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Supplemental information

Noninvasive diagnosis of secondary infections in COVID-19 by sequencing of plasma microbial cell-free DNA

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SUPPLEMENT:
Table S1: Baseline characteristics by sequencing run success, related to Table 1.

Variable	Pass	Pass Qualitatively	Fail	P-value
N	25	5	12	
Men, n (%)	20 (64.5)	1 (20.0)	8 (61.5)	0.12
Age (median [IQR])	68.7 [60.2, 76.8]	78.4 [63.1, 86.8]	58.5 [57.2, 64.7]	**0.04**
BMI (median [IQR])	31.6 [26.4, 34.0]	30.1 [26.8, 36.4]	39.6 [28.7, 42.4]	0.35
Diabetes, n (%)	11 (44.0)	3 (60.0)	4 (33.3)	0.59
COPD, n (%)	9 (36.0)	1 (20.0)	0 (0.0)	**0.05**
Immunosuppression, n (%)	2 (8.0)	1 (20.0)	1 (8.3)	0.7
Invasive Mechanical Ventilation, n (%)	14 (56.0)	4 (80.0)	9 (75.0)	0.39
ECMO, n (%)	3 (12.0)	1 (20.0)	4 (33.3)	0.3
WBC (median [IQR])	9.4 [6.3, 11.8]	11.1 [11.1, 11.5]	10.2 [7.4, 13.7]	0.46
WHO ordinal scale at ICU admission (median [IQR])	5.0 [5.0, 6.0]	5.5 [5.0, 6.5]	7.0 [6.0, 8.5]	**0.01**
RALE score (median [IQR])	22.5 [21.0, 28.0]	23.0 [22.8, 24.0]	27.5 [20.6, 35.8]	0.56
SARS-CoV-2 RNA in Plasma (cps/mL, median [IQR])	196.5 [3.0, 3349.0]	6352.0 [3286.0, 26029.3]	2491.0 [335.0, 6874.5]	0.3
IL6 pg/ml (median [IQR])	33.9 [16.9, 124.9]	165.7 [89.7, 1109.9]	51.1 [15.7, 221.3]	0.19
IL8 pg/ml (median [IQR])	22.2 [13.9, 33.6]	35.9 [29.9, 181.2]	17.6 [15.1, 62.3]	0.29
ST2 pg/ml (median [IQR])	142138.6 [78170.4, 198277.7]	253415.2 [218252.9, 578677.0]	287500.3 [216718.3, 328329.0]	**<0.01**
TNFR1 pg/ml (median [IQR])	4186.0 [3555.3, 7971.9]	9493.9 [6465.9, 12598.6]	4919.9 [3417.1, 6956.2]	0.44
SPD pg/ml (median [IQR])	24.3 [10.0, 43.0]	80.5 [20.8, 141.9]	26.0 [13.2, 53.1]	0.46
RAGE pg/ml (median [IQR])	4245.9 [2419.5, 9023.2]	4518.6 [2410.7, 9982.9]	5518.4 [2884.1, 7936.1]	0.97
Ang-2 pg/ml (median [IQR])	3905.8 [1935.6, 7130.4]	4505.1 [3942.7, 8903.3]	4543.4 [2886.8, 10443.9]	0.5
Procalcitonin pg/ml (median [IQR])	280.0 [167.0, 558.5]	3630.8 [1767.8, 4556.7]	580.8 [240.5, 1478.0]	**0.01**

Pentraxin-3 pg/ml (median [IQR])	6085.9 [2937.8, 13127.0]	19508.6 [6766.4, 32196.3]	12979.7 [7122.9, 18778.3]	0.1
Human cfDNA MPM (median [IQR])	200.3 [91.3, 424.2]	722.2 [686.2, 1299.7]	1343.1 [949.5, 3731.9]	**<0.01**
Total microbial cfDNA MPM (median [IQR])	794.8 [1.0, 1863.1]	1480.8 [818.1, 21805.9]	NA	NA
Bacterial cfDNA MPM (median [IQR])	252.3 [1.0, 1343.9]	1480.8 [537.1, 21805.9]	NA	NA
Fungal cfDNA MPM (median [IQR])	1.0 [1.0, 1.0]	1.0 [1.0, 1.0]	NA	NA
Respiratory pathogen cfDNA MPM (median [IQR])	1.0 [1.0, 1015.8]	1480.8 [1.0, 21805.9]	NA	NA

P-values significant below threshold of 0.05 are shown in bold. Abbreviations: Microbiologically-Diagnosed Secondary Infection (Micro-SI); Clinically-Diagnosed secondary infections (Clinical-SI); No Clinical Suspicion for SI (No-Suspected-SI); chronic obstructive pulmonary disease (COPD); extracorporeal membrane oxygenation (ECMO); Radiographic Assessment of Lung Edema score (RALE score); receptor for advanced glycation end products (RAGE); suppression of tumorigenicity (ST-2); tumour necrosis factor receptor (TNFR-1); Surfactant Protein D (SPD); molecules per microliter (MPMs).

Figure S1. Host response plasma biomarker comparisons between clinical groups, related to Table 1.

