Supplementary material

IMMUNOLOGICAL COROLLARY OF THE PULMONARY MYCOBIOME IN BRONCHIECTASIS: THE CAMEB STUDY

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SUPPLEMENTARY MATERIALS AND METHODS

Study Population: Patients for inclusion, at screening had confirmed radiological bronchiectasis by high resolution computed tomography (HRCT) scanning of the thorax. Patients were recruited during routine visits to the outpatient clinic and were clinically stable at recruitment. Clinical stability was defined as the absence of new symptoms and where no change to bronchiectasis therapy had occurred in the preceding six week period. Patients were excluded if they had any other major respiratory diagnosis (asthma or COPD) out-ruled by clinical symptoms and established spirometry criteria [1, 2], were pregnant or breastfeeding, had active mycobacterial disease or were on chemotherapy for malignancy. Patients with any active infection (necessitating acute use of antibiotics) or taking systemic corticosteroids in the four weeks preceding recruitment were excluded. Ten non-diseased controls were recruited in Singapore and defined as never smokers with no active or past history of any respiratory or other medical disease with normal spirometry (Easy One© NDD Medical Technologies). A total of n=138 bronchiectasis patients were recruited to the Singapore/Kuala Lumpur (SG-KL) cohort, and n=100 'matched' individually by age, sex and total bronchiectasis severity index (BSI) score (assigned at time of sample acquisition) to patients in the Dundee (DD) cohort (total CAMEB study population, n=238). Patients known to have active ABPA (defined by established criteria) at enrolment were excluded [3].

Ethical approval: This study was approved by the institutional review boards of all participating institutes as follows: CIRB 2016/2073 mutually recognised by DSRB; NTU IRB-2016-01-031; UKMMC FF-2016-440 and NHD 12/ES/0059.

Clinical data and specimen collection: Disease severity was assigned according to Bronchiectasis Severity Index (BSI) and further divided into 'Mild' (BSI; 0-4), 'Moderate' (BSI; 5-8) or 'Severe' (BSI; 9 and above) categories, which was the basis for disease severity matching in CAMEB [4]. Sputum and blood were obtained from each participant. Spontaneously expectorated 'representative' sputum from a deep cough with the assistance of a chest physiotherapist (where appropriate) was collected in sterile containers and transported (on ice) for evaluation [5]. An equal volume of Sputasol (Thermo Fisher Scientific) was added to each sample and shaken for 15 minutes at 37°C. Sputasol-homogenised samples were either stored (-80°C) or mixed with two volumes of RNAlater (Sigma-Aldrich) for DNA extraction and mycobiome analysis [6]. Blood specimens were collected in vacutainer serum tubes (BD biosciences) and centrifuged at 1300g for 10 minutes at 18°C to separate serum which was used for immunological studies. All CAMEB specimens from clinical sites were transported promptly, appropriately and processed centrally in Singapore to ensure consistency and standardisation of all experimental work. To ensure quality control of materials transported from sites outside Singapore, specimens were temperature controlled and their integrity checked on arrival to Singapore before experimental use.

Statistical analysis: All continuous data was tested for normality by the Kolmogorov-Smirnoff test. Categorical data was assessed by Chi-squared or Fisher's exact test as appropriate. For non-normal data, Mann-Whitney U-testing was used for group comparisons. For comparison of three or more groups of non-normal measures the Kruskal-Wallis test was employed with Dunn's *post hoc* test and Benjamini-Hochberg correction for multiple comparisons. For multiple group comparisons of normally distributed measures, analysis of variance (ANOVA) with Tukey's *post hoc*

analysis was applied. The Metastats statistical method for group comparison of microbiome data was employed to reveal discriminant taxa associated with identified patient groups [7]. Differences were considered significant at $p < 0.05$ and analysis performed using R Statistical Software (version 3.2.4).

