Pediatric Rheumatology

RESEARCH ARTICLE Open Access

# Joint fluid multi-omics improves diagnostic confidence during evaluation of children with presumed septic arthritis

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## Abstract

**Background** An accurate diagnosis of septic versus reactive or autoimmune arthritis remains clinically challenging. A multi-omics strategy comprising metagenomic and proteomic technologies were undertaken for children diagnosed with presumed septic arthritis to advance clinical diagnoses and care for affected individuals.

**Methods** Twelve children with suspected septic arthritis were prospectively enrolled to compare standard of care tests with a rapid multi-omics approach. The multi-omics combined bacterial 16S rRNA metagenomics, single cell transcriptomics, and proteomics on knee joint fluid specimens. The diagnostic value of the multi-omics was ascertained relative to standard of care culture and PCR-negative results.

**Results** Ten children with suspected primary septic arthritis and two with acute hematogenous osteomyelitis (AHO) diagnoses were assessed. Joint fluid bacterial cultures were positive for 6/12 (50%) patients, consistent with elevated inflammatory markers (IL-4, IL-6, IL-17A, TNF-a, etc.). Metagenomic bacterial sequencing results were 100% concordant with the culture results. Six patients were culture- and PCR-negative. Multiomics analyses of the 6 culture negative patients established that 2/6 culture-negative children had inflammatory arthritis with potential Juvenile idiopathic arthritis (JIA) and 1 had post-Streptococcal Reactive Arthritis. The children without any bacteremia had autoantibodies (IgGs) in the joint-fluid targeting several nuclear antigens (i.e., *dsDNA, histones, Jo-1, scl-70, Ro/SS-A, SmDs, CENP-A* along with non-nuclear antigens i.e. *Albumin, Collagens, Myosin, Laminin,* etc. Single cell transcriptomics confirmed an abundance of $CD4^+$ follicular helper T (Tfh), CD8 + T cells and B cells in the autoantibody positive subjects. The combination of 16S DNA sequencing ($p = 0.006$), cytokine assays ($p = 0.009$) and autoantibody profiling ($p = 0.02$) were significantly distinct between those children with and without infections. This improved the diagnostic confidence for 9 of 12 (75%) children, key for treatment decisions.

**Conclusions** The multiomics approach rapidly identified children with bacterial or autoimmune inflammatory conditions, improving diagnostic and treatment strategies for those with presumptive septic arthritis.

**Keywords** Autoimmune, Cytokine Infection, 16S rRNA bacterial sequencing, Metagenomics, Next-generation sequencing (NGS), Pediatric, Septic arthritis; Streptococcus

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## Introduction

There is significant incidence of pediatric septic arthritis in United States [1]. Definitive diagnosis of acute bacterial arthritis remains challenging due to the complex clinical presentations and limitations of current basic laboratory assays [2, 3]. Inappropriate treatment of those with reactive or inflammatory conditions can occur if a presumed bacterial infection is not rapidly confirmed [4]. For example, 34 of 355 (9.6%) children who had been treated for acute bacterial arthritis were subsequently found to have noninfectious conditions, including viral conditions, Post-Streptococcal Reactive Arthritis (PSRA), or inflammatory arthritis [4]. This indicates that unnecessary invasive procedures and antibiotic treatment courses occurred. These were likely balanced against the risk of adverse outcomes from unrecognized and untreated bacterial arthritis. There is also a concern for substantial delay in the correct assessment and treatment of children with inflammatory conditions not only during the antibiotic treatment period but also while awaiting evaluation by pediatric rheumatology.

While polymerase chain reaction (PCR) techniques have improved our ability to confirm acute bacterial arthritis and identify causative pathogens, there are limitations to this technology, including limited species level specificity and lengthy turn-around times [5]. We have previously reported joint fluid culture positivity ranging from 31.0% to 35.1% and PCR positivity ranging from 47.1% to 54.1% for an overall rate of confirmed bacterial detection of approximately 50% [4, 6]. Children with culture and PCR negative for bacterial pathogens are labeled as presumed primary septic arthritis, and remain a clinical dilemma [4, 7, 8]. Their ultimate diagnosis is often guided by elevated nucleated cell counts in joint fluid, segmented neutrophil differential, inflammatory marker trends and clinical improvements following surgical and antibiotic treatment. However, there is a wide range of immune cell ratios in the nucleated cell counts and segmented neutrophil differentials in children with confirmed or presumed primary septic arthritis [9, 10]. New technologies to discover biomarkers to evaluate and stratify children with presumed primary septic arthritis and inflammatory conditions would be extremely beneficial.

Multi-omics is an integrated approach to combine genomics, transcriptomics, proteomics, and metabolomics [11]. Clinical applications of multi-omics are rapidly expanding globally [12]. Metagenomics Next-Generation Sequencing (mNGS) offers sensitive, quick, and culture-free identification of pathogens which are difficult to grow or identify in joint fluid [13]. The small sample volume requirement makes this especially attractive for use in young children and infants [14–16]. Furthermore, ability to rapidly identify biomarkers of host response to infection or inflammation through proteomics has the potential to improve the diagnosis and treatment of affected children.

Childhood arthritis prevalence ranges from 21 to 403 per 100,000 population among U.S. children and adolescents < 18 years old. A national survey (2017–2021) estimated that 220,000 U.S. children and adolescents are diagnosed with arthritis. Current clinical and laboratory parameters do not reliably differentiate those with infectious, inflammatory, versus autoimmune arthritis. It results in presumptive surgical and/or antibiotic therapy along with prolonged follow-up care.

