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MAJOR ARTICLE

# From Chart Biopsy to Liquid Biopsy: Evaluating the Diagnostic Yield and Clinical Impact of Plasma Microbial Cell-Free DNA Next-Generation Sequencing in the Management of Fever of Unknown Origin

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***Background.*** The underlying cause of fever of unknown origin (FUO) remains unidentified in up to 51% of cases despite systematic evaluation. Microbial cell-free DNA next-generation sequencing (mcfDNA-NGS) offers an agnostic, noninvasive approach to pathogen identification, but the utility and clinical impact of this assay in FUO remain unknown.

***Methods.*** This retrospective cohort study evaluated adult patients referred for FUO evaluation at a tertiary medical center between November 2019 and November 2023. Patients underwent both standard microbiologic testing (ST) and mcfDNA-NGS. Diagnostic impact was assessed in 4 domains: new diagnoses, earlier time to diagnosis, avoidance of invasive procedures, and non-hypothesis-driven diagnoses. Logistic regression was used to identify predictors of positive mcfDNA-NGS testing.

***Results.*** Among 176 patients, mcfDNA-NGS was positive in 44.3%, with 49% of these cases considered clinically significant. Infectious cause of FUO was identified in 39% of patients, noninfectious in 35%, and unknown in 26%. mcfDNA-NGS contributed to a positive diagnostic impact in 30% of cases, mainly by earlier diagnosis (16%) and potential for avoidance of invasive procedures (10%). Positive mcfDNA-NGS was significantly associated with higher Charlson comorbidity index score (odds ratio [OR], 1.22; $P < .001$) and white blood cell (WBC) count $\leq 4.5 \times 10^9$ cells/L (OR, 8.61; $P < .001$). Conversely, FUO without localization was associated with a decreased likelihood of positive mcfNDA testing (OR, 0.18; $P < .001$).

***Conclusions.*** mcfDNA-NGS effectively complements ST in diagnosing FUO, providing earlier detection and minimizing invasive testing. Clinical predictors such as high comorbidity and low WBC count may guide the optimal use of mcfDNA-NGS in FUO. Prospective evaluation of optimal timing and use of mcfDNA-NGS and cost-benefit analysis in FUO is needed.

**Keywords.** fever of unknown origin; metagenomics; plasma microbial cell-free DNA; novel diagnostics; immunocompromised.

Fever of unknown origin (FUO) is a heterogenous syndrome that includes infections, autoimmune or autoinflammatory conditions, and malignancies [1–4]. Despite a systematic approach to determine the underlying cause, up to 51% of cases remain undiagnosed [1, 4]. Additionally, there is no current gold standard assay or approach for diagnosing FUO. Traditional hypothesis-driven microbiologic studies including cultures, serology, and molecular assays have improved the diagnostic yield but can be time-consuming, costly, and inaccessible, and have limited sensitivity and specificity [2, 3].

Pathogen-agnostic metagenomic sequencing assays, such as plasma microbial cell-free DNA next-generation sequencing (mcfDNA-NGS), are emerging diagnostic tools capable of overcoming many of these barriers. The Karius Test (Karius, Redwood City, California) is 1 such assay that detects >1250 organisms including bacteria, DNA viruses, fungi, and eukaryotic parasites, with direct applicability to conditions like FUO [5, 6]. mcfDNA-NGS has demonstrated diagnostic utility and clinical impact in various infectious syndromes including infective endocarditis [7–9], pneumonia [10, 11], febrile neutropenia [12], and invasive fungal infections (IFIs) [13, 14]. The assay is less affected by prior antimicrobial exposure and remains positive longer than conventional cultures [8, 9]. Combined with its noninvasive nature and rapid turnaround time, it is an attractive tool in identifying infectious causes of FUO.

Current evidence evaluating the role of mcfDNA-NGS in FUO is largely limited to individual case series, identifying fastidious causative pathogens including *Rickettsia typhi* [15], *Leptospira* [16], and Q fever [17]. One retrospective study

Received 30 September 2024; editorial decision 15 January 2025; accepted 22 January 2025; published online 5 February 2025

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https://doi.org/10.1093/ofid/ofaf038

evaluating mcfDNA-NGS in FUO among immunocompetent adults identified a positive test in 65.3% cases with a positive clinical impact in 40.3% of patients [18]. Nonetheless, important questions regarding the role of mcfDNA-NGS in FUO remain unanswered, including: (1) when and in whom the test should be considered, (2) the diagnostic value added by mcfDNA-NGS in adjunct to conventional microbiologic workup, and (3) the impact of the assay on clinical management.

## METHODS

### Study Design

We conducted a retrospective cohort study of adult patients (≥18 years) referred to our tertiary academic medical center for evaluation of FUO between 1 November 2019 and 31 November 2023. We included all patients who met the revised definition criterion of FUO as outlined by Durack and Street [19] or were referred to our institution for this specific indication. All patients evaluated in the inpatient or outpatient setting for FUO during the study period underwent a systematic diagnostic workup, including both standard microbiologic testing (ST; defined in Supplementary Table 1) and mcfDNA-NGS testing. Exclusion criteria are described in Supplementary Figure 1. The Mayo Clinic Institutional Review Board deemed this study exempt (IRB#23-002189).

### Data Collection

We classified patients presenting for FUO evaluation based on type of FUO and clinical indication for mcfDNA-NGS (defined in Supplementary Table 1). We abstracted patient demographics; medical comorbidities including Charlson comorbidity index (CCI) [20]; immunocompromising conditions; hospital admission and length of stay; risk factors and symptoms associated with FUO; baseline biochemical, hematological, and microbiological studies; and results of mcfDNA-NGS from patient electronic medical records using a secure REDCap electronic data capture tool [21, 22].

### Clinical Adjudication and Outcomes

Two independent clinical adjudicators (N. R. and M. F.) reviewed the medical records, results of infectious and noninfectious studies, and long-term clinical follow-up to classify each case of FUO as infectious, noninfectious, or unknown diagnosis. A third adjudicator (O. A. S.) was used in cases with discordant diagnoses. We defined positive mcfDNA-NGS results as clinically significant or insignificant based on factors including (1) detection of pathogen in relevant clinical specimens (eg, blood, respiratory samples); (2) pathogen-specific characteristics including virulence and established association with FUO syndrome (based on at least 1 or more previously published case studies); (3) likelihood of causation supported by clinical, microbiologic (including ST if positive), and radiographic findings with temporal association between pathogen identification and onset of fever; and (4) no alternate plausible etiology of FUO identified. We evaluated the diagnostic impact of mcfDNA-NGS relative to ST by classifying this outcome into 4 domains: (A) new diagnosis by mcfDNA-NGS not identified by ST, (B) earlier time to diagnosis using mcfDNA-NGS, (C) avoidance of invasive diagnostics, and (D) non-hypothesis-driven diagnosis using mcfDNA-NGS (Supplementary Table 1). We assessed the impact of ST and mcfDNA-NGS on antimicrobial therapy and immunosuppression (IS). Last, among patients with positive mcfDNA-NGS, we evaluated clinical predictors of positive testing to determine optimal use of the assay.

### Plasma mcfDNA-NGS

The Karius Test is a commercially available test developed and validated to detect and quantify mcfDNA in plasma with detailed description of test methodology and validation previously described [6]. Patient peripheral blood samples were collected in a BD or a K2–ethylenediaminetetraacetic acid Vacutainer, plasma was isolated and frozen, and shipped to Karius Clinical Laboratory Improvements Amendments–certified/College of American Pathologists–accredited laboratory (Redwood City, California) for mcfDNA sequencing and analysis.

