0.81

Table 1: Continued.

	Overall cohort ($n = 154$)	Copeptin < 17.4 pmol/l ($n = 106$)	Copeptin ≥ 17.4 pmol/l ($n = 48$)	p value
ACE inhibitor	126, 82%	91, 82.7%	35, 72.9%	0.07
Statin	135, 88%	95, 86.4%	40, 83.3%	0.26
Diuretic	45, 29%	31, 28.2%	14, 29.2%	0.97
Ca-blocker	39, 25%	27, 24.5%	12, 25.0%	0.98
Nitroglycerin	17, 11%	12, 10.9%	5, 10.4%	0.89
		Final diagnosis		
Unstable angina	30, 20%	27, 24.5%	3, 6.3%	0.005
NSTEMI	105, 68%	65, 59.1%	40, 83.3%	0.007

Data presented as n, %, or median (25th–75th interquartile range). ACE: angiotensin-converting enzyme; AMI: acute myocardial infarction; ASA: acetylsalicylic acid; BMI: body mass index; BP: blood pressure; CABG: coronary artery bypass grafting; CAD: coronary artery disease; CK-MB: creatine kinase myocardial bound; CRP: C-reactive protein; DAPT: dual antiplatelet treatment; EF: ejection fraction; GFR: glomerular filtration ratio; GRACE: Global Registry for Acute Coronary Events; Hs-TnT: high-sensitive troponin T; NSTEMI: non-ST segment elevation myocardial infarction; NT-proBNP: N-terminal pro-B-type natriuretic peptide; NYHA: New York Heart Association; PAD: peripheral artery disease; PCI: percutaneous coronary intervention.

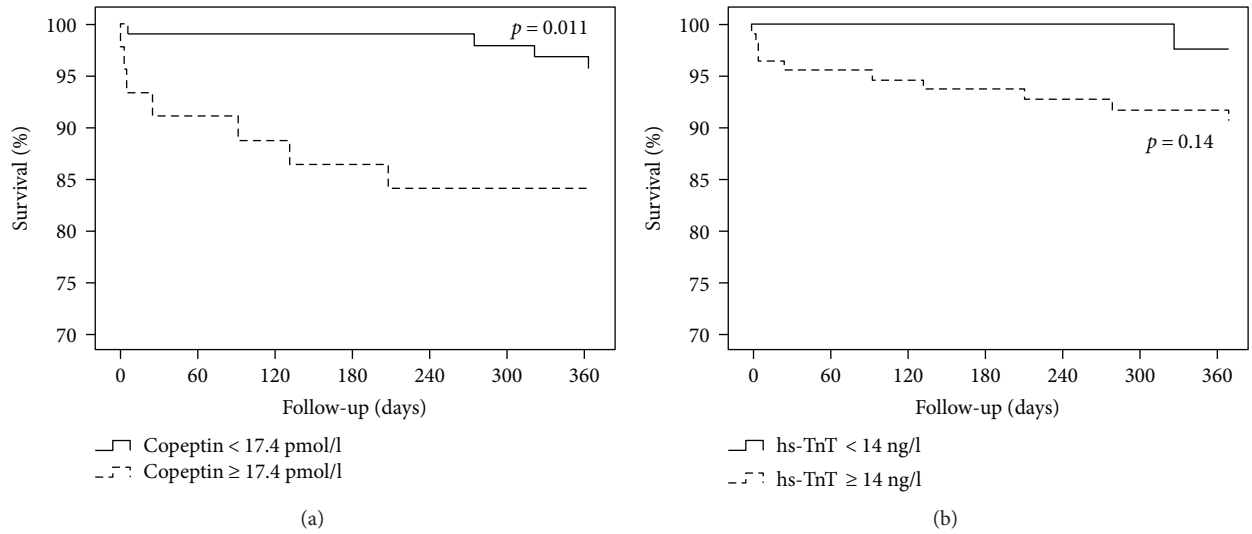

Figure 1: Survival curves or copeptin (a) and hs-TnT (b). Hs-TnT: high-sensitive troponin T.

follow-up ($p = 0.10$ and $p = 0.14$ at six months and one year, resp.) (Figure 1(b)).

Copeptin achieved a good prognostic accuracy in the ROC analysis at six months (AUC 0.86 [0.75–0.97], $p < 0.001$) and one year (AUC 0.77 [0.65–0.88], $p = 0.003$). It was higher when compared to hs-TnT and their combination at six months (0.62 [0.53–0.72], $p = 0.002$, versus 0.76 [0.53–0.99], $p = 0.42$, resp.) and one year (0.63 [0.52–0.75], $p = 0.12$, versus 0.63 [0.43–0.83], $p = 0.24$, resp.). Of note, the ROC analysis of hs-TnT did not reach statistical significance for the prediction of death at any time point. Additional analysis with the GRACE risk score for six-month prediction of death revealed that copeptin was not inferior to the GRACE risk score alone (0.86 [0.75–0.97] versus 0.96 [0.92–1.0], $p = 0.09$), and the highest AUC across all analyzed factors for six-month mortality was observed for the combination of copeptin with the GRACE risk score (0.98 [0.95–1.0], $p < 0.001$). It outperformed both of the predictors alone ($p = 0.04$) and hs-TnT ($p < 0.001$).

In the Cox regression analysis, patients with copeptin level ≥ 17.4 pmol/l were at significantly higher risk of death at six months (hazard ratio (HR) 14 (1.7–117), $p = 0.014$) and at one year (HR 4.32 [1.27–14.77], $p = 0.02$). Contrary, copeptin level ≥ 10 pmol/l was not a significant risk factor for death at six months (HR 61 [0.1–26.86], $p = 0.19$) nor at one year (HR 4.56 [0.99–21.11], $p = 0.052$). Hs-TnT measured at admission was not a predictor of mortality in the Cox analysis, neither was maximal concentration of hs-TnT. Statistical significance for the prediction of mortality was reached by age (HR 1.1 [1.04–1.18], $p = 0.002$) and GFR (HR 0.92 [0.89–0.95], $p < 0.001$). Detailed results are presented in Table 2. The combination of higher copeptin/older age/lower GFR identified a group with higher admission and follow-up NT-proBNP levels, lower baseline

Table 2: The Cox regression model for death at 6 and 12 months.

Characteristic	At six months			At one year		
	HR	95% CI	p value	HR	95% CI	p value
Copeptin ≥ 17.4 pmol/l	14.1	1.7–116.8	**0.01**	4.3	1.3–14.8	**0.02**
Age	1.3	1.1–1.5	**0.001**	1.1	1.04–1.2	**0.002**
Male sex	0.2	0.04–1.1	0.06	0.6	0.2–1.9	0.359
Diagnosis of NSTEMI	3.0	0.4–24.7	0.31	2.3	0.5–10.8	0.281
Prior AMI	1.0	0.2–5.0	0.96	1.4	0.4–4.8	0.591
Diabetes mellitus	1.1	0.2–5.8	0.89	1.1	0.3-4.1	0.909
GFR	0.9	0.86–0.95	<**0.001**	0.99	0.9–0.95	<**0.001**
EF	1.0	0.9–1.0	0.09	0.95	0.9–0.99	**0.026**
Admission NYHA class 3 or 4	7.7	1.3–46.1	**0.03**	11.8	1.9–72	**0.008**
Hs-TnT ≥ 14 ng/l at admission	33.6	0.03–366,658	0.33	4.1	0.5–32.3	0.176
Hs-TnT mx	1.0	0.99–1.0	0.82	1.0	0.99–1.0	0.642

AMI: acute myocardial infarction; CI: confidence interval; EF: ejection fraction; GFR: glomerular filtration ratio; GRACE: Global Registry of Acute Coronary Events; HR: hazard ratio; hs-TnT mx: maximal concentration of high-sensitive troponin T; NSTEMI: non-ST segment elevation myocardial infarction; NYHA: New York Heart Association.

ejection fraction, and higher risk according to the GRACE risk score (Supplemental Table S2).

4. Discussion

This prospective, observational study assessed the prognostic role of copeptin and its combination with hs-TnT in consecutive patients with acute chest pain admitted to the emergency department of a tertiary cardiologic center.

The first finding is the high prognostic accuracy of copeptin in the prediction of mortality. According to previously published data [11, 12], we report higher mortality in patients with higher plasma levels of copeptin. Secondly, copeptin had significantly better prognostic accuracy than hs-TnT in studied population. There was no benefit from a combined use of copeptin and hs-TnT over copeptin alone. Thirdly, copeptin should be considered together with other, but still simple risk factors, especially age and renal function, while assessing prognosis. Lastly, we provide indirect evidence for the significant prognostic value of copeptin in the prediction of HF at long-term assessed with NT-proBNP, a recently strongly recommended diagnostic parameter of HF [13].

The prognostic value of copeptin in patients with chest pain is the field of research interest and constant growth. In this study, we add evidence on prognostic utility of copeptin as an early marker of adverse outcome in a specific population of highly preselected patients with high rate of finally diagnosed ACS, characteristic for tertiary centers. The decision on optimal type and time frame of the management is essential for non-ST segment elevation ACS and appropriate selection of patients in whom invasive interventions are likely to be beneficial. According to the guidelines [1], the triage of patients in such circumstances should be extensive; involve clinical status of patients, medical history, the dynamics of ECG, and biomarker concentrations; and be supported by different risk scoring systems, for example, the GRACE risk score. Despite the GRACE risk score is a validated tool in risk stratification [14], the practical utility is compromised in everyday clinical practice [15]. We provide evidence that supports very early risk assessment with single measure from a blood draw at admission. The results of the Cox analysis identified copeptin, age, and renal dysfunction as risk markers of poor prognosis at one year. Whether this combination of risk factors would play a wide practical role remains to be determined in a larger cohort.

The single measurement of copeptin at admission showed also a tendency to increase the prognostic accuracy of the GRACE risk score for death at six months. It confirms previous reports on good prognostic performance of copeptin in the prediction of death, however assessed in different clinical setting including STEMI [16] or derived from measurements at later time points [6]. Nevertheless, these results might serve as a background for further research on a simple and concise prognostic evaluation methodology, which would be of clinical benefit at the bedside, without the need for extensive, repetitive examinations or online calculations.

The combination of copeptin and hs-TnT merits consideration. The estimation of prognosis based on combined use of copeptin and hs-TnT was not clinically relevant. Surprisingly, hs-TnT alone was also not a significant predictor of long-term death. It is known that cTn directly correlates with infarct size [17] and the predictive value of cTn is higher for maximal concentrations measured during the hospital stay [18]. Of note is a correlation found for copeptin with maximal concentrations of hs-TnT in our study. This leads to speculate, first, that this is the reason why hs-TnT measured at admission had low prognostic value and, second, that copeptin might be considered an early indirect predictor of infarct size. The latter hypothesis was recently evaluated and confirmed in a population of STEMI patients [19]. As a consequence might be regarded the observation of predictive value of copeptin for the development of heart failure, with higher and diagnostic for HF levels of NT-proBNP among patients stratified with copeptin, age, and GFR as high risk. It was not reflected in the value of EF but it is known that levels of NT-proBNP might be elevated also in

patients with preserved EF [20]. We would like, however, to prevent the reader from interpreting our results as a prove of direct relationship between copeptin and infarct size and/or heart failure and consider them as a hypothesis that warrants further research.

In conclusion, copeptin appears to be an independent predictor of long-term mortality in a selected population of patients suspected for an ACS. In addition, copeptin may be considered as an early marker for the identification of patients at higher risk of the development of HF at long term in this population. The outcomes warrant a confirmation in a larger cohort.

4.1. Limitations. The following limitations should be mentioned. The observational character of the study limits the interpretation of clinical benefit from risk assessment with copeptin. Further, studied troponin was the hs-TnT (Roche). It remains unknown if the use of other assays or other troponin would influence the outcomes. Next, in the study, we used a prespecified cutoff for copeptin at the 97.5th percentile, as the closest to that recommended by the guidelines (99th percentile) that was available from the manufacturer's resources; therefore, the outcomes are limited with regard to previously proposed values [6, 11]. Further studies are warranted to directly compare different cutoffs to identify the optimum for prognostic purposes.

Disclosure

Drs. Beata Morawiec and Damian Kawecki are considered the first authors.

Conflicts of Interest

The authors have no conflict of interest to disclose.

Authors' Contributions

Beata Morawiec and Damian Kawecki contributed equally to this publication.

Supplementary Materials

Table S1: baseline characteristics in patients positive and negative for copeptin with the cutoff 10 pmol/l. Table S2: outcomes of the taxonomic analysis. Figure S1: survival curves or copeptin with the cutoff 10 pmol/l. Figure S2: dendrogram—taxonomic analysis. *(Supplementary Materials)*

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Hindawi
Disease Markers
Volume 2017, Article ID 1204295, 10 pages
https://doi.org/10.1155/2017/1204295

Research Article

Is MicroRNA-127 a Novel Biomarker for Acute Pancreatitis with Lung Injury?

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Received 6 June 2017; Revised 23 September 2017; Accepted 15 October 2017; Published 24 December 2017

Academic Editor: Mina Hur

Background and Aims. The aim of this study was to determine the expression of microRNA-127 (miR-127) in both rat models and patients of acute pancreatitis (AP) with lung injury (LI). *Methods*. Rats were administrated with retrograde cholangiopancreatography injection of 0.5% or 3.5% sodium taurocholate to induce AP with mild or severe LI and were sacrificed at 6, 12, and 24 h. Rats from the control group received a laparotomy only. Plasma from a prospective cohort of AP patients was collected. The levels of miR-127 in the tissues and plasma were detected using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). *Results*. The upregulation of miR-127 in the lungs of rats was detected in the groups of AP with severe LI at 6 h and 24 h, whereas it was scarcely detectable in plasma. In the pilot study that included 18 AP patients and 5 healthy volunteers, the plasma miR-127 level was significantly downregulated in AP patients with respiratory failure compared with the healthy volunteers ($P = 0.014$) and those without respiratory failure ($P = 0.043$). *Conclusion*. miR-127 might serve as a potential marker for the identification of AP with LI.

1. Introduction

Acute pancreatitis (AP) is a life-threatening inflammatory disease characterized by significant morbidity and mortality [1–3]. During the cascade events of activation of proinflammatory cytokines and mediators in AP, the injured cells of the lungs recruit immune cells and release cytokines, chemokines, growth factors, and prostaglandins, contributing to acute inflammatory response [4–7]. Lung injury (LI) is commonly involved in 15%~60% of severe AP, and severe LI is likely to trigger acute respiratory distress syndrome (ARDS) and respiratory failure (RF). When LI persists more than 48 h, it increases mortality of severer AP to 50% and accounts for 60% of mortality within the first week [8, 9]. Since its treatment is mainly supportive without targeted intervention to modify the progression, it is critical to identify those who are at risk for developing LI. Although the roles of cytokines and chemokines in respiratory complications of AP were proposed by extensive studies in the last two decades, the definite mechanism is yet to be fully elucidated [4]. Novel markers for early identification of AP with LI remain a great challenge.

MicroRNAs (miRNAs) are a category of small nonencoding RNAs of 22 nucleotides in length. They regulate gene expression through different mechanisms [10] and play a diverse role in many cellular processes [11–14]. The over- or underexpression of miRNAs is involved in various pathophysiological processes [15–18]. The discovery of misregulated miRNAs not only broadened our biological understanding of these diseases but also provided a new class of markers. An increasing number of evidence suggests that miRNAs may act as potential biomarkers for pancreatic injury [19–23]. An improvement in our understanding of the role that miRNAs play in AP may represent an attractive way to develop

new diagnostic and prognostic tools for use in future clinical applications. Moreover, some miRNAs were reported to be correlated with the pathogenesis of lung diseases, such as lung cancer [24, 25], pulmonary fibrosis [26, 27], chronic obstructive pulmonary disease [28], and asthma [29, 30]. Here, microRNA-127 (miR-127) is one of the miRNAs that focuses on lung diseases [31–33]. miR-127 is originally found to be highly expressed in human and murine embryos and has a critical role in lung development and placental formation [34, 35]. Of note, recent study identified that miR-127 expression was aberrant in the inflammation-related pulmonary disorders [36] and further revealed that enhanced expression of miR-127 could promote the development of inflammatory macrophages and contribute to the exaggerated lung inflammation and injury [37].

Considering the potential role of miR-127 in the vital biological process of inflammation, we hypothesized that miR-127 may serve as a marker of AP with LI. To our best knowledge, it is the first study that integrated miR-127 and the inflammatory injuries of the pancreas and the lung. In this study, we sought to determine the expressions of miR-127 in the lung tissues of sodium taurocholate-induced AP models in rats and that in plasma of AP patients and to preliminarily explore the association of miR-217 levels and LI.

2. Materials and Methods

2.1. Animals. Fifty-four healthy male Sprague Dawley rats (weight, 250–300 g; age, 8–10 weeks) were purchased from Experiment Animal Center of Sichuan University. The rats were adaptively fed for one week prior to the experiments (maintained in a temperature-controlled room under a 12 h light/12 h dark cycle; fed with standard rat chow and tap water ad libitum). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocols and experiments were approved by the Ethics Committee for Animal Experiments of Sichuan University.

2.2. Experimental Groups. Rats were fasted for 12 h and were given free access to tap water until 2 h prior to experiments, at which point they were randomized into the AP and control groups. Rat models of AP with mild LI (mLI) or severe LI (sLI) were induced by retrograde cholangiopancreatography injection of either 0.5% or 3.5% sodium taurocholate (0.1 ml/100 g body weight) at a rate of 0.1 ml/min using a micropump [38, 39]. Rats from the control group received a laparotomy only. All rats were subcutaneously rehydrated (2 mL per 100 g) after surgery and provided free access to water but were fasted after awakening from surgery. Rats were sacrificed at 6, 12, or 24 h after operation, which were termed AP-mLI 6, 12, and 24 h groups; AP-sLI 6, 12, and 24 h groups; and control 6, 12, and 24 h groups ($n = 6$ for each group).