The objective of the present study was to assess the feasibility and clinical utility of multiomics approach incorporating mNGS, cytokine/antibody proteomics, and single cell transcriptomics of joint-fluid to rapidly identify causal pathogens (viral, bacteria, fungal) or autoimmune manifestations (autoantibodies and cytokine repertoires). This will guide the clinical decision making for children diagnosed with presumed septic arthritis.

## Materials and methods

### Study population

Following Institutional Review Board approval (STU-2021–0970), 12 children diagnosed with presumed septic arthritis were enrolled for the present study via written consent before the surgery (Table 1). Apart from standard of care joint-fluid specimen processing, an additional 1–1.5 ml of joint fluid was collected from each participant in a sterile collection tube and stored at -80°C. Patient demographics, clinical parameters and laboratory test data are summarized in Table 1. Data analysis team was blinded to the patient´s characteristics.

### Data collection

#### *Specimen processing*

Patient is taken to the operating room by the Orthopedic Surgeon to undergo aspiration of the joint at Children's Health, Dallas, TX. During aspiration, a 18 gauge needle is inserted into the joint space, up to 1 ml fluid is aspirated and transferred to a sterile microtainer for standard of care processing (cell count, culture and PCR). A separate 1 ml sample is collected for the multi-omics study. The research sample is placed on dry-ice within 15 min of collection and transported to the Microbiome Lab for further processing. This specimen was used for various assays including DNA extraction, immune profiling and single cell transcriptomics. 250 ul of the joint-fluid was used for DNA extraction. About 50 ul specimen was used for cytokine measurements and antibody profiling. The single cell RNA sequencing assay was performed on 100ul joint-fluid following 10X Genomics's

**Table 1** Demographics, clinical chracteristics and laboratory test results of the study cohort

| Case ID | Demographic data | | | Standard of care blood tests | | | | | | | Standard of care Joint fluid tests | | | | mNGS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sex | Age | Race | WBC count (thousands/mm3) | Absolute neutrophil count (thousands/mm3) | Platelet count (thousands/mm3) | Initial CRP (mg/L) | Initial ESR (mm/h) | Initial PCT (ng/mL) | Peak CRP (mg/L) | Nucleated cells in JF (cells/mm3) | Absolute neutrophil count in JF (cells/mm3) | Standard culture | Standard of care PCR | Total 16S sequencing read counts in JF | 16S Identified potential pathogenic species |
| **JF-1** | Male | 7 years | Hispanic | 15.5 | 12.2 | 475 | 263 | >120 | 0.39 | 263 | 14,650 | 12,452 | Negative | *Streptococcus Pyogenes* | 55,526 | *Streptococcus Pyogenes* |
| **JF-2** | Female | 2 years | Hispanic | 23.6 | 18.6 | 538 | 82 | 92 | 0.41 | 82 | 222,500 | 198,025 | *Streptococcus Pyogenes* | *Streptococcus Pyogenes* | 31,505 | *Streptococcus Pyogenes* |
| **JF-3** | Male | 3 months | White | 15.1 | 4.8 | 726 | 59 | 35 | 0.11 | 64 | 155,000 | 141,050 | *Staphylococcus aureus* | Negative | 4,232 | *Staphylococcus aureus* |
| **JF-4** | Female | 2 years | Hispanic | 9.2 | 4.8 | 188 | 100 | 38 | 0.94 | 100 | Not tested | Not tested | *Streptococcus Pyogenes* | *Streptococcus Pyogenes* | 26,366 | *Streptococcus Pyogenes* |
| **JF-5** | Male | 3 years | White | 5.4 | 1.5 | 373 | 4 | 13 | <0.04 | Not tested | 6,750 | 5,130 | Negative | Negative | 3,093 | Negative |
| **JF-6** | Male | 13 months | White | 18.6 | 5.9 | 519 | 125 | 40 | 0.06 | 125 | 65,650 | 64,337 | Negative | Negative | 904 | Negative |
| **JF-7** | Female | 7 years | Hispanic | 21.6 | 18.1 | 244 | 159 | 45 | 1.05 | 316 | 119,000 | 102,340 | *Streptococcus Pyogenes* | *Streptococcus Pyogenes* | 23,935 | *Streptococcus Pyogenes* |
| **JF-8** | Female | 17 months | Hispanic | 12.6 | 2.9 | 674 | 21 | 63 | 0.09 | Not tested | Not tested | Not tested | Negative | Negative | 5,564 | Negative |
| **JF-9** | Male | 2 years | Hispanic | 9.5 | 4.9 | 310 | 27 | 43 | 0.07 | Not tested | 93,375 | 78,435 | Negative | Negative | 548 | Negative |
| **JF-10** | Male | 12 years | White | 14.3 | 12.4 | 79 | 268 | 37 | 7.89 | 268 | Not tested | Not tested | *Staphylococcus aureus* | Not tested | 51,010 | *Staphylococcus aureus* |
| **JF-11** | Male | 11 years | White | 7.6 | 5.2 | 210 | 280 | 45 | 0.12 | 280 | 34,625 | 29,431 | Negative | Negative | 2,240 | Negative |
| **JF-12** | Female | 3 years | Black | 7.7 | 4.7 | 379 | 18 | 70 | 0.04 | Not tested | 20,563 | 18,712 | Negative | Negative | 322 | Negative |

*Abbreviations*: *ID* Identification, *mNGS* Metagenomics Next-Generation Sequencing, *WBC* White Blood Cells, *CRP* C-Reactive Protein, *ERS* Erythrocite Sedimentation Rate, *PCT* Procalcitonin, *JF* Joint Fluid, *PCR* Polymerase Chain Reaction

sample preparation instructions. Single cell viability and concentration was assessed using Countess III Invitrogen instrument.

