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Highlights

- Whole‑blood mNGS increases pathogen detection (53.26% → 58.70%) and identifies more fungi in cells (30.2% vs. 17.0%).
- Cell‑layer testing captures sedimentation‑prone/intracellular pathogens, reducing missed detection and enabling comprehensive diagnosis.
- cfDNA features predict prognosis. Richness protects; diversity/SOFA are risks. Combined SOFA‑cfDNA model predicts outcomes best (AUC 0.95).

Whole blood metagenomic next-generation sequencing in the diagnosis of bloodstream infection in patients with hematological diseases

Xinhao Chai[1], Xing Zhang[2], Dongsheng Chen[2], Dongwen Rong[1*]

[1] Department of Oncology, The first Hospital of Shanxi Medical University, Taiyuan, 030001, China.

[2] State Key Laboratory of Neurology and Oncology Drug Development, Jiangsu Simcere Diagnostics Co., Ltd., Nanjing Simcere Medical Laboratory Science Co., Ltd., Nanjing, 210042, China.

* Corresponding author: Dongwen Rong (dongwenrong2022@163.com)

Abstract

Objective: To evaluate the value of cell-free DNA (cfDNA) in plasma and genomic DNA (gDNA) in nucleated cell layer of whole blood samples detected by metagenomic next-generation sequencing (mNGS) in the diagnosis of bloodstream infection in patients with hematological diseases.

Methods: Whole blood samples collected from hematologic patients with suspected bloodstream infections were divided into the plasma and nucleated cell layers. The DNA of plasma and nucleated cell layers was extracted for mNGS. The pathogenic results were compared between whole blood (plasma plus nucleated cell layers) and plasma layer. In addition, the factors influencing the prognosis at discharge were analyzed.

Results: Totally 92 patients were included. The positive rate of mNGS in whole blood was higher than those of the single plasma layer (58.70% *vs.* 53.26%) and the culture layer (58.70% *vs*. 17.39%). The consistency of plasma and nucleated cell layers was 57.6%. The proportion of fungi detected in nucleated cell layer was higher than that in plasma layer (30.2% *vs.* 17.0%). Ten patients had extra pathogens detected in whole blood compared with the single plasma layer, and the positive rate of mNGS increased by 10.87%. gDNA microbe reads and non-host ratios in the extra-detection group were significantly higher than those in the non-extra detection group. cfDNA microbe reads, non-host ratios and microbe percent showed no significant differences between the two groups. The maximum Sequential Organ Failure Assessment (SOFA) score and age in the death group were significantly higher, while cfDNA/gDNA species richness was significantly lower compared with the survival group. The maximum SOFA score and cfDNA Shannon diversity index were found as risk factors for improved prognosis. The maximum SOFA score and cfDNA concentration were combined for the diagnosis of poor prognosis at discharge, with the highest area under the curve of 0.95.

Conclusion: Simultaneous metagenomic sequencing of plasma layer and nucleated cell layer contributes to the detection of pathogens in patients with bloodstream infection. cfDNA detection has a certain significance in predicting the prognosis of patients with bloodstream infection.

Keywords: Metagenomic next-generation sequencing; Bloodstream infection; Whole blood; cfDNA; gDNA; hematological diseases

Introduction

Patients with hematological diseases are susceptible to infection due to long-term immunotherapy. More than 80% of patients with hematologic malignancy develop fever associated with agranulocytosis after at least one course of chemotherapy, and they are likely to experience bloodstream infection, with the mortality of 7.1%-42% [1-3]. If appropriate antibiotics are not given in time, the infection-related mortality would be higher [4]. Although initial empirical anti-infective therapy can improve the prognosis of patients and reduce the mortality [5], the empirical use of antibiotics can cause bloodstream infection with pathogens difficult to culture, consequently resulting in negative culture results [6,7]. It is reported that about 35% of bloodstream infections or sepsis are difficult to be identified by conventional culture methods [8]. Currently, the bloodstream infection and mortality of hematologic malignancy patients with febrile agranulocytosis are also relatively common in China. Therefore, it is urgently needed to seek a rapid, highly sensitive method to detect the pathogens which are difficult to culture or negative for bloodstream infections.

Recently, serological reactions and molecular biology techniques are developing rapidly in addition to blood culture, a golden standard for the diagnosis of pathogens in bloodstream infection. The development and clinical application of Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), droplet digital polymerase chain reaction (ddPCR), gene chip, metagenomics next generation sequencing (mNGS) and other techniques for pathogen identification

based on nucleic acid detection have greatly improved the diagnostic efficiency of bloodstream infection, which is conducive to improving the prognosis of patients with bloodstream infection. It has been reported that the sensitivity of mNGS for the detection of pathogens in plasma cell-free DNA (cfDNA) was as high as 93%, and 62 out of 166 culture-negative blood samples were detected by mNGS [9].