### Statistical Analysis

Baseline data are expressed as median and interquartile range (IQR) for continuous variables with nonparametric distribution, mean and standard deviation for parametric continuous variables, and counts and percentages for categorical variables. Analysis of variance and Kruskal-Wallis or Pearson $\chi^2$ (or Fisher exact) tests for continuous and categorical variables, respectively, were used to compare clinical characteristics stratified by underlying diagnosis of FUO and result of mcfDNA-NGS. Univariate logistic regression was initially performed to identify clinical factors associated with an mcfDNA-NGS result. Variables with a $P$ value <.1 in univariate analysis were considered for inclusion in the multivariable logistic regression model. A backward stepwise selection method was then used to build the final multivariable model, retaining variables with a $P$ value <.05 to identify independent predictors of positive mcfDNA-NGS results. Statistical significance was indicated by a 2-tailed $P < .05$. All analyses were conducted using R software, version 4.4.0 (R Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

During the study period, a steady annual increase in use of mcfDNA-NGS for evaluation of FUO was observed (Supplementary Figure 2). Among 176 patients included, the median age of patients undergoing assessment was 57 years (IQR, 44–67 years) and 51% were immunocompromised

primarily due to pharmacologic IS, hematologic malignancy, or solid organ transplantation. Most patients presented with classic (59%) or immunodeficiency-associated (35%) FUO, with a majority evaluated in the inpatient setting. The median time from fever onset to ST and mcfDNA-NGS was 18.5 days and 39 days, respectively. Overall, mcfDNA-NGS was positive in 78 of 176 patients (44.3%), but considered clinically significant in 49% of these cases (Table 1).

#### Clinical Characteristics of Patients With FUO Classified by Underlying Etiology

Following adjudication by 2 independent reviewers, the final diagnosis was classified as infectious in 69 (39%), noninfectious in 61 (35%), and unknown in 46 (26%) patients (Table 1). Those with an infectious cause of FUO were significantly older, had higher rates of IS, and were more frequently evaluated in the inpatient setting compared to patients with noninfectious or unknown diagnosis. Baseline laboratory evaluation in these patients demonstrated lower median hemoglobin and white blood cell (WBC) count, with higher C-reactive protein (CRP). When assessing test performance of mcfDNA-NGS based on diagnosis, significantly higher rates of both positive (74%) and clinically significant (71%) mcfDNA were observed in patients with underlying infection. Interestingly, positive mcfDNA-NGS was also noted in patients with noninfectious (28%) and unknown (22%) diagnosis (Supplementary Table 2). However, pathogens isolated in only 2 of these 27 cases were considered significant (patients 70 and 71; Supplementary Table 2). Additionally, the median number of pathogens identified by mcfDNA-NGS was 1 (IQR, 1–2) in infectious and 2 (IQR, 1–5) in patients with noninfectious FUO. Overall, the rate of fever resolution was highest among those with an identifiable diagnosis (infectious or noninfectious) compared to unknown diagnosis (Table 1).

#### Diagnostic Performance of ST and mcfDNA-NGS in FUO

In our cohort, the diagnostic yield of tier 1 (minimal microbiologic evaluation) and tier 2 (hypothesis-driven evaluation) ST was low (Figure 1). Blood culture (BCX) was positive in 15 of 168 patients, 12 (7.1%) of which were felt to be "true positive" infections (Supplementary Table 3). Human immunodeficiency virus, hepatitis B and C virus, syphilis, and cytomegalovirus (CMV) testing did not identify any new cases of infection. CMV polymerase chain reaction (PCR) was positive in 5 patients, 4 of whom were felt to have CMV infection warranting treatment in the setting of IS. Tier 2 testing was performed frequently (in 50%–85% of patients), but had a low yield with infection confirmed by 4% of culture-negative studies, 0 by tick-borne panel, and 6.7% by noninvasive fungal workup (Figure 1).

Among the 69 patients with infectious FUO, ST confirmed the diagnosis in 28 patients (41%), with concordance between ST and mcfDNA-NGS observed in 21 (75%) (Figure 2). High degree of agreement was noted particularly between BCXs and mcfDNA-NGS, with 10 of 12 patients with bloodstream infection confirmed by mcfDNA-NGS (Supplementary Appendix Table 3). Fifteen patients (22%) had negative ST but positive mcfDNA-NGS (Figure 2). An overall positive diagnostic impact (stratified as Domain A–D) was noted in 21 of 69 patients (30%). This included the diagnosis of FUO not identified by ST or secondary workup (Domain A) in 3 patients (patients 36, 40, and 41) and identification of a non-hypothesis-driven diagnosis (Domain D) in 4 patients (patients 32, 35, 36, and 43). Pathogens identified by mcfDNA-NGS alone included both culturable and fastidious/difficult-to-culture bacteria (*Tropheryma whipplei*, *Coxiella burnetii*), nontuberculous mycobacteria (NTM), DNA viruses (Epstein-Barr virus [EBV], parvovirus B19, and adenovirus B), and fungi (*Histoplasma capsulatum, Pneumocystis jirovecii* [PCP]) in the setting of confirmed infection (Supplementary Figure 3 and Table 2). Interestingly, however, in 7 patients, mcfDNA-NGS was negative or noncontributory despite positive ST for similar pathogens including PCP (elevated (1-3)-β-D-glucan and positive bronchoalveolar lavage [BAL] PCR), *Histoplasma* (serology), *Cryptococcus* (positive fungal BCX), and *Coxiella* (titer 1:8192) (Table 2).

Earlier time to diagnosis using mcfDNA-NGS (Domain B) was noted in 11 patients. This included 2 cases (patient 33 and 42) wherein mcfDNA-NGS was positive for common bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) >1 week before BCXs became positive. In 4 cases of disseminated NTM infection (patients 1, 2, 4, and 17), mcfDNA-NGS provided the advantage of shortened time to diagnosis and species-level identification of pathogen. Similarly, mcfDNA-NGS led to early identification of pulmonary *Mycobacterium abscessus* and *H capsulatum* (patients 38 and 39) compared to conventional pulmonary cultures (sent concurrently with mcfDNA). In 7 patients, mcfDNA-NGS could have avoided invasive diagnosis (Domain C) as results were concordant with microbiology identified by invasive procedures like BAL, surgical biopsy, thoracentesis, liver abscess aspiration, and bone marrow biopsy (Table 2).

In 26 of 69 patients with infectious FUO, both mcfDNA-NGS and ST were negative (Table 2). Common causes of negative workup included (1) infection due to RNA viruses including Powassan virus encephalomyelitis and persistent coronavirus disease 2019 (COVID-19) in patients with immunodeficiency; (2) need for site- or tissue-specific testing such as gastrointestinal pathogen PCR panel (for *Campylobacter* and *Clostridioides difficile* colitis), synovial fluid analysis (*T whipplei*), or skin biopsy (*Mycobacterium chelonae*) suggestive of localized infection; or (3) syndromic infectious diagnosis

**Table 1. Clinical Characteristics of All Patients Evaluated for Fever of Unknown Origin and Stratified by Final Adjudicated Diagnosis (Infectious, Noninfectious, and Unknown) of FUO**[a]