#### *Joint-fluid DNA extraction*

DNA was extracted from 250 µl joint-fluid specimen using ZymoBIOMICS DNA Miniprep Kit (D4300, Zymo Research, CA, United States) according to manufacturer's instructions. The DNA concentration was measured using the Picogreen method (Invitrogen Quant-iT™ Picogreen dsDNA Assay Kit Reference No. P11496 on Perkin Elmer 2030 Multilabel Reader Victor X3). DNA integrity number (DIN) was determined on 4150 Tapestation, using Agilent's gDNA Screen Tape (Reference No. 5067–5365) using Agilent's gDNA Reagents (Reference No. 5067–5366). Quality pass DNA was used for 16S sequencing assay.

### 16S sequencing to profile joint-fluid microbiome

We performed two different 16S rRNA gene sequencing assays. The first 16S assay profiles bacterial taxa based on the v3-v4 hypervariable region (469 bp) sequence of the bacterial 16S rRNA gene. The second assay sequences a complete16S rRNA gene (1.5 Kb) in real-time, providing high resolution, species level taxonomic data. 16S rRNA v3-v4 sequencing was performed on MiSeqDx using the Zymo Research Quick-16S NGS Library Prep Kit (Catalog No D6400). The 16S rRNA gene sequencing was performed on MinION sequencer using long-read sequencing technology from Oxford Nanopore, United Kingdom [17]. All twelve samples were sequenced on one R9.4.1 flow cell for 48 h on the MinION (Oxford Nanopore Technologies). RAW quality pass FASTQ files were used for taxonomic classification and downstream analysis.

### 16S sequencing data analysis

Samples with more than 50,000 quality pass 16S sequencing reads from MiSeqDx were used for 16S operational taxonomic unit (OTU) analysis. Taxonomic classification and OTUs abundance was performed using the CLC Bio microbial genomics module (https://www.qiagenbioinformatics.com/plugins/clc-microbial-genomics-module/). Individual sample reads were annotated with the Greengenes v13 database using a 97% similarity index.

Nanopore 16S data was analyzed using the EPI2ME pipeline and WIMP workflow using the Oxford Nanopore Technology. Human sequences were excluded with the NCBI BMTagger Human Contamination Screening Tool (ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/). OTUs were based on 97% sequence similarly. Taxonomic classification and OTUs abundance analyses were performed on the filtered sequences with the computational tool MetaPhlAn. Since taxonomic abundance analysis often results in a significant portion of the reads being classified into an unknown group, the unidentified sequences were run through the de novo assembly algorithm Velvet. Assembled contigs were mapped to National Center for Biotechnology Information (NCBI) bacterial reference genome database.

Quality control included positive and negative external controls. All 8 bacteria in the Zymo community control (D6300) sample were retrieved in the expected abundance ratio. A water sample, as a negative control, generated < 500 non-specific sequencing reads. Therefore, OTUs with > 500 sequencing reads were only considered for downstream analysis. The total observed reads for a given species were normalized with the total number of reads to calculate relative abundances for comparison. Bioinformatics analyses and data visualization were performed using the R statistical package. Raw FASTQ files from Illumina and Nanopore sequencing have been deposited in the BioProject database (NCBI) with accession number PRJNA 1097662.

### Immune profiling assays

#### *48-plex cytokine assay*

The 48-plex assay measured the quantitative level of innate cytokines, chemokines, and growth factors in the joint fluid. It is a 48-plex magnetic microspheres (bead) based Luminex assay (Bio-Plex pro Human cytokine Assay, Bio-Rad) performed on a Bio-Plex 200-flow cytometer and quantified utilizing xMAP technology (Luminex Corp., Austin, TX, USA). Fifty microliters of joint fluid were used for this cytokine assay. The cytokine concentration (pg/mL) was determined by fluorescence. A standard curve was used to calculate the quantitative range of various proteins following a previously published protocol [18].

#### *Autoantibody protein array*

The antibody repertoire of knee joint-fluid was profiled using a protein array assay. Our laboratory has developed a multiplex protein array to screen autoantibody responses against common autoantigens implicated in various rheumatic and autoimmune conditions [19, 20]. It is a protein microarray that detects autoantibodies recogning autoantigens including nuclear antigens, cytoplasmic antigens, cell membrane antigens, phospholipid-associated antigens, blood cells, endothelial cells, glomerular basement membrane, mitochondria, muscle, parietal cells, thyroglobulin, nervous system antigens, plasma proteins, matrix proteins, and miscellaneous antigens, which can trigger adaptive immune response in the form of production of autoantibodies. Purified antigens including DNAs or RNAs, recombinant proteins

or synthetic peptides are embedded on a glass slide with nitrocellulose membrane (NC), hydrogel, or polymers, which hold the proteins in their native conformation. After blocking, the protein arrays are hybridized with diluted clinical or biological specimens (can include such as blood serum, plasma, body fluids, joint-fluid or cell culture supernatants). Antigen bound antibodies are then detected with the fluorophore-conjugated secondary antibodies against different isotypes of autoantibodies (IgG/IgM/IgA). Each multiplex autoantigen microarray chip can process 16 samples per chip and detect up to different 120 autoantibodies for IgG and IgM isotypes.

Using the same array and protocol, IgG and IgM reactivities against 120 common autoantigens were profiled in the joint-fluid of children. Ten microliters of joint-fluid was used for the antibody assay. The net fluorescent signal intensity of each antigen was normalized by a robust linear model using internal positive controls and standardized for heat map presentations, which were generated by the R heatmap package [21].