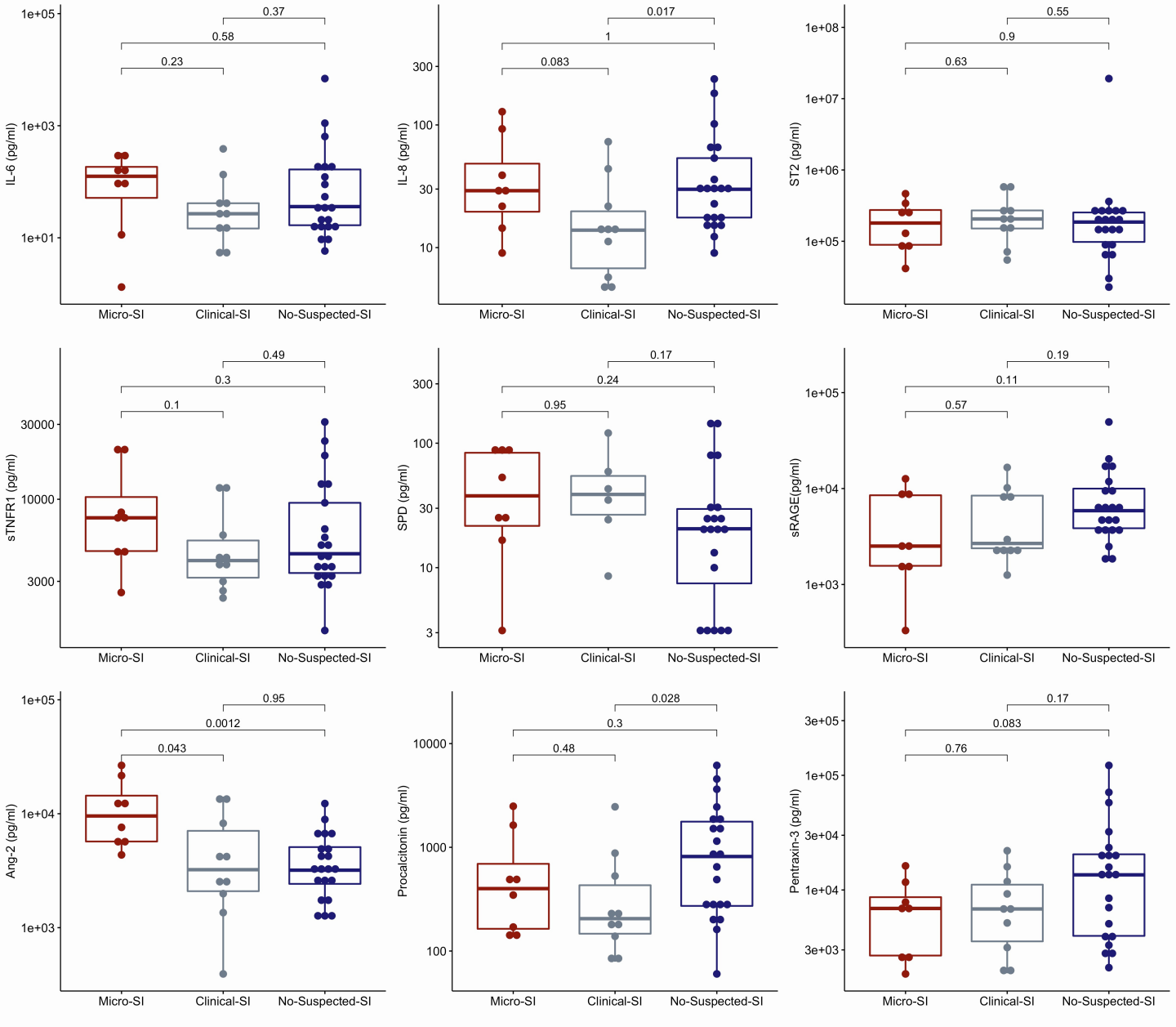

Figure S2: Comparisons of host response plasma biomarkers by WHO ordinal scale of severity at the time of admission (levels 4-9), related to Table 1.