2.3. Animal Sample Collection. Serum samples were collected and stored at −80°C. The right lung was placed in liquid nitrogen immediately after removal for the detection of myeloperoxidase (MPO) activity and miR-127 levels. The pancreas and left lung tissues were fixed in 10% neutral formalin for pathological hematoxylin and eosin (HE) staining. A blinded histological analysis of the pancreas [40, 41] and the lung [42, 43] was performed to evaluate the severity of tissue injury. For each pancreas pathological section, edema, inflammatory infiltration, and acinar cell necrosis were evaluated in 10 random visual fields under a 200x microscope (Table 1). A 4-point scale (0 = none, 1 = mild, 2 = moderate, and 3 = severe) was used to assess lung damage based on alveolar edema, inflammatory infiltration, and capillary congestion (Table 1). Serum amylase and inflammatory cytokine were determined according to instructions (amylase and MPO assay kit were provided by Nanjing Jiancheng Bioengineering Institute; Inflammatory cytokine ELISA kits were purchased from Xin Bo Sheng Biotechnology Co. Ltd.). The levels of miR-127 in serum and lung tissues were quantified using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and the expression between different time points was compared.

2.4. Patients. Plasma samples and clinical data from AP patients or healthy volunteers (HVs) were obtained from a prospective observational study that undertook measurement of various severity markers (including miRNA), in a range of patients with mild to severe AP. The study protocol and informed consent were approved by the Clinical Trials and Biomedical Ethics Committee of the West China Hospital of Sichuan University. All procedures in studies with human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki as well as its later amendments or comparable ethical standards. All subjects provided written informed consent before enrolling.

2.5. Patient Criteria. The diagnosis of AP requires two of the following three features [44]: (1) abdominal pain consistent with AP; (2) serum amylase and/or lipase ≥ 3 times the upper limit of normal; and (3) characteristic findings of AP on CT scan, magnetic resonance imaging, or transabdominal ultrasonography. Mild AP was defined as the absence of both organ failure and local or systemic complications, moderately severe AP as the presence of transient organ failure (resolved within 48 h) or local/systemic complications in the absence of persistent organ failure (persists more than 48 h), and severe AP as persistent organ failure. In accordance with the modified Marshall scoring system, organ failure was defined as a score of 2 or more for one of three organ systems (respiratory, renal, and cardiovascular) [45].

AP patients between 18 and 70 years of age who were admitted to our hospital within 48 h after the onset of disease were enrolled. Patients were excluded if they (1) were pregnant or lactating, (2) had any malignant disease, or (3) had serious primary systemic diseases. HVs were sex- and age-matched for study patients.

A total of 23 subjects were selected. AP patients comprised two groups, the presence of respiratory failure (RF, $n = 10$) and nonrespiratory failure (nRF, $n = 8$), and were compared with HVs ($n = 5$). Demographics and clinical data of patients were recorded using uniform paper charts

Table 1: Histopathological scoring criteria of the pancreas and lung.

	Condition	Score	Indication
Pancreas	Edema	0	Absent
		1	Focally increased between lobules
		2	Diffusely increased between lobules
		3	Acini disrupted
		4	Acini separated
	Inflammatory cell infiltrate	0	Absent
		1	In ducts (around ductal margins)
		2	In the parenchyma (in <50% of the lobules)
		3	In the parenchyma (in 50%–75% of the lobules)
		4	In the parenchyma (in >75% of the lobules)
	Acinar necrosis	0	Absent
		1	Periductal necrosis (<5%)
		2	Focal parenchymal necrosis (5%–20%)
		3	Diffuse parenchymal necrosis (20%–50%)
		4	Diffuse parenchymal necrosis (>50%)
Lung	Alveolar edema	0	Absent
		1	Focally alveolar septum widened (<20%)
		2	Diffusely alveolar septum widened (20%–50%)
		3	Diffusely alveolar septum widened (>50%) with alveolar cell disrupted, separated
	Inflammatory infiltration	0	Absent
		1	In interstitial
		2	In alveoli and interstitial (<50%)
		3	In alveoli and interstitial (>50%)
	Capillary congestion	0	Absent
		1	Focally in alveoli and interstitial (<25%)
		2	Diffusely in alveoli and interstitial (25%–50%)
		3	Diffusely in alveoli and interstitial (>50%)

and electronic medical records, which were reviewed by 2 independent physicians.

2.6. Human Plasma Collection. Peripheral blood samples were collected from patients with AP using BD Vacutainer EDTA tubes within 24 h after admission. The processing of all blood samples began within 30 minutes after collection and was performed according to the following procedures: maintained upright for 20–25 minutes, centrifugation at room temperature (22–24°C) at 600g for 30 minutes, further centrifugation of the supernatant at 24°C and 1500g for 10 minutes, and storage of the supernatant at −80°C.

2.7. Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA from lung tissues was extracted using TRIzol reagent. Total RNA was extracted from 100 μl serum from rats or 400 μl plasma from human subjects using the mirVana™ PARIS™ Kit (Ambion, USA) following the manufacturer's protocol.

QRT-PCR was performed to detect and quantify miR-127 with TaqMan Small RNA Assays (Applied Biosystems, USA). Reverse transcription was performed using TaqMan miRNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. PCR amplification was performed using TaqMan Universal Master Mix (Applied Biosystems, USA) on an AB7900HT with cycling conditions as recommended by Applied Biosystems. Each reaction was performed in triplicates containing 0.75 μl cDNA, 0.5 μl 20 × TaqMan microRNA Assay Mix, 5 μl 2 × TaqMan Universal PCR buffer, and 3.75 μl ddH_2O. The amplification was performed as follows: denaturation at 50°C for 2 min and at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. miR-16 in plasma and U6 snRNA in the lung tissues were used as an internal control. The miRNA levels that were not detected after 45 cycles of real-time PCR were considered to have a threshold cycle (Ct) equivalent to 45.

2.8. Statistical Analysis. Statistical analyses were performed using SPSS 21.0 software. The Ct was defined as the fractional cycle number of fluorescence that passed through a given threshold. The target miRNA expression was analyzed by $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{\text{target miRNA}} - Ct_{\text{internal control}}$) using REST 2009 software (Qiagen Inc.). Continuous variables were expressed as the mean ± standard error, and categorical variables were expressed as proportions. One-way ANOVA, Kruskal-Wallis test, chi-square test, or Fisher's exact test were used, when appropriate, to determine significant

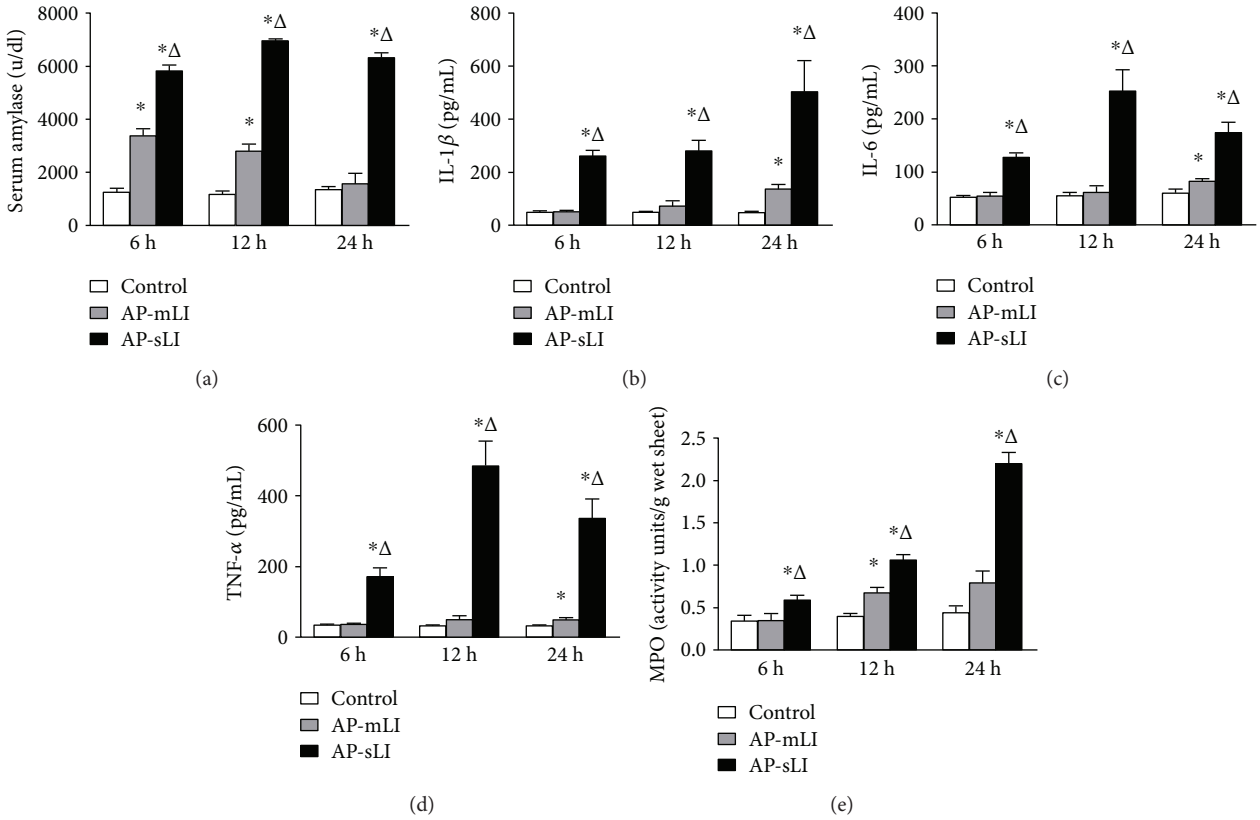

Figure 1: The levels of serum amylase (u/dl), serum cytokines, and MPO activity in the lung tissues in rats. (a) Serum amylase; (b) interleukin-1β (IL-1β); (c) interleukin-6 (IL-6); (d) tumor necrosis factor-α (TNF-α); and (f) myeloperoxidase (MPO). (mean with SEM, $N = 6$ for each group) $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus AP-mLI group.

differences between the groups. A two-sided P value less than 0.05 was considered statistically significant.

3. Results

3.1. Normal Markers in Serum and Tissues of Rats with AP-mLI and AP-sLI. The levels of serum amylase (u/dl) in rats with AP were significantly increased compared to those in the control rats at each time point (Figure 1(a)). Serum amylase levels were significantly higher in the AP-mLI 6 and 12 h groups than in the control group and nearly returned to a normal level at 24 h. They were significantly elevated in the AP-sLI 6, 12, and 24 h groups than in both AP-mLI and control groups at each time point (all $P < 0.05$).

The levels of serum IL-1β, IL-6, and TNF-α were significantly elevated in AP-mLI group at 24 h than in the control group, and they were all significantly higher in the AP-sLI 6, 12, and 24 h groups than in both control and AP-mLI groups at each time point (all $P < 0.05$) (Figures 1(b), 1(c), and 1(d)).

As shown in Figure 1(e), MPO activity of the lung tissues in AP-mLI group was significantly higher than that in the control group at 12 h ($P < 0.05$), and no significant difference was found at 6 and 24 h. In AP-sLI group, MPO activity of the lung tissues was significantly higher than those in both control and AP-mLI groups at each time point (all $P < 0.05$).

3.2. Histopathologic Severity of the Pancreas and the Lung of Rats with AP-mLI and AP-sLI. The histopathological changes in the pancreatic tissues are shown in Figure 2. The pancreatic tissues in the control group lacked obvious changes (Figures 2(a), 2(b), and 2(c)). Interstitial edema, inflammatory cell infiltration, and acinar cellular necrosis that are indicative of AP were seen in light micrographs (Figures 2(d), 2(e), 2(f), 2(g), 2(h), and 2(i)). Higher histopathologic scores were shown in both AP-mLI and AP-sLI groups at 6, 12, and 24 h after sodium taurocholate administration than in the control group at each time point (Figure 2(j)). Furthermore, the AP-sLI group exhibited more severe pancreatic injury than AP-mLI and control groups (all $P < 0.05$).

The histopathological changes in the lung tissues are shown in Figure 3. Rats in the control group exhibited no obvious changes (Figures 3(a), 3(b), and 3(c)). Interstitial edema, inflammatory cell infiltration, and widened alveolar septum in the lung tissue were observed in the AP-mLI group (Figures 3(d), 3(e), and 3(f)). Rats in the AP-sLI group exhibited a variety degree of alveolar and interstitial edema, hemorrhaging with atelectasis, widened alveolar septum, and the infiltration of inflammatory cells and red blood cells that are indicative of obvious LI (Figures 3(g), 3(h), and 3(i)). As shown in Figure 3(j), pathologic severity scores of the lung tissues were higher in both AP-mLI and AP-sLI groups than the control group at 6, 12, and 24 h. The AP-sLI group

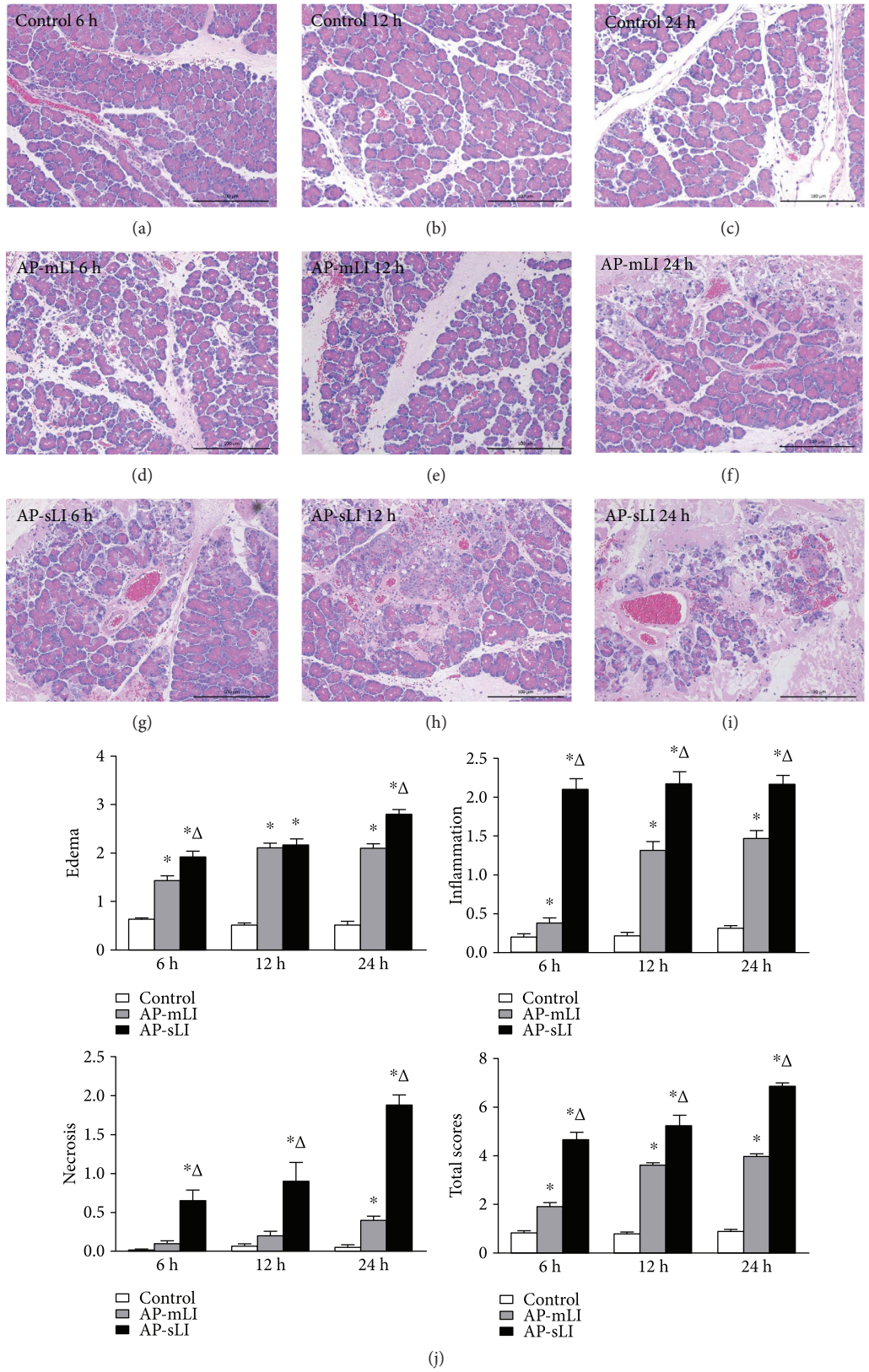

Figure 2: Histopathological changes of the pancreatic tissues. Light micrographs (×200) of the pancreas in the control rats at 6 h (a), 12 h (b), and 24 h (c), AP-mLI rats at 6 h (d), 12 h (e), and 24 h (f), and AP-sLI rats at 6 h (g), 12 h (h), and 24 h (i). (j) Blinded histopathological analysis for edema, inflammatory infiltration, acinar cellular necrosis, and total histological scores. $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus AP-mLI group.