#### Single cell RNA sequencing and analysis

Based on the 16S sequencing and antibody results, we selected one infection positive subject (JF7) and two infection negative subjects (JF8 & JF12) to perform single cell RNA sequencing analysis. Joint-fluid specimens were immediately transported to the Microbiome Research Laboratory at UT Southwestern Medical Center to perform the single cell rRNA sequencing assay. We used 100ul of joint-fluid for single cell RNA sequencing assay. A single cell viability score was > 80%. The 10 × Genomics Chromium controller instrument was used for Gel Bead- in Emulsion (GEMs) preparation using Chromium Next GEM Single Cell 3' Kit v3.1 (PC-1000269), Chromium Next GEM Chip G Single Cell Kit (PC-1000127) and Dual Index Kit TT Set A Kit (PC-1000215). Complementary DNA (cDNA) and barcoded sequencing libraries were generated as per the manufacturer's specifications. The quality and concentration of each library was assessed using the Bioanalyzer 2100 and qPCR, respectively. Quality pass single cell libraries were sequenced on NovaSeq6K sequencer using the paired end 150 bp sequencing kit. About 30–40 K sequencing reads were generated per single cell. Unique Molecular Identifier (UMI) counts for each cellular barcode were quantified and used to estimate the number of successfully captured and sequenced cells. Cell Ranger Single-cell Software Suit (v 5.0.0) was used for sample demultiplexing and single-cell 3' gene counting. SoupX R package (constantAmateur/SoupX: R package to quantify and remove cell free mRNAs from droplet based scRNA-seq data (github.com)) was used for ambient RNA reads cleaning. Doublets were identified using Scrublet (Scrublet: Computational Identification of Cell Doublets in Single-Cell Transcriptomic DataScienceDirect) and removed from downstream analysis.

#### Statistical analysis

Quantitative data were expressed by the median and interquartile range (IQR 25%-75%) and compared using the U-Mann Whitney test. Spearman correlation was used for correlation analysis between statistically significant variables. Blood biomarkers from patients diagnosed with concomitant osteomyelitis were excluded from the analysis. Differences with a $p < 0.05$ were considered statistically significant. Parametric and non-parametric tests were performed and values are provided in associated Supplemental Tables. All statistical analyses were performed using R (version 4.3).

## Results

#### Patient characteristics and clinical presentation

Demographics, clinical characteristics, and standard of care results for the 12 children in the study group are summarized in Table 1. Ten of twelve joint fluid samples were from suspected primary septic arthritis. Two children presented as acute hematogenous osteomyelitis (AHO) with contiguous joint involvement (JF10 and JF11), which was affirmed by positive blood cultures for *Staphylococcus aureus* and Magnetic Resonance Imaging (MRI) findings.

Overall, 6/12 (50%) joint fluid samples were PCR and/or culture positive, determined with standard-of-care assays. *Streptococcus pyogenes* and *Staphylococcus aureus* infections were confirmed in 4 and 2 subjects, respectively. The remaining 6/12 (50%) samples had no indication of bacterial infections. Of these 12, 2 cases were classified as presumed acute bacterial arthritis with no pathogen identified in the joint fluid (JF6, JF9) and based on an absolute neutrophil count in the joint fluid (> 50,000 cells/mm$^3$) [22]. The remaining four (JF5, JF8, JF11, JF12) were classified as undetermined. Overall clinical characteristics of infection-positive vs infection-negative subsets were similar, except PCT (procalcitonin) level which was significantly higher ($p = 0.04$) in infection positive group (Suppl. Table 1).

#### Results from multiomics assays

##### *Bacterial pathogens were detected in the knee joint-fluid of children*

16S sequencing analysis detected the presence of *Streptococcus pyogenes* DNA signature in the joint fluid of the 4 children, while 2 children had *Staphylococcus aureus* (Table 1, Fig. 1A). No significant pathogenic bacterial signature was detected in the remaining 6 children (50%). The median number of 16S sequencing read counts in

infection negative joint-fluids was significantly lower than those with infection (2112 [322–5564] vs 32,096 [4232–55526], $p=0.003$) (Suppl. Figure 1). The 16S rRNA bacterial reads stratified our study cohort into two groups, one with and one without infectious pathogen in the knee joint-fluid.

#### *Elevated level of inflammatory cytokines in infection positive joint-fluids*

Joint fluid cytokine analysis revealed significant differences in the innate immune response between children with versus without bacterial identification (Fig. 1B). The level of 36 inflammatory cytokines such as IL-4, GM-CSF, IL-12p70, IL-17A, MCP-1, IL-5, IL-8, IL-2, IL-1b, IL-6, TNF-a etc. were significantly elevated in children with positive identification of bacteria in the joint—fluid as compared to those without (T-test $p=0.005$) (Suppl. Table 2). Twenty two of 48 cytokines (46%) correlated significantly with the number of 16S sequencing reads. The strongest correlations were with LIF ($r=0.84$, $p=0.001$), IL-2Ra ($r=0.82$, $p=0.002$), MIP-1a ($r=0.79$, $p=0.004$) and GRO-a ($r=0.78$, $p=0.004$). Other cytokines with undetectable values in the negative group were GM-CSF, b-NGF, IL-5 and IL-12p70. The comparison of cytokine profiles between the *Streptococcus pyogenes* positive verses the *Staphylococcus aureus* positive group showed higher levels of Macrophage migration inhibitory factor (MIF), IL-15, SDF-1a, and IL-6 in the *Staphylococcus aureus* positive group, these changes were statistically significant ($p=0.01$) for IL-16 (Suppl. Figure 1).