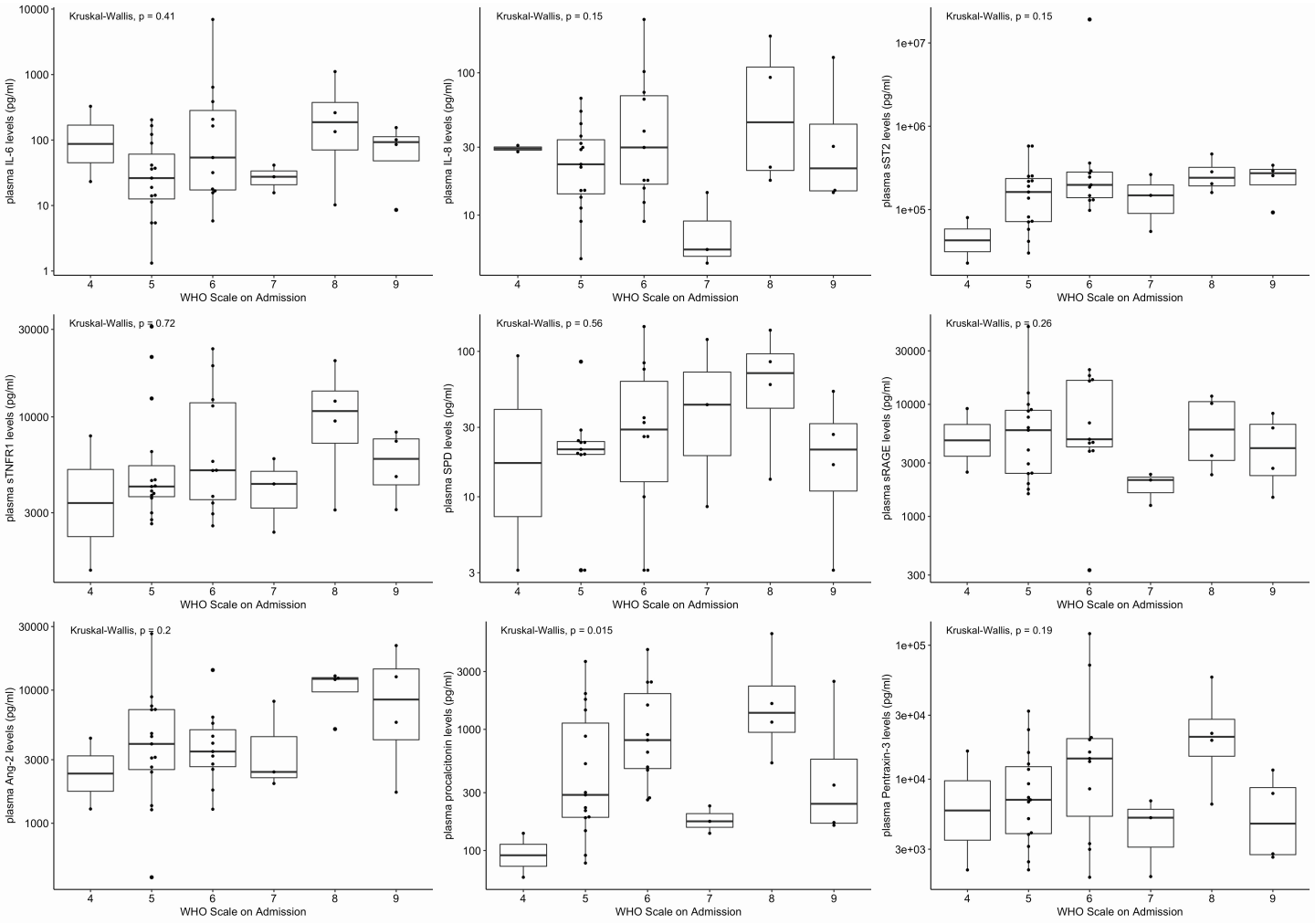

Figure S3: Comparisons of host response plasma biomarkers by the level of respiratory support required at the time of sampling (ECMO vs. Invasive Mechanical Ventilation [IMV] or non-invasive support), related to Table 1.

