

# Dynamic Changes in Plasma Mitochondrial DNA Concentration in Patients with Severe Acute Pancreatitis

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## Research article

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# Abstract

**Background:** The elevated plasma mitochondrial DNA (mtDNA) is associated with prognosis in patients with severe acute pancreatitis (SAP). However, it is not clear that the dynamic process of plasma mtDNA during the early stage of SAP and the correlation between mtDNA and clinical features.

**Methods:** Twenty-six eligible patients with SAP in the general intensive care unit of our institution were enrolled in this study. The mtDNA concentration were assessed at admission and on days 3, 5, and 7.

**Results:** The mtDNA concentration of the patients with SAP was elevated at each time point compared with that in the healthy controls. The mtDNA levels increased rapidly, peaking on day 3 after admission, and began to decrease on day 5. The trend remained statistically consistent among the acute physiology and chronic health evaluation (APACHE II) score, the sequential organ failure assessment (SOFA) score, C-reactive protein (CRP) levels and mtDNA levels. Contrastingly, the changes were not statistically consistent among the procalcitonin (PCT), calciumion ( $\text{Ca}^{2+}$ ) and mtDNA concentrations. The mtDNA level correlated significantly with the APACHE II score, SOFA score, and Ranson score, but not with the CRP, PCT, and  $\text{Ca}^{2+}$  concentrations.

**Conclusions:** The dynamic change of plasma mtDNA correlated significantly with SAP development. The elevated mtDNA levels could be used as a biomarker for the early stage of SAP.

**Trial registration:** NCT: 04079777. Registered 4 September 2019 - Retrospectively registered, <https://register.clinicaltrials.gov/prs/app/action/SelectProtocol?sid=S00096E5&selectaction=Edit&uid=U000205I&ts=2&cx=-e6bci8>

## Background

Acute pancreatitis (AP), an inflammatory disorder of the pancreas, is the leading cause of admission to hospital for gastrointestinal disorders in the USA and many other countries[1]. Severe acute pancreatitis (SAP) is defined as acute pancreatitis complicated by persistent organ failure, regardless of whether the organ failure occurs in the early or late phase of the disease[2]. In the United States, AP results in health care costs of \$2.5 billion and accounts for 275,000 admissions each year[3]. Admissions have increased by at least 20% over the past 10 years[4]. Mortality associated with AP has decreased over time[5], and the overall mortality is now approximately 2%[4]. However, up to 25% of all patients with AP develop severe complications and are classified as having SAP, with an associated death rate of 30–50%[6]. Although advances in diagnosis and management have been made, SAP still represents a major health problem in many countries.

Acute pancreatitis is an inflammatory process that causes a local and systemic inflammatory response syndrome (SIRS)[7]. The pathogenesis of SAP is mainly blockage of pancreatic secretion, which impedes exocytosis of zymogen granules (containing digestive enzymes) from acinar cells[1]. The resulting accumulation of active trypsin within vacuoles can activate a cascade of digestive enzymes, leading to

autodigestive injury[1]. Organ failure is the most important determinant of outcome in SAP[8]. The development of organ failure appears to be related to the development and persistence of SIRS in patients with SAP[9].

Mitochondrial DNA (mtDNA) is found in the matrix or inner membrane of mitochondria[10]. Normally, it is strictly contained in mitochondria and is not exposed to the innate immune system, even after cell apoptosis[11]. However, cellular injury caused by SAP can release endogenous damage-associated molecular patterns (DAMPs) that activate innate immunity[12]. Injury releases mitochondrial DAMPs into the circulation, which cause SIRS, with functionally important clinical immune consequences[12].

Many predictors, including clinical and laboratory markers, such as white blood cells (WBC), erythrocyte sedimentation rate (ESR), serum amylase, and lipase; and various scoring systems, such as the acute physiology and chronic health evaluation (APACHE II) score, the sequential organ failure assessment (SOFA) score, and Ranson score systems, have been developed to improve clinical judgment; however, current research results show that no scoring system has an absolute advantage in terms of prediction ability[13, 14]. Whether plasma mtDNA, as a plasma biomarker, could better reflect the development of SAP and its therapeutic effect requires further study. In the present study, we aimed to investigate the dynamic process of plasma mtDNA during the early stage of SAP, and to determine the relationships between mtDNA and clinical features.