#### *Infection negative patients exhibit strong autoantibody (IgG) signatures*

All 12 patients were screened for presence of autoantibodies in their joint-fluid. As shown in the heatmap in Fig. 1C, patients without any infection exhibited a higher prevalence of multiple autoantibodies that targeted nuclear (dsDNA, Chromatin, Histones, sm, snRNPs, Jo-1, etc.) and non-nuclear autoantigens (Collagens, Laminins, Albumin, etc.). Such antibodies are previously implicated in autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Rheumatoid arthritis, etc. [23]. We found that 33 autoantibodies were significantly (*Mann Whitney* U-test $p<0.05$) elevated in the infection negative group (Suppl. Table 2). Autoantibodies targeting Collagens, *Albumin, Factor 1 and Nup62* protein showed significant enrichment in infection negative patients (Suppl. Figure 2). Most strikingly, JF8 and JF12 exhibited the strongest autoantibody signatures with very high concentrations of many anti-nuclear antibodies (ANAs) including those targeted to *dsDNA, histones, Jo-1, scl-70, Ro/SS-A, SmDs, smRNPs, and CENP-A*. Similarly, JF5 & JF6 presented strong titers of anti- U1-snRNPs, complement, myosin, laminin, nucleosome and Sm autoantibodies, which represent non-nuclear protein targets previously reported in patients with autoimmune conditions. Interestingly, 3 of 6 infection negative subjects showed significantly ($p=0.02$) elevated level of autoantibodies targeting collagen proteins (i.e. Collagen I, II, III, IV) (Suppl. Figure 2).

#### *Single cell RNA sequencing data confirmed the presence of autoreactive T and B cells in infection negative patients*

To further confirm and investigate the autoantibody findings, we performed single cell RNA sequencing analysis in few representative patient joint-fluids.

These included JF7 (infection positive patient) and JF8 and JF12 (infection negative, autoantibody positive patients). As shown in Fig. 1D, single cell transcriptomics analysis detected 15,629 neutrophils (88%) 1,550 monocytes (16%), 238 macrophages (2%), 123 T cells (1%) and < 100 (1%) Dendritic Cells and Natural Killer cells in JF7 patient. JF8 joint fluid was significantly (~30-fold) enriched for T and B cells. We detected 4,920 neutrophils (49%) 332 monocytes (3%), 103 macrophages (1%), 3,286 T cells (33%), 282 B cells (3%), 157 Dendritic Cells (2%) and < 100 (1%) other cell types. Similarly, JF12 sample showed significant abundance of T cells [3003 cells (53%)], B cells [132 cells (2%)], Monocytes [1369 cells (24%)] and 10% (597) Neutrophils. More strikingly, JF8 and JF12 joint fluid showed > twofold abundance of CD4 + CXCR5 + follicular helper T cells (Tfh) as compared to JF7 (Fig. 2, S. Figure 5). Thus, consistent with the autoantibody

(See figure on next page.)

**Fig. 1** Multiomics data analysis in the study cohort. **Panel A** shows 16S sequencing result on 12 joint-fluid DNAs. The x-axis shows samples and y-axis shows number of 16s sequencing reads. Detected bacterial species is shown on the top of each bar. **Panel B** shows a heatmap on 48-plex cytokine expression in all 12 joint-fluid specimens. Blue and red color of the heatmap indicate relatively low and high cytokine expression, respectively. The blue bar-plot underneath heatmap shows sum of cytokine responses in each sample. **Panel C** shows a heatmap on IgG antibody protein array data. Each sample presents joint-fluid IgG antibody reactivities against 120 autoantigens labeled on the side axis. Blue and red color of the heatmap indicate relatively low and high autoantibody level, respectively. The pink bar-plot underneath heatmap shows sum of autoantibody responses in each sample. Some clinically significant autoantigens are listed in boxes on the side. **Panel D** presents single cell RNA sequencing data-based t-SNE plot on JF7, JF8 and JF12 sample. Each dot represents individual single cell. Detected immune cell types are indicated with different colors