Nowadays, most of mNGS detection processes for blood are to centrifuge the blood and take the supernatant to detect cfDNA, which will lead to missed detection due to the particle precipitation of some pathogens. On the other hand, some intracellular parasites cannot be detected in plasma cfDNA at some time. Consequently, we developed a mNGS detection process of whole blood (plasma cfDNA+ nucleated cell gDNA). The comprehensive detection of nucleic acid in the supernatant and sediment of whole blood is expected to improve the positive rate of pathogenic microorganism detection in blood samples, which will bring great clinical benefits for patients with bloodstream infection. Therefore, by comparing the pathogen detection of whole blood and plasma samples in patients with hematological diseases, we further evaluated the value of mNGS in plasma and nucleated layers in bloodstream infection.

Methods

Study population and design

The whole blood samples were collected from patients with clinically suspected bloodstream infections at the time of fever in Department of Medical Oncology, First

Hospital of Shanxi Medical University. Inclusion criteria were as follows: (a) diagnosis of hematological diseases; (b) unilateral axillary temperature ≥ 38.3 ℃ or ≥ 38.0 ℃ for 1 h; (c) blood collected at the time of the fever. Patients without the results of blood culture, mNGS, and clinical information were excluded.

DNA extraction

The sterilized samples from the original containers were labeled (such as name and original tube number). After centrifugation at 1 500 g for 15 min, all the plasma was aspirated into 2 mL EP tubes and labeled. The suction operation was as follows: use a 1000 uL pipettor to suck the middle nucleated cell layer, and gently rotate and suck about 200 uL along the tube wall from outside to inside into the 2 mL centrifuge tube. Notably, the tip of pipettor was always controlled on the surface of the liquid to avoid immersing the blood cell layer. The separated plasma and nucleated cell layers were used for nucleic acid extraction. Then, 2 mL plasma was centrifuged at 14 000 g for 10 min (4 °C). After centrifugation, the supernatant was collected into a new 10 mL centrifuge tube and cfDNA was extracted directly with the Apostle MiniMaxTM kit (Apostle,A17622-ACRG-LV) without wall breaking. The precipitate from the bottom of the tube was mixed with 200 uL of the nucleated cell layer and added to the sequenced MP Lysing Matri E tube. 200 μL of GB lysate was added, and the MP wall-breaking machine FastPrep-24™ 5G was shaken at 6 m/s for 120 s. Next, the MP Lysing Matri E tube was centrifuged at 14 000g for 5 min. After centrifugation, all the supernatant was added to a new 2 mL EP tube, and gDNA nucleic acid was extracted using the Microsample genomic DNA extraction kit (DP316, Tiangen). The

processing flow of the samples is shown in **Fig. 1**.

Library preparation and sequencing

The extracted nucleic acids were subjected to quantitative nucleic acid quality inspection using Qubit 4.0. The qualified nucleic acids were entered into the library construction step, and the DNA was broken to about 200 bp by enzyme digestion, followed by end repair, A-tail addition, adapter ligation and tag labeling. Finally, the DNA was enriched by PCR, the Agilent 4200 was used to detect the fragment size of the library, and the Qubit 4.0 was used to check the quality of the library concentration. Fragments and libraries with qualified concentrations were sequenced on NextSeq 550 DX sequencers (Illumina, CA, United States) with single end ≥50 bp.

Bioinformatics analyses

Low-quality and short sequences (<50 bp) were removed. Fastp was used to remove duplicated reads. Human sequences were removed using Burrows-Wheeler Alignment with reference to human Genome (GRCh38). And remaining reads were aligned to NCBI nt database by SNAP and considered to have potential value in detection of microorganisms. Microbial classification and verification were performed with Kraken2 and BLAST. All the mapped reads were processed to taxonomy annotation, genome coverage calculation and abundance calculation with in-house scripts.

Statistical analysis

SPSS 22.0 statistical software was used for data analysis, and Graphpad Prism 8 were used for plotting. Normally distributed data were expressed as mean ± SD and compared between groups using the *t* test. Non-normally distributed data were

expressed as the median [first quartile (Q1), third quartile (Q3)], and non-parametric Mann-Whitney U test was used for comparison. The counting data were expressed as the number of cases (percentage) [n (%)], and the data between groups were compared by chi-square test or Fisher's exact test. A two-tailed value of $p<0.05$ indicated statistical significance.

Results

Baseline information

From January to September in 2022, a total of 100 patients were enrolled, among whom 8 were excluded due to unqualified samples and lack of clinical information. Finally, 92 patients were included in this study. The average age of patients was 52 ± 16 years. 77 patients were diagnosed with hematological tumors and 15 with other hematological diseases. The clinical baseline data of these patients are shown in **Table 1**.

The distribution and positive rate of mNGS in plasma and nucleated cell layers

The detection and relative abundance of pathogens in different types of blood diseases are shown in **Fig. 2A**. The positive rate of mNGS in whole blood was higher than those of the single plasma layer (58.70% *vs.* 53.26%) and the culture layer (58.70% *vs*. 17.39%) (**Fig. 2B**). The consistency of plasma and nucleated cell layers was 57.6%, among which the positive and negative concordant rates were 16.3% and 41.3%, respectively. The proportion of fungi detected in the nucleated cell layer was higher than that in the plasma layer (30.2% *vs.*17.0%, **Fig. 2C**).