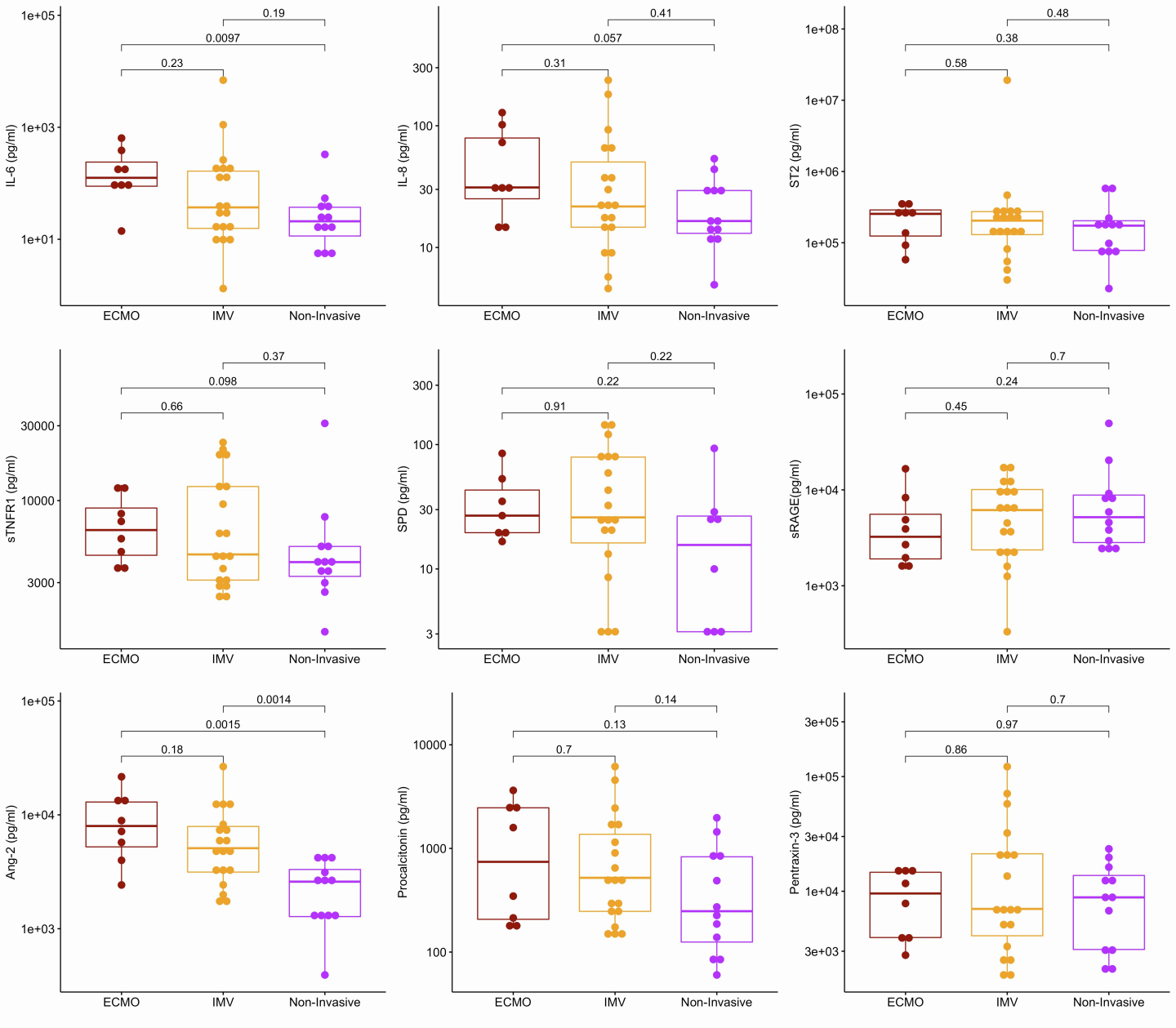

Figure S4: Successful plasma metagenomic sequencing runs had significantly lower levels of human cell-free DNA compared to unsuccessful runs, related to Table 1 and Figure 2. We classified the derived metagenomic sequences as human (hcfDNA) vs. microbial (mcfDNA), expressed as molecules per microliter (MPMs). Based on meeting minimum sequencing coverage metric required for quality control, we classified sequencing runs as successful ("Pass"), "Qualitatively Pass" or "Failed". Baseline "Pass" samples had significantly lower hcfDNA compared to "Qualitatively Pass" or "Failed" samples (Wilcoxon test pairs p<0.001, panel A). We also found that among subjects with both Day 1 and Day 5 samples, those samples that failed on both time points per subject (i.e. "0" sequencing success in panel B) had significantly higher hcfDNA levels compared to "Pass" samples on both days (Wilcoxon p-value <0.001). Data in boxplots are represented as individual values with median values and interquartile range depicted by the boxplots.

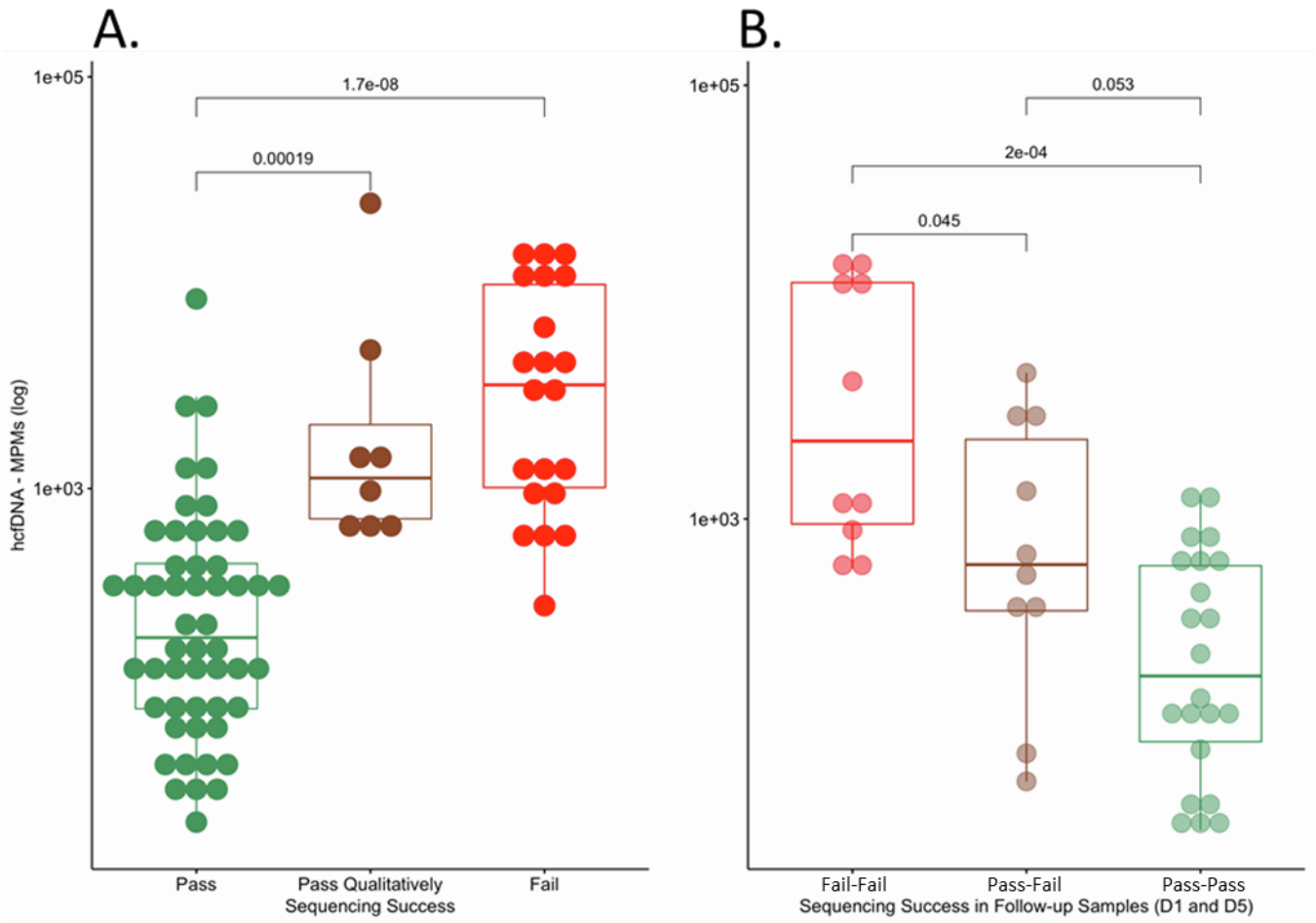

Figure S5: hcfDNA levels by sequencing run success, stratified by ECMO status, related to Table 1 and Figure 2. Data in boxplots are represented as individual values with median values and interquartile range depicted by the boxplots.