## **1 Methods**

### **1.1 Ethics Statement**

This study protocol was approved by the Institutional Review Committee on Human Research of the First People's Hospital of Chenzhou, Hunan Province, China (reference number 2018-012). Informed consent was obtained from all individual participants included in the study, including all patients and the healthy controls. The study was registered with the US National Institutes of Health Clinical Trials Register (NCT: 04079777) and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

### **1.2 Study Population**

From July 1, 2018 to January 31, 2020, a total of 26 consecutive patients with SAP were recruited for the prospective study after they were admitted to the general intensive care unit (ICU) of the First People's Hospital of Chenzhou. Ten healthy adult volunteers who received physical examination were recruited as the control group.

### **1.3 Diagnosis and Definition of Severity**

The diagnosis of acute pancreatitis requires two of the following three features: (1) Abdominal pain consistent with acute pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back); (2) serum lipase activity (or amylase activity) at least three times higher than the upper limit of normal; and (3) characteristic findings of acute pancreatitis on computed tomography (CT) and less commonly, magnetic resonance imaging (MRI) or transabdominal ultrasonography. Severe acute pancreatitis is characterized by persistent organ failure, which is defined as organ failure that persists for > 48 h[15].

## 1.4 Inclusion Criteria and Exclusion Criteria

Consecutive adult (at least 18 years old) patients with SAP admitted to the ICU were assessed for inclusion. The following patients were excluded: (1) Those who suffered SAP for more than 48 h before admission; (2) those who did not give their consent; (3) those with cancer; (4) pregnant patients; (5) those with hepatosis, defined as a Child Pugh C score of more than grade II; (6) those who had received or were receiving high-dose steroid treatment; (7) those who required renal replacement therapy (RRT); (8) those who had contracted AIDS; (9) those who had participated in other studies; and (10) those with a history of renal transplant. The following patients withdrew from the study during the period of observation: (1) Those who declined treatment or who died; (2) those who received blood transfusion of more than 1000 ml; (3) The patient's clinical experimental data were incomplete; or (4) The patients or their family members requested to withdraw.

## 1.5 Clinical Data Collection and Treatment

Upon admission to the general ICU, data on the patients' baseline characteristics were collected, including age, sex, etiological factors, and underlying diseases. Clinical biomarkers of SAP were also collected, such as the WBC count, and C-reactive protein (CRP), procalcitonin (PCT), and  $\text{Ca}^{2+}$  levels. In addition, other physiological and clinical information was collected and the condition of the disease was assessed using the APACHE II, SOFA, and Ranson scores. The clinical treatment of patients with SAP included in the study were based on the clinical guidelines for acute pancreatitis[16].

## 1.6 Blood Sample Collection

After admission, samples were taken at four time points: At admission, and on days 3, 5, and 7 after admission. Blood samples were collected into Ethylene Diamine Tetra-acetic Acid (EDTA)-containing tubes and processed within 1 h. The samples were centrifuged at  $1000 \times g$  for 10 min. The freshly isolated plasma was transferred into a clear polypropylene tube and then centrifuged at  $12,000 \times g$  for another 10 min to prepare the cell free plasma. The plasma was stored at  $-80 \text{ }^\circ\text{C}$  until further mtDNA extraction and detection.

## 1.7 Extraction and Detection of mtDNA

The mtDNA was extracted using a mtDNA isolation kit following the manufacturer's standard protocol[17]. Detection of plasma mtDNA was performed as reported previously[18]. Briefly, 200 µl cell-free plasma per patient was used for DNA isolation using an animal mtDNA column extraction kit (Sequencing Grade) (LMAI Bio - 120501) according to manufacturer's instructions. The extracted DNA was eluted using 200-µl gallium (GA) buffer and detected for nucleic purity using SMA4000 spectrophotometer (Merinton, Ann Arbor, MI, USA). The plasma mtDNA concentration was measured by determining the level of the human mitochondrial cytochrome B (h-mt-cytB) genes (ID: NC\_012920.1) using a real-time quantitative PCR assay performed on a 7500 real-time PCR system (ABI-7500, Applied Biosystems, Foster City, CA, USA). Two microliters (2.0 µg) of plasma DNA solution was added into a reaction solution containing 2.0 µl of PCR forward primer (2 µM), 2.0 µl of PCR reverse primer (2 µM), 0.4 µl of 50 × Rox Reference Dye and 3.6 µl of ddH<sub>2</sub>O to a final volume of 10 µl. Primers for the h-mt-cytB genes were: Forward: 5' - CTAGGCGACCCAGACAATTATAC- 3' and reverse: 5' - TTAGGGACGGATCGGAGAAT - 3'. The thermal profile was set up as follows: An initial denaturation step at 95 °C for 10 min, followed by 40 cycles of a denaturation step at 55 °C for 20 s and an annealing step at 95 °C for 10 s. The melting curve was obtained when the temperature was raised from 55 °C to 95 °C. The purity and quantity of the mtDNA amplicons were assessed using the absorbance at 260/280 nm and 260 nm, separately. This pure mtDNA was used for standard curve establishment. The absolute mtDNA concentration was calculated according to the standard curve. Each sample was run in triplicate, and the mean value was used for further analysis.

