CLINICAL INVESTIGATIONS

Mucosal Microbiome in Patients with Recurrent Aphthous Stomatitis

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Appendix

Methods

DNA Extraction

Swabs were immersed directly in DNA extraction buffer (300 µL), and the suspension was stored at 4°C until DNA extraction was performed, which was a maximum of 24 h postcollection. DNA extraction was conducted with the commercially available QIAamp Mini Kit (Qiagen, Crawley, UK), with minor amendments as described previously (Thomson et al. 2011). Following storage, 30 µL of proteinase K was added for an initial lysis period of 2 h at 56°C to ensure complete lysis before DNA extraction. Incubation was extended to 30 min at 70°C following the addition of AL buffer. A polymerase chain reaction (PCR) test was performed with all DNA samples, utilizing universal primers for bacteria to confirm the suitability of the DNA for further analysis (Weisburg et al. 1991).

Preparation of Samples for High-throughput Sequencing

Bacterial DNA was quantified by spectrophotometry (NanoDrop 1000, Labtech, East Sussex, UK) prior to analysis. Initial PCR amplification was undertaken with FastStart High Fidelity PCR reagents (Roche Diagnostics, West Sussex, UK) as described previously (Hansen et al. 2012). The 16S rDNA primers were obtained from Dethlefsen et al. (2008), although we elected to utilize the 338f primer partnered with the 1064r primer, spanning the V3 to V6 region of the 16S rRNA gene and providing a base pair (bp) product of ~726. Fusion primer sequences were obtained from Roche Unidirectional Sequencing for Amplicon Libraries. For the forward primer, we utilized multiplex identifiers from Roche Amplicon Fusion Primer Design Guidelines to allow multiplexing of paired samples during sequencing. No identifier was added to the reverse primer. Therefore, the ~726-bp PCR product was flanked by a 40-bp fusion primer/multiplex identifier sequence at the forward end and a 30-bp fusion primer at the reverse end resulting in a ~796-bp sequence. PCR cycling conditions were as previously described (Dethlefsen et al. 2008): 1 cycle of 3 min at 94°C; 30 cycles of 30 s at 94°C, 45 s at 55°C, 60 s at 72°C; 1 cycle of 2 min at 72°C. After confirmation of adequate and appropriately sized product, the product was purified as per recommended AMPure purification method for 454 sequencing. The PCR product was quantified by spectrophotometry and sequenced on Roche 454 Titanium by NewGene (Newcastle, UK).

Bioinformatic and Statistical Analysis

Data analysis of the 454 sequence data was performed with the QIIME 1.1.0 workflow (Caporaso et al. 2010). Data were preprocessed to remove sequences with mean quality scores <25, mismatches in primer sequences >0, ambiguous bases >0, homopolymers >6, and sequences outside the bounds of 150 to 700 bp. Sequences were binned according to sample-specific barcode, denoised (fast denoiser), and clustered with uclust (Edgar 2010) into operational taxonomic units (OTUs) at 97% sequence similarity. Representative sequences were picked for each OTU and aligned with Pynast (Caporaso et al. 2010) via the Greengenes (DeSantis et al. 2006) template alignment core_set_aligned.fasta.imputed. Chimera check was performed with ChimeraSlayer (Haas et al. 2011), and potential chimeric sequences were removed. Taxonomy assignment of each OTU was performed according to ribosomal database project taxonomy (Wang et al. 2007), followed by construction of OTU tables at different taxonomic levels. The alignment of the representative sequences was filtered with Greengenes (DeSantis et al. 2006) lanemask_in_1s_and_0s. For analysis of diversity within samples (alpha diversity), OTU tables were rarefied at 3,000 reads, and diversity indices Chao 1,