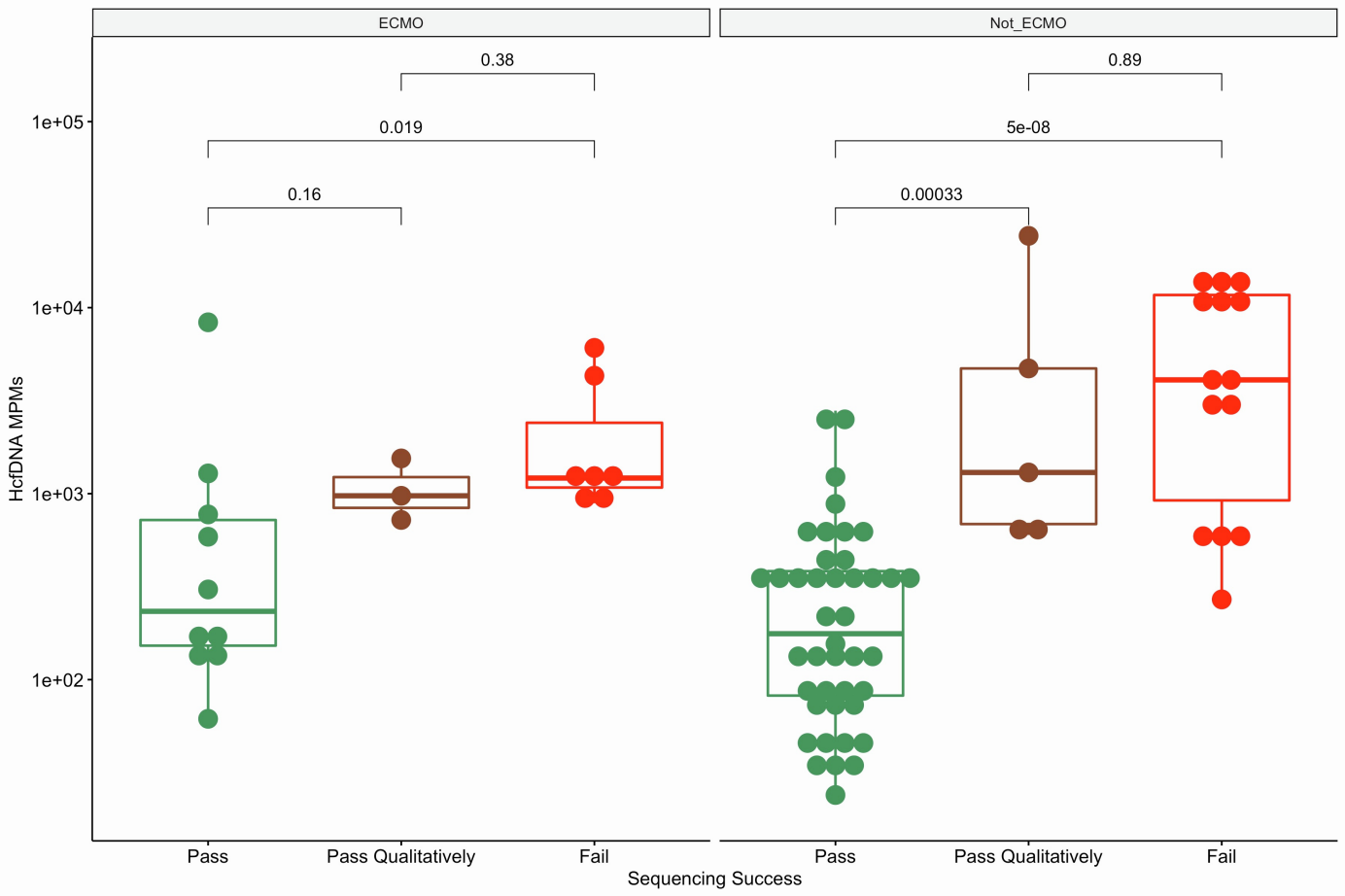

Figure S6: Subjects with COVID-19 have much higher levels of human cell-free DNA compared to non-COVID subjects with and without pneumonia, related to Table 1 and Figure 2. To contextualize the circulating cfDNA load in our COVID-19 cohort, we compared hcfDNA, total mcfDNA and pathogen mcfDNA MPMs between the COVID-19 SI categories against available published data from our group for mechanically ventilated patients with microbiologically-confirmed pneumonia (n=26, MCP), clinically-diagnosed pneumonia (n=41, CDP) and uninfected controls (n=16, intubated for airway protection or due to cardiogenic pulmonary edema). We found markedly higher levels of hcfDNA in subjects with COVID-19 compared to all non-COVID patient groups (p-values shown for the No-Suspected-SI only for parsimony). Non-COVID patients with microbiologically-confirmed pneumonia had higher mcfDNA levels compared to patients with COVID-19 with No-Suspected-SI, who in turn had markedly higher mcfDNA levels compared to uninfected controls. Data in boxplots are represented as individual values with median values and interquartile range depicted by the boxplots.