Sputum DNA extraction: Sputum DNA was extracted using methods previously described [6]. Briefly, sputum samples in RNAlater were centrifuged at 13000rpm for 10 minutes and resultant pellets resuspended in 500µL sterile phosphate-buffered saline (PBS) (GE Lifesciences) and transferred to sterile bead mill tubes (VWR) containing 1mm sterile glass beads (Sigma-Aldrich). Next, homogenisation using a bead mill homogeniser (VWR) was performed and DNA purified using the Roche High-pure PCR Template Preparation Kit (Roche) according to manufacturers' instructions.

Mycobiome analysis

Construction of Shotgun Sequencing Libraries of Amplified Fungal Internal Transcribed Spacer (ITS) Regions ITS1 and ITS2: The previously described flanking primer pair ITS1 and ITS4 was chosen amplify the ITS1-5.8S-ITS2 Internal Transcribed Spacer (ITS) regions (Table E1) [8]. To assess the ability of this primer pair to target the ITS region in diverse fungal taxa relevant to our investigations, the Polymerase Chain Reaction (PCR) efficiency of the primers was determined against different fungal species (Table E2). Briefly, dilution series (1:10) of isolated DNA forming six taxonomically distinct fungi including both clinical and non-pathogenic species were performed (ranging from 10^5-10^2 estimated genome equivalents per reaction) and amplified by qPCR. Amplifications were performed in optical 96-well

plates using a StepOnePlus Real-time PCR instrument and PowerUP SYBR Green chemistry (Applied Biosystems). The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C (30 s), 57°C (15 s), and 72°C (60 s) followed by the generation of a melt curve to verify amplification specificity. Reactions contained 4.8 µL template DNA, 5 µL 2x PowerUP SYBR Green Master Mix (Applied Biosystems), 0.1 µl forward and reverse primers (500 nM final concentration) in a total volume of 10 µL. The PCR efficiency (E) was calculated as $E = (10^{-1/slope} - 1) \times 100$ (Table E2).

For the preparation of amplicon shotgun sequencing libraries, purified sputum DNA served as a template for PCR using the ITS1/ITS4 primers. Each PCR reaction contained 50 ng of extracted DNA in a master mix containing the following components (Thermo Fisher Scientific); 2.5 µl of 10X buffer, 1.5 µl of 50 mM $MgCl_2$, 0.14 µl of Platinum Taq DNA polymerase, 0.5 µl of 25mM dNTPs and 2.5 µl of each primer at a concentration of 1 µM. Reaction volumes were adjusted to 25 µl with molecular grade water. PCR conditions were as follows: denaturation at 94°C for 2min, followed by 35 cycles at 94°C for 30s, annealing at 57°C for 30s and extension at 72°C for 1min. Agencourt AMPure XP beads (Beckman Coulter) were used to purify amplicons using 1.5 times the volume of PCR product with final elution in 20 µl of EB buffer (Qiagen). Purified products were then visualised using Agilent Bioanalyser, prepared with an Agilent DNA1000 Kit (Agilent Technologies). PCR product was topped-up to 50µl and subjected to shearing using Adaptive Focused Acoustics™ (Covaris). Fragment size ranged from 150 – 600 bp. DNA libraries were prepared by using the Gene Read DNA Library I Core Kit (Qiagen) according to manufacturers' instructions except for the use of a custom adaptor in place of

GeneRead Adapter I Set (Table E1). Fourteen cycles of enrichment PCR was carried out with index-primers according to a protocol adapted from the Multiplexing Sample Preparation Oligonucleotide kit (Illumina). Libraries were quantified using Agilent Bioanalyser, prepared with Agilent DNA1000 kit (Agilent Technologies). Paired-end sequencing (2 x 101 bp reads) was performed on DNA libraries using the Illumina HiSeq2500 platform.