## 1.8 Statistical Analysis

The data were processed using Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) software. The measured data that followed a normal distribution in this analysis were expressed as the mean ± the standard deviation, such as age, body mass index (BMI), APACHE II score, SOFA score, and the concentration of mtDNA, Ca<sup>2+</sup>. Other measured data, such the time of symptom onset, and the levels of CRP and PCT, were expressed as median values (25th and 75th percentiles). The changes in plasma mtDNA levels over time and the plasma mtDNA concentration of patients with SAP with different etiologies or BMI were compared using an analysis of variance (ANOVA). Two-way ANOVA analysis was used to assess whether the trend of clinical data, score systems, and mtDNA levels remained statistically consistent. For correlation analysis among mtDNA, clinical data, and score systems, Pearson correlation coefficient analysis was used. To compare the statistical differences between the different ages and sexes, a *t* test was used. A *P* value < 0.05 was considered to indicate statistical significance.

## 2 Results

A total of 86 consecutive patients with SAP who admitted to the hospital and diagnosed with SAP were screened in the prospective study. Among them, 57 patients were later excluded according to the exclusion criterion and three patients withdrew during the observation period. Thus, a total of 26 patients with SAP, together with 10 healthy volunteers (5 male and 5 female), were included. The selection process for this experiment is shown in Fig. 1. The mean age of the patients with SAP was  $56.65 \pm 17.56$  years old, and 11 patients (42.31%) were male. Two patients died during 28 days of follow-up, giving a fatality rate of 7.69%. The baseline characteristics of the study patients are listed in Table 1.

Table 1  
Baseline Characteristics of the Patients

Characteristics	SAP patients ( n = 26 )
Age (years)	56.65 ± 17.56
Male, n (%)	11 (42.31)
BMI (kg/m <sup>2</sup> )	23.08 ± 3.29
Etiology, n (%)	
Biliary	11 (42.31)
Hypertriglyceridemia	7 (26.92)
Alcohol	3 (11.54)
Others	5 (19.23)
Underlying disease, n (%)	
Hypertension	4 (15.38)
Diabetes	7 (26.92)
CHD	6 (23.08)
Cerebral infarction	4 (15.38)
Others	13 (50.00)
Time of symptom onset (h)	17.58 (8.75, 24)
mtDNA (pg/mL)	9.96 ± 3.82
APACHE II score	18.50 ± 5.02
SOFA score	6.24 ± 1.96
Ranson score	3.96 ± 1.34
CRP (mg/L)	109.02 (36.75, 152.00)
PCT (ng/mL)	7.35 (0.26, 4.01)
Ca <sup>2+</sup> (mmol/L)	2.15 ± 0.29
Mortality, n (%)	2 (7.69)
<p><i>APACHE II</i> acute physiology and chronic health evaluation II, <i>BMI</i> body mass index, <i>Ca<sup>2+</sup></i> calciumion, <i>CRP</i> C-reactive protein, <i>CHD</i> coronary arteryheart disease, <i>mtDNA</i> mitochondrial DNA, <i>PCT</i> procalcitonin, <i>SAP</i> severe acute pancreatitis, <i>SOFA</i> sequential organ failure assessment</p>	

We detected plasma mtDNA levels in all the collected samples. We assessed the changes in plasma mtDNA levels during the early stage of SAP (0 to 7 days) d (Fig. 2A). The results showed that the mtDNA

levels were significantly higher at each time point compared those of the volunteers (all  $P < 0.0001$ ). The mtDNA levels increased rapidly, reaching a peak on day 3 after admission ( $13.23 \pm 3.24$  vs.  $8.61 \pm 3.49$  pg/mL,  $t = 6.285$ ,  $P < 0.0001$ ) and began to decrease on day 5 ( $9.81 \pm 3.59$  pg/mL) and again on day 7 ( $8.20 \pm 2.86$  pg/mL). Similarly, the APACHE II and SOFA scores and the CRP concentration increased on day 3, and decreased continuously on day 5 and day 7 (Fig. 2B, 2C and 2D). Two-way ANOVA analysis showed that the trend remained statistically consistent among the APACHE II score, SOFA score, CRP levels, and the mtDNA levels ( $F = 26.40$ ,  $P < 0.0001$ ;  $F = 23.49$ ,  $P < 0.0001$ ;  $F = 6.605$ ,  $P = 0.0005$ , respectively). By contrast, the changes were not consistent among the concentrations of PCT,  $\text{Ca}^{2+}$ , and mtDNA ( $F = 2.679$ ,  $P = 0.053$ ;  $F = 1.127$ ,  $P = 0.2884$ , respectively, Fig. 2E and 2F).