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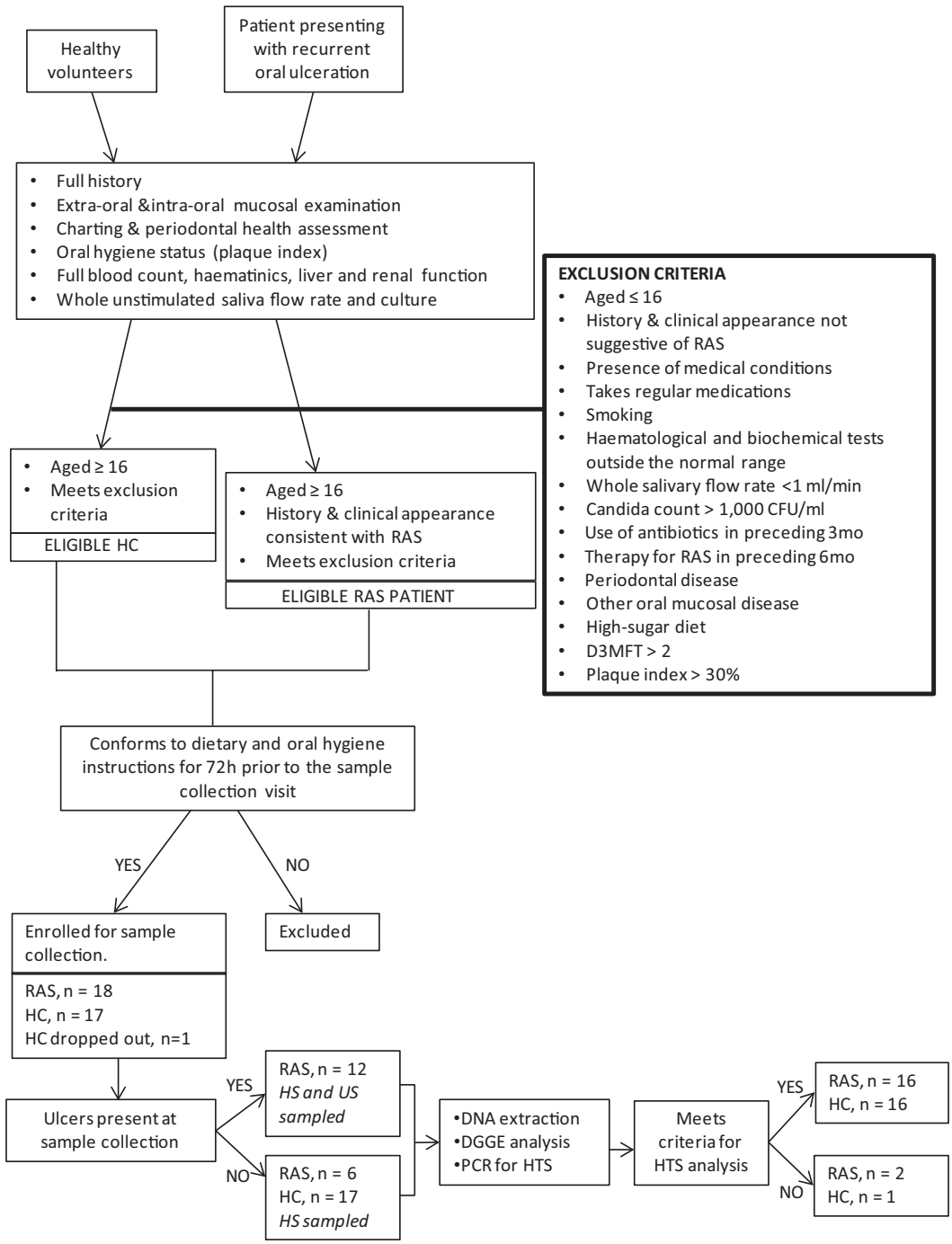
Appendix Figure.

Recruitment and sample collection. Flowchart illustrating the recruitment method for recurrent aphthous stomatitis (RAS) patients and healthy controls (HCs). Exclusion criteria are highlighted. Numbers of patients recruited initially and those included in high-throughput sequencing (HTS) analysis are indicated. Sample types are indicated in italics for healthy site (HS) and ulcerated site (US). CFU, colony-forming unit.