Bioinformatic methods: Raw sequence reads (3.29 +/- 0.184 million reads per sample) were quality trimmed using famas (v0.0.7) and analysed using a modified version of the pipeline described in Ong *et al*. [9]. The depth of coverage achieved saturation of sequencing libraries as determined using 'rarecurve' from the R package 'vegan' (Figure E2) [10]. Given the high level of sampling depth, rarefication was not performed mitigating against the loss of statistical power associated with subsampling approaches [11]. The rarefaction curve represents, for each sample, the number of genera identified given the fraction of sequencing data analyzed. The complete UNITE database (UNITE_public_22.08.2016) was clustered at 97% using VSEARCH (v1.9.3) and ITS reads were assembled into full-length ITS sequences by EMIRGE using the pre-clustered UNITE database sequences [12, 13]. The full length ITS sequences were subsequently mapped to the UNITE representative sequences (sh_general_release_dynamic_22.08.2016) using both GraphMap (version 0.2.2) and BWA MEM (version 0.7.12) [14, 15]. For all alignments, hits covering less than 40% of the reference sequence and below the predefined percent identity (94.5% at the genus level and 70% at kingdom level) were not considered for classification purposes and filtered out. The results from both GraphMap and BWA MEM were assessed with preference given to GraphMap hits in the event of disagreement

between alignments. Beta-diversity, expressed as Bray-Curtis distance between samples was assessed using 'vegdist' from the 'vegan' R package [10].

Quantitative PCR (qPCR) for detection and conidial quantification of *Aspergillus spp.* in sputum: The presence of four major *Aspergillus spp.* in sputum was assessed using a probe-based qPCR assay previously described by Walsh *et al.* [16]. *A. fumigatus* and *A. flavus* probes tagged with a 5' 6-carboxyfluorescein (FAM) reporter dye and a 3' tetramethyl rhodamine isocyanate (TAMRA) quencher and *A. niger* and *A. terreus* probes tagged with a 5' HEX reporter dye and a 3' NFQ-MGB quencher (Integrated DNA technologies) were used. Details of the primers and probe sequences are provided in Table E3. qPCR assays with crossover thresholds (CT) values of <40 for were considered positive. For quantification of sputum conidial burden, the 18S ITS1 region was amplified as described and cloned using the TA-cloning system in the pGEM-T easy vector (Promega) in order to generate standard curves ranging from a 10^2 – 10^6 gene copies, against which the conidial burden per gram of sputum for each sample was determined [6]. For each qPCR reaction the following components were included (Applied Biosystems): 10µL of 2X Taqman gene expression master mix; 1µL of primer-probe mix containing 750 nM of each primer and 300nM of probe; 5µL of template DNA; 2.5 µL of internal positive control (IPC) master mix including IPC target DNA and 1.5µL of molecular grade water. Inclusion of the IPC allowed assessment of PCR inhibitors in the DNA extract for each sputum sample. Reactions were setup in Microamp fast optical 96-well reaction plates and run on a Quantstudio 6-flex system (Applied biosystems) under the following conditions: denaturation at 95°C for 30s followed by 40 cycles at 95°C for 3s and annealing/extension at 60°C for 30s.

Immunological bioassays

Total serum IgE: Total serum IgE was measured using the Human IgE ELISA kit (Abcam) according to the manufacturer's instructions. All samples were run in duplicate with one set of IgE reference standards per microplate. Samples above the cut-off value of 100 Au/mL were considered positive.

Immuno-dot blot assay for Aspergillus-specific IgE (sIgE) measurement: Immuno-dot blot assay for specific immunoglobulin-E (sIgE) response to *A. fumigatus* and *A. terreus* respectively was assessed using established published methodologies by our group [17-21]. Briefly, crude protein extracts from *A. fumigatus* and *A. terreus* (both obtained as defatted mold allergens from Greer Laboratories Inc., Lenoir, N.C.) were prepared by homogenisation and suspended in phosphate buffered saline (PBS). Protein concentration of the crude extracts was determined by Bradford assays [22] and NanoDrop quantification (Thermo Fisher Scientific). Serum titres of sIgE against *A. fumigatus* and *A. terreus* crude extracts were then determined as follows: 1 µg of each allergen was dotted in duplicate onto a nitrocellulose membrane coupled to serial dilutions of IgE standards (1000 IU/mL serially diluted two-fold to 0.195 IU/mL; National Institute for Biological Standards) for standard curve determination. One microgram each of bovine serum albumin (BSA) and protein buffers were employed as a negative protein control and a negative control, respectively. Membranes were air-dried, blocked with PBS-T 0.1% (1 X PBS with 0.1 % Tween 20) and incubated overnight with patient serum (1:4 in PBS) at 4°C. After washing, membranes were incubated with anti-human IgE antibodies conjugated with alkaline phosphatase (1:1000 in PBS; Sigma Aldrich). Alkaline phosphatase activity was subsequently detected by addition of nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-