To assess the correlations between mtDNA and clinical features, Pearson correlation coefficient analysis was used. Correlation analysis showed that the APACHE II score, SOFA score, and Ranson score correlated statistically with the mtDNA level ( $r = 0.4065$ ,  $P < 0.0001$ ;  $r = 0.2581$ ,  $P = 0.0081$ ;  $r = 0.7302$ ,  $P < 0.0001$ , respectively) (Fig. 3A, 3B and 3C). However, there were no statistical correlations between the changes in CRP, PCT, and  $\text{Ca}^{2+}$  and the mtDNA level ( $r = 0.1824$ ,  $P = 0.0639$ ;  $r = -0.0224$ ,  $P = 0.8218$ ;  $r = -0.1038$ ,  $P = 0.2945$ , respectively, Fig. 3D, 3E and 3F).

Next, we determined the plasma mtDNA levels in patients with SAP under different conditions. There was statistically significant difference between the mtDNA concentration in the older ( $\geq 65$  years old) group and that in the younger ( $< 65$  years) group ( $t = 2.643$ ,  $P = 0.0095$ ) (Fig. 4A). Interestingly, the plasma mtDNA levels of female patients with SAP was significantly higher than that in the male patients ( $t = 2.471$ ,  $P = 0.0151$ ) (Fig. 4B). No statistically significant difference was found among patients with SAP with different etiologies with respect to plasma mtDNA levels ( $F = 1.431$ ,  $P = 0.2382$ ) (Fig. 4C). There was no statistically significant difference in plasma mtDNA levels according to body mass index (BMI) when the patients with SAP were divided into underweight (BMI  $< 18.5$ ), normal (BMI 18.5–23.9), and overweight (BMI  $\geq 24$ ) groups ( $F = 1.849$ ,  $P = 0.1627$ ).

### 3 Discussion

This study was an observational study designed to explore the dynamic changes in plasma mtDNA concentration in patients with SAP. We found that the circulating mtDNA concentration was elevated significantly in patients with SAP compared with that of the healthy control subjects. The levels of plasma mtDNA concentration increased significantly after 3 days before gradually decreasing on days 5 and days 7. Furthermore, the change trend of mtDNA concentration was statistically consistent with the APACHE II, SOFA, and Ranson scores.

Previous studies on the changes of mtDNA levels in pancreatitis reported a significant increase in cell-free mtDNA in plasma during pancreatitis compared with that in normal controls [19, 20]. In addition, the mtDNA concentrations in the early stage were elevated significantly, which was consistent with the findings of the present study. However, the relationship between the plasma mtDNA concentration and the severity of SAP remains largely unknown. The mtDNA in circulation is believed to originate from

necrotic cells, because mtDNA in apoptotic cell is broken down within the lysosomes without being released[21]. SAP is a process of autodigestion of the gland caused by prematurely activated pancreatic enzymes; therefore, the elevated mtDNA in patients with SAP might originate from necrotic pancreatic and peripancreatic cells[20]. In last 10 years, accumulating evidence revealed that mtDNA might act as an alarm or a DAMP when released from injured cells into the circulation, resulting in functionally important immune consequences[22]. Combined with the systemic treatment of the in hospital, such as fluid resuscitation therapy, the inflammatory response would be remitted. The plasma mtDNA concentration gradually decreased on days 5 and 7, remaining statistically consistent with the changes of APACHE II and SOFA scores and CRP levels.

In the present study, we found that the changes in plasma mtDNA levels showed a statistical consistency with the APACHE II, SOFA, and Ranson scores. Previous studies have confirmed that the APACHE II and Ranson scores are useful tools to evaluate the severity and prognosis of SAP[23–25]. The calculated Pearson correlation coefficients showed that that the mtDNA level correlated positively with the APACHE II, SOFA, and Ranson scores, suggesting that the mtDNA concentration might represent a new marker to evaluate SAP.