| Variable | No. | All Patients Evaluated for FUO (n = 176) | Infectious Diagnosis (n = 69) | Noninfectious Diagnosis (n = 61) | Unknown Diagnosis (n = 46) | *P* Value |
|---|---|---|---|---|---|---|
| Age, y | 176 | 57.0 (43.8, 67.0) | 61.0 (46.0, 68.0) | 59.0 (51.0, 67.0) | 50.0 (36.3, 61.0) | .020[a] |
| Sex (female) | 176 | 73 (41.5) | 27 (39.1) | 23 (37.7) | 23 (50.0) | .389[b] |
| CCI score | 176 | 4.0 (2.0, 5.0) | 4.0 (3.0, 6.0) | 4.0 (3.0, 5.0) | 2.0 (1.0, 4.0) | .002[c] |
| Immunocompromising condition | 176 | 89 (50.6) | 43 (62.3) | 33 (54.1) | 13 (28.3) | .001[b] |
| Type of immunocompromising condition | 89 | | | | | .275[b] |
| Pharmacologic immunosuppression | | 35 (39.3) | 11 (25.6) | 15 (45.5) | 9 (69.2) | |
| Hematologic malignancy | | 21 (23.6) | 13 (30.2) | 7 (21.2) | 1 (7.7) | |
| SOT | | 16 (18.0) | 10 (23.3) | 4 (12.1) | 2 (15.4) | |
| HCT | | 10 (11.2) | 5 (11.6) | 4 (12.1) | 1 (7.7) | |
| Solid organ malignancy | | 6 (6.7) | 4 (9.3) | 2 (6.1) | 0 (0) | |
| Primary immunodeficiency | | 1 (1.1) | 0 (0) | 1 (3.0) | 0 (0) | |
| Classification of FUO[d] | 176 | | | | | .031[b] |
| Classic | | 104 (59.1) | 32 (46.4) | 39 (63.9) | 33 (71.7) | |
| Immunodeficiency-associated | | 61 (34.7) | 32 (46.4) | 21 (34.4) | 8 (17.4) | |
| Nosocomial | | 9 (5.1) | 4 (5.8) | 1 (1.6) | 4 (8.7) | |
| Travel-associated | | 2 (1.1) | 1 (1.4) | 0 (0) | 1 (2.2) | |
| Inpatient admission | 176 | 115 (65.3) | 57 (82.6) | 39 (63.9) | 19 (41.3) | <.001[a] |
| Hospital length of stay, d | 115 | 11 (5.0, 20.0) | 2.0 (6.0, 25.0) | 10.0 (6.5, 18.0) | 6.0 (3.5, 19.5) | .508[a] |
| Bloodwork | | | | | | <.001[a] |
| Hemoglobin | | 11.1 (8.9, 13.0) | 9.8 (8.7, 11.8) | 10.8 (8.9, 12.4) | 13.3 (11.1, 14.4) | |
| WBC | | 6.8 (4.1, 10.0) | 5.4 (2.9, 8.1) | 7.6 (4.9, 11.6) | 7.2 (4.9, 9.5) | .003[a] |
| ALT | | 28 (18.0, 44.0) | 29.0 (21.0, 44.0) | 30.0 (18.8, 53.3) | 24.0 (17.0, 39.3) | .712[a] |
| CRP | | 46.6 (8.2, 125.6) | 68.5 (27.8, 117.3) | 46.4 (5.0, 147.2) | 14.0 (3.5, 96.2) | .215[a] |
| Abnormal CRP | 176 | 130 (73.9) | 58 (84.1) | 43 (70.5) | 29 (63.0) | .032[b] |
| Rheumatologic workup pursued | 176 | 103 (58.5) | 24 (34.8) | 44 (72.1) | 35 (76.1) | <.001[b] |
| Abnormal rheumatologic workup | 103 | 32 (31.1) | 9 (37.5) | 16 (36.4) | 7 (20.0) | .219[b] |
| Time from fever onset to microbiologic workup, d | 176 | 18.5 (3.0, 92.5) | 6.0 (0.0, 30.0) | 28.0 (3.0, 94.0) | 55.5 (12.3, 210.8) | .107[a] |
| Time from fever onset to mcfDNA-NGS, d | 176 | 39.0 (14.0, 168.8) | 20.0 (11.0, 46.0) | 59.0 (17.0, 150.0) | 142.5 (33.5, 376.0) | .009[a] |
| Clinical indication for mcfDNA-NGS[e] | 176 | | | | | <.001[b] |
| FUO without localization | | 52 (29.5) | 6 (8.7) | 20 (32.8) | 26 (56.5) | |
| FUO without clear pathogen, but radiographic foci | | 37 (21.0) | 21 (30.4) | 10 (16.4) | 6 (13.0) | |
| FUO with disseminated infection in ICH | | 47 (26.7) | 26 (37.7) | 16 (26.2) | 5 (10.9) | |
| FUO with dissemination in immunocompetent host | | 23 (13.1) | 9 (13.0) | 9 (14.8) | 5 (10.0) | |
| FUO with suspected endovascular infection | | 17 (9.7) | 7 (10.1) | 6 (9.8) | 4 (8.7) | |
| Positive mcfDNA-NGS | 176 | 78 (44.3) | 51 (73.9) | 17 (27.9) | 10 (21.7) | <.001[b] |
| Clinically significant pathogen identified using mcfDNA-NGS | 78 | 38 (48.7) | 36 (70.6) | 2 (11.8) | 0 (0.0) | <.001[b] |
| No. of pathogens identified | 176 | 1.0 (1.0, 2.0) | 1.0 (1.0, 2.0) | 2.0 (1.0, 5.0) | 1.000 (1.0, 1.0) | <.001[a] |
| Resolution of fever on follow-up | 176 | 132 (75.0) | 63 (91.3) | 50 (82.0) | 19 (14.3) | <.001[b] |
| Duration of follow-up, d | 176 | 372 (82.8, 553.3) | 244 (68.0, 549.0) | 410 (182.0, 642.0) | 343.5 (98.8, 531.5) | .253[a] |

Values represent median (quartile 1, quartile 3) for continuous variables and frequency (percentage) for categorical variables. Abbreviations: ALT, alanine aminotransferase; CCI, Charlson comorbidity index; CRP, C-reactive protein; FUO, fever of unknown origin; HCT, hematopoietic cell transplant; ICH, immunocompromised host; mcfDNA-NGS, plasma microbial cell-free DNA next-generation sequencing; SOT, solid organ transplant; WBC, white blood cell count.

[a]Analysis of variance.

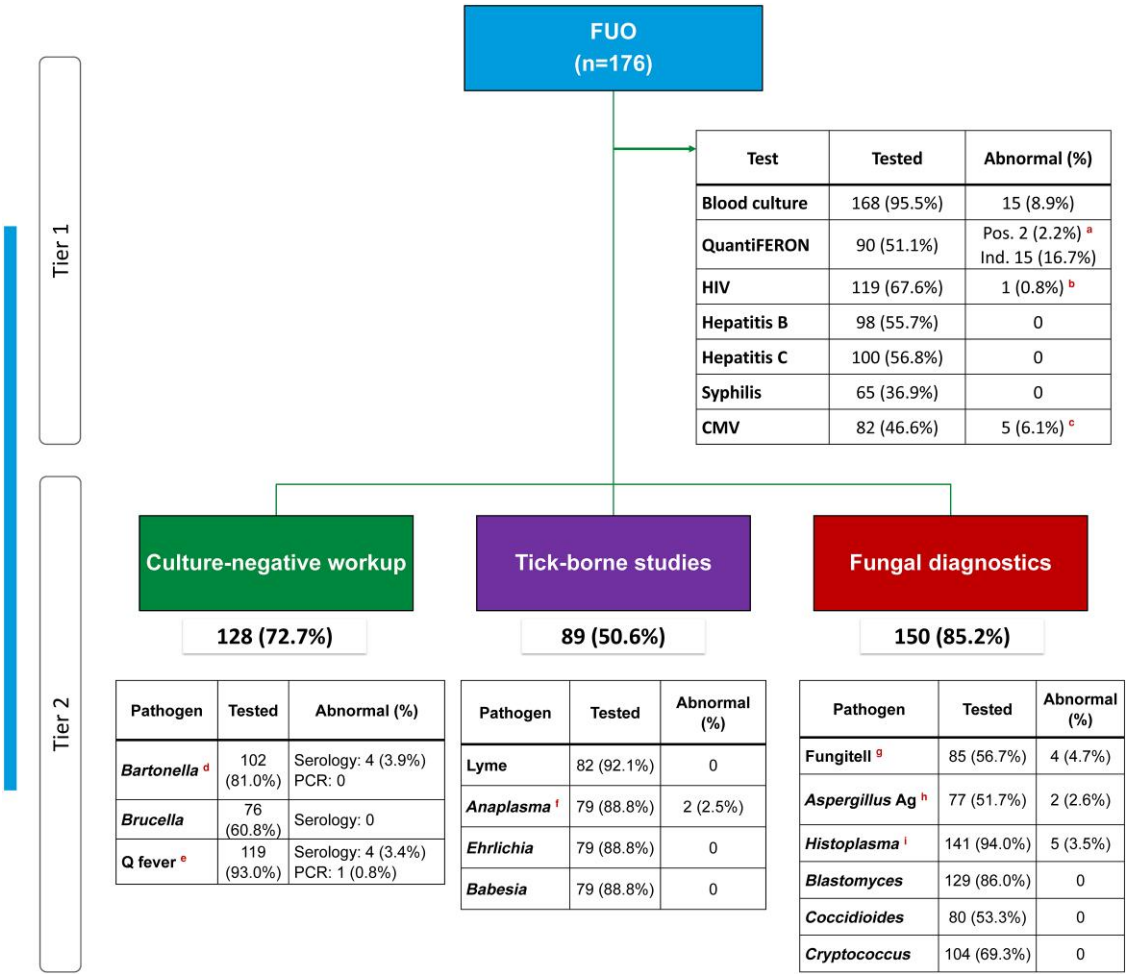
[b]Pearson $\chi^2$ test.