Extra pathogens detected by mNGS in whole blood

Of the 92 patients, extra pathogens were detected in the whole blood of 10 cases compared with the single plasma layer, and the positive rate of mNGS increased by 10.87%. Extra pathogens in 3 cases were verified by qPCR, and the positive rate of pathogens proven in whole blood increased by 3.3%. Cases of extra pathogens detected in whole blood are listed in **Table 2**. In the extra-detection group, gDNA microbe reads and non-host ratio were significantly higher than those in the non-extra detection group. There were no significant differences in the plasma cfDNA non-host percent, microbe reads and non-host ratios between extra and non-extra detection group (**Fig. 3**). Comparison of DNA indexes between culture-negative (n=16) and culture-positive (n=73) groups further showed no statistical differences (p>0.05).

Correlation between infection severity and cfDNA and gDNA detection

Patients were classified into non-agranulocytosis group (n=37) and agranulocytosis group (n=55) according to presence or absence of agranulocytosis during hospitalization. It could be observed that there were significant differences between the two groups in hospital stays (18 *vs.* 27 d), procalcitonin (PCT, 0.35 *vs.* 0.89 ng/mL), high-sensitivity C reactive protein (hsCRP, 82.6 ± 66.4 *vs.* 124.2 ± 71.9 mg/L), cfDNA species richness (defined as the count of distinct species detected) (7 *vs.* 11), gDNA species richness (10 *vs.* 20), cfDNA non-host ratio (0.02% *vs.* 0.04%), and gDNA non-host ratio (0.02% *vs.* 0.16%)(**Fig. 4**).

Patients' prognosis at discharge

According to the physiological characteristics and symptoms at discharge, the patients

were divided into the improved prognosis group (n=70) (improved symptoms upon discharge) and poor prognosis group (n=22) (no significant changes in symptoms, or worse or death at discharge). The distribution of hormone use (68.2% *vs.* 37.1%), kidney damage (27.3% *vs.* 8.6%), cardiac insufficiency (36.4% *vs.* 10%) and respiratory failure (27.3% *vs.* 1%) were statistically significant between the poor and improved prognosis groups. The maximum SOFA score, age and PCT at discharge in the poor prognosis group were significantly higher than those in the improved prognosis group (**Fig. 5A**). To further analyze the clinical characteristics between dead (n=11) and survived (n=81) patients, we found the maximum SOFA score and age in the dead group were significantly higher, while the cfDNA/gDNA species richness was significantly lower compared with those in the survived group. No significant differences were presented in non-host radios and Shannon diversity indexes [10] (**Fig. 5B)**.

Analysis of the prognostic factors

The factors associated with prognostic improvement at discharge were analyzed in **Table 3**. The maximum SOFA score (OR=0.45, 95%CI: 0.28-0.73, p=0.01) and cfDNA Shannon diversity index (OR=0.04, 95%CI: 0.00-0.80, p=0.04) were significantly associated with a higher likelihood of prognostic improvement. In contrast, cfDNA species richness (OR=1.27, 95%CI: 1.00-1.61, p=0.05) was associated with a lower likelihood of prognostic improvement.

Evaluation of diagnostic models

The maximum SOFA score has the highest diagnostic efficiency in predicting the

prognosis of patients(**Fig. 6**). The model combining the maximum SOFA score and cfDNA concentration for diagnosing poor prognosis at discharge achieved an area under the curve (AUC) of 0.95.

Discussion

In this study, the value of whole blood metagenomic sequencing in pathogen diagnosis was evaluated by analyzing the pathogen detection distribution in plasma and nucleated cell layers through metagenomic sequencing and culture in 92 patients with hematological diseases who were suspected of bloodstream infection during fever. In pathogen diagnosis, the metagenomic sequencing of whole blood identified additional pathogens compared with cfDNA, especially in the detection of fungi at the level of nucleated cells. Both cfDNA and gDNA sequencing results were far superior to culture methods. Notably, in assessing the outcome of patients, the maximum SOFA score during hospitalization was found to be a key and highly effective predictor of poor outcomes or death, and cfDNA might be expected to be a potential marker.

As previously reported, the positivity rate of mNGS in cfDNA from plasma in patients with hematologic malignancy accompanied by febrile agranulocytosis was totally 43% and 32% except for viruses compared with 14% of blood culture [11]. In this study, the positive rate of cfDNA detection was 53.26%, and the positive rate of pathogens detected by mNGS based on cfDNA and gDNA was 58.7%, suggesting the improvement of the detection rate of pathogens in blood samples. It has been reported that the positive rate of whole blood samples was higher than that of plasma samples

when tNGS was used to detect pathogens of infective endocarditis. However, when the results of whole blood and plasma samples were combined, the positive rate of pathogen diagnosis could increase to 66% [12]. In the positive detection results of mNGS, whether in plasma layer or nucleated cell layer, the positive rate of viruses was the first. Excluding viruses, bacteria were mostly detected in the plasma layer, while fungi were mostly detected in the nucleated cell layer. Unfortunately, this study did not provide guidance on drug treatment for patients because the patients' blood was frozen, and the last testing was performed. Previous studies reported that the plasma mNGS results led to a positive impact in 57.1% of patients by initiating targeted therapy [13].