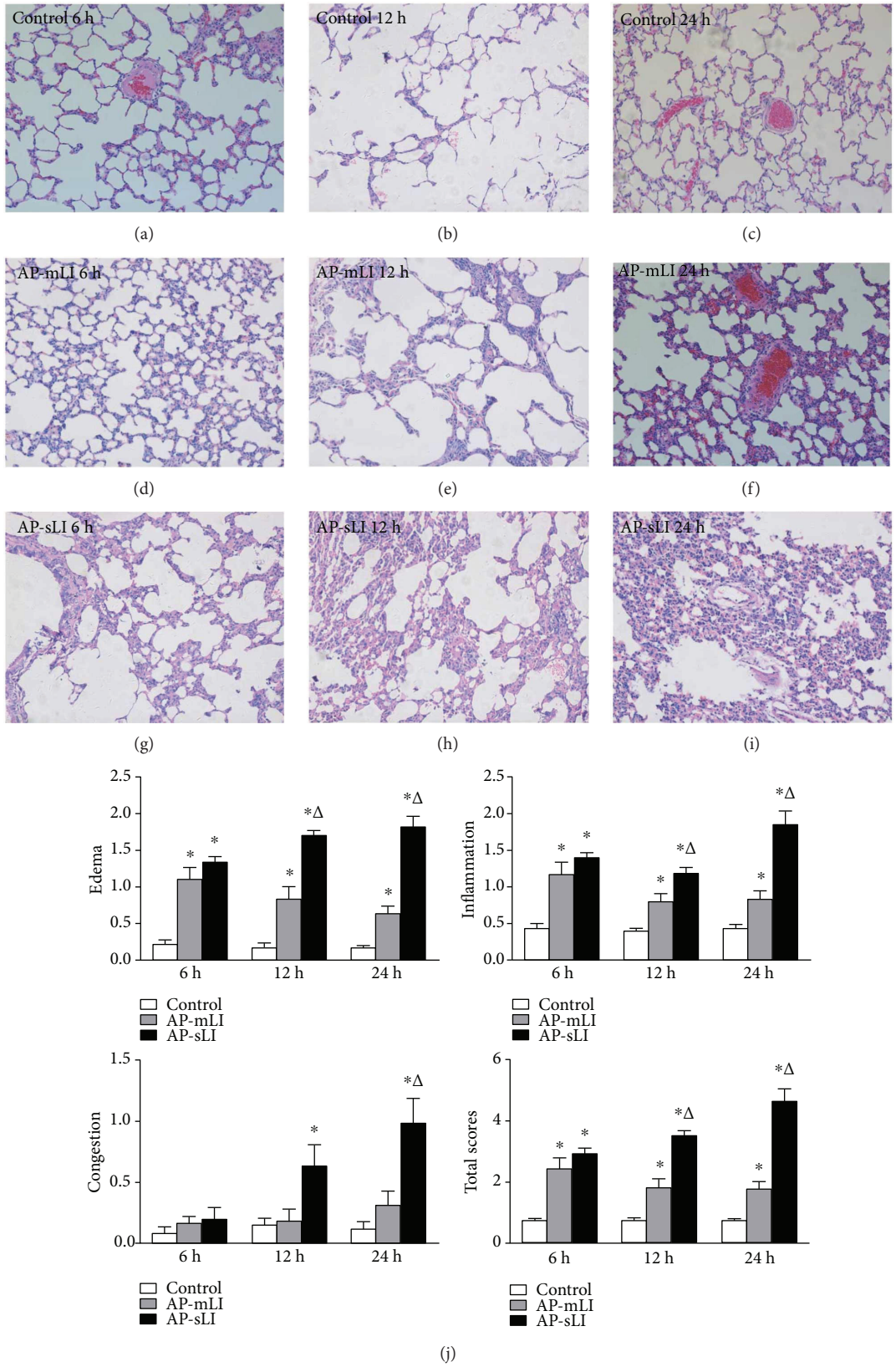

Figure 3: Histopathological changes of the lung tissues. Light micrographs (×200) of the lungs in the control rats at 6 h (a), 12 h (b), and 24 h (c), AP-mLI rats at 6 h (d), 12 h (e), and 24 h (f), and AP-sLI rats at 6 h (g), 12 h (h), and 24 h (i). (j) Blinded histopathological analysis for alveolar edema, inflammatory infiltration, capillary congestion, and total histological scores. $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus AP-mLI group.

exhibited higher severity scores of the lungs than the AP-mLI group at 12 and 24 h (all $P < 0.05$).

3.3. miR-127 Was Significantly Upregulated in Lung Tissues of AP Rats. The expression levels of miR-127 in the lung tissues of the rats in AP-mLI group were significantly higher than those in the control group at 24 h (Figure 4, $P < 0.05$) and were insignificantly different at 6 and 12 h (all $P > 0.05$). The upregulation of miR-127 in the AP-sLI group was significant than that in both AP-mLI and control groups at 6 and 24 h ($P < 0.05$). The levels of miR-127 in AP-mLI and AP-sLI groups at 12 h were slightly increased, but they did not significantly differ from those in the control group. No difference was observed in the expression level of miR-127 between AP-mLI and AP-sLI groups at 12 h. U6 snRNA in the lung tissues was insignificantly different in any of the groups.

3.4. miR-127 Was Lowly Expressed in Serum of Rats. We determined the expression levels of miR-127 in serum of the rats using miR-16 as an internal control. The expression of serum miR-16 was successfully detected, and no significant difference was observed among the AP-mLI, AP-sLI, and control groups. However, miR-127 could not be detected by qRT-PCR in serum samples of the rats even with the modifications to the methods allowed their reliable quantitation (all nearly Ct = 45).

3.5. miR-127 Levels Positively Correlated with Pathological Severity in the Lung Tissues of Rats. A Pearson's correlation coefficient analysis showed that the miR-127 levels in the lung tissues were significantly positively correlated with pancreatic edema, inflammatory infiltration, acinar cell necrosis, and total scores (Table 2, all $P < 0.05$). The levels of lung miR-127 also demonstrated a significant positive correlation with lung edema, inflammatory infiltration, capillary congestion, and total scores (Table 2, all $P < 0.05$).

3.6. miR-127 in the Plasma of AP Patients and Healthy Controls. The age, body mass index (BMI), and routine biochemical parameters (including amylase and lipase) were similar between RF and nRF groups (all $P > 0.05$). In terms of single markers on admission, the patients in the RF group exhibited significantly lower oxygenation index (PaO_2/FiO_2), and higher C-reaction protein (CRP) and interleukin-6 (IL-6), than those in the nRF group. The modified Marshall scores in the RF group were significantly higher than those in the nRF group (2.40 ± 0.70 versus 1.13 ± 1.25; $P = 0.014$). All patients survived, and those who developed RF had a higher rate of infection and necrosis and longer length of hospital stay.

The expression of miR-127 was successfully detected in plasma of both AP patients and the HVs (Figure 5). Similar expressions were detected in terms of plasma miR-127 between nRF and HV groups. Significant downregulation of plasma miR-127 was shown in the RF group than in the HV group ($P = 0.014$) and the nRF group ($P = 0.043$). No detectable difference was shown in the expression of plasma miR-16 as an internal control between AP patients and HVs.

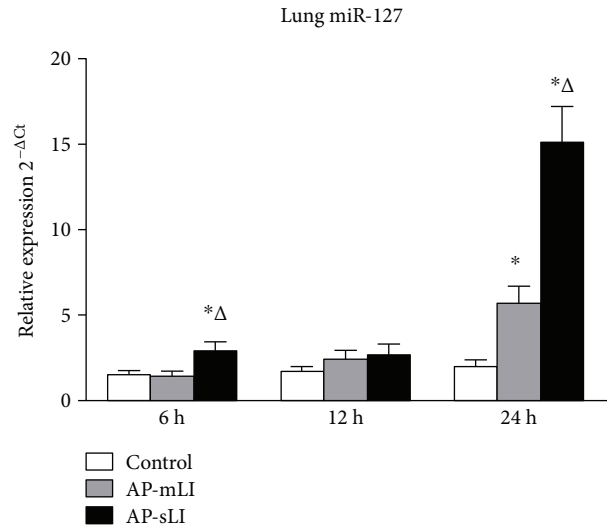

Figure 4: The relative expression of miR-127 in the lung tissues of rats. The level of miR-127 was expressed as $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{miR\text{-}127} - Ct_{U6\ snRNA}$) using REST 2009 software (Qiagen Inc.). $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus AP-mLI group.

Table 2: The correlations of miR-127 levels and pathological severity in the lung tissues of rats.

	Histopathologic scores	Lung miR-127	
		r	P
Pancreas	Edema	0.571	<0.001
	Inflammatory infiltration	0.495	<0.001
	Acinar cellular necrosis	0.703	<0.001
	Total scores	0.627	<0.001
Lung	Edema	0.470	<0.001
	Inflammatory infiltration	0.662	<0.001
	Capillary congestion	0.637	<0.001
	Total scores	0.641	<0.001

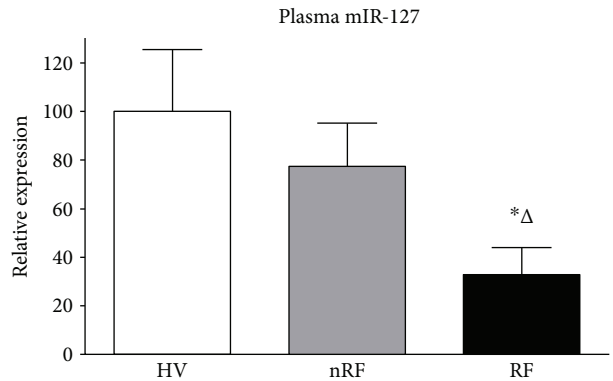

Figure 5: The relative expression of plasma miR-127 in the patients with AP and HVs. The level of miR-127 was expressed as $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{miR\text{-}127} - Ct_{miR\text{-}16}$) using REST 2009 software (Qiagen Inc.). Normalized HV group as 100. $^{*}P = 0.014$ versus HVs; $^{\Delta}P = 0.043$ versus nRF group.

4. Discussion

The identification of LI in AP patients for the potential improvement of the outcome remains a challenging issue. The discovery of miRNAs [46] provided a new class of disease markers. Studies in recent years have revealed that miRNAs might be novel biomarkers for AP. miR-127 has been reported to play a pivotal role in organic development [35, 47], ischemia/reperfusion injury [48], cancer [49], lung disease [37], and so forth. Nevertheless, the expressions of miR-127 and its role in AP with LI are unknown. This study explored the relationship between miR-127 and AP with LI from the perspective of blood and tissue.

In this study, retrograde cholangiopancreatography injection of sodium taurocholate was used to induce a rat model with AP complicated by LI, which is widely accepted by investigators of pancreatic diseases. Various degrees of injury in the pancreas and the lung could be achieved by adjustments of the dose and concentration of sodium taurocholate, rate of administration, and perfusion pressure by a syringe pump [50]. Based on the previous reports and our preliminary experiments, we used two concentration and dosages of sodium taurocholate for retrograde infusion. The results of biochemical tests and histopathological scoring of the pancreas and the lungs proved that sodium taurocholate successfully evoked a dose- and time course-dependent changes in terms of histopathological processes. Furthermore, higher dose of sodium taurocholate could induce more severe injuries with higher levels of serum IL-6, IL-1β, and TNF-α and MPO of the lungs. The histopathological scoring of the pancreas and the lungs showed that AP-mLI group exhibited obvious pancreatic injuries and mild LI, while AP-sLI group presented both more extensive and severe injuries in the pancreas and the lungs.

The levels of miR-127 were upregulated in the lung tissues of AP rats. Recent studies demonstrated the similar result that miR-127 was prominently induced in an exaggerated pulmonary inflammation and injury [32]. However, another study [37] showed that miR-127 was downregulated in lipopolysaccharide-stimulated LI, which appeared to be paradoxical to the previous study [32] and our findings. These results indicate the exact role of miR-127 in pulmonary inflammation, and injury is still controversial.

In this study, miR-127 levels presented a significant positive correlation with histopathological severity scores of the pancreas and the lungs. Furthermore, both miR-127 and proinflammatory cytokine (IL-1β, IL-6, and TNF-α) were increased in AP with LI. Although the mechanism involved has been largely undefined, the studies indicated that miR-127 promoted lung inflammation and injury by activating the inflammatory pathway [32]. Therefore, these results suggested that miR-127 in the lungs might reflect pancreatic and lung tissue injuries and play a potential role in the inflammatory signaling and lung pathology.

With regard to its potential as a marker for LI in AP, we verified its expressions in plasma of patients with AP-induced LI and HVs. The results showed that plasma miR-127 in the RF group tended to be significantly lower than those in the nRF group and the HV group. To the best of our knowledge, it was the first study reporting plasma miR-127 in AP patients. Among the limited studies on miR-127 in human pancreatic diseases, it was transiently expressed in pancreatic duct progenitor cells during the embryonic stages [51] and deregulated in pancreatic intraepithelial neoplasia [52] and in human pancreatic cancer [53]. Circulating miRNAs have been reported as promising novel noninvasive biomarkers in pathophysiological conditions, but little is known about the sources of circulating miRNAs and their relations with tissue miRNAs. Circulating miRNAs may be a result of the leakage of miRNAs into body fluid from tumor tissues or damaged tissues [54–56]. Furthermore, extensive studies in the last two decades have demonstrated that the first 24 h after the onset of symptoms are critical for identifying the at-risk group of patients and initiate aggressive treatment. Herein, we determined its expression in AP patients with early-stage AP. Thus, miR-127 might be a potential biomarker for early identification of RF in severe AP patients.

The major findings of this study were that miR-127 correlated to the severity of LI in AP rats and differentially expressed in plasma of AP patients with RF. Despite the potential interest in our findings, this study had limitations. First, miR-127 was undetectable in serum of rats, and the correlations of serum and tissue miR-127 were not analyzed. Second, miR-127 was detected in a small number of sample size ($n = 23$) for analysis in our study, and it was still lowly expressed. Third, pancreatitis is a complex and heterogeneous disease that evolves over time to time, and the discriminative ability of miR-127 at multiple time points should be determined in future studies.

5. Conclusions

miR-127 might be of help for the identification of AP with LI. A prospective, consecutive cohort study with a large sample is required to validate its value as a disease marker for AP in the future.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Na Shi and Lihui Deng contributed equally to this paper. These two authors share first authorship. Lihui Deng and Qing Xia contributed to the conception and designed the study; Na Shi and Ruijie Luo contributed to the animal experiment; Na Shi, Weiwei Chen, Xiaoxin Zhang, Chen Du, and Yun Ma contributed to the acquisition of clinical data; Lihui Deng, Tao Jin, Ziqi Lin, Kun Jiang, Jia Guo, and Xiaonan Yang contributed to the clinical observation and treatment; Na Shi, Lihui Deng, Tao Jin, and Qing Xia contributed to the analysis and interpretation of data; Na Shi and Lihui Deng wrote the manuscript; and all authors read and approved the final manuscript.

Acknowledgments

This work was supported by the National Natural Science Foundation Program of China (No. 81300358) and Research Fund for the Doctoral Program of Higher Education of China (No. 20130181120050).

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Hindawi
Disease Markers
Volume 2017, Article ID 9126560, 10 pages
https://doi.org/10.1155/2017/9126560

Research Article

Increased Levels of S100A8/A9 in Patients with Peritonsillar Abscess: A New Promising Diagnostic Marker to Differentiate between Peritonsillar Abscess and Peritonsillitis

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Received 19 June 2017; Revised 22 August 2017; Accepted 24 August 2017; Published 17 October 2017

Academic Editor: Patrizia Cardelli

Peritonsillar abscess (PTA) is a very frequent reason for urgent outpatient consultation and otolaryngological hospital admission. Early, correct diagnosis and therapy of peritonsillar abscess are important to prevent possible life-threatening complications. Based on physical examinations, a reliable differentiation between peritonsillar cellulitis and peritonsillar abscess is restricted. A heterodimeric complex called calprotectin consists of the S100 proteins A8 and A9 (S100A8/A9) and is predominantly expressed not only in monocytes and neutrophils but also in epithelial cells. Due to its release by activated phagocytes at local sites of inflammation, we assumed S100A8/A9 to be a potential biomarker for peritonsillar abscess. We examined serum and saliva of patients with peritonsillitis, acute tonsillitis, peritonsillar abscess, and healthy controls and found significantly increased levels of S100A8/A9 in patients with PTA. Furthermore, we could identify halitosis, trismus, uvula edema, and unilateral swelling of the arched palate to be characteristic symptoms for PTA. Using a combination of these characteristic symptoms and S100A8/A9 levels, we developed a PTA score as an objective and appropriate tool to differentiate between peritonsillitis and peritonsillar abscess with a sensitivity of 92% and specificity of 93%.

1. Introduction

The palatine tonsils are related to the mucosa-associated lymphatic tissue (MALT) of the upper respiratory tract. Immunologically, four important microcompartments can be defined: the crypt epithelium which consists of a non-uniform epithelium; the follicular germinal center; the mantle zone, which is characterized by a high density of lymphocytes; and the interfollicular area, populated by T-lymphocytes [1]. As a part of Waldeyer's ring, tonsils play an important role in nasopharyngeal immunity and present the first line in host defense against pathogens particularly in children. In some cases, however, they trigger severe head and neck infections causing life-threatening complications [2, 3]. The most common severe head and neck infection is the peritonsillar abscess (PTA) which is a very frequent reason for nonelective otolaryngological hospital admission [4]. Extension of an acute tonsillitis or an infection of Weber's salivary glands in the supratonsillar fossa was discussed to be the associated pathomechanisms of PTA which is characterized by an accumulation of pus between the fibrous capsule of the palatine tonsil and the pharyngeal constrictor muscle [5, 6]. Due to the level of the uvula tip, peritonsillar abscesses can be divided into superior and inferior types. With a percentage of 75%, the superior type is described to be the more common sort of peritonsillar abscesses [3].

Bacteria could be detected in abscess aspirates in 98% of the cases and 76% of those cases showed a combination of anaerobic and aerobic isolates [7, 8]. *Fusobacterium necrophorum* and *Prevotella* were described to be the most frequent anaerobic bacteria, and *Streptococcus pyogenes*,

Staphylococcus aureus, and *Haemophilus influenzae* were the common aerobic isolates from peritonsillar abscess [7–11].