[c]Kruskal-Wallis test.

[d]FUO was classified as classic (FUO despite reasonable initial investigations in the inpatient or outpatient setting, in the absence of meeting criteria for alternate types of FUO), immunodeficiency-associated (FUO occurring in patients with severe immunocompromise including solid organ transplant or hematopoietic cell transplant recipients, patients with neutropenia [absolute neutrophil count is <500 cells/μL], solid or hematologic malignancy, or patients with human immunodeficiency virus infection not on antiretroviral therapy), nosocomial (FUO that develops in a hospitalized patient in whom infection was not apparent on admission and underwent at least 3 days of investigation in the inpatient setting), or travel-associated (acute febrile illness in a patient following recent high-risk local or international travel).

[e]Clinical indication for mcfDNA-NGS was defined as:

- FUO without localization: FUO presenting with nonspecific symptoms (eg, malaise, fatigue, dyspnea, generalized abdominal pain, nonproductive) without localizing clinical or radiographic foci of infection.
- FUO with radiographic foci of infection: FUO with a radiographic focus of infection (eg, pulmonary nodule, hepatic abscess).
- FUO with suspected endovascular infection: FUO in the setting of suspected endocarditis (native or prosthetic valve) or vascular graft infection.
- FUO with disseminated infection in immunocompromised host: FUO in immunocompromised host with clinical and/or radiographic suspicion for multisystem involvement due to infection.
- FUO with disseminated infection in immunocompetent host: FUO in immunocompetent host with clinical and/or radiographic suspicion for multisystem involvement due to infection.




| Test | Tested | Abnormal (%) |
|---|---|---|
| Blood culture | 168 (95.5%) | 15 (8.9%) |
| QuantiFERON | 90 (51.1%) | Pos. 2 (2.2%) [a] Ind. 15 (16.7%) |
| HIV | 119 (67.6%) | 1 (0.8%) [b] |
| Hepatitis B | 98 (55.7%) | 0 |
| Hepatitis C | 100 (56.8%) | 0 |
| Syphilis | 65 (36.9%) | 0 |
| CMV | 82 (46.6%) | 5 (6.1%) [c] |

| Pathogen | Tested | Abnormal (%) |
|---|---|---|
| *Bartonella* [d] | 102 (81.0%) | Serology: 4 (3.9%) PCR: 0 |
| *Brucella* | 76 (60.8%) | Serology: 0 |
| Q fever [e] | 119 (93.0%) | Serology: 4 (3.4%) PCR: 1 (0.8%) |

| Pathogen | Tested | Abnormal (%) |
|---|---|---|
| Lyme | 82 (92.1%) | 0 |
| *Anaplasma* [f] | 79 (88.8%) | 2 (2.5%) |
| *Ehrlichia* | 79 (88.8%) | 0 |
| *Babesia* | 79 (88.8%) | 0 |

| Pathogen | Tested | Abnormal (%) |
|---|---|---|
| Fungitell [g] | 85 (56.7%) | 4 (4.7%) |
| *Aspergillus* Ag [h] | 77 (51.7%) | 2 (2.6%) |
| *Histoplasma* [i] | 141 (94.0%) | 5 (3.5%) |
| *Blastomyces* | 129 (86.0%) | 0 |
| *Coccidioides* | 80 (53.3%) | 0 |
| *Cryptococcus* | 104 (69.3%) | 0 |

**Figure 1.** Diagnostic yield of standard microbiologic assays among patients presenting for fever of unknown origin (FUO) evaluation. [a]Two cases of positive QuantiFERON Gold assays were consistent with latent tuberculosis (TB) infection without concern for active TB. [b]One positive human immunodeficiency virus (HIV) test was in a patient with known HIV infection on antiretroviral therapy and was not felt to be related to the underlying FUO. [c]Four of 5 cases were considered true cytomegalovirus viremia necessitating treatment in setting of immunosuppression. [d]Only 1 of 4 cases with positive *Bartonella* serology was considered true positive with disseminated bartonellosis; 1 case was cross-reactive in setting of Q fever, with 2 cases considered false positive with subsequent convalescent serology negative. [e]All 4 cases were consistent with Q fever; 1 patient had both serum serology (1:32 768) and polymerase chain reaction positive. [f]Both cases of positive anaplasma serology were false positive in the setting of Q fever. [g]Of the 4 cases with positive (1-3)-β-D-glucan, 3 were diagnosed with pneumocystis pneumonia on bronchoscopy and 1 patient had concurrent pulmonary aspergillosis (positive serum galactomannan). [h]Both cases with positive serum galactomannan had probable pulmonary aspergillosis. [i]Four of 5 patients with positive *Histoplasma* serology and/or antigen testing had probable histoplasmosis (2 pulmonary, 2 disseminated), with 1 case of possible histoplasmosis. Abbreviations: Ag, antigen; CMV, cytomegalovirus; Fungitell, (1-3)-β-D-glucan; FUO, fever of unknown origin; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

without a clear identifiable pathogen (eg, colitis, lung abscess, intrabdominal abscess).

#### Impact of mcfDNA-NGS on Antimicrobial and Immunosuppressive Therapy

Patients with infectious diagnosis had significantly higher baseline use of empiric antimicrobials, with lower rate of IS compared to those with noninfectious diagnoses (Supplementary Figure 4). Diagnostic studies primarily led to a modification in antibiotics in 49.3% of patients with infection, with minimal impact noted on antiviral or antifungal therapy. Immunosuppression was reduced or discontinued in 13% of patients with infection. Interestingly, initiation of IS was noted in 4 patients with infection in the setting of Q fever, parvovirus B19, T-cell chronic active EBV, and COVID-19. Among patients with noninfectious FUO, negative or noncontributory microbiologic workup predominantly led to de-escalation or stoppage of antimicrobials. The most prominent treatment impact was noted in the IS group, with augmentation or initiation observed in 21 patients (34.5%).