Increased cfDNA levels have been reported in septic patients and are indicative for mortality associated with sepsis [14-17]. In our study, the concentration of cfDNA in the blood of patients at the time of fever was also confirmed to have a prognostic value in patients at discharge. Of course, SOFA score still played a key and dominant role in evaluating the prognosis. In pediatric patients with hematological malignancy, mNGS has been demonstrated a novel approach to determine the microbiological etiology of fever of unknown origin, and integration of interleukin-6 can improve the diagnostic precision of bacterial infection [18]. Species richness was significantly decreased in patients, especially in non-survivors. However, inconsistent with other reports [19], we found no statistical differences in Shannon diversity index between the two groups. The possible reasons might be associated with the inconsistency in the calculation method of Shannon diversity index and different settings of the

signal-generating threshold.

Recently, scholars have integrated host and pathogen metagenomic RNA and DNA next generation sequencing of whole blood and plasma from critically ill patients to diagnose sepsis [20]. In this study, by analyzing the human sequence of the host in whole blood with metagenomic DNA next-generation sequencing, we further established a model of whole blood human gene sequence for the diagnosis of prognosis in patients with hematological diseases. Similarly, there are reports that integrate plasma cfDNA quantity, human cfDNA fragmentation patterns, infecting pathogens, and overall microbial composition to diagnose sepsis and predict mortality as soon as the first day of ICU admission [19].

This study had some limitations that should be cautiously interpreted. First, the positive rate of whole blood metagenomic sequencing was low in the overall samples, affecting the evaluation on the diagnostic value of whole blood metagenomic sequencing in additional pathogens. Meanwhile, this also led to a small sample size in the extra detection group when analyzing the extra detection group and the non-extra detection group, which may impact the statistical power. Second, the blood of suspected patients was frozen and finally tested uniformly, leading to difficulty in conducting prospective clinical treatment. In the future, we will conduct a prospective study to further evaluate the value of whole blood mNGS in guiding clinical treatment.

In summary, simultaneous metagenomic sequencing of plasma and nucleated cell layers contributes to the detection of pathogens in patients with bloodstream infection.

cfDNA detection has a certain significance in predicting the prognosis of patients with bloodstream infection.

Figure legends

Fig. 1 The flow chart of metagenomic sequencing of plasma and nucleated cell layers.

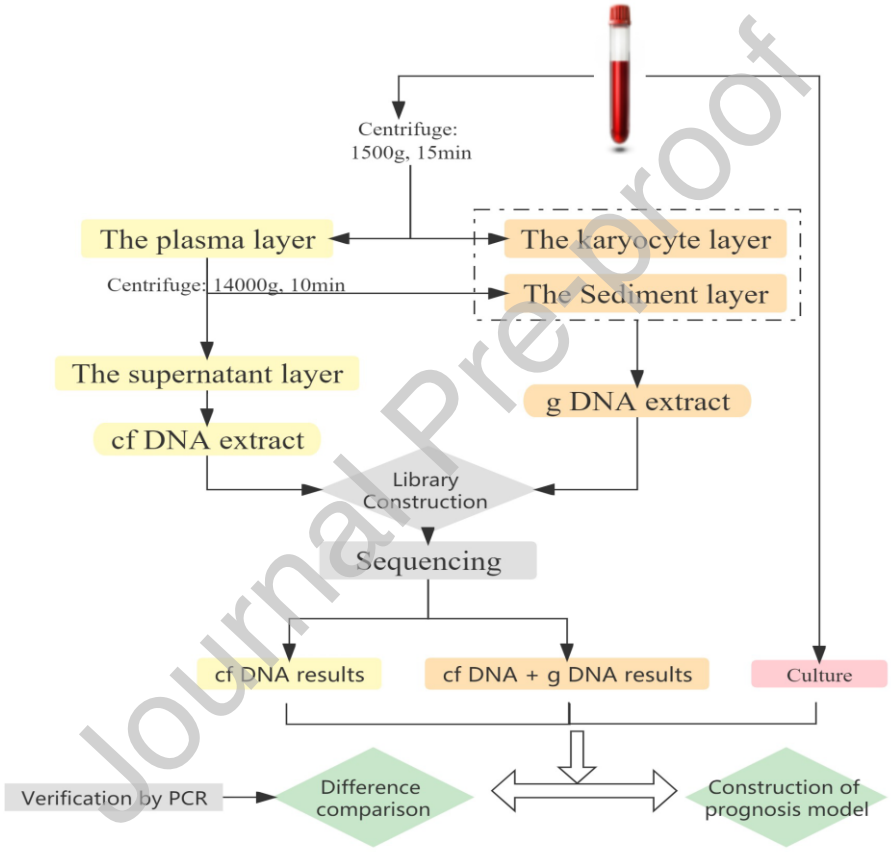


Fig. 2 Microbial detection in plasma and nucleated cell layers. **A**: Pathogen detection distribution in plasma and nucleated cell layers; **B**: Comparison on the positive rates of mNGS and culture in plasma, nucleated cell layer and whole blood. **C**: Comparison on the distribution of pathogen species in plasma and nucleated cell layers.