In the present study, statistically significant consistency was not detected between the levels of mtDNA and CRP. The levels of CRP, an inflammatory factor and sensitive indicator for inflammation diagnosis, increase markedly during tissue damage and acute inflammation[26]. Serum CRP levels of patients with SAP generally peak at 36–72 hours after disease onset, then gradually decreased with time[27]. Indeed, CRP lacked independent predictive value[28]. Our data were consistent with these previous findings. In addition, there was no statistical correlation between the changes in plasma PCT and  $\text{Ca}^{2+}$  levels and the mtDNA concentration. Despite their routine use in the clinic, PCT and  $\text{Ca}^{2+}$  are not specific predictors for SAP, because their levels could be affected by many other factors. PCT has been shown to be a biomarker with the potential to differentiate bacterial infections from viral infections and noninfectious inflammatory conditions[29]. Nonetheless, many conditions result in increased PCT serum concentrations, even in the absence of bacterial infections, which can make interpretation of PCT concentrations challenging[29]. The serum  $\text{Ca}^{2+}$ , in additional, may reflect the development of organ dysfunction in SAP[28]. However, the best predicted match between the serum  $\text{Ca}^{2+}$  and severity of organ failure in SAP was only after 24 h of symptom onset[28].

Interestingly, we found that, as reported previously, the mtDNA content of elderly patients with SAP was significantly higher than that of young and middle-aged patients[30]. Additionally, we found that the plasma mtDNA levels in female patients with SAP were higher than those in male patients, which was consistent with the findings of a previous study[31]. In terms of etiology, the whole data were analyzed to verified that there was no statistically significant difference between different etiologies with regard to plasma mtDNA concentration. All the well-known causes of AP trigger pathological cellular pathways and organelle dysfunction that culminate in the hallmarks of acinar cell death and local and systemic inflammation[32]. However, there was no statistically significant difference in plasma mtDNA levels in patients with SAP with different BMIs. This contrasts with previous research[33]. This might have been

because the number of included patients with obesity was too small and because the group with a low BMI contained more elderly patients.

However, there were several limitations to our study. First, this experiment is a single center study, which meant that the sample size was relatively small and the selected samples might lack clinical representation. Second, the experimental researchers did not participate in the clinical treatment of the patients with SAP and thus could not fully guarantee the homogeneity of treatment. To reduce the interference of this factor as much as possible, we formulated strict inclusion, exclusion, and withdrawal criteria. Besides, more rigorous expert consensus guidelines have been formed, with the accumulation of medical research and knowledge, to ensure the accuracy and timeliness of clinical treatment measures. Third, the number of deaths was too small; therefore, the comparison of relevant data between the deceased group and the surviving group was not of great clinical significance. Considering the limited sample size and the experimental conditions, the above deficiencies will be addressed in future studies to further support our research conclusions.

## Conclusions

In conclusion, the results of the present study suggest that plasma mtDNA is significantly elevated in patients with SAP and could be used as a biomarker for early prediction and monitoring of SAP.

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## Abbreviations

ANOVA: analysis of variance; AP: Acute pancreatitis; APACHE II: acute physiology and chronic health evaluation II; BMI: body mass index;  $Ca^{2+}$ : calcium ion; CHD: coronary artery heart disease; CRP: C-reactive protein; DAMPs: damage-associated molecular patterns; EDTA: Ethylene Diamine Tetra-acetic Acid; ESR: erythrocyte sedimentation rate; ICU: intensive care unit; mtDNA: mitochondrial DNA; PCT: procalcitonin; RRT: renal replacement therapy; SAP: severe acute pancreatitis; SIRS: systemic inflammatory response syndrome; SOFA: sequential organ failure assessment; WBC: white blood cells.

## Declarations

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## Acknowledgements

Not applicable.

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

This study protocol was approved by the Institutional Review Committee on Human Research of the First People's Hospital of Chenzhou T (reference number 2018-012). All participants or their family members gave written informed consent.

## Competing Interest

The authors declare that they have no competing interests.

## Contributions

XD and ZD designed the study, participated in the acquisition of the data, performed the data analysis, and drafted the manuscript. ZD and YW carried out the biochemical assays and contributed to the conception. HL and QL carried out the biochemical assays, performed the data analysis and interpretation of the data. XD guided the data analysis and the use of medical statistics, responded for protocol revisions, and final draft revision. All authors have read and approved the final manuscript for publication.