#### Clinical Predictors of Positive mcfDNA-NGS Testing in FUO

Patients with positive mcfDNA-NGS had higher median CCI score and baseline IS, lower median hemoglobin, lower WBC count, and higher CRP compared to patients with negative

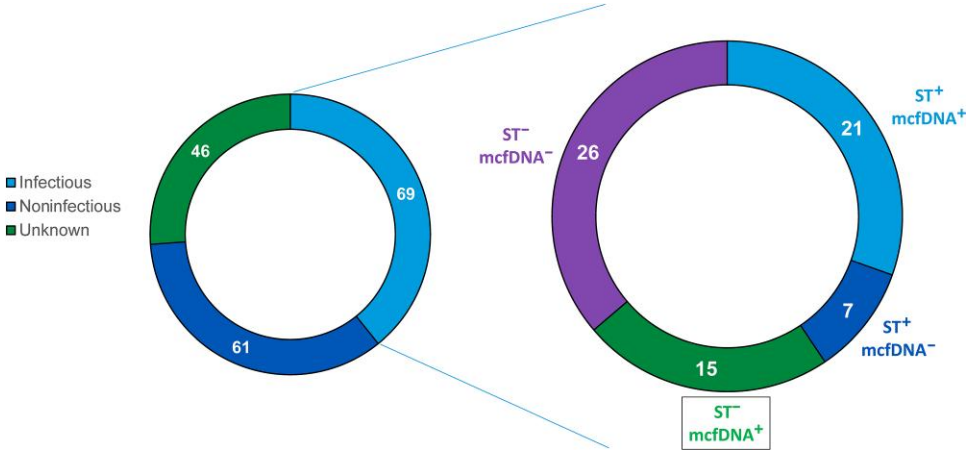



**Figure 2.** Diagnostic impact of plasma microbial cell-free DNA next-generation sequencing in patients with a final infectious diagnosis of fever of unknown origin (diagnostic impact domain A). Abbreviations: ⁻, negative; ⁺, positive; mcfDNA, microbial cell-free DNA next-generation sequencing; ST, standard microbiologic testing.

mcfDNA-NGS testing (Table 3). The rate of preceding antimicrobial exposure in this group was higher (74% vs 62%), albeit nonsignificant. These patients also had significantly shorter time from fever onset to both standard testing and mcfDNA-NGS. Notably, patients with negative mcfDNA-NGS had higher rate of FUO without localization as the primary indication for testing. These clinical predictors were confirmed in the unadjusted logistic regression model (Table 4). In the multivariable analysis, higher CCI score (odds ratio [OR], 1.22 [95% confidence interval {CI}, 1.01–1.48]; $P < .001$) and WBC $\leq 4.5 \times 10^9$ cells/L (OR, 8.61 [95% CI, 3.26–25.13]; $P < .001$) remained significant positive predictors, with the indication of FUO without localization (OR, 0.18 [95% CI, .06–.48]; $P < .001$) noted to be a negative predictor of pathogen detection by mcfDNA-NGS. Among patients with either of the 2 latter predictors (indication other than FUO without localization or WBC count $\leq 4.5 \times 10^9$ cells/L), the model provided a sensitivity (SN), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) of 92.3%, 63.3%, 54.5%, and 86.3%, respectively, for identifying cases with positive mcfDNA-NGS testing. Only 8 of 52 patients (15%) with FUO without localization had a positive mcfDNA-NGS assay, with 3 considered clinically significant.

## DISCUSSION

In this retrospective study of patients referred to our tertiary medical center for evaluation of FUO, we assessed the diagnostic and clinical impact of mcfDNA-NGS in conjunction with ST. Overall, mcfDNA-NGS was positive in 44.3%, and it was considered clinically significant in 49% of these cases.

Patients with infectious FUO had higher median age, were frequently immunocompromised, and had bloodwork suggestive of an inflammatory phenotype (high CRP, low hemoglobin, low WBC count). These are likely important factors that impact clinician pretest probability as a majority of these patients were evaluated in the inpatient setting, had shorter time from fever onset to ST and mcfDNA-NGS, and had higher rates of both positive (74%) and clinically significant (71%) mcfDNA identification. mcfDNA-NGS has been repeatedly demonstrated to have diagnostic and clinical utility in evaluation of infectious syndromes including pneumonia [10, 11], febrile neutropenia [12], and IFI [13, 23] in immunocompromised hosts (ICHs). Only 1 study has evaluated the assay in FUO, identifying a similarly high rate of positive mcfDNA-NGS in infectious compared to noninfectious FUO (89.5% vs 43.8%), but was limited to non-ICHs [18]. A pediatric study evaluating utility of Karius Test in identifying clinically relevant pathogens noted higher yield in ICH compared to immunocompetent patients (61% vs 35%), but demonstrated a lower specificity (64%) in this group. Consistent with both studies, mcfDNA-NGS was positive but nonspecific in a subset of patients with noninfectious or unknown diagnosis. These "false positive" pathogens identified by mcfDNA-NGS were frequently commensal oropharyngeal flora or represented viral reactivation (CMV, EBV, or herpes simplex virus type 1) occurring as a consequence of the underlying noninfectious condition (autoimmune or malignancy) rather than as the cause of FUO [24]. Consequently, cautious interpretation of mcfDNA-NGS results in collaboration with infectious diseases clinicians is warranted to accurately distinguish signal from noise.

Overall, mcfDNA-NGS in adjunct to ST had a positive diagnostic impact in 30% of patients, with minimal impact noted in the remainder. The areas of greatest impact included earlier time to diagnosis (Domain B; 16%) and potential for avoidance of invasive workup (Domain C; 10%). We noted important advantages and limitations of mcfDNA-NGS in FUO. First, the

**Table 2. Descriptive Analysis of Concordant and Discordant Results Between Standard Microbiologic Workup and Plasma Microbial Cell-Free DNA Next-Generation Sequencing in Evaluation of Patients With Infectious Etiology of Fever of Unknown Origin**

| Test | Patient | FUO Definition | Standard Microbiologic Workup Yield | 2nd Microbiologic Confirmation | mcfDNA-NGS Pathogen ID[a] | Diagnosis Impact Domain | Final Diagnosis |
|---|---|---|---|---|---|---|---|
| ST positive, mcfDNA-NGS positive | 1[b] | Classic | BCX: MAC | Heart valve CX: *Mycobacterium chimaera* | *M chimaera* | B | *M chimaera* PV IE |
| | 2[b] | Classic | BCX: *Mycobacterium* spp | BAL: *Mycobacterium immunogenum* | MAC, *Mycobacterium kyorinense*, *Mycobacterium celatum* | B | Disseminated NTM infection |
| | 3 | Classic | *Histoplasma* Ab, urine Ag positive | N/A | *Histoplasma capsulatum* | NI | Disseminated histoplasmosis |
| | 4[b] | Classic | BCX: *M chimaera* | Aortic tissue CX: MAC | *M chimaera* | B | *M chimaera* PV IE with aortic graft involvement |
| | 5 | Immunodeficiency | Serum CMV PCR positive | N/A | CMV | NI | CMV infection |
| | 6 | Immunodeficiency | BCX: *Rothia mucilaginosa* | N/A | *R mucilaginosa* | NI | *Rothia* BSI with neutropenic enterocolitis |
| | 7 | Immunodeficiency | BCX: *Enterococcus faecium* | N/A | *E faecium* | NI | VRE BSI with pelvic abscess |
| | 8 | Classic | Q fever serology: 1:32 768<br>Q fever PCR: positive | N/A | *Coxiella burnetii* | NI | Chronic Q fever with PV IE |
| | 9 | Classic | Serum CMV PCR positive | Serum CMV IgM positive | CMV | NI | Primary CMV infection |
| | 10 | Classic | Q fever serology: 1:2048 | N/A | *C burnetii* | NI | Acute Q fever |
| | 11 | Nosocomial | BCX: *Enterobacter cloacae* | N/A | *E cloacae* | NI | *Enterobacter* BSI with infected vascular graft |
| | 12 | Classic | Q fever serology: 1:2048 | N/A | *C burnetii* | NI | Acute Q fever |
| | 13 | Immunodeficiency | BCX: *E faecium*<br>Fungitell: >500 pg/mL<br>Serum galactomannan: 2.24 | Induced sputum CX: *Aspergillus fumigatus* | *E faecium*, *A fumigatus* | NI | *E faecium* BSI (cholecystitis)<br>Probable invasive pulmonary aspergillosis |
| | 14 | Immunodeficiency | *Histoplasma* Ab, urine Ag positive | BCX: *Histoplasma capsulatum* (ordered after mcfDNA-NGS) | *H capsulatum* | NI | Disseminated histoplasmosis with secondary HLH |
| | 15 | Immunodeficiency | Serum galactomannan: 1.59 | BAL galactomannan: positive | *Aspergillus flavus* | NI | Probable invasive pulmonary aspergillosis |
| | 16 | Classic | *Bartonella* serology: 1:65 536 | N/A | *Bartonella henselae* | NI | Disseminated bartonellosis with vasculitis phenomenon |
| | 17[b] | Classic | BCX: MAC | N/A | *M chimaera* | B | Disseminated MAC infection |
| | 18 | Classic | BCX: *Staphylococcus epidermidis* | N/A | *S epidermidis* | NI | Lumbar vertebral OM |
| | 19 | Immunodeficiency | BCX: *Streptococcus pneumoniae* and *Klebsiella pneumoniae* | Urine streptococcal antigen | *S pneumoniae* | NI | Bilateral PNA with BSI due to *S pneumoniae* |
| | 20 | Immunodeficiency | Fungitell: >500 pg/mL | BAL: *Pneumocystis* PCR positive | *Pneumocystis jirovecii* | C | PCP PNA |
| | 21 | Classic | *Histoplasma* Ab, urine Ag positive | Induced sputum and BM biopsy CX: *H capsulatum* | *H capsulatum* | C | Disseminated histoplasmosis |
| ST positive, mcfDNA-NGS negative | 22 | Immunodeficiency | Fungitell: 173 pg/mL | BAL: PCP PCR positive | Negative | NI | PCP PNA |
| | 23 | Classic | Q fever serology: 1:8192 | N/A | Negative | NI | Acute Q fever |
| | 24 | Classic | *Histoplasma* Ab positive | N/A | Negative | NI | Pulmonary histoplasmosis |
| | 25 | Classic | BCX: *Cryptococcus neoformans*, *Candida dublinensis* | N/A | CI-P | NI | Cryptococcemia, *Candida* CRBSI |
| | 26 | Immunodeficiency | *Histoplasma* Ab positive | BM: parvovirus B19 PCR positive | CI-P | NI | Parvovirus B19 infection, possible histoplasmosis |