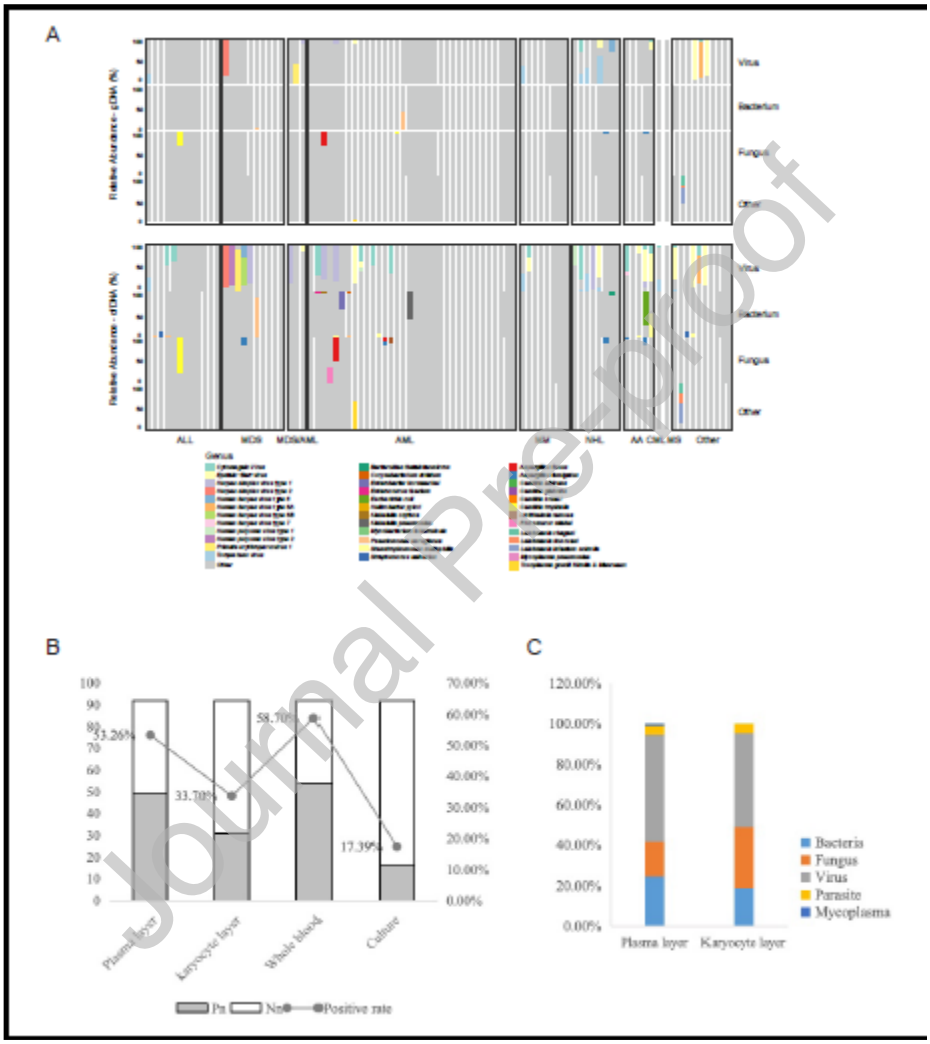


Fig. 3 Analysis of DNA indexes between whole blood extra-detection group and non-extra detection group.