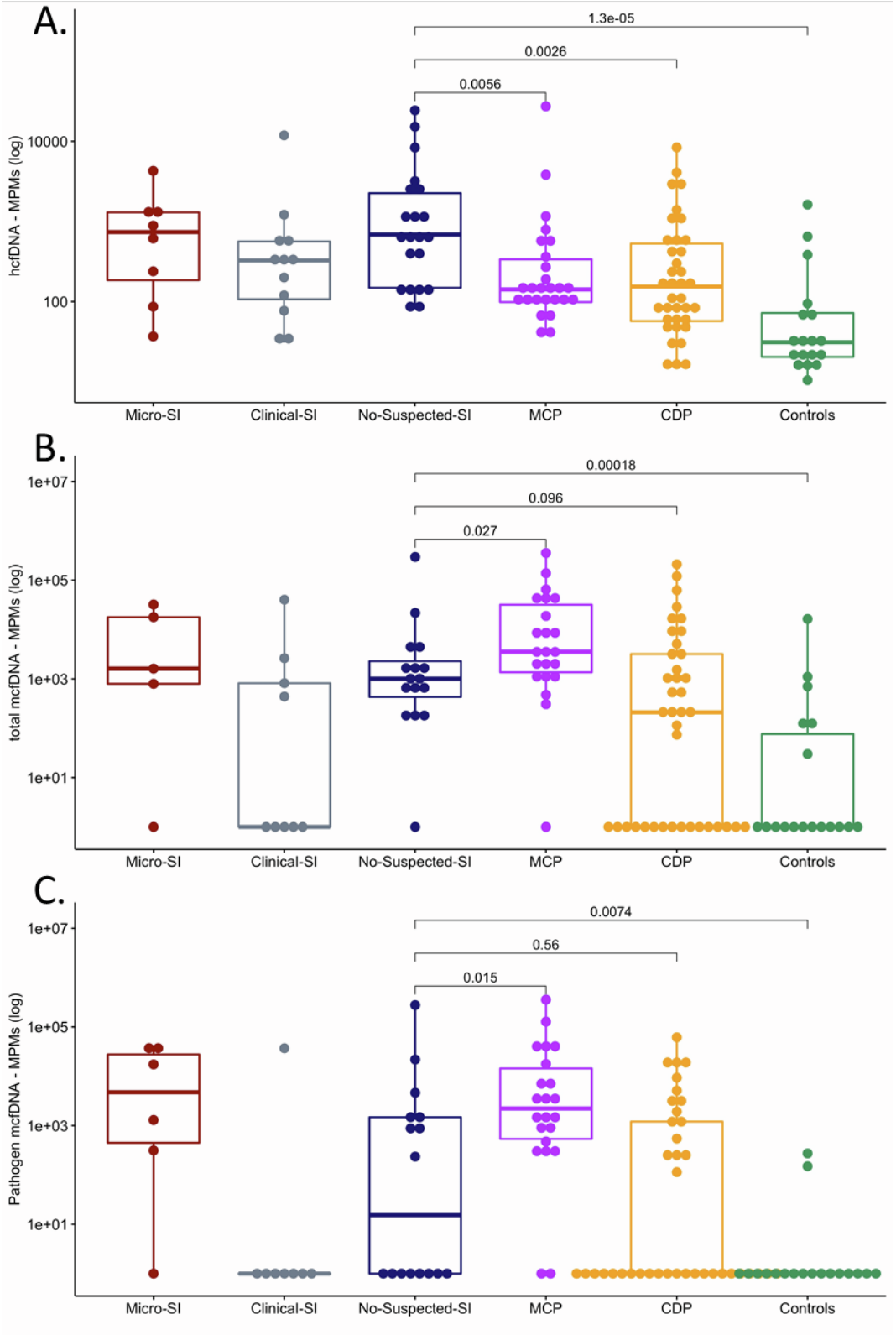
A.
1.3e-05
0.0026
0.0056
hcfDNA - MPMs (log)
10000
100
Micro-SI
Clinical-SI
No-Suspected-SI
MCP
CDP
Controls
B.
0.00018
0.096
0.027
total mcfDNA - MPMs (log)
1e+07
1e+05
1e+03
1e+01
Micro-SI
Clinical-SI
No-Suspected-SI
MCP
CDP
Controls
C.
0.0074
0.56
0.015
Pathogen mcfDNA - MPMs (log)
1e+07
1e+05
1e+03
1e+01
Micro-SI
Clinical-SI
No-Suspected-SI
MCP
CDP
Controls

Figure S7: Longitudinal evaluation of SI classifications with corresponding changes in mcfDNA and hcfDNA levels, related to Figure 4. The Sankey plot shows the subjects who transitioned between SI categories throughout the study from days 1, to 5, and 10 (A). Height of bars represents number of subjects. Attrition occurred throughout the study leading to decreased height of day 5 and 10 nodes. HcfDNA was not significantly different among SI groups across the study period (B). Total mcfDNA was not significantly different among SI categories on enrollment (C). Micro-SI subjects had significantly higher total and pathogen mcfDNA versus No-Suspected-SI subjects at Day 5 (C, D).