Appendix Table 1.
Demographic and Clinical Details of RAS Patients

Patient Code	Sex	Age, y	Ulcer Severity Score [a]	Oral Mucosal Sites Sampled [b]
MU11	Male	23	38	Ventral surface of tongue (US) Lower buccal sulcus (HS)
MU12	Female	58	39	Lateral border of tongue (US) Lower buccal sulcus (HS)
MU13	Female	53	29	Buccal mucosa (US) Lower buccal sulcus (HS)
MU14	Female	18	31	Lower buccal sulcus (US) Contralateral lower buccal sulcus (HS)
MU15	Male	16	30	Upper labial mucosa (US) Lower buccal sulcus (HS)
MU17	Male	42	29	Lateral border of tongue (US) Lower buccal sulcus (HS)
MU18	Male	33	32	Upper labial mucosa (US) Lower buccal sulcus (HS)
MU22	Male	32	27	Lower labial mucosa (US) Lower buccal sulcus (HS)
MU23	Female	40	29	Buccal mucosa (US) Lower buccal sulcus (HS)
MU25	Female	37	34	Ventral surface of tongue (US) Lower buccal sulcus (HS)
MU27	Female	23	31	Buccal mucosa (US) Lower buccal sulcus (HS)
MU31	Male	51	22	Buccal mucosa (US) Lower buccal sulcus (HS)
MU16[b]	Female	39	17	Lower buccal sulcus (HS)
MU19[b]	Female	58	25	Lower buccal sulcus (HS)
MU20[b]	Female	27	20	Lower buccal sulcus (HS)
MU21[b]	Male	25	19	Lower buccal sulcus (HS)
MU24[b]	Male	35	18	Lower buccal sulcus (HS)
MU26[b]	Female	21	23	Lower buccal sulcus (HS)

HS, healthy site; RAS, recurrent aphthous stomatitis; US, ulcerated site.
[a]As described in Tappuni et al. (2013).
[b]RAS patients presenting with active ulceration at the time of recruitment but no ulcers at the visit for sample collection.

Appendix Table 2.
Indices of Bacterial Alpha Diversity from High-throughput Sequencing

Bacterial Diversity Index	Patients, Mean ± SD				Statistical Comparison[a]		
	HC	RAS-HS	RAS-US	RAS-HS (No Ulcers)[b]	HC vs RAS-HS	HC vs RAS-US	RAS-HS vs RAS-US
Shannon	3.9 ± 1.0	3.7 ± 1.5	4.0 ± 1.0	3.9 ± 0.7	0.782	0.798	0.656
Simpson	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.10	0.395	0.370	0.209
Chao 1	175.3 ± 47.7	172.2 ± 66.6	175.1 ± 68.6	158.7 ± 25.0	0.894	0.995	0.920
Observed species	123.4 ± 35.0	120.8 ± 48.5	121.9 ± 46.3	113.3 ± 16.5	0.876	0.931	0.955
Phylogenetic diversity	9.9 ± 2.5	9.3 ± 3.1	9.1 ± 3.0	9.2 ± 0.8	0.607	0.503	0.881

HC, healthy control; HS, healthy site; RAS, recurrent aphthous stomatitis; US, ulcerated site.
[a]Two-sample *t* test based on Monte Carlo permutations allowing for nonparametric data.
[b]RAS patients who presented with active ulceration at the time of recruitment but no ulcers at the visit for sample collection.

Shannon, Simpson, observed species, and phylogenetic diversity (PD_whole_tree) were determined. To assess differences between microbial communities (beta diversity), weighted Unifrac analysis (Lozupone et al. 2006) on rarefied data with 97% (equivalent to species level) and 95% (equivalent to genus level) OTU clustering was performed, followed by principal components analysis. Statistical analysis of pyrosequencing phylum and genus data was undertaken between pairs of phenotypic groups through analysis of variance. Additional sample comparisons were done between groups with *t* tests and Mann-Whitney *U* test where appropriate. *P* values <0.05 were considered statistically significant. All statistical tests were undertaken with PASW Statistics 22.

Appendix References

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