phosphate (BCIP) solution for 10 minutes (Thermo Fisher Scientific). Spot intensities on the membrane were then measured using Syngene imaging software and normalised. Inter- and intra-assay concordance exceeded 90% and 95% respectively, demonstrating strong assay reproducibility. Multiple dilution experiments were performed to demonstrate linear parallelism between the specific IgE and total IgE standard curves over the linear range of the specific IgE dilutions.

Thymus and Activation Regulated Chemokine (TARC) ELISA: Serum TARC (CCL17) levels were measured using the Human TARC/CCL17 sandwich ELISA kit (Sigma-Aldrich) according to the manufacturer's instructions. The detection limit was 10-2500 pg/mL. All samples were run in duplicate with one set of TARC standards per microplate. Samples above the cut-off value of 386 pg/mL were considered positive [23].

Aspergillus-specific *IgG:* Serum *Aspergillus*-specific specific Immunoglobulin-G (IgG) antibodies were measured using the Platelia Anti-*Aspergillus* IgG kit (Bio-rad) according to the manufacturer's instructions. The assay detection range is 0-80 AU/mL and all samples were run in duplicates with one set of *Aspergillus* IgG calibrators per microplate. Values between 5-10 AU/mL and >10 AU/mL were considered intermediate and strong positives respectively.

Sputum galactomannan (GM): Aspergillus-associated sputum GM antigen was measured using the Platelia *Aspergillus* Ag kit (Biorad) according to the manufacturer's instructions and as previously described [24]. Duplicate samples were run and values ≥ 0.5 considered positive [24].

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SUPPLEMENTARY TABLE AND FIGURE LEGENDS

Table E1. Primers and adaptor sequences used to generate ITS libraries.

Primer	Sequence
ITS 1	5'–TCCGTAGGTGAACCTGCGG –3'
ITS 4	5'– TCCTCCGCTTATTGATATGC –3'
Custom adaptor	5' P-GATCGGAAGAGCACACGTCT
	5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Table E2. Calculated PCR efficiencies of the ITS1-ITS4 primer pair for the ITS regions of phylogenetically distinct fungal species.

Genus	Representative species	Slope	Y-intercept	R^2	PCR efficiency (E)
Aspergillus	*A. fumigatus*	-4.02	29.62	0.999	77.34
	A. terreus	-3.74	29.54	0.993	85.24
Penicillium	*Penicillium* spp. M29	-3.80	29.65	0.996	83.40
Candida	*C. tropicalis*	-3.90	28.54	0.996	80.50
Curvularia	*C. lunata*	-3.57	31.19	0.998	90.61
Byssochlamys	*B. spectabilis*	-3.97	29.50	0.997	78.72

Table E3. Primers and probes for detection of specific *Aspergillus species*.

Species	Primers	Probe
A. fumigatus	5'–GCCCGCCGTTTCGAC–3' 5'–CCGTTGTTGAAAGTTTTAACTGATTAC–3'	5'–CCCGCCGAAGACCCCAACATG–3'
A. terreus	5'–CATTACCGAGTGCGGGTCTTTA–3' 5'–CCCGCCGAAGCAACAAG–3'	5'–CCCAACCTCCCACCCGTGACTATTG–3'
A. niger	5'–GCCGGAGACCCCAACAC–3' 5'–TGTTGAAAGTTTTAACTGATTGCATT–3'	5'–AATCAACTCAGACTGCACGCTTTCAGACAG–3'
A. flavus	5'–CGAGTGTAGGGTTCCTAGCGA–3' 5'–CCGGCGGCCATGAAT–3'	5'–TCCCACCCGTGTTTACTGTACCTTAGTTGCT–3'