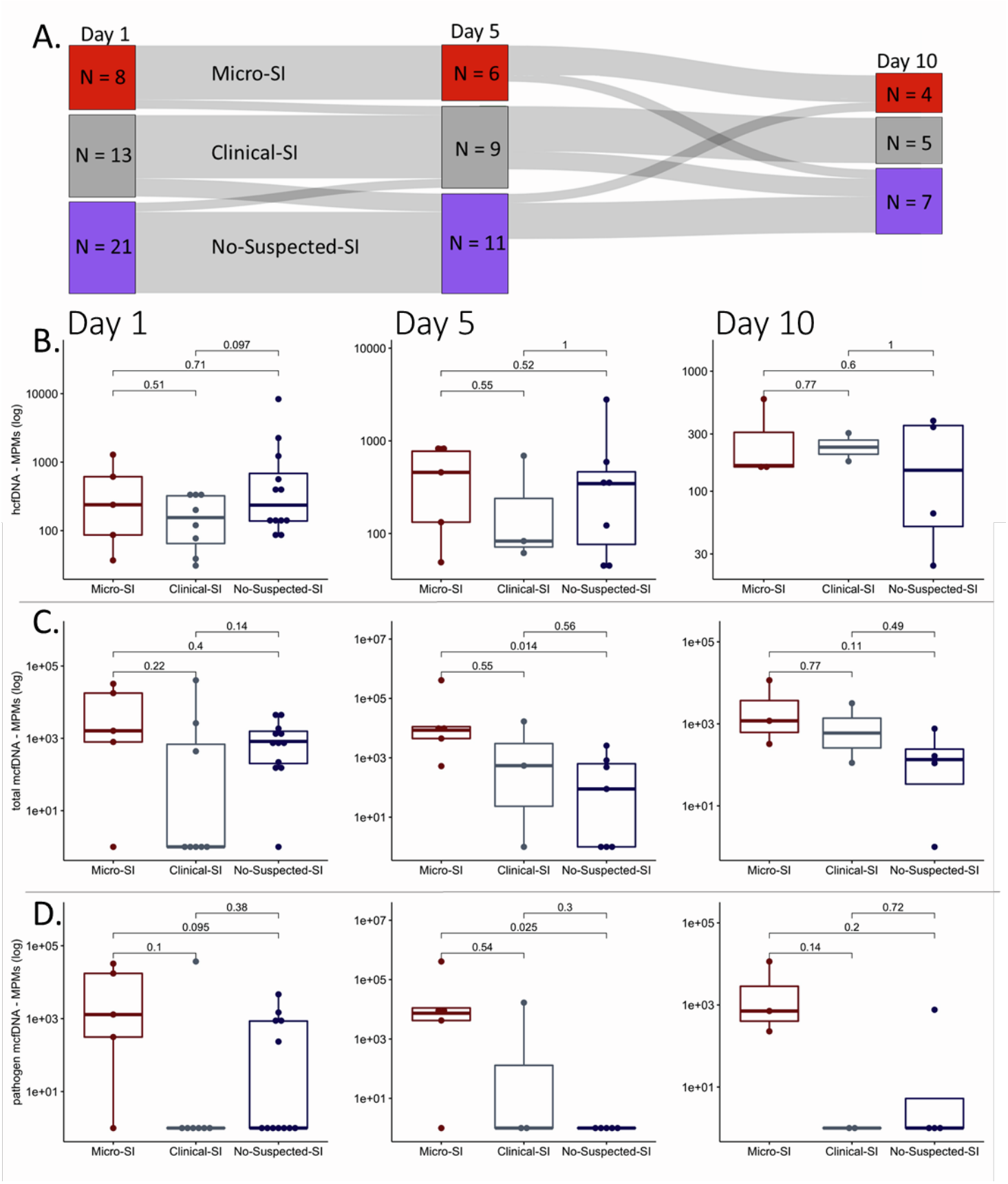
A.
Day 1
Day 5
Day 10
N = 8
Micro-SI
N = 6
N = 4
N = 13
Clinical-SI
N = 9
N = 5
N = 21
No-Suspected-SI
N = 11
N = 7
B.
Day 1
Day 5
Day 10
hcfDNA - MPMs (log)
0.097
0.71
0.51
1
0.52
0.55
1
0.6
0.77
Micro-SI
Clinical-SI
No-Suspected-SI
C.
total mcfDNA - MPMs (log)
0.14
0.4
0.22
0.56
0.014
0.55
0.49
0.11
0.77
D.
pathogen mcfDNA - MPMs (log)
0.38
0.095
0.1
0.3
0.025
0.54
0.72
0.2
0.14

Figure S8: Human and total mcfDNA trajectories among survivors and non-survivors, related to Figure 6. Trajectories of human cfDNA and total mcfDNA were not significantly different across the study period in survivors and non-survivors (A, B).

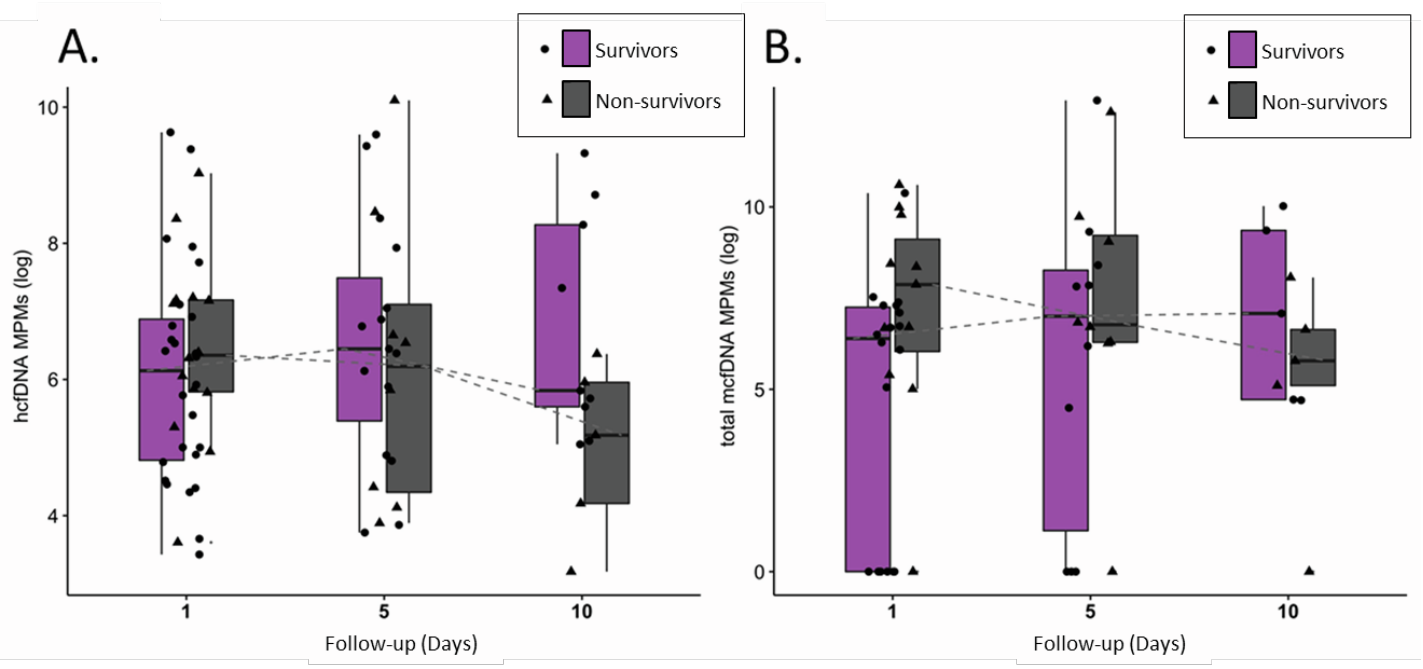

Table S2: Detailed results of mcfDNA positive/negative findings by SI category, among all sequenced samples and among “Pass” samples only, related to Figure 2.