Incisional drainage, needle aspiration, and tonsillectomy are common treatment strategies in peritonsillar abscesses. Indications of abscess tonsillectomy became more doubtful over the years because this procedure is associated with an increased risk of spread of infection and postoperative hemorrhage [3]. Thus, needle aspiration was established as a less invasive, alternative treatment approach for peritonsillar abscess with success rates between 72 and 95% [12–14]. Recurrence of a peritonsillar abscess could be observed in 10–19% after needle aspiration and the need of multiple attempts for adequate abscess relief indicate the strains for the patients and the limitation of this therapeutic regime [12, 15]. Incision and drainage of the peritonsillar abscess is considered to be the more definite but also more painful procedure [16]. Only medical therapy has been described to be as successful as initial surgical treatment in patients with less severe initial infection [4]. Due to these highly variable and partially expensive therapeutic regimes, the optimal treatment strategy of patients with PTA at reasonable cost level still remains controversial [12, 16].

Furthermore, there is a small group of patients who show symptoms suspicious of PTA such as trismus, uvula edema, and swelling of the arched palate. Still, aspiration or incisional drainage revealed no pus. In this group, diagnosis changed from PTA to peritonsillar cellulitis (PC), also known as peritonsillitis [17]. Reliable differentiation between PTA and PC is of great importance to avoid delay of the appropriate treatment and consequently life-threatening complications like airway obstruction, aspiration and pneumonia, or erosion of major vessels [9, 18]. However, 50% of the PTA patients are treated by nonotolaryngologists and diagnosis based on clinical examination is associated with a sensitivity of 78% and a specificity of just 50% [13, 18]. Hence, objective criteria or biomarkers to identify patients with PTA and to discriminate PTA from PC, to assess the severity of infection, or to identify patients who benefit from medical or surgical treatment would be helpful but still remain desirable [14].

S100A8 and S100A9, also known as myeloid-related proteins 8 and 14 (MRP 8/14), are members of the S100-protein family and show proinflammatory activities in a variety of different diseases. Both proteins form heterodimers also known as calprotectin or tetramers in the presence of calcium ions and are not only predominantly expressed in monocytes and neutrophilic granulocytes but are also inducible in epithelial cells [19, 20]. They are related to the group of danger-associated molecular patterns (DAMP) or alarmins and activate leukocytes via a toll-like receptor 4 (TLR4) pathway resulting in increased cytokine and chemokine expression and thereby trigger inflammatory reactions [21–23]. There have been several reports about the pivotal role of S100A8/A9 as a biomarker in inflammatory diseases like rheumatoid arthritis, acute myocardial infarction, or chronical inflammatory bowel diseases [24–26].

In this prospective study, we examined S100A8/A9 levels in the serum and saliva and its potential role as a promising and helpful biomarker to differentiate between acute tonsillitis (AT), PC, and PTA.

2. Material and Methods

2.1. Patients and Healthy Controls. This prospective study was performed in the Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Münster. 25 patients with acute tonsillitis (AT) (11 males, 14 females, 27.8 ± 2.5 years [mean $\pm$ SEM]), 36 patients suffering from peritonsillar abscess (19 males, 17 females, 34.4 ± 2.8 years), and 16 patients with peritonsillitis (8 males, 8 females, 34.8 ± 4.8 years) were included. Peritonsillar abscess was diagnosed by needle aspiration, abscess drainage in local or tonsillectomy in common anesthesia. Patients with clinical presentation similar to PTA but negative needle aspiration, incision, or tonsillectomy without revealing any purulent fluid and a good response to systemic antimicrobial treatment were assigned to the peritonsillitis group. Diagnosis was proven retrospectively. Healthy volunteers ($n = 15$, 32.9 ± 10.5 years) without any history of acute or recurrent tonsillitis served as controls. Symptoms and observations of the physical examination were documented during initial outpatient consultation. The study was approved by the institutional ethics committee [2015-217-f-S], and written informed consent was obtained from all subjects.

2.2. S100A8/A9 Sandwich ELISA of the Serum and Saliva Samples. Serum samples were centrifuged at $2000g$ for 10 minutes within 2 hours after acquisition, and supernatant was aliquoted and stored at −20°C until analysis. Saliva acquisition was performed with untreated Salivette® (Sarstedt, 51.1534) as described in the manufacturer's datasheet or by collecting saliva in a 50 ml Falcon tube and centrifugation at $1000g$ for 15 minutes. Supernatant was aliquoted and stored at −20°C. S100A8/A9 concentrations were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) for human S100A8/A9 as described earlier [27].

2.3. Bead-Based Immunoassay. Quantification of cytokines/chemokines in serum and saliva was performed with the LEGENDplex™ assay "Human Inflammation Panel" (BioLegend) as described in the manufacturer's manual. The "Human Inflammation Panel" allows simultaneous measurement of IL-1β, IL8, and other cytokines and chemokines. Fluorescent signal intensities were detected by NAVIOS™ Flow Cytometer (Beckmann Coulter).

2.4. Laboratory Parameters. As common inflammatory parameters, C reactive protein (CRP, [mg/dl]) and whole white blood cells [$\times 10^3/\mu$l] were analyzed by clinical routine methodology.

2.5. Histological and Immunohistochemical (IHC) Analyses. Tonsils of 10 patients undergoing tonsillectomy because of peritonsillar abscess were used for further histological examinations. As a healthy control, hypertrophic tonsils of patients without any history of recurrent tonsillitis were obtained ($n = 10$). The tonsils were divided into two parts immediately after surgical extirpation. One-half was snap-frozen in liquid nitrogen and stored at −80°C, whereas the other part was fixed in 4% formalin for 2-3 days. Samples

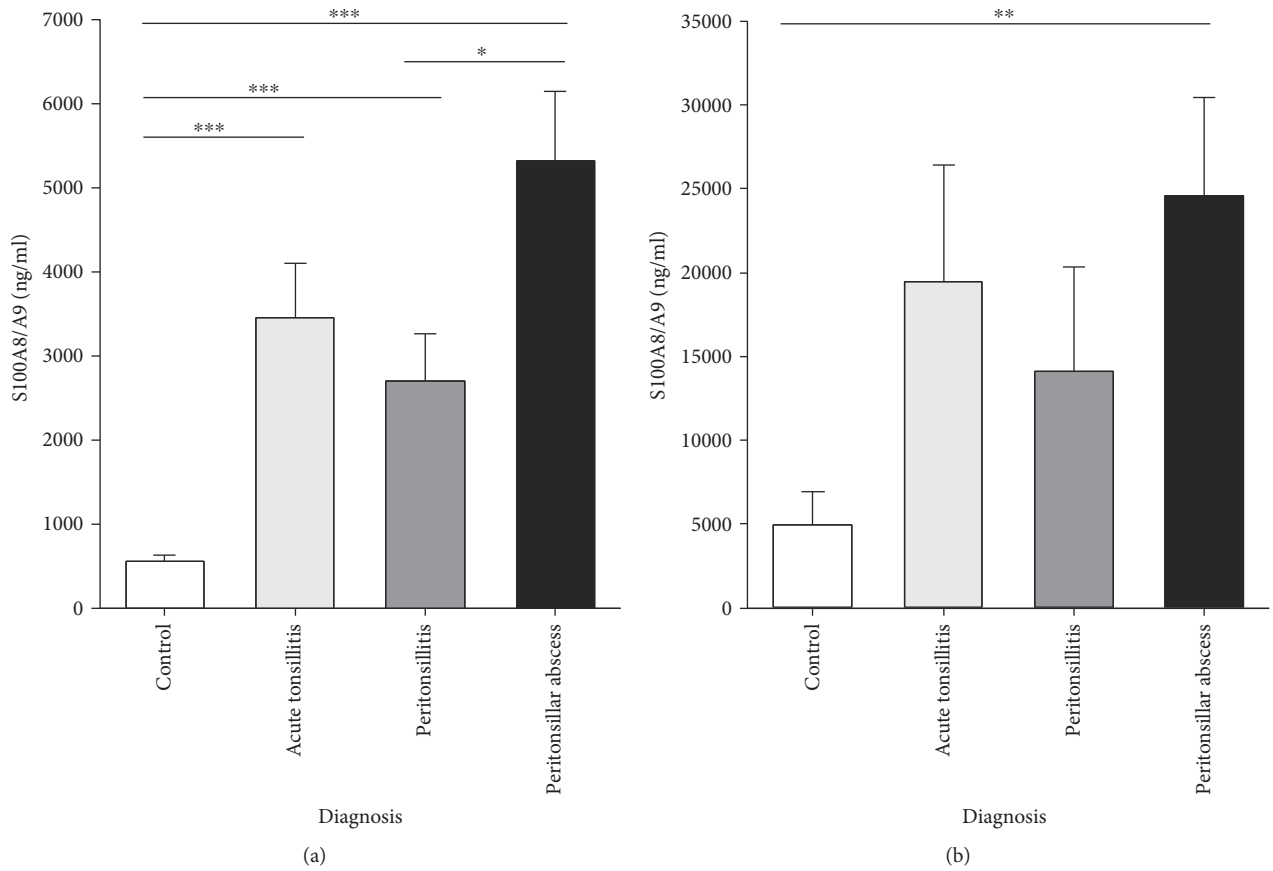

Figure 1: S100A8/A9 levels in serum (a) and saliva (b) of controls and patients with acute tonsillitis, peritonsillitis, or peritonsillar abscess (mean ± SEM). All entities show significantly increased serum levels compared to the controls. Furthermore, significantly higher S100A8/A9 levels were detectable in sera of PTA patients compared to PC patients. There was a significant increase of salivary S100A8/A9 levels in patients with PTA compared to healthy controls ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

were embedded in paraffin and cut into 3 μm thick sections followed by staining with hematoxylin and eosin. Furthermore, polyclonal rabbit anti-human S100A8 and anti-human S100A9 were used as primary antibodies and a biotinylated goat anti-rabbit IgG was added as a secondary antibody. Streptavidin peroxidase binding to biotin and its reaction with 3′-amino-ethyl-carbazole (Sigma, Germany) was utilized to identify S100A8/A9 localization in the tonsils. Additionally, nuclear counterstaining was performed with Mayer's hämalaun (Merck, Germany). Microphotographs of the complete tonsils were performed with the AxioVision MosaiX module for the Axio Observer Z1 microscope (Zeiss, Germany). Using a four-point Likert scale (0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = high positive staining), semiquantitative analysis of the stained sections was performed by two independent investigators who were blinded regarding the diagnosis.

2.6. Statistical Analysis. Results are mean values ± standard error of the mean (mean ± SEM) or mean value ± standard deviation (mean ± SD) as indicated in the figures. Chi-square analysis was used to identify possible relations between variables. Student *t*-test was used to detect significant differences in parametric results and Mann–Whitney *U* test was performed to analyze differences between nonparametric groups. p values > 0.05 are considered not to be significant. Significant results are marked with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). The capacity of the model to differentiate between positive and negative results is illustrated by receiver operating characteristic (ROC) curves which allow calculation of area under the curve values (*A* values) and cut-off values. Discriminative power of the model is considered to be excellent with an *A* value of >0.9, good > 0.8, acceptable > 0.7, and poor > 0.6. Statistical analyses and creation of figures were performed with IBM® SPSS® Statistics 24 and SigmaPlot®12.

3. Results

3.1. Serum and Saliva Analysis. Systemic S100A8/A9 levels were increased in the serum of patients with acute tonsillitis compared to healthy controls (3450 ± 650 ng/ml versus 550 ± 90 ng/ml, $p < 0.001$). Furthermore, S100A8/A9 levels in patients with PTA (5330 ± 820 ng/ml) were significantly higher than in patients with PC (2710 ± 550 ng/ml, $p < 0.05$) and healthy controls ($p < 0.001$). There was no significant difference in S100A8/A9 levels in the sera between patients suffering from AT and PC or PTA (Figure 1(a)). Analysis of S100A8/A9 levels in saliva revealed no significant difference of the S100A8/A9 level in patients with AT

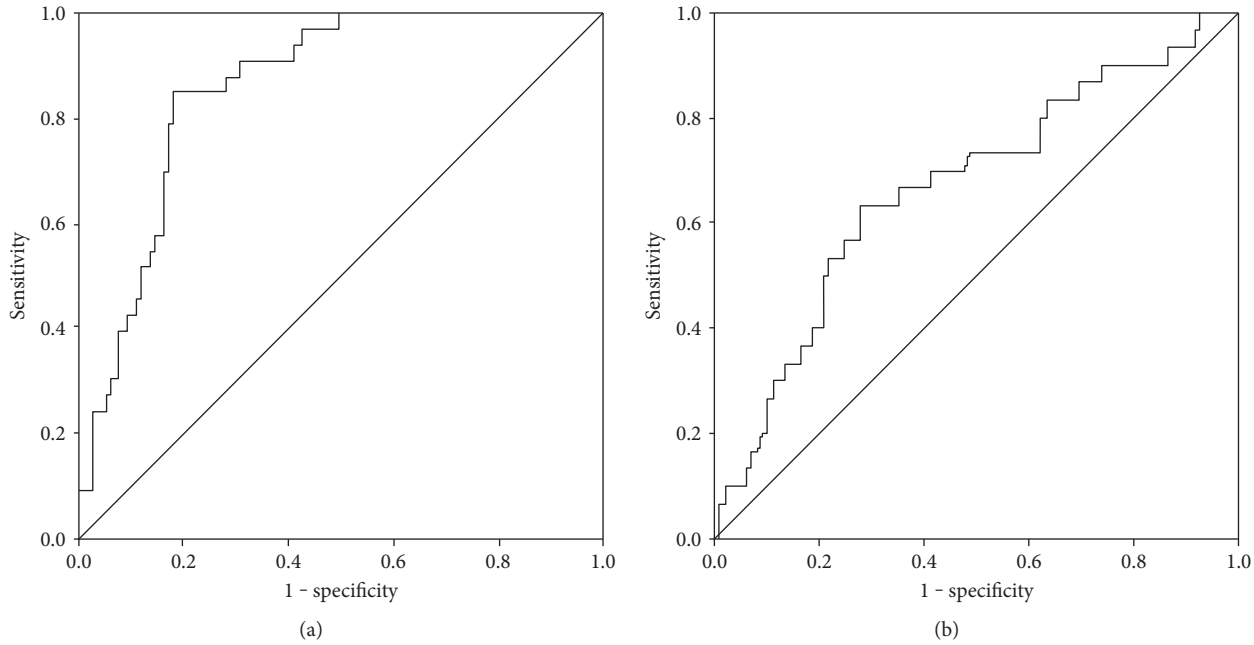

Figure 2: Receiver operating characteristic (ROC) curve in black, sensitivity on y-axis, and 1 − specificity on x-axis of S100A8/A9 in serum (a) and saliva (b) to identify patients with peritonsillar abscess. Grey: diagonal association line. Area under the curve (a) = 0.86 and (b) = 0.67.

(19480 ± 6920 ng/ml, $p = 0.08$) or PC (14110 ± 6220 ng/ml, $p = 0.265$) in comparison to the control group (4940 ± 1980 ng/ml). However, the S100A8/A9 level in patients' saliva with peritonsillar abscess was significantly higher than in controls (24590 ± 5850 ng/ml, $p = 0.002$) (Figure 1(b)). Although a difference between the PTA and the PC group could be observed, the results were not significant ($p = 0.087$). Receiver operating characteristic (ROC) curve analysis provided a cut-off value of 8180 ng/ml in saliva (sensitivity = 0.63, specificity = 0.72, $p = 0.019$) and 2550 ng/ml in serum (sensitivity = 0.85, specificity = 0.82, $p = 0.001$) for the existence of a peritonsillar abscess (Figures 2(a) and 2(b)). Neither levels of CRP (AT: 9.82 ± 1.82 mg/dl, PTA: 11.99 ± 1.45 mg/dl, and PC: 12.69 ± 2.43 mg/dl, $p > 0.05$) nor levels of white blood cells (AT: $13.38 \pm 0.90 \times 10^3/\mu$l, PTA: $14.11 \pm 0.76 \times 10^3/\mu$l, and PC: $13.08 \pm 1.15 \times 10^3/\mu$l, $p > 0.05$) showed any significant differences between the diagnosis groups (Figures 3(a) and 3(b)). Also, analysis of IL-1β and IL-8 in sera and saliva revealed no significant differences between the different cohorts (Figure 4).

3.2. Histological and Immunohistochemical Examinations. PTA is a localized process with an accompanying inflammatory reaction. Hence, to avoid misinterpretation of sectional S100A8/A9 expression, the MosaiX module was utilized to analyze each tonsil in toto. A normal microarchitecture with crypt epithelium, follicular germinal centers was observed in the tonsil from patients with PTA. Staining for S100A8/A9 was strongly positive in tonsils of patients with PTA (3.30 ± 0.23 [mean score $\pm$ SD]) in contrast to hyperplastic tonsils without any history of tonsillitis (1.68 ± 0.22, $p < 0.001$). Above all, there was a sectional positive staining which might be due to abscess localization (Figures 5(a), 5(b), 5(c), and 5(d)).