	Non-diseased (ND)	***Aspergillus* Colonized (AC)**	***Aspergillus* Sensitized (AS)**	**Serologic ABPA (sABPA)**	**Suspected CPA (sCPA)**
Aspergillus qPCR (*A. fumigatus* and/or *A. terreus*)	-	+	+/-	+/-	+/-
Aspergillus specific IgE (> 0.35 KU/L)	-	-	+	+	-
Aspergillus specific IgG (> 5 IU/mL)	-	-	-	+	+
Sputum Galactomannan (OD > 0.5)	-	+/-	+/-	+/-	+

Table E4. Summary of the immunologic classification used to stratify patients by category of Aspergillosis including Non-diseased (ND); *Aspergillus*-colonized (AC); *Aspergillus*-sensitized (AS); serological allergic bronchopulmonary aspergillosis (sABPA) and suspected chronic pulmonary aspergillosis (sCPA). These criteria have been modified from that published for use in cystic fibrosis by Baxter *et al.* [24]

	All vs;					ND vs;			AC vs;		AS;
	ND	AC	AS	ABPA	AS+ABPA	AC	AS	ABPA	AS	ABPA	ABPA
Candida	0.799	0.638	0.334	0.725	0.415	0.754	0.783	0.852	0.686	0.680	0.581
Saccharomyces	*0.061*	*0.099*	0.992	0.742	0.632	0.364	*0.071*	*0.055*	0.100	*0.073*	0.821
Penicillium	0.316	0.141	0.819	0.552	0.155	1.000	0.324	0.276	**0.002**	0.120	0.561
Aspergillus	0.187	*0.096*	0.841	0.375	**0.033**	1.000	0.200	0.155	0.112	0.073	0.440
Phellinus	0.668	0.413	**0.000**	**0.000**	0.545	0.534	0.664	0.686	1.000	1.000	**0.034**
Trichosporon	1.000	0.627	0.431	**0.000**	**0.021**	0.392	1.000	1.000	0.619	0.636	0.208
Cryptococcus	0.633	0.195	1.000	**0.013**	**0.000**	1.000	0.631	0.405	0.190	*0.053*	**0.038**
Clavispora	0.412	0.185	0.394	0.412	**0.000**	1.000	1.000	0.387	1.000	0.253	0.390
Magnusiomyces	0.669	1.000	**0.019**	1.000	**0.000**	0.557	0.666	0.697	1.000	1.000	0.683
Mycosphaerella	1.000	1.000	0.438	0.650	**0.001**	1.000	1.000	1.000	1.000	1.000	0.767
Phlebia	1.000	0.637	0.491	0.225	1.000	1.000	1.000	1.000	0.647	1.000	0.223
Botrytis	1.000	1.000	**0.000**	**0.003**	**0.000**	1.000	1.000	1.000	0.771	0.248	**0.001**
Wickerhamomyces	0.630	0.056	**0.000**	**0.000**	**0.000**	1.000	0.645	1.000	**0.016**	1.000	**0.000**
Cladosporium	1.000	*0.060*	**0.000**	**0.026**	**0.000**	1.000	1.000	1.000	**0.018**	**0.009**	0.456
Alternaria	1.000	0.182	**0.000**	**0.022**	**0.000**	1.000	1.000	1.000	0.125	0.604	**0.008**
Trametes	1.000	1.000	*0.058*	0.345	0.689	1.000	1.000	1.000	1.000	1.000	0.227

Table E5. Reported p-values (metastats analysis) for observed discriminant taxa within identified immunological classifications (ND, AC, AS, sABPA) (Figure 6). Distinct taxa of each immunological classification (compared to all others; "All vs") are shown on the left (columns 2-6) including an analysis of all sensitized patients ("AS+ABPA" – a composite grouping of both AS and ABPA). Columns 7-12 detail p-values obtained from pairwise comparison of individual immunological classification against each other. Significant p-values ($p < 0.05$) are indicated in bold while p values < 0.1 are underscored and in italics.