	Samples	All patients	Micro-SI	Clinical-SI	No-Suspected-SI	Fischer’s p-value
All		82	22	23	37	
	Pass (% total)	52 (63)	16 (73)	13 (57)	23 (62)	0.54
	Pass, positive for mcfDNA (% of total pass samples)	40 (77)	15 (94)	7 (53)	18 (78)	**0.04**
Baseline		42	8	13	21	
	Pass (% baseline samples)	25 (60)	5 (63)	8 (62)	12 (57)	1
	Pass, positive for mcfDNA (% of baseline pass samples)	18 (72)	4 (80)	3 (38)	11 (92)	**0.02**

Table S4: Linear regression models for the adjusted effects of cfDNA (human and microbial) on host biomarkers when controlling for the plasma viral load variable, related to Figure 5. Significant results are highlighted in bold.

Biomarker	mcfDNA variable p-value	vRNA variable p-value
IL-6	**0.007**	0.132
IL-8	0.085	0.095
SPD	**0.028**	0.206
Biomarker	hcfDNA variable p-value	vRNA variable p-value
IL-6	0.076	0.525
IL-8	0.093	0.310
Procalcitonin	**0.003**	0.943
Pentraxin-3	**0.0007**	**0.037**

Table S5: Microbial species classifications, related to STAR methods.

Taxon	Class	Anatomic Compartment
Actinomyces graevenitzii	Recognized pathogen	Oral
Actinomyces odontolyticus	Recognized pathogen	Oral
Bacteroides ovatus	Microbe with unclear clinical importance	GI
Bacteroides vulgatus	Microbe with unclear clinical importance	GI
Burkholderia cepacia complex	Recognized pathogen	Respiratory, Nosocomial
Candida parapsilosis	Microbe with unclear clinical importance	Skin, GI
Candida tropicalis	Microbe with unclear clinical importance	Skin, GI
Corynebacterium striatum	Microbe with unclear clinical importance	Skin
Enterococcus faecalis	Recognized pathogen	GI
Enterococcus faecium	Recognized pathogen	GI
Escherichia coli	Recognized pathogen	GI
Haemophilus influenzae	Recognized pathogen	Oral
Haemophilus parahaemolyticus	Microbe with unclear clinical importance	Oral
Helicobacter pylori	Microbe with unclear clinical importance	GI
Human herpesvirus 1	Microbe with unclear clinical importance	Unknown
Human herpesvirus 4	Microbe with unclear clinical importance	Unknown
Klebsiella pneumoniae	Recognized pathogen	Oral, GI
Klebsiella variicola	Recognized pathogen	GI
Lactobacillus gasseri	Microbe with unclear clinical importance	GI
Pantoea ananatis	Microbe with unclear clinical importance	Unknown
Pediococcus acidilactici	Microbe with unclear clinical importance	GI
Prevotella bivia	Microbe with unclear clinical importance	Oral
Prevotella denticola	Microbe with unclear clinical importance	Oral
Prevotella oris	Microbe with unclear clinical importance	Oral
Proteus mirabilis	Recognized pathogen	GI
Pseudomonas aeruginosa	Recognized pathogen	GI, Pulmonary, Nosocomial
Raoultella ornithinolytica	Recognized pathogen	GI
Rothia mucilaginosa	Microbe with unclear clinical importance	Oral
Staphylococcus aureus	Recognized pathogen	Skin
Staphylococcus epidermidis	Microbe with unclear clinical importance	Skin
Streptococcus gordonii	Microbe with unclear clinical importance	Oral
Streptococcus infantarius	Microbe with unclear clinical importance	Oral
Streptococcus mitis	Recognized pathogen	Oral, Skin
Streptococcus parasanguinis	Recognized pathogen	Oral
Streptococcus salivarius	Microbe with unclear clinical importance	Oral
Streptococcus sanguinis	Microbe with unclear clinical importance	Oral
Streptococcus thermophilus	Microbe with unclear clinical importance	Oral
Scardovia wiggsiae	Microbe with unclear clinical importance	Oral

Table S6: References for pathogenic microbe classifications, related to STAR Methods.

Species	Reference
Actinomyces graevenitzii	[1]
Actinomyces odontolyticus	[1]
Burkholderia cepacia complex	[2]
Pediococcus acidilactici	[3]
Prevotella bivia	[4]
Prevotella denticola	[4]
Raoultella ornithinolytica	[5]
Scardovia wiggsiae	[6]
Staphylococcus epidermidis	[7]
Streptococcus gordonii	[8]
Streptococcus infantarius	[9]
Streptococcus sanguinis	[10]

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S1. Hall, V. (2008). Actinomyces—gathering evidence of human colonization and infection. Anaerobe *14*, 1-7.

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S10. Zhu, B., Macleod, L.C., Kitten, T., and Xu, P. (2018). Streptococcus sanguinis biofilm formation & interaction with oral pathogens. Future Microbiol *13*, 915-932. 10.2217/fmb-2018-0043.