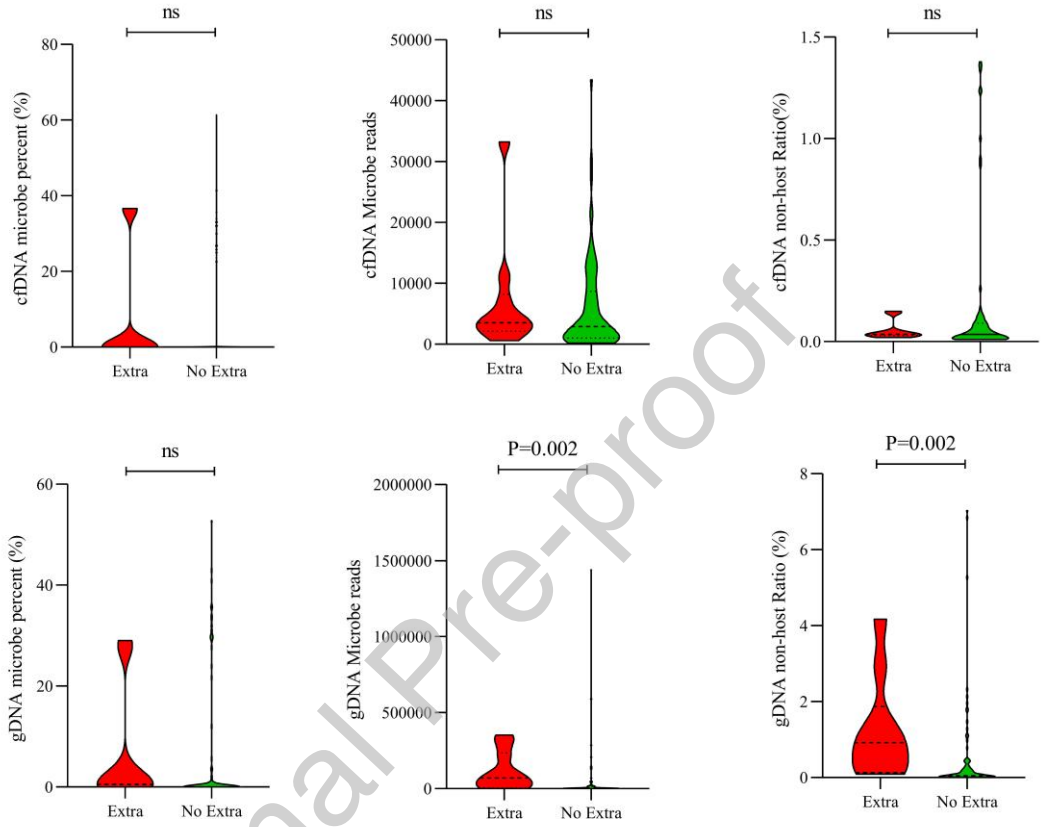


Fig. 4 Comparison of infection severity and DNA indexes between agranulocytosis and non-agranulocytosis groups. **A**. Comparison of inflammatory indexes between agranulocytosis and non-agranulocytosis groups. **B**. Comparison of the number of species detected by mNGS and non-host ratio between agranulocytosis and non-agranulocytosis groups.

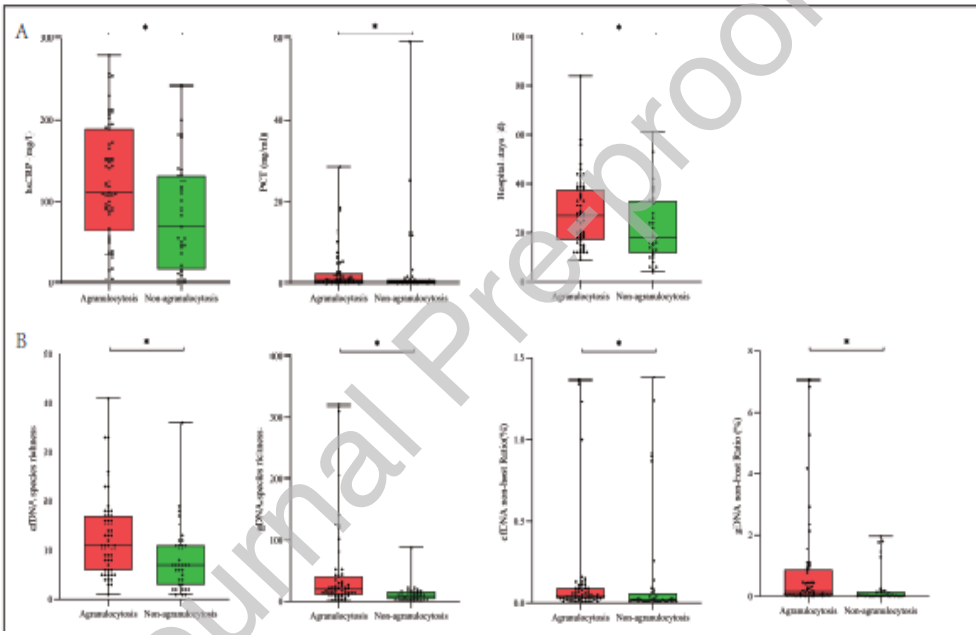


Fig. 5 Comparison of patients' prognosis at discharge.