3.3. Development of a PTA Score. Diagnosis of PTA is made by clinical examination and depends on subjective assessment of the clinician. Chi-square analysis revealed no significant relations between CRP ($\chi^2 = 124.05$, $p = 0.431$), leukocyte levels ($\chi^2 = 135.8$, $p = 0.301$), and patients with peritonsillar abscess. Symptoms like trismus ($\chi^2 = 30.39$, $p < 0.001$), halitosis ($\chi^2 = 12.14$, $p = 0.007$), uvula edema ($\chi^2 = 27.01$, $p < 0.001$), and unilateral swelling of the arched palate ($\chi^2 = 60.11$, $p < 0.001$) were observed to be helpful clinical characteristics to identify peritonsillar abscess. Hence, by addition of one point for each symptom and for S100A8/A9 levels above the cut-off value of 2550 ng/ml in serum or 8180 ng/ml in saliva, a PTA score (S_{PTA}) with the lowest value of 0 and a maximum value of 6 points was developed (Table 1). PTA ($S_{\mathrm{PTA}} = 3.84 \pm 0.15$, $p < 0.001$), PC ($S_{\mathrm{PTA}} = 2.13 \pm 0.38$, $p < 0.001$), and AT ($S_{\mathrm{PTA}} = 0.93 \pm 0.22$, $p = 0.002$) showed significantly increased S_{PTA} values in contrast to the control group ($S_{\mathrm{PTA}} = 0.2 \pm 0.11$). Furthermore, the differences between AT and PTA or PC were significant (PTA: $p < 0.001$, PC: $p = 0.007$) as well as the difference between patients suffering from PTA and PC ($p < 0.001$) (Figure 6(a)). A ROC curve analysis revealed a cut-off value of 2.5 (sensitivity = 0.92, specificity = 0.93, $p < 0.001$) for the existence of a peritonsillar abscess (Figure 6(b)). The likelihood of PTA increases with higher S_{PTA} values (Table 2). For S_{PTA} values ≥ 3, the overall probability of PTA is about 89.2%.

4. Discussion

Several studies analyzed the influence of lymphocytes and the adaptive immune system on acute and recurrent tonsillitis and the triggering pathogens [2, 5, 11]. To our knowledge,

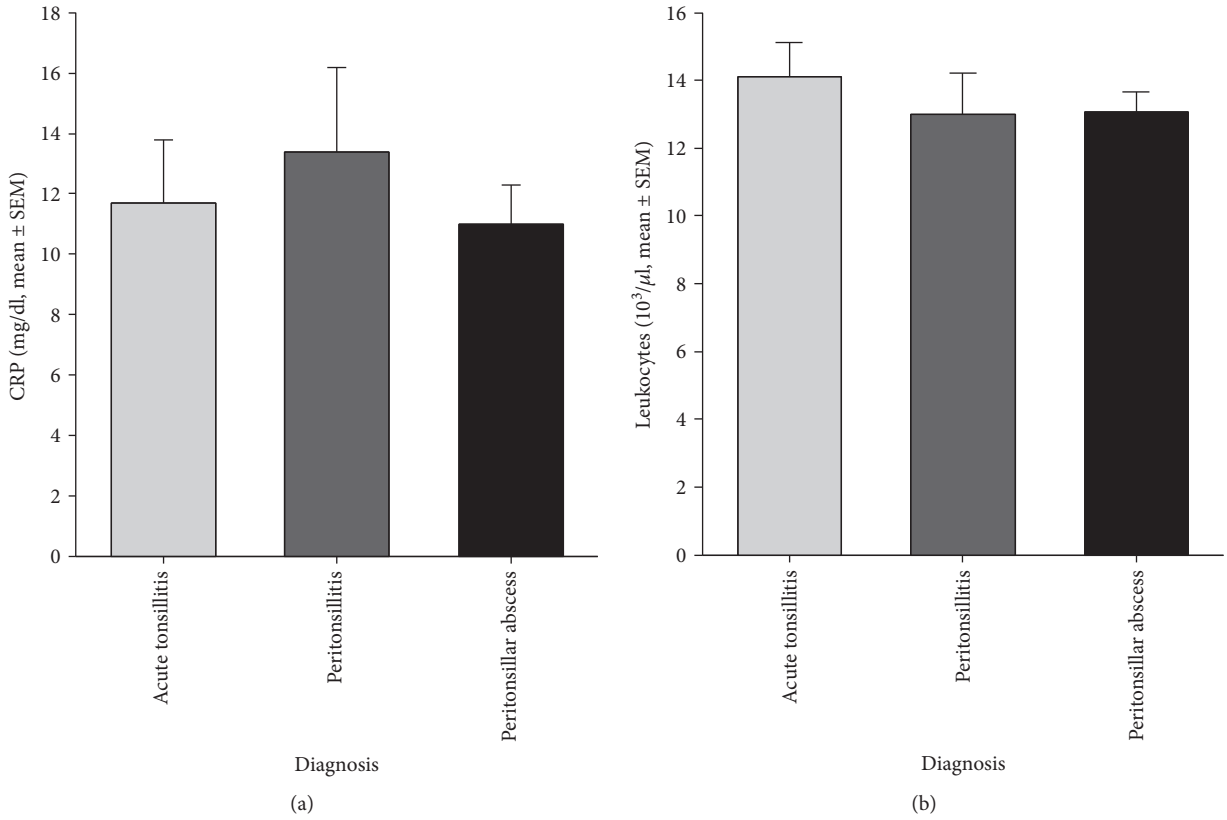

Figure 3: CRP levels (a) and leukocytes (b) in patients with acute tonsillitis, peritonsillitis, and peritonsillar abscess (mean ± SEM). No significant differences were observed between cohorts.

this is the first report of a correlation between elevated expression of S100A8/A9 and AT, PC, and PTA suggesting an important role of the innate immune response in disease development. As already mentioned above, S100A8/A9 has been described as a biomarker in several inflammatory and malignant diseases [24, 28–31]. In this study, we could quantify increased S100A8/A9 levels in patients' sera and saliva suffering from PTA.

Although increased levels of CRP (>15.5 mg/dl) and age > 35 years are described to be predictors of retropharyngeal abscesses and necrotizing fasciitis, it is proven, and our data confirm, that neither CRP nor leukocytes in sera are appropriate markers to distinguish between PTA and AT [10]. Additionally, there are also no useful markers to differentiate between PTA and PC. Nevertheless, low correlations of S100A8/A9 levels in serum with CRP and leukocytes were detectable in patients with tonsillitis which is in concordance with recent findings in patients with myocardial infarction or rheumatoid arthritis [32, 33]. Recently increased levels of IL-8 in the tissue of tonsils with PTA compared to tonsils derived from patients with recurrent tonsillitis were observed, and strong positive correlations of S100A8/A9 and IL-8 have been described in association with congestive heart failure [34, 35]. Although we could observe a correlation between S100A8/A9 and IL-8 levels in the serum and saliva as well, neither IL-8 nor any other cytokine or chemokine we determined in the serum and saliva provides the potential to differentiate between acute tonsillitis, peritonsillitis, and peritonsillar abscess. Thus, we can assume S100A8/A9 to be a useful biomarker to identify patients with PTA.

Saliva of both patients and controls are macroscopically very inhomogeneous, and consequently, the content of S100A8/A9 shows a great variety in all cohorts. Although the data analysis has a high standard deviation, a comparative analysis was possible. Immunohistochemical findings revealed higher concentrations of S100A8/A9 in the tonsils of patients with PTA in contrast to hyperplastic tonsils without any history of tonsillitis. These findings impressively demonstrate a pivotal role in the pathomechanism as well as local expression and release of these DAMPs during PTA. However, the influence of S100A8/A9 on the development of PTA and its function regarding the tonsillar epithelium have not been elucidated so far. Hypothetically, S100A8/A9 is expressed by tonsil epithelial cells due to disruption of barrier function. A positive feedback mechanism with amplification of inflammation, induction of proinflammatory cytokine production, and simultaneous proliferation of keratinocytes as described in patients with systemic lupus erythematosus or psoriasis is assumable in tonsils and tonsillitis [36]. Thus, associated with leukocyte recruitment, pathogens could be eliminated and invasion could be averted [37]. Granulocyte migration through the gut wall into the feces allows determination of fecal S100A8/A9 in patients with inflammatory bowel diseases like Crohn's disease or appendicitis whereas release of S100A8/A9 by

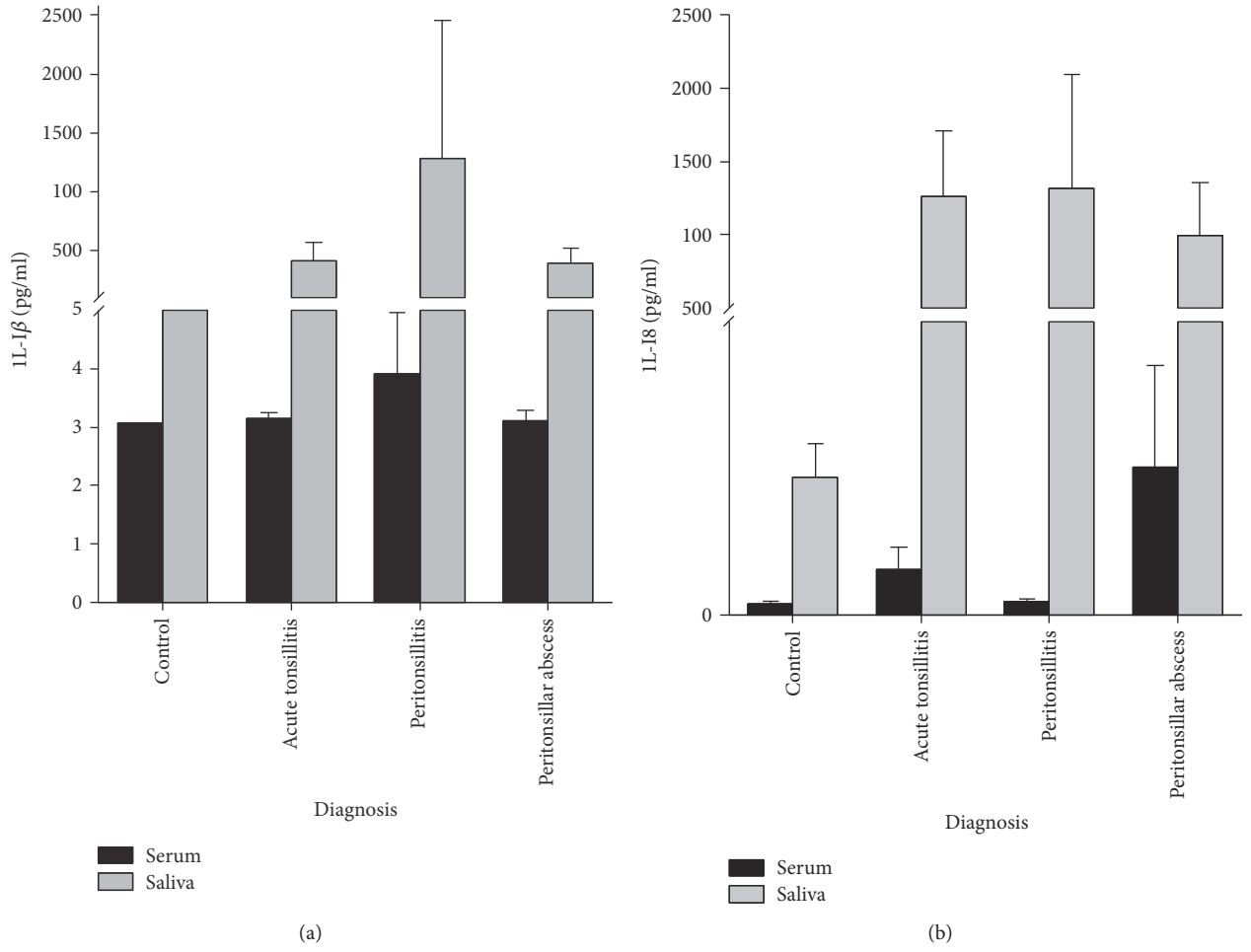

Figure 4: Cytokines and chemokines in saliva and serum. Levels of IL-1β (a) and IL-8 (b) in controls, patients with acute tonsillitis, peritonsillitis, or peritonsillar abscess. No significant differences between the diagnosis groups were detectable ($p > 0.05$). Black bars = serum levels, grey bars = saliva levels, mean ± SEM.

endothelial cells could be observed due to leukocyte interaction [26, 27, 37, 38]. According to these findings, two possible mechanisms for elevated salivary levels of S100A8/A9 could be assumed: (1) active secretion by the tonsillar keratinocytes and (2) migration of activated leukocytes through the tonsillar epithelium.

Patients with peritonsillitis show symptoms suspicious of peritonsillar abscess but early, correct, and reliable diagnosis is important to determine the adequate treatment approach and to prevent the progress of the PTA attended with severe complications. The difficulty to differentiate between PTA and PC in clinical examination points out the necessity of an objective, reliable parameter [14]. Computed tomography scan (CT) was considered to be a helpful tool to distinguish between PC and PTA [39]. CT scan might enhance the diagnostic accuracy and avoid unnecessary drainage procedures but there are some limitations which should be taken into consideration [40]. Grant et al. evaluated the CT scan in children with PTA and observed a delay of less-invasive interventions and no influence of the CT scan on the intervention chosen by the clinician [41]. Furthermore, in 13% of the 3 to 5-year-old children, common anesthesia or sedation was required for CT scan execution enhancing the risk of associated complications [41]. Due to the most important disadvantages of additional high costs, the radiation exposure and a specificity of just 50%, the value of the CT scan as an appropriate tool to differentiate between PTA and PC is doubtful. Thus, CT scan should be limited to special, isolated cases and not be part of routine PC or PTA management, particularly in children [41]. Hence, the intraoral and transcutaneous ultrasound of the tonsil and the peritonsillar abscess were established as a cost-efficient and lower-risk method compared to CT scan [18]. Intraoral ultrasound was described to be more sensitive than the transcutaneous ultrasound (89–95% versus 10–91%), and specificity of intraoral ultrasound varied from 70 to 83% [18, 42–45]. Although the ultrasound represents a noninvasive and rapid tool to differentiate between PTA and PC, there exist also some limitations restricting the use of this methodology. Albeit, transoral ultrasound was described to be well-tolerated; examination's result depends on the general cooperativeness as well as the physical condition of the patient [18]. Especially, children and compromised patients may not tolerate the procedure. Furthermore, in cases of severe trismus, entry of the probe into the oral cavity might be difficult or impossible [44]. Correct interpretation of the

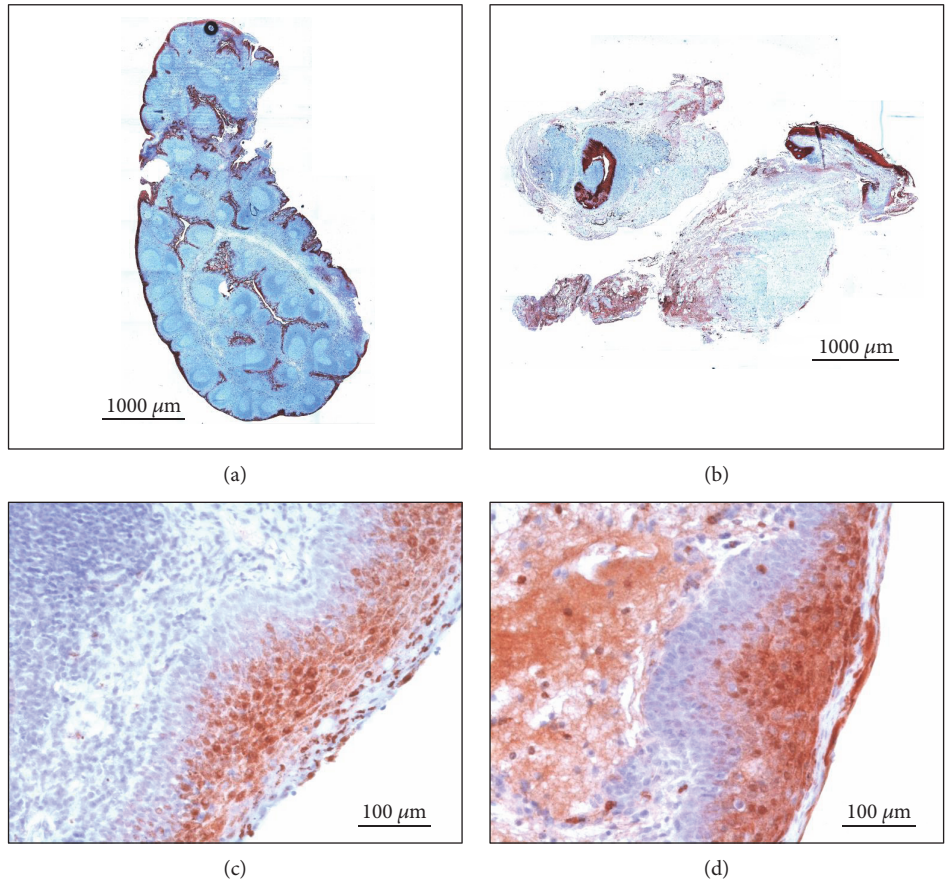

Figure 5: IHC staining for S100A8 of human palatine tonsils of patients with hypertrophic tonsils without any history of recurrent tonsillitis (a, c) and suffering from peritonsillar abscess (b, d). S100A8 expression is limited to the surface and crypt epithelium of the hypertrophic tonsil. The tonsil of the PTA patient shows an intensified staining for S100A8 in the epithelium and the parenchyma. (a) and (b): MosaiX module, original magnification ×40. (c) and (d): magnification ×200.

Table 1: PTA score: Addition of one point each for symptoms like halitosis, trismus, uvula edema, unilateral swelling of the arched palate, and S100A8/A9 levels in serum and saliva higher than the cut-off values results in a score with a range from 0 to 6. High S_{PTA} values are associated with an increased probability of PTA.