**Table 2. Continued**

| Test | Patient | FUO Definition | Standard Microbiologic Workup Yield | 2nd Microbiologic Confirmation | mcfDNA-NGS Pathogen ID[a] | Diagnosis Impact Domain | Final Diagnosis |
|---|---|---|---|---|---|---|---|
| | 27 | Immunodeficiency | Fungitell: 393 pg/mL | BAL: PCP PCR positive | CI-P | NI | PCP PNA |
| | 28 | Classic | BCX: *Pa* and *Enterococcus faecalis* | N/A | Negative | NI | Polymicrobial BSI |
| ST negative, mcfDNA-NGS positive | 29 | Immunodeficiency | Negative | BAL: PCP PCR positive | *P jirovecii* | C | PCP PNA |
| | 30 | Classic | *Bartonella* serology: 1:20 (false positive) | 2-wk convalescent Q fever Ab: positive | *C burnetii* | B | Acute Q fever |
| | 31 | Nosocomial | Negative | Left neck fluid CX: *Staphylococcus aureus* | *S aureus* | C | Neck necrotizing SSTI |
| | 32 | Classic | Negative | Pleural fluid: *Tropheryma whipplei* PCR positive | *T whipplei* | C, D | Whipple disease |
| | 33* | Immunodeficiency | Negative | BCX: *Escherichia coli* (7 d after mcfDNA-NGS) | *E coli* | B | Colitis with *E coli* BSI |
| | 34 | Classic | Negative | 2-wk convalescent Q fever Ab: positive | *C burnetii* | B | Acute Q fever |
| | 35 | Immunodeficiency | Negative | Urine/serum adenovirus PCR positive (2 d after mcfDNA-NGS) | Human adenovirus B | D | Disseminated adenovirus infection |
| | 36 | Classic | Negative | N/A | *Haemophilus influenzae* | A, D | *H influenzae* PV IE |
| | 37 | Classic | Negative | Liver fluid CX: *E coli* (1 d after mcfDNA-NGS) | *E coli* | B, C | Hepatic abscess due to *E coli* |
| | 38 | Immunodeficiency | Negative | Sputum CX: *Mycobacterium abscessus* (24 d after mcfDNA-NGS) | *M abscessus* | B | Disseminated *M abscessus* infection |
| | 39 | Classic | Negative | BAL CX: *H capsulatum* (13 d after mcfDNA-NGS) | *H capsulatum* | B, C | Pulmonary histoplasmosis |
| | 40 | Classic | BCX: *Staphylococcus hominis* (false positive) | N/A | EBV | A | T-cell chronic active EBV with secondary HLH |
| | 41 | Nosocomial | Negative | N/A | EBV | A | EBV-associated lymphoproliferative disorder |
| | 42[c] | Immunodeficiency | Negative | BCX: *Pseudomonas aeruginosa* (9 d after mcfDNA) | *Pa* | B | *Pseudomonas aeruginosa* BSI |
| | 43 | Immunodeficiency | Negative | BM: parvovirus B19 PCR positive | Parvovirus B19 | D | Parvovirus B19 infection |
| ST negative, mcfDNA-NGS negative | 44 | Immunodeficiency | Negative | Skin biopsy CX: *Mycobacterium chelonae* | CI-P | NI | *M chelonae* disseminated infection |
| | 45 | Immunodeficiency | Negative | Liver aspirate: hyphae, CX negative | Negative | NI | Intra-abdominal abscess (presumed fungal) |
| | 46 | Immunodeficiency | Negative | Lymph node biopsy CX: *Bacillus circulans* | CI-P | NI | *B circulans* infection |
| | 47 | Immunodeficiency | Negative | N/A | CI-P | NI | Infectious colitis |
| | 48 | Immunodeficiency | Negative | CSF metagenomic panel: Powassan virus | Negative | NI | Powassan virus encephalitis/ cerebritis |
| | 49 | Immunodeficiency | Negative | Rectal biopsy: *E faecium* | CI-P | NI | Perirectal abscess |
| | 50 | Classic | Negative | CSF arbovirus panel: Powassan IgM positive | CI-P | NI | Powassan virus encephalomyelitis |
| | 51 | Immunodeficiency | Negative | COVID-19 RNA PCR positive | Negative | NI | Persistent COVID-19 PNA |

**Table 2. Continued**

| Test | Patient | FUO Definition | Standard Microbiologic Workup Yield | 2nd Microbiologic Confirmation | mcfDNA-NGS Pathogen ID[a] | Diagnosis Impact Domain | Final Diagnosis |
|---|---|---|---|---|---|---|---|
| | 52 | Immunodeficiency | Negative | Sputum PCP PCR positive | Negative | NI | PCP PNA |
| | 53 | Immunodeficiency | Negative | N/A | CI-P | NI | Lung abscess |
| | 54 | Immunodeficiency | Negative | Q fever serology positive (6 wk after mcfDNA-NGS) | Negative | NI | Q fever endocarditis |
| | 55 | Classic FUO | Negative | Synovial fluid: *T whipplei* PCR positive | Negative | NI | Whipple arthritis |
| | 56 | Immunodeficiency | Negative | N/A | Negative | NI | Presumed hepatosplenic candidiasis |
| | 57 | Immunodeficiency | Negative | N/A | CI-P | NI | Aortic graft infection, presumed polymicrobial |
| | 58 | Classic | Negative | N/A | CI-P | NI | Culture-negative IE |
| | 59 | Nosocomial | Negative | GI pathogen PCR positive | Negative | NI | *Campylobacter* gastroenteritis |
| | 60 | Travel-associated | Negative | GI pathogen PCR positive | CI-P | NI | *Clostridioides difficile* infection |
| | 61 | Classic | Negative | BAL *Legionella* PCR positive | CI-P | NI | *Legionella* pneumonia |
| | 62 | Classic | Negative | N/A | Negative | NI | Culture-negative pulmonary TB |
| | 63 | Classic | Negative | COVID-19 RNA PCR positive | Negative | NI | COVID-19 PNA |
| | 64 | Immunodeficiency | Negative | COVID-19 RNA PCR positive | Negative | NI | Persistent COVID-19 PNA |
| | 65 | Classic | Negative | N/A | Negative | NI | Community-acquired PNA |
| | 66 | Classic | Negative | COVID-19 RNA PCR positive | Negative | NI | COVID-19 PNA |
| | 67 | Classic | Negative | CSF Powassan virus PCR positive | CI-P | NI | Powassan virus encephalomyelitis |
| | 68 | Immunodeficiency | Negative | N/A | Negative | NI | Possible invasive pulmonary fungal infection |
| | 69 | Immunodeficiency | Negative | N/A | CI-P | NI | Chronic sinusitis |