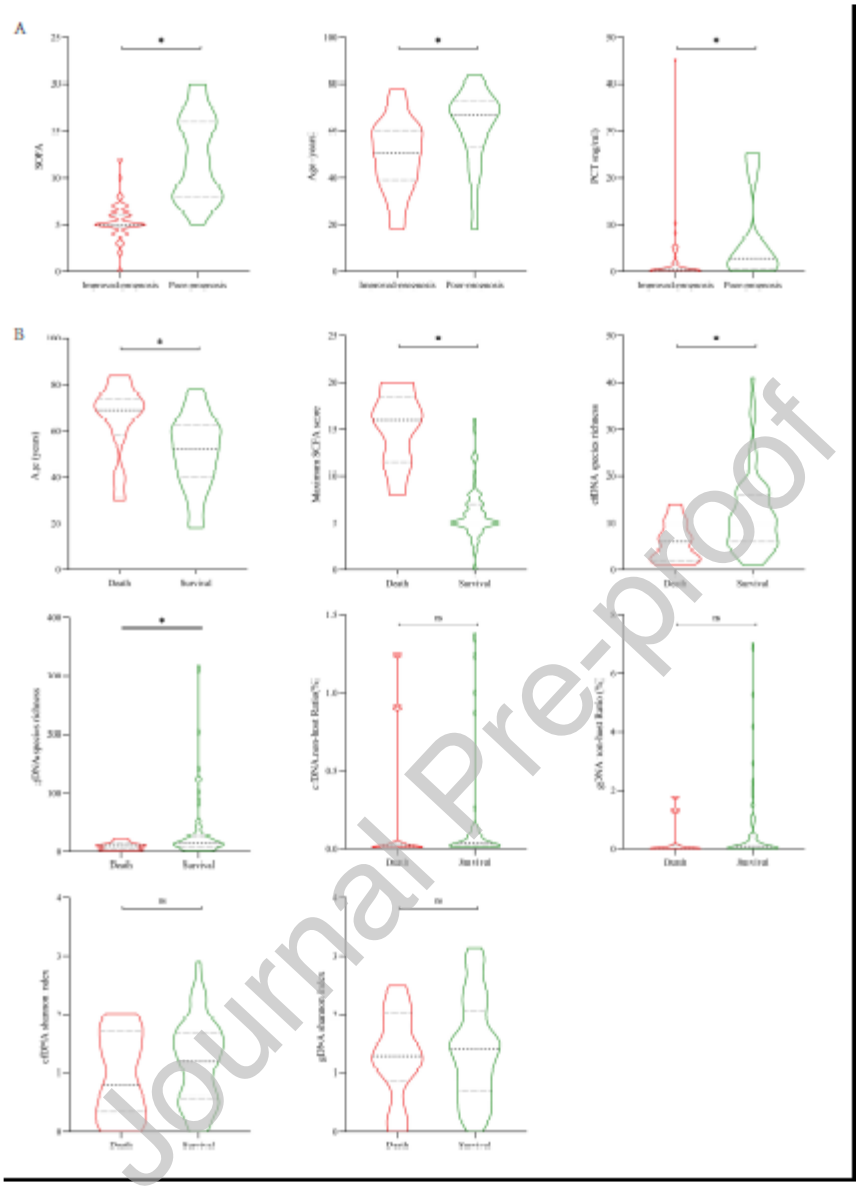
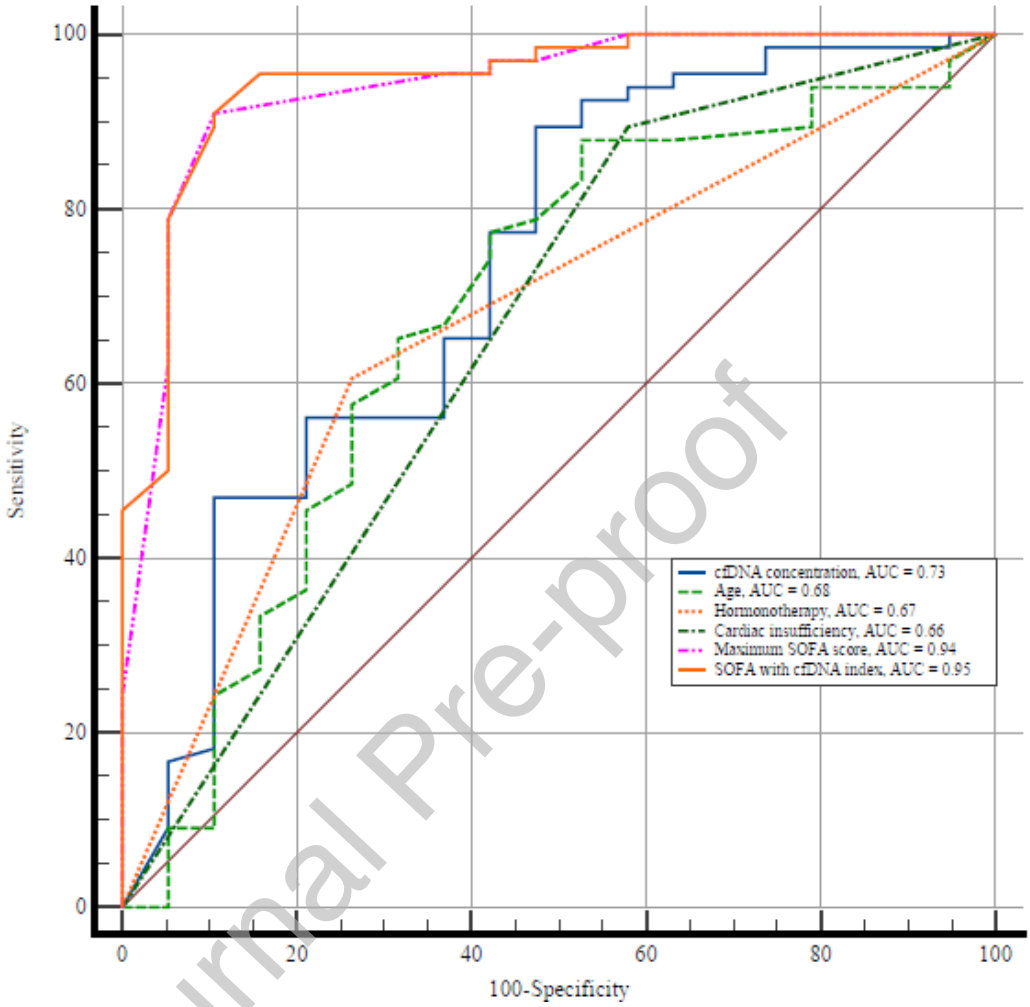


Fig. 6 ROC in diagnosis of poor prognosis at discharge.