## Author details

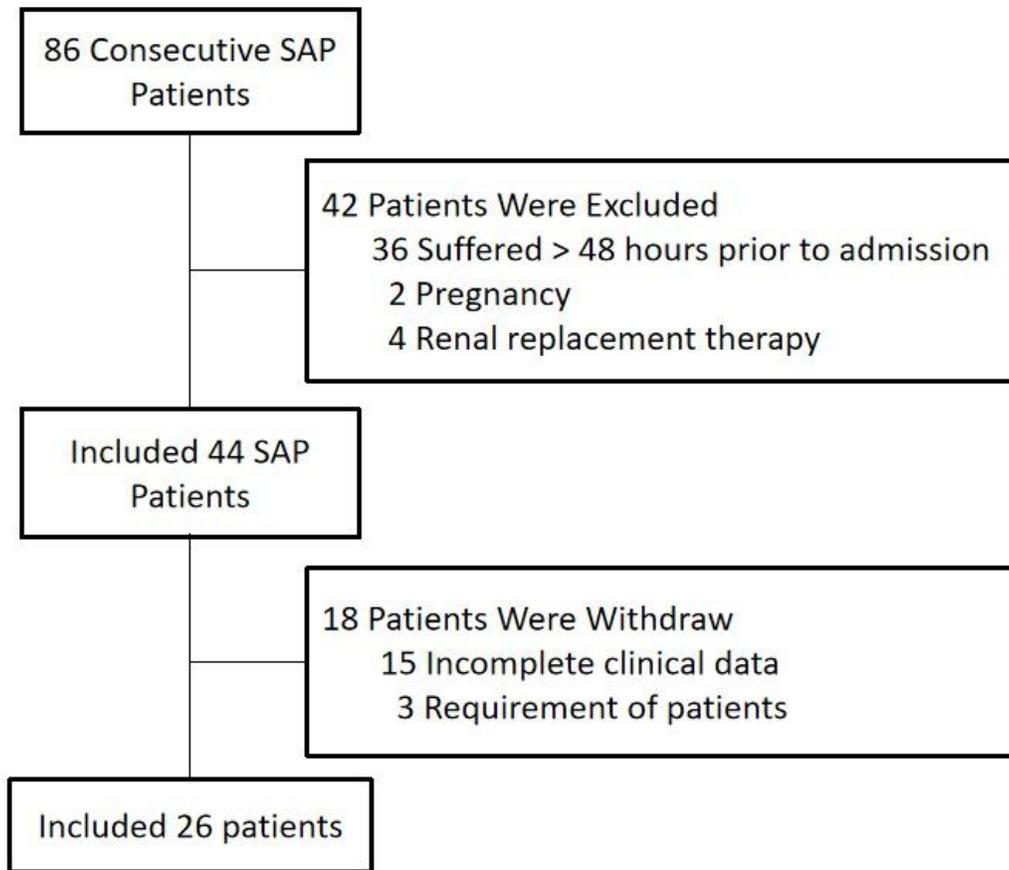
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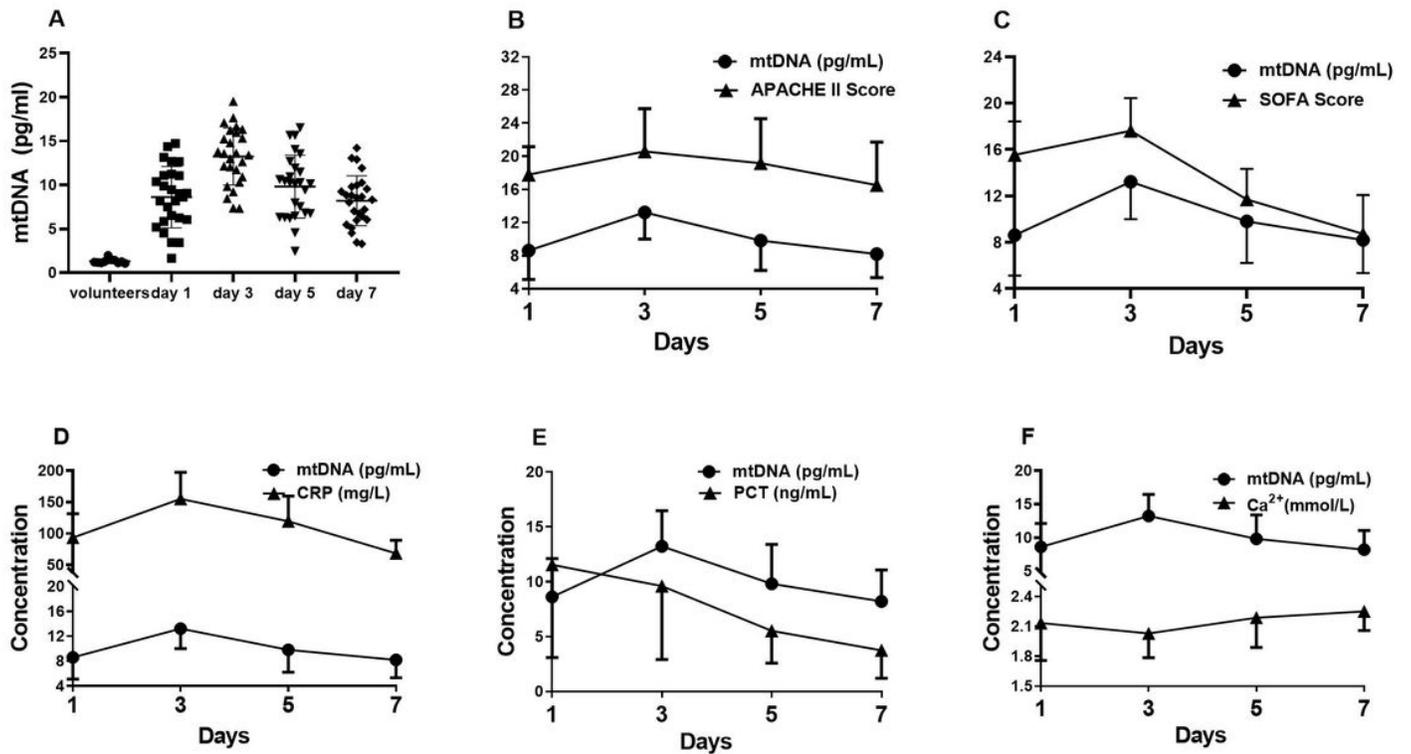
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## Figures