Symptoms	Points
Halitosis	1
Trismus	1
Uvula edema	1
Unilateral swelling of the arched palate	1
Serum S100A8/A9 > 2550 ng/ml	1
Saliva S100A8/A9 > 8180 ng/ml	1

0–2: acute tonsillitis/peritonsillitis; ≥3: peritonsillar abscess.

findings depends on the quality of ultrasound images as well as the advanced degree of technical and diagnostic expertise [18, 46]. Hence, further limitation arises due to limited access to trained ultrasonographers, a significant interuser variability, and limited availability of adequate equipment [18, 43, 44]. As mentioned above, 50% of the patients with PTA were treated by nonotolaryngologists who were probably not familiar with ultrasound of the head and neck region, and ultrasound is not available at every ambulance [13, 47]. Thus, for improving quality of care, it was the aim of the study to analyze the potential of S100A8/A9 as an objective marker to identify patients with peritonsillar abscess. We could show that the determination of S100A8/A9 levels in the serum and saliva in combination with symptoms suspicious of PTA is a helpful tool to distinguish between PTA and PC with a sensitivity of 92% and a specificity of 93%. Sample acquisition is not associated with any risks or complaints for the patients, and data analysis is independent from the degree of expertise. Hence, the newly developed PTA score seems to be an appropriate screening method for peritonsillar abscess. It should be mentioned that both the S100A8/A9 level in serum and saliva should be included in the new PTA score for improving sensitivity and specificity. If S100A8/A9 levels were excluded, a cut-off value of 0.5 for the existence of PTA was determined which is inadequate for differentiation between acute tonsillitis, peritonsillitis, and peritonsillar abscess. Analyzing the power of the PTA score without S100A8/A9 to differentiate PTA from PC and AT revealed a cut-off value of 1.5 with a sensitivity of 0.89 but a low specificity of 0.71. Thus, with a sensitivity of 92% and a specificity of 93%, the PTA score including S100A8/A9 levels in the serum and saliva is more

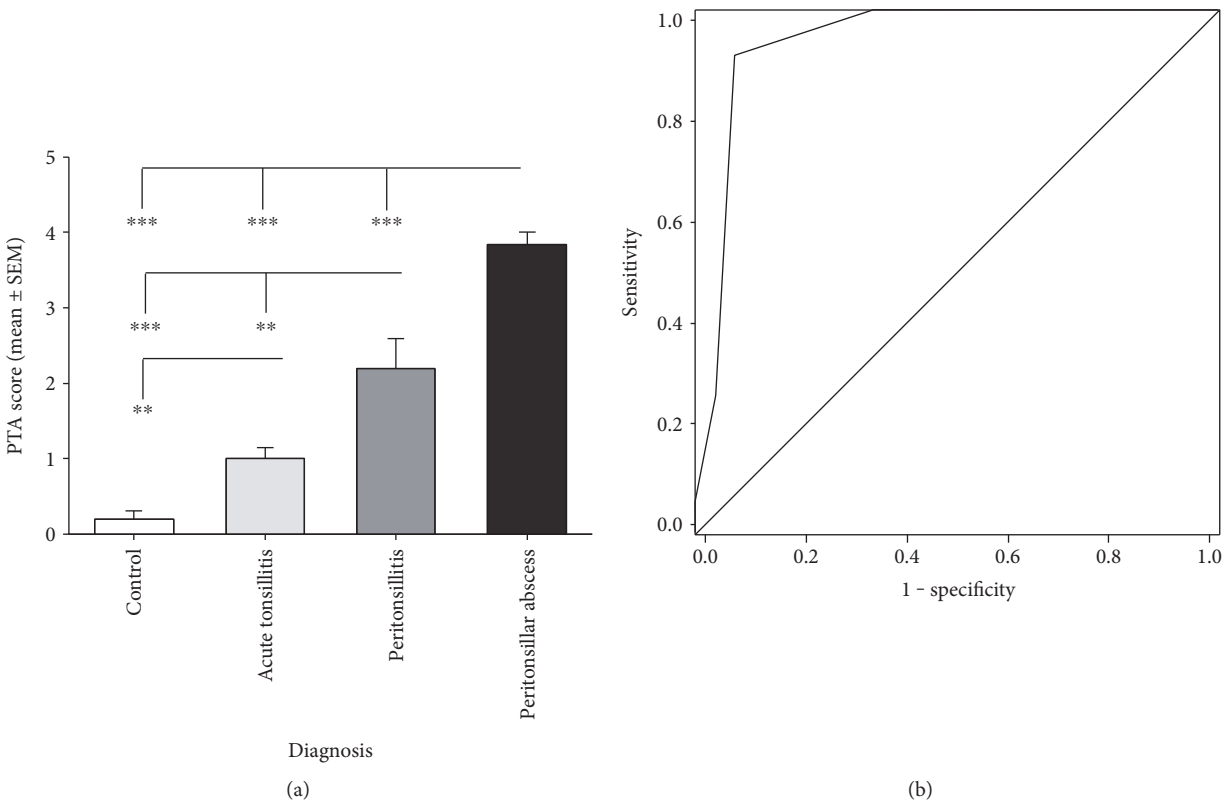

Figure 6: PTA score (S_{PTA}) was developed by the addition of one point each for symptoms like trismus, halitosis, uvula edema, unilateral swelling of the arched palate, S100A8/A9 > 2550 ng/ml in the serum, and S100A8/A9 > 8180 ng/ml in the saliva. Patients with acute tonsillitis, peritonsillitis, and peritonsillar abscess show significant higher S_{PTA} values in comparison with the controls. Furthermore, a significant difference of S_{PTA} values between the PTA and the PC cohort was detectable (a). ROC analysis of the PTA score revealed a cut-off value of $S_{PTA} = 2.5$ (sensitivity = 0.92, specificity = 0.93, $p < 0.001$) to identify patients suffering from PTA (black: ROC curve in black, grey: diagonal association line) ($^{**}p < 0.01$, and $^{***}p < 0.001$).

Table 2: Probability of PTA in dependence on the S_{PTA} values. The likelihood for PTA increases with higher PTA score values. For S_{PTA} values ≥ 3, the overall probability of PTA is about 89%.

PTA score value	Probability of PTA	n
0	0%	21
1	0%	16
2	18%	17
3	92%	12
≥4	88%	25

reliable. Additionally, as mentioned above, increased levels of S100A8/A9 could be observed in serum of patients with varied diseases concerning the bowel, the joints, the heart, the skin, and multiple other organs, and enhanced S100A8/A9 levels have been observed in crevicular fluid of patients suffering from periodontitis [26, 27, 34, 48, 49]. Therefore, including typical symptoms of PTA, the PTA score indicates the probable association between elevated S100A8/A9 levels and PTA and is more reliable than the determination of S100A8/A9 levels solely.

However, this methodology also has its limitations. S100A8/A9 values were determined by ELISA which is time-consuming and not suitable for the outpatient consultation. Hence, a rapid test for the immediate and easy measurement of salivary and serum S100A8/A9 levels which can be performed by every person independent from degree of expertise, with a low interuser variability within a short time, is under development. Another limitation of our study is an incertitude concerning the diagnosis of peritonsillitis. Although needle aspiration or incision revealed no pus and patients showed a good response to intravenous antibiotics, an abscess could not be certainly excluded.

Further randomized clinical trials with bigger cohorts are necessary to verify the importance, sensitivity, specificity, and accuracy of the PTA score as a prognostic and diagnostic parameter in the future. Particularly, the possibility of the new developed S_{PTA} including S100A8/A9 to identify patients who might profit from medical treatment or whether tonsillectomy or abscess relief is required has to be elucidated. Furthermore, the influence of S100A8/A9 and the associated immune cells on abscess formation needs further investigations.

5. Conclusion

The elevated levels of S100A8/A9 in the sera and saliva of patients with peritonsillar abscess represent a new

application of the well-established biomarker calprotectin. Hence, ascertainment of S100A8/A9 levels in the serum and saliva and application of the new PTA score is a useful diagnostic tool to differentiate between peritonsillar abscess, peritonsillar cellulitis, or acute tonsillitis during urgent outpatient consultation.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

Christoph Spiekermann is supported by a fellowship of the medical faculty at the University of Muenster, Germany. This work was supported by Grants of the IMF (Innovative Medizinische Forschung) of the medical faculty at the University of Muenster to Christoph Spiekermann (SP 2 1 15 11), the Interdisciplinary Center of Clinical Research at the University of Muenster to Thomas Vogl and Johannes Roth (Vo2/014/09, Ro2/003/15), the German Research Foundation (DFG) CRC 1009 B8 and B9 to Thomas Vogl and Johannes Roth, and by the Federal Ministry of Education and Research (BMBF), project AID-NET to Johannes Roth. This work was supported by a fellowship of the Graduate School of the Cells-in-Motion Cluster of Excellence (EXC 1003-CiM), University of Muenster, Germany to Antonella Russo. The authors thank A. Dietrich, M. Menke, C. Terwesten-Solé. A. Stadtbäumer, H. Berheide, and H. Hater for excellent technical assistance and acknowledge the support from the Open Access Publication Fund of University of Muenster.

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Hindawi
Disease Markers
Volume 2017, Article ID 5434310, 5 pages
https://doi.org/10.1155/2017/5434310

Research Article

Longitudinal Assessment of Transorbital Sonography, Visual Acuity, and Biomarkers for Inflammation and Axonal Injury in Optic Neuritis

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Received 25 June 2017; Accepted 17 August 2017; Published 11 September 2017

Academic Editor: Patrizia Cardelli

Background and Objective. To investigate the relationship between optic nerve sheath diameter, optic nerve diameter, visual acuity and osteopontin, and neurofilament heavy chain in patients with acute optic neuritis. *Patients and Methods*. Sonographic and visual acuity assessment and biomarker measurements were executed in 23 patients with unilateral optic neuritis and in 19 sex- and age-matched healthy controls. *Results*. ONSD was thicker on the affected side at symptom onset (median 6.3 mm; interquartile range 6.0–6.5) than after 12 months (5.3 mm; 4.9–5.6; $p < 0.001$) or than in controls (5.2 mm; 4.8–5.5; $p < 0.001$). OND was significantly increased in the affected side (3.4 mm; 2.9–3.8) compared to healthy controls (2.7 mm; 2.5–2.9; $p < 0.001$) and was thicker at baseline than after 12 months (2.8 mm; 2.7–3.0; $p < 0.01$). Visual acuity improved significantly after 12 months (1.00; 0.90–1.00) compared to onset of symptoms (0.80; 0.40–1.00; $p < 0.001$). OPN levels were significantly higher in patients at presentation (median 6.44 ng/ml; 2.05–10.06) compared to healthy controls (3.21 ng/ml, 1.34–4.34; $p < 0.03$). Concentrations of NfH were significantly higher in patients than in controls. *Conclusion*. ONSD and OND are increased in the affected eye. OPN and NfH are elevated in patients, confirming the presence of any underlying inflammation and axonal injury.

1. Introduction

Optic neuritis can be easily investigated because of its well-defined onset and accessibility to different diagnostic methods [1]. The most common pathophysiological mechanism of optic neuritis is inflammatory demyelination of the optic nerve. However, axonal degeneration and inflammation coexist in these patients and are associated with visual function impairment [2, 3].

Although optic neuritis is a clinical diagnosis, investigations such as retinal optical coherence tomography (OCT) or MRI may be proven useful in supporting the diagnosis. These techniques have provided fascinating insights into the pathophysiology of optic neuritis in its various forms [4, 5]. Transorbital sonography is a sensitive, highly accessible, user-friendly, and reliable technique for detecting optic nerve diameter (OND) and optic nerve sheath diameter (ONSD) [6] and is also able to show a significant thickening

of OND and ONSD in the affected side, which is probably due to inflammation with subsequently increased perineural subarachnoid fluid [7].

Transorbital sonography and OCT provide different and complementary information on the pathophysiology of optic neuritis. Compared to OCT, nerve ultrasonography has an inferior resolution but can provide a better depiction of the nerve and the orbita, because OCT is limited to surface analysis [7].

In a recent systematic review assessing the role of optic ultrasonography in the diagnosis of acute optic neuritis, this technique was able to detect an increase of the optic nerve and sheath diameters in about 80% of affected patients [8]. This finding is consistent with the development of vasogenic edema and the presence of the early optic nerve lesion, recognizable by the gadolinium leakage often shown in MRI or the lesions identified with particular sequences of MRI such as FLAIR sequences [5, 9].

Conversely, OCT accurately documents changes in the thickness of retinal layers such as swelling in case of acute optic neuritis with optic disc swelling; this might be proven further useful, although only one-third of patients with acute optic neuritis show papillitis [4, 8].

Osteopontin (OPN), a proinflammatory cytokine expressed in several tissues and pathological conditions [10, 11], was shown to be increased in the cerebrospinal fluid of patients with optic neuritis [12]. In multiple sclerosis (MS), OPN levels correlate with disease severity and relapse rate [13]. On the other hand, blood levels of neurofilament heavy chain (NfH), a biomarker for neurodegeneration, are increased in patients with acute optic neuritis and inversely correlated with visual loss [14]. Simultaneous assessment of visual acuity, transorbital sonography, and biomarkers may provide an opportunity to further explore inflammation and neurodegeneration in optic neuritis and was therefore the aim of this study.

2. Methods

Written informed consent was obtained from all persons before entering the study. The study was approved by the local ethics committee (Bolzano, number 19-2014) and performed in accordance with the Declaration of Helsinki.

2.1. Patients' Inclusion. All consecutive patients presenting to the Neurology Outpatient Clinic of Merano Hospital between December 2014 and November 2015 with a clinical diagnosis of optic neuritis were enrolled. Inclusion criteria were those adopted in the Optic Neuritis Treatment Trial by the Opticus Neuritis Study Group: 18–46 years of age; acute unilateral optic neuritis with visual symptoms for 8 days or fewer; a relative afferent pupillary defect and a visual field defect in the affected eye; no previous episodes of optic neuritis in the affected eye; no previous corticosteroid treatment for optic neuritis or multiple sclerosis; and no systemic disease other than multiple sclerosis that might be the cause of the optic neuritis [3].

All patients underwent neurologic and ophthalmologic examinations, including visual acuity assessment and direct ophthalmoscopy. Laboratory examinations included vasculitis screening, anti-neuromyelitis optical antibodies (aquaporin4-antibodies), and antibodies against myelin oligodendrocyte glycoprotein. MRI was done to exclude other causes of optic neuritis or compressive lesions [3]. All patients were followed up for 12 months.

2.2. Procedure of the Study and B-Ultrasound Sonography. Transorbital sonography, visual acuity, and biomarkers were assessed at onset (T0) and after 12 months (T12). At first presentation, transorbital sonography was evaluated by an expert neurosonologist (PL), who was accredited by the Italian Medical Ultrasound Society. We did not evaluate the sensitivity of sonographic data, but high attention was paid in order to assure quality control for the sonographic data. The high reliability of this technique has been reported in previous studies [15, 16]. In order to reduce the variability of measurements, the ONSD was measured three times in each eye and the mean values were calculated.

Transorbital sonography was always performed prior to the initiation of steroid treatment. Afterwards, all included patients received a single course of one gram of intravenous methylprednisolone sodium succinate over five days. The sonographer was unaware of the condition of case or control and of the affected side. To ensure blinding, patients and healthy controls were asked not to reveal their status (or their affected side) during examinations and were always placed on the examination table before the sonographer's arrival.

Transorbital sonography was carried out in B-mode using a Toshiba Applio XG equipped by a 4-11 Megahertz 5 S1 Linear Probe (Toshiba Medical System, Applio Nasu, Japan). We adopted the same procedure described elsewhere [6, 7].

2.3. Assessment of OPN and NfH. OPN and NfH levels were determined for all patients presenting with symptoms suggestive of acute unilateral optic neuritis and before the beginning of the infusion of methylprednisolone. After collection, the clotted venous blood was centrifuged at 3000 rpm for 15 min to obtain the serum that was then stored at −80°C until use. OPN and NfH serum levels were measured using the human OPN DuoSet ELISA development kit and the ELISA-pNFH-V1 (R&D Systems, Minneapolis, MN, USA; EnCor Biotechnology Inc., Gainesville, FL, USA, resp.) according to the manufacturer's instructions. The optical density (OD) of each sample was determined at 450 nm with a Spectra Count (Bio-Rad, Hercules, CA, USA), subtracting the lowest mean OD of the negative control. Sensitivity for OPN ELISA was 0.0625 ng/ml and for NfH ELISA was 0.2 ng/ml. Specificity of OPN ELISA kit was tested with recombinant human MMP-3 prepared at 50 ng/ml that showed no cross-reactivity or interference. Specificity of NfH ELISA kit was tested with HPLC-purified bovine NfM (7.8%), with bovine NfL (6.5%), and with bovine GFAP (0.06%) [17]. All samples were stored at the Clinic of Merano Hospital and then sent to the University of Novara. Due to a shortage of patients' samples, we decided to test OPN in all samples and NfH in 19 patients.

2.4. Statistical Evaluation. Continuous variables were described using median and interquartile range (IQR) for

Table 1: Characteristics of patients and controls.

	Optic neuritis	Healthy controls	p value
Number of patients, n (%)	23 (100)	19 (100)	
Sex			
Females, n (%)	19 (83)	13 (68)	0.47*
Males, n (%)	4 (17)	6 (32)	
Time from onset days	14 (8–26)	—	
Follow-up days	360	—	
Age, years (mean ± SD)	33.0 ± 8.0	34.3 ± 9.2	0.63**
Isolated optic neuritis, n (%)	12 (52.2)		
MS-optic neuritis, n (%)	11 (47.8)		

Data were expressed in frequency (percentage) or means ± standard deviation. *Fisher's exact test. **t-test for independent groups.

nonparametrically distributed variables and mean with standard deviation for parametrically distributed variables.

Comparisons between groups were assessed using the nonparametric Mann–Whitney U test and Wilcoxon signed-rank test. Correlations were assessed with the Spearman test; the level of statistical significance was set at $p = 0.05$.

All analyses were performed using dedicated statistical software (IBM Statistical Package for Social Science (SPSS), version 23.0.0.2, Armonk, New York, USA).