Abbreviations: Ab, antibody; Ag, antigen; BAL, bronchoalveolar lavage; BCX, blood culture; BM, bone marrow; BSI, bloodstream infection; CI-P, clinically insignificant positive microbial cell-free DNA next-generation sequencing; CMV, cytomegalovirus; COVID-19, coronavirus disease 2019; CRBSI, catheter-related bloodstream infection; CSF, cerebrospinal fluid; CX, culture; EBV, Epstein-Barr virus; Fungitell, (1-3)-β-D-glucan; FUO, fever of unknown origin; GI, gastrointestinal; HLH, hemophagocytic lymphohistiocytosis; IE, infective endocarditis; IgM, immunoglobulin G; mcfDNA-NGS, microbial cell-free DNA next-generation sequencing; MAC, *Mycobacterium avium* complex; N/A, not available; NI, no diagnostic impact; NTM, nontuberculous mycobacteria; OM, osteomyelitis; *Pa*, *Pseudomonas aeruginosa*; PCP, *Pneumocystis jirovecii* pneumonia; PCR, polymerase chain reaction; PNA, pneumonia; PV, prosthetic valve; SSTI, skin and soft tissue infection; ST, standard microbiologic testing; TB, tuberculosis; VRE, vancomycin-resistant *Enterococcus*.

[a]For the mcfDNA-NGS pathogen column, only the clinically significant pathogen has been enumerated; as noted by *, several other pathogens may have been identified but have not been included to focus on relevant microbiologic data.

[b]Significantly earlier time to identification by mcfDNA-NGS compared to routine blood cultures.

[c]Although mcfDNA-NGS was positive for common pathogens, pathogen was identified earlier than conventional BCX.

**Table 3. Clinical Characteristics Among Patients With Positive Versus Negative Plasma Microbial Cell-Free DNA Next-Generation Sequencing[a]**

| Variable | No. | Negative mcfDNA-NGS (n = 98) | Positive mcfDNA-NGS (n = 78) | *P* Value |
|---|---|---|---|---|
| Age | 176 | 55.5 (43.0, 73.0) | 59.0 (42.3, 71.0) | .278[a] |
| Sex (Female) | 176 | 47 (48.0) | 26 (33.3) | .070[b] |
| CCI score | 176 | 3.0 (2.0, 4.8) | 4.0 (3.0, 6.0) | <.001[c] |
| Immunocompromising condition | 176 | 40 (40.8) | 49 (62.8) | .004[b] |
| Classification of FUO | 176 | | | .001[b] |
| Classic | | 68 (69.4) | 36 (46.2) | |
| Immunodeficiency-associated | | 27 (27.6) | 34 (43.6) | |
| Nosocomial | | 1 (1.0) | 8 (10.3) | |
| Travel-associated | | 2 (2.0) | 0 (0) | |
| Prior antibiotic therapy | 176 | 61 (62.2) | 58 (74.4) | .088[b] |
| Bloodwork | | | | |
| Hemoglobin | 176 | 12.1 (9.9, 14.0) | 9.7 (8.2, 11.7) | <.001[a] |
| WBC | 176 | 7.5 (5.5, 10.1) | 4.6 (2.5, 9.2) | .007[a] |
| CRP | 162 | 29.6 (4.7, 117.5) | 68.8 (23.2, 129.2) | .261[a] |
| ESR | 126 | 36.0 (10.0, 70.0) | 42.0 (21.0, 72.0) | .525[a] |
| Abnormal rheumatologic workup | 103 | 21 (29.2) | 11 (35.5) | .525[b] |
| Time from fever onset to microbiologic workup, d | 176 | 35.0 (6.0, 182.8) | 7.5 (1.0, 40.8) | .023[a] |
| Time from fever onset to mcfDNA-NGS, d | 176 | 73.5 (17.8, 293.8) | 23.0 (10.0, 55.3) | .010[a] |
| Service ordering mcfDNA-NGS | 176 | | | .143[b] |
| Infectious diseases | | 74 (75.5) | 67 (85.9) | |
| Other services (HIM, rheumatology, pulmonology) | | 24 (24.5) | 11 (14.1) | |
| mcfDNA-NGS ordered in inpatient setting | 176 | 37 (37.8) | 65 (83.3) | <.001[b] |
| Clinical indication for mcfDNA-NGS | 176 | | | <.001[b] |
| FUO without localization | | 44 (44.9) | 8 (10.3) | |
| FUO without clear pathogen, but radiographic foci | | 19 (19.4) | 18 (23.1) | |
| FUO with disseminated infection in ICH | | 16 (16.3) | 31 (39.7) | |
| FUO with disseminated infection in immunocompetent | | 12 (12.2) | 11 (14.1) | |
| FUO with suspected endovascular infection | | 7 (7.1) | 10 (12.8) | |
| Final diagnosis | 176 | | | <.001[b] |
| Infectious | | 18 (18.4) | 51 (65.4) | |
| Noninfectious | | 44 (44.9) | 17 (21.8) | |
| Unknown | | 36 (36.7) | 10 (12.8) | |

Values represent median (quartile 1, quartile 3) for continuous variables and frequency (percentage) for categorical variables.

Abbreviations: CCI, Charlson comorbidity index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; FUO, fever of unknown origin; HIM, hospital internal medicine; ICH, immunocompromised host; mcfDNA-NGS, plasma microbial cell-free DNA next-generation sequencing; WBC, white blood cell count.

[a]Analysis of variance.

[b]Pearson $\chi^2$ test.

[c]Kruskal-Wallis test.

quick turnaround time facilitated early and species-level identification of critical, but slow-growing pathogens like NTM and fungi. Similar rapid diagnosis with moderate sensitivity and high specificity has been previously demonstrated in infection due to NTM [25], *Mycobacterium tuberculosis* (SN, 68%; SP, 98%) [26], and *Aspergillus* and non-*Aspergillus* molds (SN, 44%–51%; SP, 95%) [13, 27]. Second, in a subset of patients, mcfDNA-NGS was positive ≥7 days prior to detection by BCX or culture from other sources. A pediatric study of patients with relapsed/refractory malignancy demonstrated a 75% predictive sensitivity of mcfDNA-NGS (detecting pathogens >3 days prior to BCX), supporting mcfDNA-NGS as a sensitive marker, particularly in hematogenous infection [28]. Third, disseminated infection due to difficult-to-diagnose pathogens like *C burnetii*, *T whipplei*, PCP, and NTM were readily detected by mcfDNA-NGS [5]. In several cases these pathogens were not routinely part of the differential diagnosis of FUO (Domain D), but identification of these critical pathogens warranted consideration of therapy as mcfDNA-NGS has high specificity in this context [10, 15, 17, 29]. However, the assay has limitations as demonstrated by our cohort, wherein 7 patients who had positive ST for these organisms had negative mcfDNA. The reason for this failure of detection is unclear, but may be impacted by timing of assay relative to the infectious course (too early vs too late) [13], exposure to antimicrobials, or limitations in assay performance/microbial data library [6]. While mcfDNA detection provides a noninvasive tool, further study of its diagnostic performance remains an important future direction.