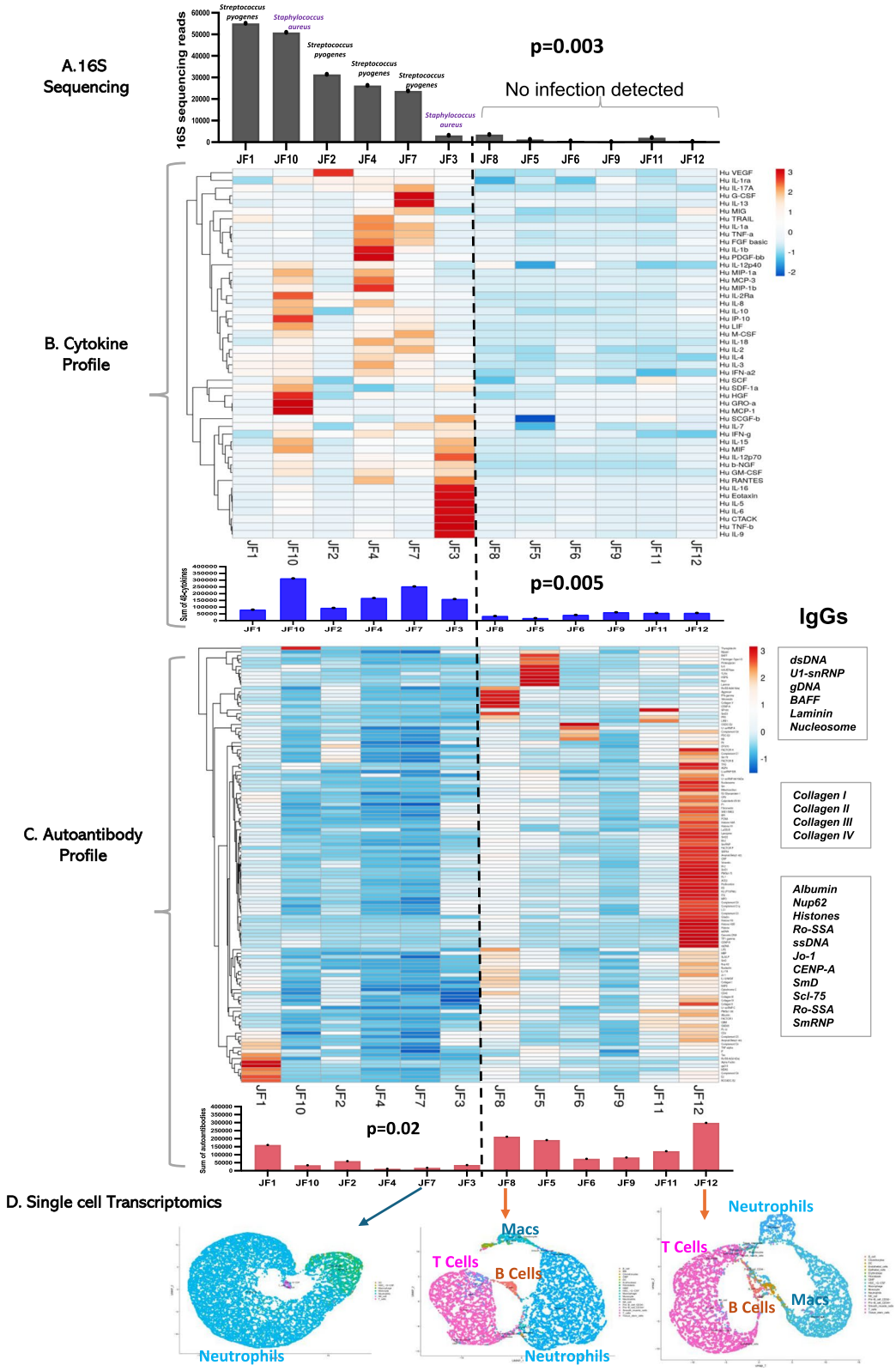


**Fig. 1** (See legend on previous page.)

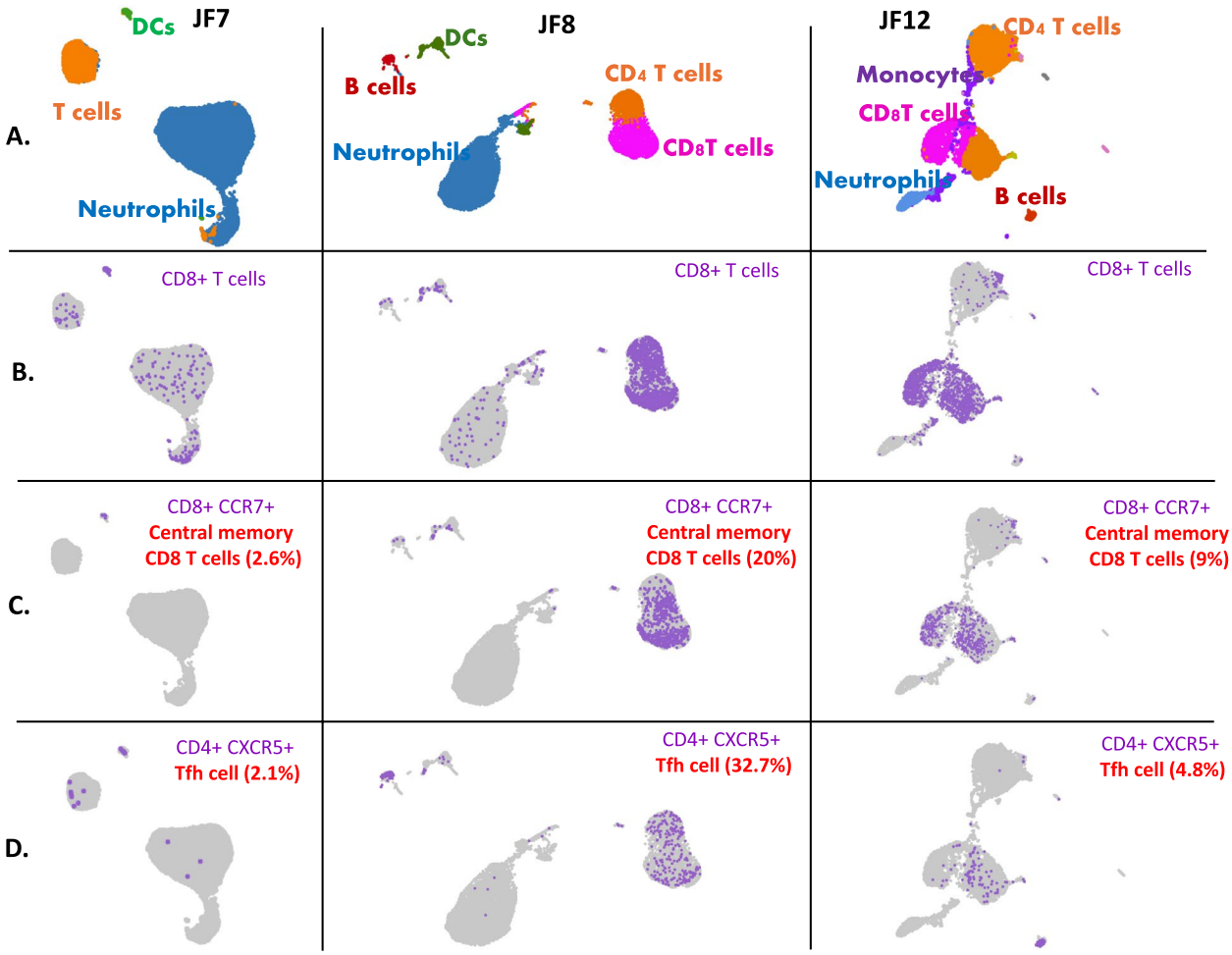


**Fig. 2** Single cell RNA sequencing unravel immune cell composition in infection negative and infection positive joint-fluids. **Panel A** shows t-SNE plot on various immune cell clusters detected in one infection positive (JF7) and two infection negative and autoantibody positive subjects (JF8 & JF12). Various cell clusters are generated based on the cell-specific gene expression signatures. Neutrophils are indicated with blue color, T cells with pink (CD8) and orange (CD4) and B cells with red color. Other small cell types are indicated by other colors. Each dot represent one individual cell detected by 10X genomics cell-ranger data analysis pipeline. **Panel B** shows distribution and abundance of central memory CD8+ T cells in three samples. **Panel C** presents percentage of CD8+CCR7+ autoreactive T cells in three samples. **Panel D** presents percentage of CD4+CXCR5+ Tfh cells in three samples. Single cell gene expression matrix was generated using 10X Genomics's Cell Ranger pipeline. Various cell clusters are visualized using 10x Genomics's Loupe browser software

data, single cell sequencing detected presence of antibody producing CD19 + B cells and abundance of autoreactive and apoptosis resistant CD8 + CCR7 + T cell subset selectively in JF8 and JF12 subjects and not JF7 (Fig. 2, Suppl. Figure 4&5).

Taken together, these data suggest that a subset of presumed septic arthritis cases present non-infectious etiologies and distinct innate and adaptive immune signatures in the joint-fluid, which are suggestive of conditions like Inflammatory arthritis, Juvenile idiopathic arthritis (JIA) and post-Streptococcal Reactive Arthritis.

## Discussion

The present study demonstrates the power of applying multiomics approaches to identify children with infectious versus non-infectious septic arthritis, including autoimmune pathogenesis. Our results provide strong evidence in support of clinical application of multiomic approaches for mainstream diagnostic practices [12, 24, 25].

We observed 100% concordance between bacterial culture positive samples and 16S sequencing results. This concurs with existing literature that support the use of 16S sequencing in identifying infectious pathogens in pediatric patients [26]. We also tested long-read

sequencing technologies to confirm the 16S rRNA gene sequencing strategies to establish a taxonomic characterization of knee joint-fluid bacterial populations [27]. The 16S sequencing results findings were 100% concordant with hospital PCR testing.