3. Results

3.1. Participants. Of the 28 patients who presented with a newly diagnosed optic neuritis during the study period, 5 did not meet the study criteria and were excluded from further analysis (1 missed during follow-up, 4 had potentially confounding conditions: 1 pituitary adenoma, 1 meningeal carcinomatosis, 1 neurosarcoidosis, and 1 tuberculum sellae meningioma). Hence, we had data from 23 patients, of whom 11 had prior MS relapses without previous optic neuritis and 12 had isolated optic neuritis. We analyzed data from 19 age-matched healthy controls. Demographic and clinical characteristics of the included patients and controls are reported in Table 1.

3.2. Ultrasound and Visual Acuity. Table 2 reports findings on ONSD, OND, and visual outcomes of both patients and controls. We observed a significant thickening of ONSD in the affected side (median 6.3 mm, IQR 6.0–6.5) compared to healthy controls (median 5.2 mm, IQR 4.8–5.5; $p < 0.001$) and after 12 months (median 5.3 mm, IQR 4.9–5.6; $p < 0.001$). The median OND in the affected side (3.4 mm, IQR 2.9–3.8) was significantly increased compared to the controls (median 2.7 mm, IQR 2.5–2.9; $p < 0.001$) and after 12 months of follow-up (median 2.8 mm, IQR 2.7–3.0; $p < 0.01$). Visual acuity in the affected eye was significantly worse at presentation (median 0.80, IQR 0.40–1.00) than after 12 months (median 1.00, IQR 0.90–1.00; $p < 0.001$).

3.3. Biomarkers. Serum OPN levels were significantly higher in patients with optic neuritis at presentation (median 6.44 ng/ml, IQR 2.05–10.06) compared to healthy controls (median 3.21 ng/ml, IQR 1.34–4.34; $p < 0.03$) (Table 3). Concentrations of NfH were significantly higher in patients (median 0.39 ng/ml, IQR 0.21–0.55) than in controls (median 0.02 ng/ml, IQR 0.02–0.08; $p < 0.001$) (Table 3). OPN and NfH concentrations remained elevated after 12 months with no statistically significant difference in OPN ($p = 0.24$) or NfH ($p = 0.94$) between T0 and T12.

Table 2: Sonographic features and visual outcomes of patients and OPN and NfH values assessed at baseline (T0) and after 1 year (T12) of follow-up.

Parameter	T	ON patients affected eye ($n = 23$)	Healthy controls ($n = 19$)	p value
ONSD, mm	T0	6.3 (6.0–6.5)	5.2 (4.8–5.5)	<0.001
	T12	5.3 (4.9–5.6)		
	p value	<0.001		
OND, mm	T0	3.4 (2.9–3.8)	2.7 (2.5–2.9)	<0.001
	T12	2.8 (2.7–3.0)		
	p value	0.001		
Visual acuity	T0	0.80 (0.40–1.00)		
	T12	1.00 (0.90–1.00)		
	p value	<0.001		

T: time; ON: optic neuritis; ONSD: optic nerve sheath diameter, mm; OND: optic nerve diameter, mm; mm: millimeter; OPN: osteopontin; NfH: neurofilament heavy chain; ng/ml: nanogramm/milliliter; n: number of patients. Data are reported as median (IQR).

Table 3: OPN and NfH values assessed at baseline (T0) and after 1 year (T12) of follow-up.

Parameter	T	Patients*	Healthy controls ($n = 19$)	p value
OPN, ng/ml	T0	6.44 (2.05–10.06)	3.21 (1.34–4.34)	<0.03
	T12	6.70 (3.85–10.90)		
	p	0.24		
NfH, ng/ml	T0	0.39 (0.21–0.55)	0.02 (0.02–0.08)	<0.001
	T12	0.3 (0.21–0.48)		
	p	0.94		

OPN: osteopontin; NfH: neurofilament heavy chain; ng/ml: nanogramm/milliliter; n: number of patients. Data are reported as median (IQR). *23 patients underwent OPN measurements at T0 and T12, and 19 patients underwent NfH measurements at T0 and T12.

3.4. Correlations between OPN, NfH, and ONSD. We found a statistically significant correlation between the ONSD value in the affected side and serum levels of OPN at T0 ($r = 0.68$; $p < 0.05$). Conversely, no statistically significant correlation between ONSD in the affected side and NfH level was observed ($r = -0.43$; $p = 0.09$).

3.5. OPN, NfH and Visual Acuity. Statistically significant differences were found in serum levels of OPN at T0 between patients with residual visual deficit and patients showing complete recovery (medians 7.7 versus 2.6 ng/ml; $p = 0.03$).

Regarding the serum levels of NfH at T0, we observed no statistically significant difference between patients with visual deficit at follow-up compared with patients with a good visual outcome (medians 0.39 versus 0.28 ng/ml; $p = 0.291$).

OPN values at T12 were significantly higher in patients with worsened visual acuity compared to patients with complete recovery of visual function (medians 10.8 versus 4.6 ng/ml; $p = 0.019$).

Regarding the serum levels of NfH at T12, we observed no statistically significant difference between the 7 patients with worsened visual acuity and patients who had regained normal visual acuity (medians 0.36 versus 0.25 ng/ml; $p = 0.236$).

4. Discussion

This is the first longitudinal study assessing circulating biomarkers of inflammation and axonal injury in parallel with transorbital sonography and visual acuity in patients with acute optic neuritis. Our approach is quite novel since we explored translational aspects that are well established in other neuroinflammatory conditions such as MS [18] but definitely less studied in optic neuritis [19].

Our study confirms that transorbital sonography is a sensitive technique for visualizing optic nerve thickening in the affected eye [7]. Nerve swelling is indeed the direct expression of the hallmark of optic neuritis, that is, inflammation. Furthermore, transorbital sonography can provide quantitative observations of the increase of nerve sheaths determined by the perineural subarachnoid fluid.

NfH and OPN levels were higher in patients with optic neuritis than in controls and remained elevated even after 12 months. Moreover, OPN concentration was significantly higher in patients with residual visual deficit after 12 months, thus supporting its detrimental effect in neuroinflammatory conditions [20]. Conversely, we did not find a higher concentration of NfH in patients with residual deficit compared to patients with a complete recovery after 12 months.

Our findings are in line with a previous study showing higher OPN levels during MS relapses and a direct correlation between OPN levels and disability [21]. Two further studies confirmed that OPN levels were higher in MS relapse compared to remission [13, 22].

Other studies found higher concentrations of NfH in patients with optic neuritis compared to controls or higher concentrations in patients with optic neuritis and neuromyelitis optica with more serious visual impairment compared to MS patients with optic neuritis or controls [2, 23]. Our study failed to show a significant relation between higher NfH concentrations at symptom onset and residual visual impairment. Conversely, we found a direct correlation between OPN and both ONSD and OND. Such parameters can provide complementary information on the dynamics of inflammation, and therefore, we suggest that they should be further studied in optic neuritis.

Notwithstanding the significant improvement in visual acuity, the persistence of high biomarkers concentrations over time suggests the persistence of underlying inflammation and neurodegeneration. The ONTT trial has indeed shown a spontaneous recovery in many but not all patients over a few weeks in acute idiopathic optic neuritis, even though the effect of treatment in subgroups was not analyzed [3].

The main limitation of this study is the small sample size. Therefore, our results need to be cautiously interpreted and replicated on a larger population. The use of transorbital sonography together with biomarkers would be of particular interest to monitor subgroups of patients with visual loss progressing for more than 2 weeks and with the absence of recovery for more than 3 weeks. Our findings were obtained by a single operator, but they can be easily replicated thanks to the low inter-rater variability of this technique [6].

Overall, our results support the usefulness of examining biomarkers in optic neuritis, since they provide complementary information on pathophysiology. The increase of OPN and ONSD seems to be correlated with the intensity of inflammation and might have a prognostic value, in analogy to what was suggested in MS [24]. Moreover, a similar study design could be easily applied to test correlations between ultrasound findings and other circulating molecules with a potential role in optic neuritis [25].

In conclusion, this study suggests that OPN levels predict visual outcome and neuronal loss after optic neuritis, whereas the role of NfH remains to be fully elucidated.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Authors' Contributions

All authors provided substantial contributions to the conception or design of the work or the acquisition, analysis, or interpretation of data. Piergiorgio Lochner, Roberto Cantello, Raffaele Nardone, and Francesco Brigo are responsible for the study organisation, execution, manuscript review, and critique. Antonio Siniscalchi and Lorenzo Coppo are responsible for the study execution, manuscript review, and critique. Nausicaa Clemente is responsible for the study organisation, execution, and critique. Martin Lesmeister and Andrea Naldi are responsible for the statistical analysis, manuscript review, and critique. Cristoforo Comi is responsible for the study conception, manuscript writing, review, and critique. Francesco Brigo and Cristoforo Comi contributed equally to this work. Klaus Fassbender is responsible for manuscript review and critique.

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Hindawi
Disease Markers
Volume 2017, Article ID 3649693, 7 pages
https://doi.org/10.1155/2017/3649693

Research Article

Overexpression of miR-24 Is Involved in the Formation of Hypocoagulation State after Severe Trauma by Inhibiting the Synthesis of Coagulation Factor X

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Received 22 January 2017; Revised 16 April 2017; Accepted 14 May 2017; Published 14 June 2017

Academic Editor: Mina Hur

Background. Dysregulation of microRNAs may contribute to the progression of trauma-induced coagulopathy (TIC). We aimed to explore the biological function that miRNA-24-3p (miR-24) might have in coagulation factor deficiency after major trauma and TIC. *Methods*. 15 healthy volunteers and 36 severe trauma patients (Injury Severity Score ≥ 16 were enrolled. TIC was determined as the initial international normalized ratio >1.5. The miR-24 expression and concentrations of factor X (FX) and factor XII in plasma were measured. In vitro study was conducted on L02 cell line. *Results*. The plasma miR-24 expression was significantly elevated by 3.17-fold ($P = 0.043$) in major trauma patients and reduced after 3 days ($P < 0.01$). The expression level was significantly higher in TIC than in non-TIC patients ($P = 0.040$). Multivariate analysis showed that the higher miR-24 expression was associated with TIC. The plasma concentration of FX in TIC patients was significantly lower than in the non-TIC ones ($P = 0.030$) and controls ($P < 0.01$). A negative correlation was observed between miR-24 and FX. miR-24 transduction significantly reduced the FX level in the supernatant of L02 cells ($P = 0.030$). *Conclusions*. miR-24 was overexpressed in major trauma and TIC patients. The negative correlation of miR-24 with FX suggested the possibility that miR-24 might inhibit the synthesis of FX during TIC.

1. Introduction

Traumatic injury is life threatening and it is the fourth leading cause of death in high-income countries [1, 2]. Trauma-induced coagulopathy (TIC) is recognized as an early hypocoagulable state leading to exacerbation of bleeding and associated with fourfold-increased mortality among severely injured patients [3]. TIC was first described during the Korean War [4]. Its complex fundamental mechanisms remain elusive up till now [5]. Current opinions suggest that the pathogenesis is multifactorial and mechanisms like activation of the protein C pathway, endothelial injury, coagulation factor deficiency, hyperfibrinolysis, and platelet dysfunction participate in the development of TIC [6–8].

Coagulation factor deficits after severe trauma have been recognized by a number of studies for many decades [9–13]. It is verified by the successful therapies of repletion of clotting factors by infusion of higher ratio of flesh frozen plasma (FFP) and additional prothrombin complex concentrate (PCC) in trauma patients [14–16]. Studies have shown that coagulation factor deficiency occurred early on the scene and aggravated with time [12, 17]. Theories explaining this

phenomenon are direct loss, ongoing consumption, and dilution [6, 18]. However, reduction in the synthesis of coagulation factor can also be present.

MicroRNAs (miRNAs) are noncoding small RNA molecules that are 20–23 nucleotides in length, which play important regulatory roles in plants and animals by targeting mRNAs for cleavage or translational repression. They regulate diverse cellular functions including proliferation, differentiation, apoptosis, and plasticity [19]. miRNAs have lately been recognized to play a role in severe injuries. Yet, few reports have expounded on that subject [20, 21]. A recent study implied that miRNA-24-3p (miR-24) can reduce the FX and FXII mRNA levels by downregulating protein hepatocyte nuclear factor 4α (HNF-4α) [22]. The clinical relevance of this particular finding is however still not clear. So in the present study, we investigated the relations between plasma miR-24 expression and coagulation factor X (FX) and XII (FXII) levels in major trauma and TIC patients. We assumed that the overexpression of miR-24 in trauma patients is involved in the hypocoagulation state by inhibiting the synthesis of FX and/or FXII.

2. Methods

2.1. Patients and Normal Donors. Upon approval from the ethics committee and obtaining written informed consents, blood samples were obtained from 36 severely injured patients from June to August, 2016 at Wuhan Union Hospital, a tertiary hospital located in the center of China. The patients were only included when the researchers were on shifts. All patients enrolled had an Injury Severity Score (ISS) of at least 16 points within 12 h of injury and without any previous medical history. Exclusion criteria were patients aged less than 16 years old, pregnant women, or those who were on anticoagulant medications or had surgeries prior to attending the emergency department (ED). 15 healthy adult donors served as the control group. The patients' standard blood test values, ISS, and shock index (SI) were collected and calculated based on clinical records upon their ED arrivals. TIC was defined by international normalized ratio (INR) of more than 1.5 [23, 24]. The ISS calculation was based on the ordinal scale developed by Baker et al. [25] and updated in 2008.

2.2. Blood Collection and Measurements. Blood samples were collected from each patient via venous puncture into tubes containing EDTA at 2 time points: upon arrival to ED and 3 days later if they were still hospitalized. All blood samples were processed for the isolation of plasma within 4 h after collection. Plasma samples was transferred to an RNase-free tube and stored at −80°C. Samples were analyzed by researchers who were blinded to all patients' data.

2.3. RNA Isolation and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). For measuring miR-24, total RNA was isolated from 200 μL plasma samples using the RNAmisi microRNA fast extraction kit (Aidlab, Beijing, China) according to the manufacturer's instructions. A synthetic miRNA cel-miR-39 (Biomics, Nantong, China) was added to each plasma specimen at a final concentration of 1 μM as a reference before isolation [26]. Relative quantification of the total RNA was conducted with a 2-step method: reverse transcription (RT) and qRT-PCR. RT reaction was performed using a RevertAid First Strand cDNA Synthesis Kit (Invitrogen, Shanghai, China). 4 μL of extracted RNA, 1 μL, 2 μmol/L stemloop RT primer (2 μM; Invitrogen, Shanghai, China), and 1 μL RNase-free H_2O, were incubated at 65°C for 10 min. Next, 5× buffer (2 μL), 10 mM dNTP (0.5 μL), RiboLock RNase Inhibitor (0.5 μL), and RevertAid Reverse Transcriptase (1 μL; Thermo Fisher Scientific Inc., Shanghai, China) were added. The samples were incubated at 42°C for 1 h, followed by 10 min at 70°C for enzyme inactivation, after which the reactions were held at 4°C until use. qRT-PCRs were processed in 96-well plates on an ABI StepOnePlus analyzer (Applied Biosystems, CA, USA). In a final reaction volume of 20 μL, the following were added: 0.5 μL of cDNA, 10 μL of qPCR Master Mix (Invitrogen, Shanghai, China), 1.6 μL of miRNA-specific forward and reverse primer (10 μM), and 7.9 μL of nuclease-free water. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Each qRT-PCR was performed in triplicate. Table 1 summarizes the sequences of RT and PCR primer used. Expression values were normalized using the mean threshold cycle (Ct) obtained from the spiked-in controls cel-miR-39. Delta Ct is the difference in the cycling threshold between miR-24 and cel-miR-39. In this article, the relative expression value of miR-24 is either expressed as $\log_{10}2^{-\text{Delta Ct}}$ [27] or −Delta Ct.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was used to detect the plasma levels of FX and FXII; the kits were purchased from Abcam, USA. All the plasma sample processing, measurement, and content calculation were done according to kit instructions.

2.5. Cell Culture and Transfections. L02 cells (China Cell Culture Center, Shanghai, China) were cultured in RPMI1640 medium at 37°C under 5% CO_2. Medium was supplemented with 10% fetal calf plasma, 100 μg/mL streptomycin, and 100 U/mL penicillin. The miR-24 mimics and negative control (NC) sequences were obtained from RIBOBIO (Guangzhou, China). The cells were seeded at a density of 80,000 cells/well one day before transfection using a Lipofectamine 2000 Reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. The final concentration of miR-24 mimics was 100 nM. Cell supernatants were harvested at 24 h after transfection. Each group contains 5 examples.

2.6. Statistical Analyses. Statistical analysis was performed by SPSS 22 software and P values of <0.05 were defined as statistically significant. Fisher's exact test was used for comparisons of proportions. Kolmogorov-Smirnov test was conducted to determine whether the data were normally distributed. For normally distributed variables, data were expressed as mean and standard deviation (SD); the comparisons of 2 groups were analyzed by Student's t-test, and 3

Table 1: Sequences of RT primers and PCR primers.

miRNA	RT primer		PCR primer
has-miR-24-3p	***CTGTTC	Forward	CCGTGGCTCAGTTCAGCAG
		Reverse	CAGTGCAGGGTCCGAGGTAT
cel-miR-39	***CAAGCT	Forward	CGCTCACCGGGTGTAAATCAG
		Reverse	CAGTGCAGGGTCCGAGGTAT

***Mean sequence of 'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC'. RT: reverse transcription.

Table 2: Demographics.