**Table 4. Univariate Logistic Regression Model of Clinical Predictors Associated With Positive Plasma Microbial Cell-Free DNA Next-Generation Sequencing Test in Patients Evaluated for Fever of Unknown Origin**

| Variable | Unadjusted OR | (95% CI) | *P* Value |
|---|---|---|---|
| Age | 1.01 | (.99–1.03) | .277 |
| CCI score | 1.32 | (1.15–1.54) | **<.001** |
| Presence of immunocompromising condition | 2.45 | (1.34–4.55) | **<.001** |
| Prior antibiotic therapy | 1.76 | (.92–3.42) | .090 |
| Time from fever onset to mcfDNA-NGS ≤30 d | 2.42 | (1.32–4.50) | **.004** |
| Bloodwork | | | |
| Hemoglobin ≤10 g/dL | 3.58 | (1.92–6.83) | **<.001** |
| WBC ≤4.5 × $10^9$ cells/L | 7.17 | (3.48–15.7) | **<.001** |
| CRP ≥50 mg/L | 1.91 | (1.02–3.61) | **.043** |
| Definition of FUO | | | |
| Classic FUO | 0.38 | (.20–.70) | **.002** |
| Immunodeficiency-associated FUO | 2.03 | (1.09–3.84) | **.027** |
| Indication for FUO | | | |
| Without localization | 0.14 | (.06–.31) | **<.001** |
| Disseminated infection in ICH | 3.38 | (1.70–6.95) | **<.001** |
| Disseminated infection in immunocompetent | 1.18 | (.48–2.85) | .716 |
| Without clear pathogen, but radiographic foci | 1.24 | (.60–2.59) | .551 |
| Suspected endovascular infection | 1.91 | (.70–5.51) | .211 |

The bolded values refer to values observed to be statistically significant ($P < .05$).

Abbreviations: CCI, Charlson comorbidity index; CI, confidence interval; CRP, C-reactive protein; FUO, fever of unknown origin; ICH, immunocompromised host; mcfDNA-NGS, plasma microbial cell-free DNA next-generation sequencing; OR, odds ratio; WBC, white blood cell count.

In FUO, both positive and negative infectious workup can impact antimicrobial and immunosuppressive therapy. Patients with infectious FUO had higher empiric use of antimicrobials, while those in the noninfectious group had higher baseline IS. In the infectious group, diagnostic studies primarily resulted in antibiotic optimization (49.3%), with initiation of targeted antiviral/antifungal therapy noted in a minority. Benamu et al demonstrated a similar modification of antimicrobials in 47% of patients with febrile neutropenia (20% antibiotics, 14.5% antivirals, 3.6% antifungals) [12]. In patients with noninfectious FUO, the negative diagnostic workup primarily resulted in antimicrobial de-escalation/discontinuation, with augmentation/initiation of IS (34.5%). Further prospective evaluation is warranted because delineating the direct impact of mcfDNA-NGS on management of FUO was challenging in our cohort as patients concurrently underwent ST and treatment decision-making was often multifactorial.

Last, to better understand when and in whom to perform mcfDNA-NGS, we evaluated clinical factors associated with positive mcfDNA, with higher CCI score, WBC count ≤4.5 × $10^9$ cells/L, and an indication for mcfDNA-NGS other than FUO without localization noted to be significant predictors in the multivariable model. To date, no other studies have evaluated predictors of positive mcfDNA-NGS in FUO. Based on these clinical factors and previously noted SN and NPV, we propose an algorithm for consideration of mcfDNA-NGS as adjunct to ST in FUO with the assay performed either sequentially or concurrently based on logistical considerations (Supplementary Figure 5). mcfDNA-NGS assays like the Karius Test can be cost prohibitive ($2200/test) [30]. Therefore, cost-benefit analysis, similar to that performed for mcfDNA-NGS use in IFI [30], is an important consideration in FUO. Particular emphasis should be placed not only on the cost of the test relative to the need for comprehensive workup of FUO (clinical, radiographic, and invasive diagnostic studies), but also on the impact of false-positive mcfDNA-NGS on clinical care and the overall low yield of the assay in this clinical condition. While our study was not designed to address this analysis, we did observe an approximate cost of $10 185 per clinically significant mcfDNA-NGS test among all patients assessed for FUO. Consistent with the proposed algorithm, patients with FUO without localization had the lowest diagnostic yield with a cost of $28 600 per clinically significant mcfDNA-NGS. A comprehensive cost-benefit analysis incorporating timing of mcfDNA-NGS relative to standard testing represents an important future direction when developing a strategy for diagnostic stewardship of mcfDNA-NGS.

#### Limitations

Several important limitations warrant consideration when interpreting the study findings. First, the retrospective evaluation limits our ability to capture potential confounders impacting decision-making regarding timing and use of mcfDNA-NGS relative to ST in FUO. Second, the majority of patients with suspected FUO were referrals to our tertiary medical center and often had extensive prior workup at external institutions with granular data not always available. The low yield of ST noted in our study should be interpreted in this context as our cohort likely differs from patients presenting for initial FUO evaluation. Third, definition of standard testing of FUO is heterogenous. The 2-tiered ST approach highlighted in this study is the approach to FUO at our center and limits generalizability to all institutions. For instance, we did not consider invasive studies like BAL, thoracentesis, or biopsy as ST but rather secondary syndrome–driven workup. Similarly, COVID-19 testing was not considered ST, as our study predated the pandemic, but would now be routine practice. Fourth, despite having clear criteria for adjudication of clinical significance of mcfDNA-NGS by 2 independent reviewers, classifying the result as "clinically significant" is prone to subjective interpretation that is inherent to this type of assay in distinguishing signal from noise. The comprehensive narrative review in Table 2 is therefore aimed

at providing further insight into how concordance between ST and mcfDNA-NGS was assessed. Concurrently, molecular diagnostic assays such as mcfDNA-NGS cannot distinguish between active and prior infections and necessitate cautious interpretation in the context of additional microbiologic and syndromic information. Fifth, our study lacks a contemporary control as all patients in this study underwent both mcfDNA-NGS and ST. This limits our ability to comment on the independent additive diagnostic value of mcfDNA-NGS relative to ST. Rather, we focus on presenting the diagnostic impact of mcfDNA-NGS in parallel with ST with emphasis on concordance, timing, and potential avoidance of invasive diagnostics. Last, the study was limited in its ability to comment on appropriate timing of mcfDNA-NGS relative to ST and provide additional performance characteristics (SN/SP, PPV/NPV) due to a lack of gold standard approach in the diagnostic workup of FUO.

## CONCLUSIONS

Within this study we highlight 3 important conclusions: (1) Among patients with infectious cause of FUO, mcfDNA-NGS in adjunct to ST had an overall positive diagnostic impact in 30% of patients (primarily due to earlier diagnosis and potential for avoidance of invasive tests); (2) clinical predictors of positive mcfDNA testing included higher CCI score, WBC $\leq 4.5 \times 10^9$ cells/L, and an indication other than FUO without localization, with the latter 2 included in the proposed decision-making algorithm; and (3) positive mcfDNA in patients with infection and negative/noncontributory mcfDNA testing in those with noninfectious FUO impacted antimicrobial and immunosuppressive therapy. Future prospective studies should aim to delineate optimal timing and utilization of mcfDNA-NGS in FUO, determine strategies to minimize invasive diagnostic workup, and understand the cost-benefit ratio of mcfDNA in this syndrome.

### Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

### Notes

***Acknowledgments***. N. R., B. B. G., O. A. S., and M. F. contributed to the conception and design of this study. N. R., B. B. G., J. V., S. K., and M. K. contributed to data extraction. Statistical design and analysis were performed by N. R. Figures were created by N. R. All authors contributed significantly to the data analysis, writing, and review of the manuscript and approved the submission of this manuscript.

***Data availability.*** Data are not publicly available but can be made available upon reasonable request.

***Disclaimer.*** The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

***Financial support***. N. R., B. B. G., S. K., J. V., and M. K. were supported by the National Center for Advancing Translational Sciences (grant number UL1-TR002377).

***Potential conflicts of interest***. All authors: No reported conflicts.

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