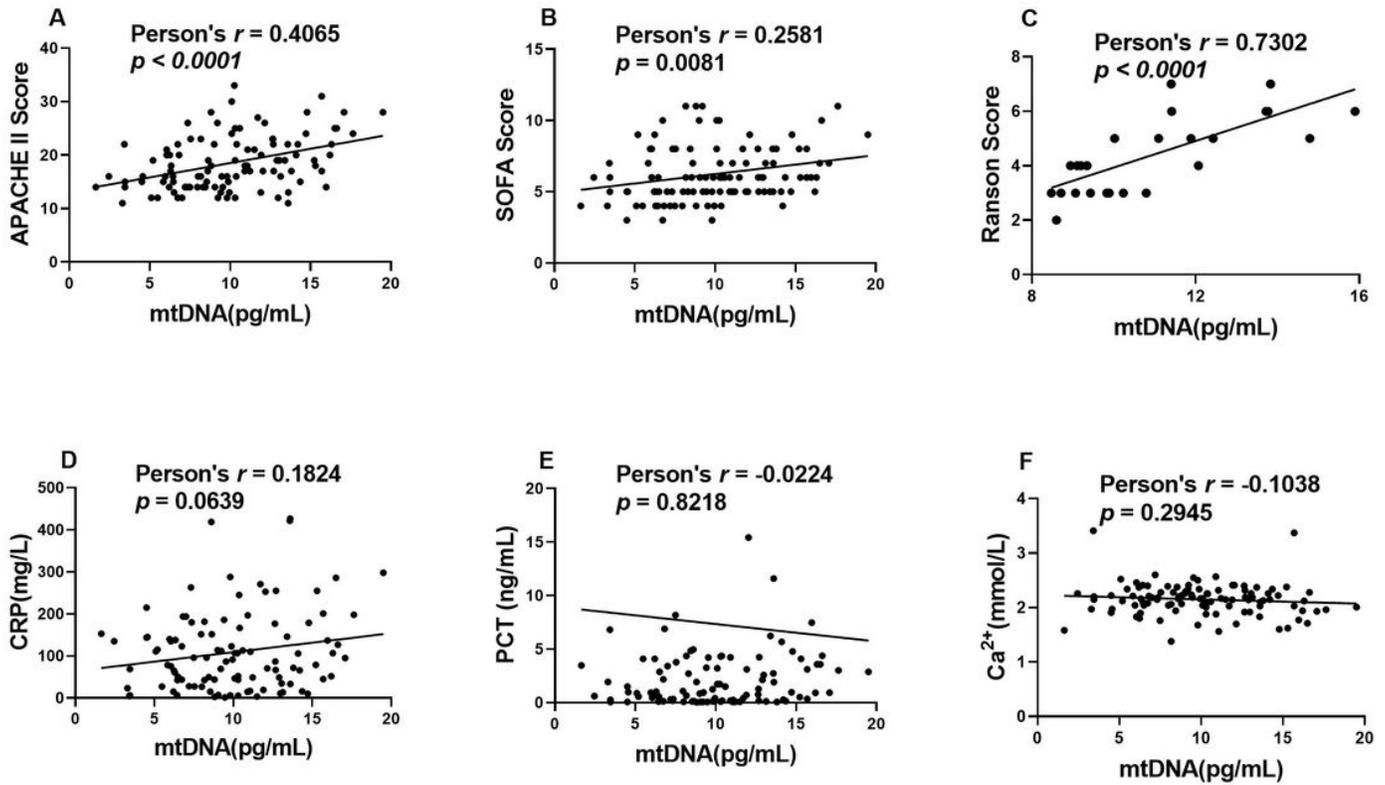
**Figure 1**

Flow chart depicting the selection process. SAP severe acute pancreatitis.



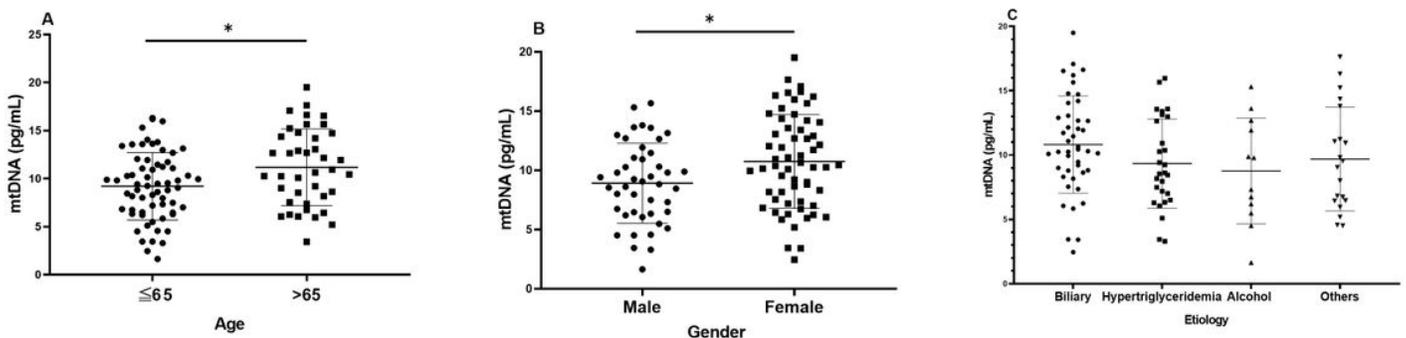
**Figure 2**

Dynamic Change in plasma concentrations of mtDNA in patients with SAP. A: Dynamic change of plasma mtDNA levels in patients with SAP and healthy volunteers. B-F: The changes in APACHE II score, SOFA score, and Ranson score, and CRP, PCT, and Ca<sup>2+</sup> levels over time. APACHE II acute physiology and chronic health evaluation II, Ca<sup>2+</sup> calciumion, CRP C-reactive protein, mtDNA mitochondrial DNA, PCT procalcitonin, SAP severe acute pancreatitis, SOFA sequential organ failure assessment.



**Figure 3**

Correlation analysis of plasma mtDNA, APACHE II score, SOFA score, and Ranson score, CRP, PCT, and calcium ions. A-C: APACHE II score, SOFA score and Ranson score showed a statistical consistency with the mtDNA concentration. D-F: There was no statistical correlation between the levels of plasma CRP, PCT, Ca<sup>2+</sup>, and mtDNA levels. APACHE II acute physiology and chronic health evaluation II, Ca<sup>2+</sup> calcium ion, CRP C-reactive protein, mtDNA mitochondrial DNA, PCT procalcitonin, SOFA sequential organ failure assessment.



**Figure 4**

The plasma mtDNA levels in patients with SAP with different conditions. A-B: There was a statistical difference in the mtDNA concentration among different ages and sexes in the patients with SAP. C: The

plasma mtDNA levels in patients with SAP with different etiologies. No statistically significant differences were found among patients with SAP with different etiologies regarding plasma mtDNA levels. \*P < 0.05. mtDNA mitochondrial DNA, SAP severe acute pancreatitis.