The joint fluid samples in the 6 children with confirmed bacteria infections had significantly higher expression of pro-inflammatory cytokines, including IL-1b, IL-6, IL-4, IL-2Ra, and GRO-a consistent with published literature [28–30]. One of the most strongly elevated cytokines was GRO-alpha (CXCL1), a chemoattractant that recruits neutrophils to the site of infection [31]. Interestingly, it was exclusively expressed in joint fluids with confirmed bacterial presence. Among elevated cytokines, IP-10, IL-1B, IL-18 and IL-6 are well-established markers of pathogenic *S. pyogenes* infections in early innate immune responses [32, 33]. IL-15 and IL-16 were significantly elevated in *Staphylococcus aureus* positive joint fluid (JF-3 & JF-10) as compared to samples positive for *Streptococcus pyogenes*. These 2 cytokines are implicated in *Staphylococcus aureus* induced arthritis [34], Overall, the cytokine data clearly distinguished children with and without bacterial infection.

Six children without a pathogenic bacterial signature by standard of care testing or (JF5, JF6, JF8, JF9, JF11, JF12), reveals a common diagnostic dilemma [10]. The cytokines levels in this cohort were consistent with negative 16S sequencing results, strongly supporting a non-infectious cause [35]. Autoantibodies are established biomarkers of rheumatic and autoimmune diseases, such as systemic lupus erythematosus (SLE) [23]. The autoantibody signatures in infection negative group suggest an autoimmune pathology in this subset. This is consistent established autoreactive antibodies in rheumatic conditions such as SLE (*SmD, U1-snRNPs, dsDNA Albumin)* [36], arthritis *(Albumin)* [37], myositis *(Nup62)* [38] and collagen-induced arthritis *(Collagen II, Collagen III & IV)* [39]. In this context, sample JF8, JF9 and JF12 had the highest levels of these autoantibodies. This suggested an autoimmune pathology in these three subjects. It is known that individuals can producet autoantibodies several years prior to the onset of clinical autoimmune symptoms [40]. Consistent with this, the antibody data was corroborated in three children during afollow up visit. Two of these subjects (JF8 & JF12) were confirmed to be JIA following a rheumatological work-up. The strong autoantibody signatures in JF8 and JF12 were further supported by single cell RNA sequencing data showing abundance of autoreactive Tfh, CD8 + CCR7 + T cells and B cells in these subjects. Follicular T helper cells (Tfh) are crucial for B cell differentiation and autoantibody production in autoimmune diseases and has been correlated with disease activity and anti-DNA antibody titer [41]. Consistent with this lietaure, observed enrichment of Tfh and CD8 + CCR7 + T cells in JF8 and JF12 joint fluid suggest role of Tfh and CD8 + T cells in induction, progression and pathogenesis of autoimmune diseases in infection negative and autoantibody positive cases [42]. One subject (JF11) was confirmed as PSRA as evidenced by elevated Anti-Streptolysin O and Anti-DNAse B titers.