Declaration of competing interest

Author XZ, DC were employed by Jiangsu Simcere Diagnostics Co. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

Not applicable.

Ethics statement

This study was reviewed and approved by Nanjing Simcere Medical Laboratory Institutional Review Board (NSML-IRB-202210-MS22).

Author contributions

HC, DR participated in study design and writing the manuscript. XZ, DC analyzed the biomolecular data. All authors contributed to the article and approved the submitted version.

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Table 1 Clinical characteristics of patients (n=92)

Characteristics	Value
Gender , [n(%)]	
Male	47 (51.1)
Female	45 (48.9)
Age, [Years, Mean±standard]	52±16
Hypertension, [n(%)]	16 (17.4)
Diabetes, [n(%)]	13 (19.1)
Hospital stays, [Days, median (IQR)]	24 (14.8, 34.2)
SOFA score within 48 hours of admission [median (IQR)]	4 (3, 5)
Highest SOFA score during hospitalization [median (IQR)]	6 (5, 8)
Liver damage, [n(%)]	27 (29.3)
Kidney damage, [n(%)]	12 (13.0)
Cardiac insufficiency, [n(%)]	15 (15.8)
Gastrointestinal bleeding, [n(%)]	4 (4.3)
Respiratory failure, [n(%)]	7 (7.6)
Other malignant tumours, [n(%)]	6 (6.5)
Abdominal infection, [n(%)]	18 (19.6)
Classification of hematological diseases, [n(%)]	
AML	37 (40.2)
ALL	12 (13.0)
MDS	10 (10.9)
MS	1 (1.1)
MM	8 (8.7)
NHL	8 (8.7)
CML	1 (1.1)
AA	5 (5.4)
AIHA	1 (1.1)

HPS	4 (4.3)
IDA	1 (1.1)
ITP	1 (1.1)
Undefined diagnosis	3 (3.3)
Inflammatory markers on admission, [median (IQR)]	
White blood cell count (* 10^9 /L)	0.55 (0.22, 4.49)
Neutrophils count (* 10^9 /L)	0.13 (0.01, 2.56)
Lymphocyte count (* 10^9 /L)	0.33 (0.09,0.62)
Platelet count (* 10^9 /L)	23 (9,43)
hsCRP (mg/L)	108.21 (46.01,154.65)
PCT (ng/ml)	0.67 (0.20,1.75)
Prognosis at discharge, n(%)	
Improved	70
Death	11
Poor	11

Abbreviations: AML, Acute myelocytic leukemia; ALL, Acute lymphoblastic leukemia; MDS, Myelodysplastic-syndromes; MS, Myeloid sarcoma; MM, Multiple myeloma; NHL, Non-Hodgkinlymphoma; CML, Chronic myelognous leukemia; AA, Aplastic anemia; AIHA, Autoimmune hemolytic anemia; HPS, Hemophagocytic syndrome; IDA, Iron Deficiency Anemia; ITP, Primary immune thrombocytopenia.

Table 2 Characteristics of extra pathogens detected in whole blood

Patient number	Extra pathogens	qPCR validation	Culture	Reads	Relative abundance (%)	RPM
P5	Herpes simplex virus type 1	Positive	Negative	3	2.05	5
P26	Candida tropicalis	Negative	Negative	23	7.14	207
P27	Pseudomonas aeruginosa	Unvalidated	Negative	36940	43.29	140248
P28	Human herpesvirus 6	Unvalidated	Fusobacterium nucleatum	6	30	365
P35	Candida krusei	Unvalidated	Negative	4	2.02	170
P37	Candida tropicalis, Herpes simplex virus type 1	Negative, Unvalidated	Negative	3,4	0.29, 9.30	16, 21
P39	Candida tropicalis	Negative	Negative	7	0.43	18
P49	Aspergillus flavus	Positive	Capnocytophaga sputigena	507	31.85	3038
P55	Aspergillus flavus	Positive	Negative	55	0.74	70
P79	Primate erythrocyte parvovirus type 1	Unvalidated	Negative	17	47.22	1013

Table 3 Factors influencing the prognostic improvement of patients at discharge

Variables	B	S.E.	P	OR	95% CI	
Age	-0.01	0.03	0.75	0.99	0.94	1.05
Respiratory failure	-0.43	3.14	0.89	0.65	0.00	309.31
Maximum SOFA score	-0.79	0.24	0.01	0.45	0.28	0.73
PCT (At discharged)	-0.09	0.05	0.08	0.91	0.82	1.01
cfDNA Shannon diversity index	-3.14	1.49	0.04	0.04	0.00	0.80
cfDNA species richness	0.24	0.12	0.05	1.27	1.00	1.61

Abbreviations: B, regression coefficient; S.E., standard error; P, p-value; OR, odds ratio; 95% CI, 95% confidence interval.

Declaration of Interest Statement

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author XZ, DC were employed by Jiangsu Simcere Diagnostics Co. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.