Variable	Healthy controls ($n = 15$)	Non-TIC ($n = 28$)	TIC ($n = 8$)	P value
Age (y)	42.27 ± 14.59	47.96 ± 12.68	46.50 ± 15.21	0.43
Male, n (%)	11 (73.33%)	20 (71.43%)	6 (75%)	0.98
Blunt, n (%)	N/A	23 (82.14%)	6 (75%)	0.64
Hours from scene	N/A	8.2 ± 3.1	7.6 ± 4.0	0.80
Injury Severity Score	N/A	25 (16–54)	34 (26–45)	**0.03**
Shock index	N/A	0.74 (0.53–2.00)	0.98 (0.60–1.52)	**0.04**
INR	N/A	1.13 (0.93–1.43)	1.70 (1.56–2.11)	**<0.01**
Transfused, n (%)	N/A	17 (60.71%)	8 (100%)	**0.03**
Packed red blood cells (U)	N/A	3.5 (0–20)	9 (0–19)	0.13
Fresh frozen plasma (mL)	N/A	0 (0–1150)	425 (0–2000)	0.14
Platelets (U)	N/A	0 (0–3)	1 (1–3)	**<0.01**
In-hospital mortality, n (%)	N/A	2 (7.14%)	2 (33.33%)	0.21
LOS in the hospital (d)	N/A	19.93 ± 14.02	23.63 ± 15.84	0.53
LOS in the ICU (d)	N/A	0 (0–11)	0 (0–9)	0.61

INR: international normalized ratio; LOS: length of stay; $p < 0.05$: values in boldface.

groups or more by one-way ANOVA test; the correlation study was conducted by using Pearson's correlation analysis. For the data with a non-normal distribution, data were expressed as mean and range; the comparison of 2 independent groups was analyzed by nonparametric Mann-Whitney U test. Binary multivariate logistic regression analysis was used to identify the association of TIC with ISS, SI, and miR-24 level. Repeated measures ANOVA was used to compare the changes of relative values of miR-24 over time. All experiments in the study were performed in duplicate.

3. Results

3.1. Demographic Data. The present study included 36 trauma patients with the average age of 47.6 ± 13.0 years, of whom 72.22% ($n = 26$) were men. ISS was 29.6 ± 10.7; 80.56% ($n = 29$) of the patients had blunt trauma. Among the 36 patients, 8 patients (22.22%) had TIC, and 9 patients (25%) received massive transfusion (≥10 RBC during the initial 24 hours). Overall mortality was 11.11% ($n = 4$). 2 deaths were due to traumatic brain injury (3 h and 4 d after ED visit), 1 exsanguination (5 h after ED visit), and 1 sepsis syndrome (29 d after ED visit). 15 healthy volunteers were enrolled and with the average age of 42.27 ± 14.59 years; 73.33% were male. Coagulopathic patients have higher ISS, SI, transfusion rate, and platelet requirement as compared to noncoagulopathic patients (Table 2).

3.2. Overexpression of Plasma miR-24 Is Found in Major Trauma and TIC Patients. Using qRT-PCR, plasma miR-24 levels were analyzed in the patients and controls. It was identified that average plasma miR-24 expression exhibited a 3.17-fold increase ($P = 0.043$) in all major trauma patients (Figure 1(a)). Plasma expression of miR-24 was significantly higher in TIC patients than in the non-TIC ones ($P = 0.040$). There were 1 death and 3 discharges within 3 days after ED visits in the non-TIC group and 1 death in the TIC group, so that left $n = 24$ and 7 in the two day 3 groups. MiR-24 expression was obviously downregulated on day 3 compared with that on day 0 ($P < 0.01$; Figure 1(b)). In multivariate logistic analysis, greater ISS and higher miR-24 expression were associated with TIC (Table 3).

3.3. Concentrations of FX and FXII Were Markedly Reduced in TIC Patients. Major trauma patients were reported to have coagulation factor deficiency. As shown in Figure 2(a), the average level of FX was significantly reduced by 48.63% in TIC patients than in healthy controls (4.06 ± 2.14 versus 7.91 ± 2.00 μg/L, $P < 0.01$). The FX level was lower in TIC patients than in the non-TIC ones (4.06 ± 2.14 versus 6.46 ± 2.76 μg/L, $P = 0.030$). Reduction in the average level of FXII in TIC patients than in control was also noted (25.00 ± 12.72 versus 34.71 ± 9.34 μg/L, $P = 0.049$; Figure 2(b)).

3.4. Concentrations of FX and FXII Correlate with Clinical Data. As summarized in Table 4, we next analyzed the

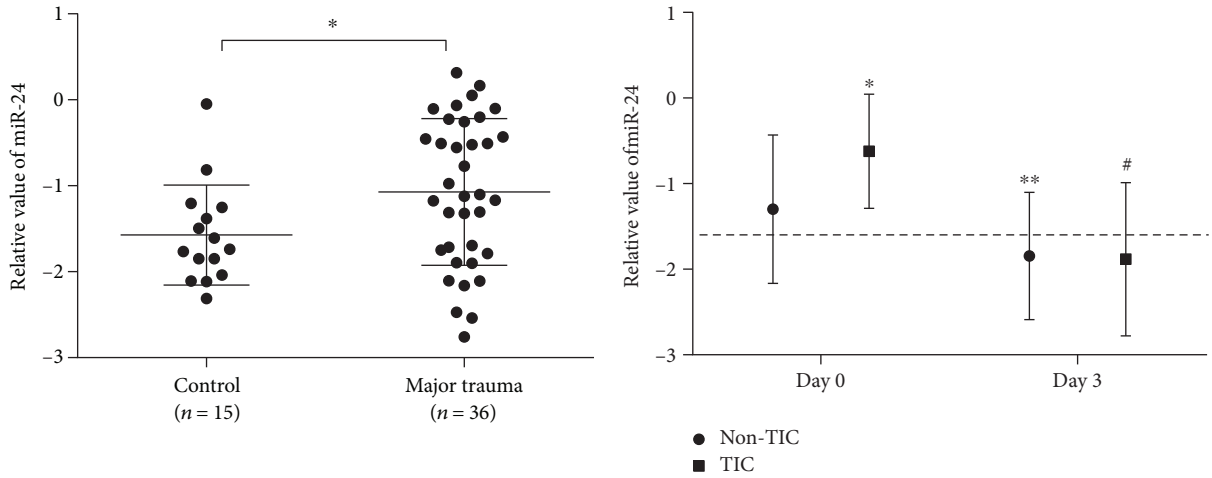

Figure 1: (a) Comparison of plasma expression levels of miR-24 in 36 major trauma patients and 15 controls. Plasma expression levels of miR-24 were higher in all major trauma patients than in healthy controls. $^{*}P < 0.05$. (b) Plasma expression levels of miR-24 were significantly increased in 8 TIC patients than in the 28 non-TIC ones on ED arrival. MiR-24 expression levels were significantly downregulated on day 3 compared with those on day 0 ($n = 24$ in the non-TIC day-3 group and $n = 7$ TIC day 3 group). Compared to the non-TIC day 0 group, $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the TIC day 0 group, $^{\#}P < 0.01$.

Table 3: Multivariate analysis for the association of TIC with ISS, SI, and miR-24 expression level.

Variable	Odds ratio (95%CI)	P value
Injury Severity Score	1.25 (1.01–1.54)	**0.04**
Shock index	1.20 (0.80–1.80)	0.37
miR-24	2.86 (1.24–6.60)	**0.01**

$p < 0.05$: values in boldface.

correlation between FX, FXII, and several key clinical and laboratory data including age, SI, ISS, time to admission, hemoglobin level, platelet count, INR, partial thromboplastin time (PT), activated partial thromboplastin time (APTT), and albumin, all of which can reflect the clinical conditions of patients. As a result, the FX level was shown to have a moderate to strong negative correlation with SI, ISS, APTT, INR, PT, and albumin and a moderate positive correlation with hemoglobin. The FXII level was shown to have a moderate negative correlation with age, INR, PT, and APTT.

3.5. MiR-24 Expression Negatively Correlates with the Concentration of FX. Former studies indicated that miR-24 could reduce the FX and FXII mRNA levels by downregulating HNF-4α in liver cancer cell line HepG [22]. Hence, we wanted to determine whether the miR-24 level correlated with FX or FXII levels in trauma patients on their ED arrivals. Pearson's correlation test showed the expression levels of miR-24 were inversely correlated with levels of FX (rho = -0.40, $P = 0.016$; Figure 3) but not with FXII (rho = -0.24, $P = 0.164$).

3.6. MiR-24 Can Inhibit FX Synthesis in Human Hepatocyte Cell Line. To investigate the effect of miR-24 on FX of the supernatant of L02 cells, we altered the miR-24 level in the cells. After transfection of miR-24 mimics or negative control, ELISA analysis showed that compared to negative control, the level of FX was reduced by transfection of miR-24 mimics, from (55.38 ± 5.78) ng/mL to (43.30 ± 8.49) ng/mL ($P = 0.030$).

4. Discussion

Despite dozens of years of intense research in this field, the management of TIC remains largely supportive without the therapeutic breakthroughs. The present study showed that the plasma miR-24 level was significantly elevated in major trauma patients, especially in TIC patients. It reduced to under average value in 3 days after homeostasis. The level of miR-24 was inversely correlated with that of FX. In vitro study showed that miR-24 transduction significantly reduced the FX level in the supernatant of L02 cells. They altogether indicated that overexpression of miR-24 was involved in the hypocoagulation state by inhibiting the production of FX after severe trauma. This is the first study which tries to explore the relationship between miRNA and coagulation factors in the trauma setting.

miRNAs can target hundreds to thousands of proteins and significant differences in the expression levels are altered in almost every type of disease [28]. Downregulation of miR-24 has been detected in the procoagulant states including myocardial infarction [29], acute cerebral infarction [27], osteomyelitis [30], and rats' acute respiratory distress syndrome [31]. These findings suggested that miR-24 might have an anticoagulant function. A study by Uhlich et al. revealed that the miR-24 level was increased among the 69 differentially expressed miRNAs in the severely injured [21]. In the present study, we confirmed that the miR-24 was overexpressed in major trauma patients. We further displayed that the difference in the miR-24 increase was

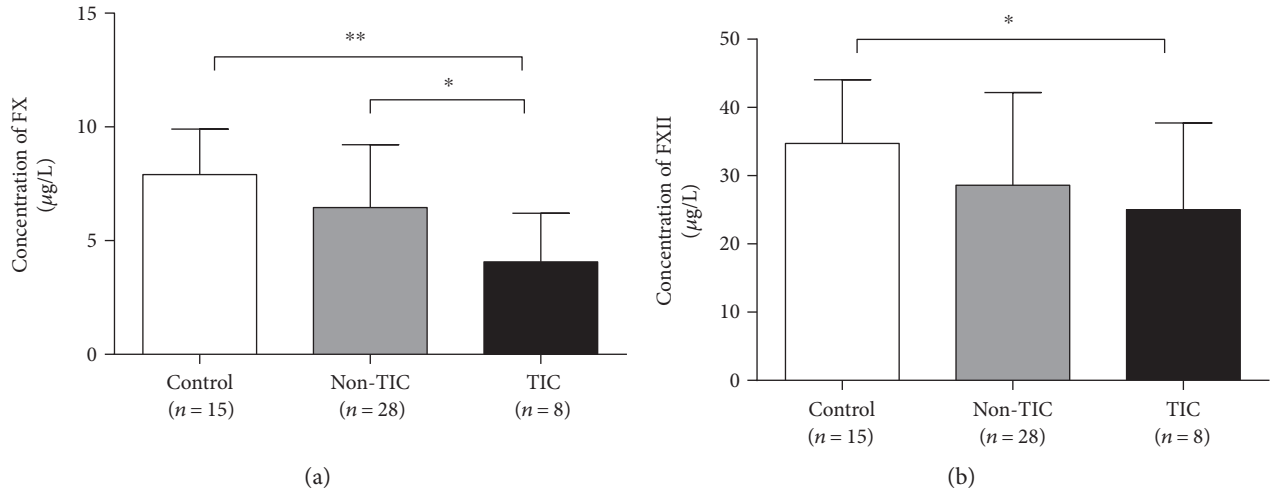

Figure 2: (a) The average level of FX in 8 TIC patients was significantly lower than that in 15 controls and 28 non-TIC patients. (b) Reduction in the average level of FXII in TIC patients compared to that in healthy controls was also noted. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 4: Correlations of coagulation factor levels with patients' clinical and laboratory data.

	Age	SI	ISS	Time to admission	Hemoglobin	Platelet count	INR	PT	APTT	Albumin
FX	0.26 (0.12)	**−0.39 (0.02)**	**−0.56 (<0.01)**	0.22 (0.20)	**0.44 (<0.01)**	0.21 (0.21)	**−0.59 (<0.01)**	**−0.59 (<0.01)**	**−0.36 (0.03)**	**−0.54 (<0.01)**
FXII	0.24 (0.16)	−0.21 (0.21)	−0.10 (0.58)	0.09 (0.61)	0.27 (0.11)	0.25 (0.15)	**−0.37 (0.03)**	**−0.37 (0.03)**	**−0.35 (0.03)**	−0.20 (0.25)

Table shows Pearson's rank correlation coefficient rho with P value (in the bracket). SI: shock index; ISS: Injury Severity Score; INR: international normalized ratio; PT: partial thromboplastin time; APTT: activated partial thromboplastin time.

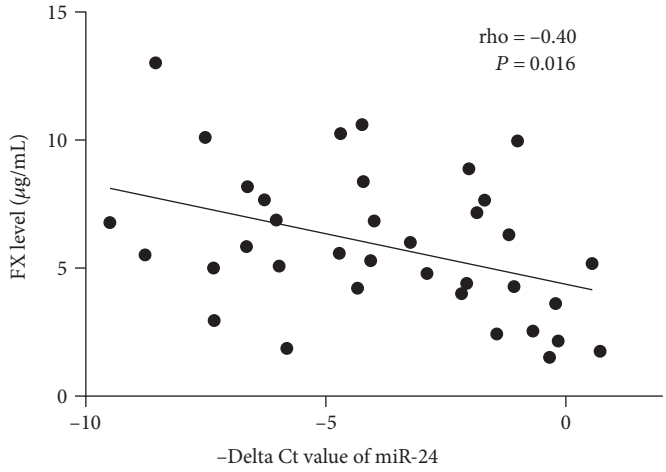

Figure 3: Correlation analysis for miR-24 expression and the level of FX ($n = 36$).

associated with TIC, which implied that miR-24 might be a useful diagnostic marker for TIC.

FX is synthesized in the liver and requires vitamin K. It plays a pivotal role at the convergence of the intrinsic and extrinsic pathways in the coagulation cascade [32]. Congenital FX deficiency can result in severe bleeding disorders [33]. A recently published cohort study proved that the activity of FX could predict the 28-day mortality after controlling for confounding factors following trauma [34]. Our measurement of FX in the injured by ELISA, which can determine the exact quantification of FX and activated FX combined, was not reported before. We showed that FX concentration was clearly reduced in TIC patients, which was consistent with previous reports showing the decrease of the activity of multiple coagulation factors after major trauma [11, 13]. The FX level was negatively correlated with ISS, SI, and positively with the hemoglobin level, which implied that the FX level might aggravate with the severity of the disease [35, 36]. Our findings added to the current evidence of coagulation factor deficiency after major trauma and

provided another proof for the rationale of the use of high ratio of FFP and PCC for the supplement of the depleted coagulation factors.

The liver is the primary source of a number of circulating coagulation factors. Dramatic changes in the homeostasis of the body can gravely impair its synthetic function. For example, fibrinogen production reduces to half after 2 hours of hypermetabolic state [37]. The disturbed coagulation system can be detected early as a result of reduced clotting factor production as evidenced by severe bleeding in acute hepatic failure [38]. miRNAs play a critical role in the fine-tuning of fundamental biological liver processes including glucose, lipid, iron, and drug metabolism [39]. However, studies regarding miRNAs and liver in the coagulation factor production are scanty [22, 40]. In our study, plasma miR-24 expression was negatively correlated with the FX level in trauma patients, and pretreatment with miR-24 significantly suppressed the level of FX in the supernatant of normal liver cell line L02, which agreed with the result of Salloum-Asfar et al. showing that miR-24 could reduce the FX mRNA level in HepG cell line [22]. Considering the synthesis of coagulation factors by the hepatocytes is an ongoing state which can be affected by miRNAs; our results in combination with others indicated that the elevation of miR-24 in the severely injured may have a role in the development of TIC by inhibiting the synthesis of FX. Therefore, miRNAs can be manipulated as therapeutic agents and miR-24 inhibitor may be developed into a novel therapeutic treatment for patients with TIC.

This study has several limitations. The patients were inconsecutive and the sample size was small; therefore, some of the values only reached borderline significance. The average hours from the scene were 8.1 hours, which is a lot longer than those of similar studies outside China. The main reason was that our hospital is a referral one located in the center of a large city and many patients were transferred from remote parts both inside and outside our province. The relatively long time from the scene to the hospital affected the effect that reduction in synthesis may have in the hypocoagulation state after severe trauma. The exact data on blood transfusion before ED visit was not available, which may be the main reason that the transfusion of PRBC and FFB difference between non-TIC and TIC patients did not reach significance. We did not collect data on lactate and BD values because blood gas analysis is not a routine test unless the patient has decreased oxygen saturation in our ED. Lastly, correlation does not mean causality and further basic studies are warranted to confirm if the reduction in synthesis of coagulation factors can be the cause of TIC.

In summary, we demonstrated that the miR-24 level is upregulated in trauma patients, especially in patients who developed TIC. The elevated miR-24 level is negatively correlated with the decreased level of FX. MiR-24 mimics can significantly reduce FX secretion in the cultured hepatocytes. These findings not only provide new insights from a different point of view into coagulopathy during major trauma but also suggest potential new diagnostic and treatment measures for TIC patients.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Lu-Jia Chen and Lian Yang contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81271609). There are no other sources of support such as pharmaceutical and industry support.

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