Altogether, these findings reveal the value of multiomics approaches for infection negative children diagnosed with septic arthritis. The multiomics tests utilized have the benefit of a rapid diagnostic turnaround time (within 48 h of specimen procurement), a considerable improvement over current practices [43]. This supports the multi-omics will be informative for both culture positive and culture negative cases [44]. For the child with JIA (JF8 & JF12), the single cell sequencing analysis was consistent with a strong antibody profile. This would support a clinical decision that high dose antibiotics should not have been administered, and the child would have benefited from an expedited rheumatology consultation. Similarly, JF11 would have undergone testing for Anti-Streptolysin O and Anti-DNAse B titer. This is particularly important in young children with acute monoarthritis as clinical and laboratory parameters do not reliably differentiate between infectious, inflammatory, or reactive arthritis, which necessitates ongoing follow-up and presumptive antibiotic therapy while observing for resolution or evolution toward alternative diagnostic considerations [45].

The present study is limited by small sample size and demographic variables. This ability to rapidly characterize the joint fluid of children beyond routinely used parameters has tremendous potential for clinical application. However, the small sample size and missing clinical data contributed to a lack of statistical significance of some known biomarkers of acute bacterial arthritis. This limits the broad conclusión on diagnostic application of this approach at population level. Another limitation is previous antibiotic administration prior to treatment, which may impact clinical read outs as well as immune profile. Another limitation was the lack of serum samples to profile cytokine and antibody systemically to compare the joint-fluid results. An ongoing follow up in the subset of patients with autoimmune phenotypes is underway. Other causal etiologies in culture/PCR negative subset could be infections by rare pathogens that are missed. For example, *Kingella kingae* has been implicated in musculoskeletal infections in young children [46]. JF-5 subject in our study is suspected to have *Kingella kingae* infection given the young age of the child, male sex, indolent presentation with minimally elevated inflammatory markers, and history of antibiotic pre-treatment with amoxicillin,

consistent with published literature [47, 48]. Similarly, JF-6 and JF-9 are thought to be presumed primary septic arthritis associated with Kingella as they had clinical and laboratory improvement to normalcy within the course of treatment with antibiotics and no recurrence in the period of follow-up. The infection negative samples showed small number of 16s sequencing reads mapping to non-pathogenic organisms such as *Phyllobacterium, Rhizobiaceae and Micrococcus*, which may be sequencing artifacts or contaminants that need further investigation and confirmation.

In summary, our study support the idea that 16S sequencing and single cell analysis combined with cytokine and antibody characterizations enable a quick evaluation and management of suspected primary septic arthritis and AHO. Beyond rapidly confirming or eliminating infectious etiologies, a multi-omics approach may reveal developing inflammatory, autoimmune or reactive conditions early on. Further studies with larger cohorts of children are needed to confirm and expand upon our current findings.

## Conclusions

The multiomics approach, including a comprehensive autoantibody screening of joint-fluid, rapidly identified those children with either pathogen reactive versus autoimmune inflammatory conditions. This will help guide appropriate treatment strategies in a higher percentage of cases than previously possible using standard evaluation strategies. Based on our results, a rapid autoantibody screening of joint fluid, especially in case of culture/PCR negative cases, is of clinical value.

### Abbreviations

| | |
|---|---|
| AHO | Acute Hematogenous Osteomyelitis |
| DNA | Deoxyribonucleic acid |
| IL | Interleukin |
| JF | Joint Fluid |
| mNGS | Metagenomics Next-Generation Sequencing |
| NCBI | National Center for Biotechnology Information |
| OTU | Operational Taxonomic Unit |
| PCR | Polymerase Chain Reaction |
| PSRA | Post-Streptococcal Reactive Arthritis |
| RNA | Ribonucleic acid |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12969-025-01060-z.

Additional file 1

Additional file 2

### Acknowledgements

Authors are grateful to The Hartwell Foundation for funding this research study. Authors gratefully acknowledge and thank study participants and their families for providing invaluable specimens to conduct the present research. Authors also acknowledge Microbiome, Microarray and Genomics Core Facility in the department of Immunology at UT Southwestern Medical Center.

### Authors' contributions

YPL: Interpretation of data, Investigation, Manuscript writing and editing, NGT: Particpant consent and sample aquisition, BZ: Single cell sequencing assay and analysi, IR: Autoantibody CAA: Sequencing and data análisis, CZ: Cytokine and Antibody data statistics and methods, YL: 16S sequencing and análisis, method, PS: Cytokine assay and analysis, NSCvO: Manuscript editing and interpretation, SM: Autoantibody data interpretation, discusión, manuscript editing, LVH: Methodology, Investigation, Resources; LAC: Patient enrollment, Clinical diagnosis, Data Interpretation, P.R., Resource, Study Conceptualization, investigation, data analysis, manuscript writing & editing. All authors have read and agreed to the publication of this manuscript.

### Funding

This study was funded by the Hartwell Foundation grant to Dr. Prithvi Raj.

### Data availability

All 16S rRNA raw sequence data were deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) under the BioProject ID -PRJNA1097662 and is available at the following URL: https://urldefense.com/v3/http://www.ncbi.nlm.nih.gov/bioproject/1097662_-BioProject—NCBI(nih.gov). Single cell data, cytokine and antibody arrays are available from the Microbiome, Microarray and Immunophenotyping Core at UT Southwestern Medical Center upon request. Further array data from our study are included in Supplemental information.

## Declarations

### Ethics approval and consent to participate

This research involved human subjects. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from the parents or guardians of the children who served as subjects of the investigation. All families had knowledge that the findings would be published in a scientific journal. The Institutional Review Board at UT Southwestern Medical Center approved this study (IRB# STU-2021–0970).

### Consent for publication

All participants and investigators/ co-authors have agreed and provided the consent for publication of the present manuscript.

### Competing interests

None.

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Received: 5 July 2024 Accepted: 8 January 2025
Published online: 24 January 2025

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