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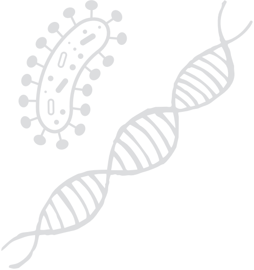
Title: 16S rRNA gene profiling: Direct and indirect applications for clinical microbiology
Issue Date: 2020-12-08



CHAPTER 8

Balanopreputial sac and urine microbiota in patients with male genital lichen sclerosus

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International Journal of Dermatology (2020); in press

ABSTRACT

Male genital lichen sclerosus (MGLSc) is a chronic inflammatory scarring dermatosis associated with penile carcinoma. The prepuce is pivotal in its aetiology. Other proposed aetiological factors are the subject of dispute, and include occluded urinary exposure, autoimmunity, immune-dysregulation and infectious agents. The objective of this study was to determine whether the bacterial microbiota of the balanopreputial sac and urine are associated with MGLSc. 20 uncircumcised patients with MGLSc and 20 healthy uncircumcised males were enrolled in a prospective case-control study. Balanopreputial swabs and urine specimens were subjected to 16S rRNA gene amplicon sequencing. Microbiota analysis indicated differences between the groups. In the balanopreputial sac, the median relative abundance of *Finegoldia* spp. was lower (9% [range 0% – 60%] in MGLSc patients than in controls (28% [range 0% – 62%]). Conversely, the median relative abundance of *Fusobacterium* spp. was higher in MGLSc patients (4% [range 0% – 41%]) than in controls (0% [range 0% – 28%]). In the urine, the median relative abundance of *Finegoldia* spp. was comparable between groups, whereas that of *Fusobacterium* spp. was higher in MGLSc patients (0% [range 0% – 18%] vs 0% [range 0% – 5%]). There was a strong association between the microbiota composition of the balanopreputial sac and urine in MGLSc. In conclusion, dysbiosis could be involved in the aetiopathogenesis of MGLSc. Further studies are required to confirm the association suggested herein and determine its nature.

INTRODUCTION

Lichen sclerosus (LSc) is a chronic lichenoid inflammatory fibrosing disorder with a predilection for genital skin (1, 2). Genital (G)LSc can cause substantial dermatological, sexual and urological morbidity and predisposes to intraepithelial neoplasia and squamous cell carcinoma (SCC) (1, 2). The aetiology is disputed (1, 2). Claims have been advanced for autoimmunity, immunodysregulation, and infective agents (1-4). The obligate role of the foreskin in male (M)GLSc is acknowledged; MGLSc is exceptionally rare in those circumcised at birth and circumcision is usually curative. The exact role of the foreskin is unclear, although occlusion and koebnerisation appear relevant. Unlike in women, male GLSc does not affect perianal or perineal skin; the exception to this rule is the observation of peri-urethrostomy LSc in males, indicating a link with urinary exposure. Furthermore, MGLSc is associated with high rates of post-micturition 'micro-incontinence' (2, 5). Together, these observations have led to the hypothesis that MGLSc arises from occluded exposure of a susceptible epithelium to urine. However, to date, no specific constituent or property of urine nor specific susceptibility factor has been identified (2, 6, 7).

The defining characteristics of the LSc are inflammation, sclerosis and neoplasia, but the pathogenesis remains poorly defined at a molecular level. It is plausible that a unified pathogenic pathway could account for each of these characteristics. In recent years, evidence has emerged i) that variations in the composition of the human microbiota may contribute to the development of many hitherto pathogenically unexplained chronic inflammatory diseases (8-10) and ii) that commensal bacteria can beneficially regulate host immunity, decreasing the risk of infection-induced autoimmune diseases and/or inflammation (11). Conversely, maladaptive alterations in microbiota composition (dysbiosis) may lead to perturbed immune homeostasis and disease (11). In addition, dysbiosis is implicated in fibrotic disease (12) and carcinogenesis (8, 13).

The ecosystem of the skin is divided into multiple anatomical 'niches' each with a site-specific microbiota (14). Various inflammatory dermatoses manifest preferentially at certain sites; for example, acne predominantly affects the face, chest and back (14), while LSc usually affects the genitalia. It is conceivable that the genital predilection of LSc could reflect site-specific dysbiosis. Previous studies of the penile microbiota indicate that circumcision dramatically changes its composition, with significant decreases in the abundance of anaerobic bacteria (15). Anaerobic bacteria in the coronal sulcus of uncircumcised men correlate with the presence of specific inflammatory cytokines (16), several of which are elevated in LSc (17, 18).

The microbiota composition of the balanopreputial sac has not previously been investigated in MGLSc. Dysbiosis may account for unresolved questions about the exact nature of the relationship between urine and epithelial susceptibility in MGLSc, and the pathways from lichenoid inflammation to fibrosis and carcinogenesis. Hence this analysis of the microbiota of the balanopreputial sac and urine in MGLSc.

MATERIALS AND METHODS

Study design

Ethical approval for this prospective case-control study was obtained through the NHS Health Research Authority Research Ethics Service (RES); London – Riverside Research Ethics Committee references 07/H0706/62, amendment number 4.

Twenty uncircumcised adult males with untreated, clinically pathognomonic MGLSc were recruited from the Male Genital Dermatoses Clinic at our institution. The clinical diagnosis of MGLSc was based on a comprehensive history and examination by highly experienced clinicians. Controls were recruited from general dermatology clinics and the Male Genital Dermatoses Clinic. They were uncircumcised, with clinically healthy balanopreputial tissue. No participant was immunocompromised.

From each participant, a swab of the balanopreputial sac (glans and inner prepuce) and a first void urine sample was collected. The swab was obtained by rubbing a sterile cotton swab (Puritan, Guilford, ME, USA) soaked in 1 mL 0.9% NaCl Tween Solution firmly five times against across the balanopreputial skin. First-void urine was collected using a Colli-Pee (Novosanis, Wijnegem, Belgium). All samples were stored at -20 °C until DNA extraction.

DNA extraction

DNA was extracted from 200 µL samples and eluted in a final volume of 50 µL with the MagNA pure 96 instruments using the MagNA pure 96 DNA and Viral NA small volume kit and the pathogen universal protocol (Roche Diagnostics, Basel, Switzerland).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed as described elsewhere (19). Briefly, the total bacterial load of the samples was established by targeting the 16S rRNA gene. Amplification reactions were performed using a LightCycler 480 Instrument (Roche Diagnostics) under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 sec, 60 °C for 15 sec and 72 °C for 1 sec, and a final step of 10 sec at 40 °C. For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis. A concentration of ≥ 5.00E+02 copies/µL was considered positive.

Amplicon sequencing of the 16S rRNA gene

Amplicon sequencing of the 16S rRNA gene to determine the microbiota composition was performed as described elsewhere (19). Briefly, a fragment of approximately 464 bp of the V3-V4 region of the 16S rRNA gene was amplified using the universal primers described by Klindworth et al. (20). PCR products with a positive agarose gel result were further processed and subsequently sequenced with the MiSeq desktop sequencer (Illumina, San Diego, CA, USA).

Sequencing data was processed following the standard QIIME pipeline. High quality sequences (length > 100 bp, quality score > 20) were clustered at a 97% similarity level using a pre-clustered version of the Augustus 2013 Greengenes database. Low abundance OTUs (a fraction < 0.005 of all sequences) were removed. A sample was considered positive for a specific genus when more than 1% of the sequences were assigned to that genus.

Statistical analysis

The software package SPSS version 26 was used for statistical analysis. For bacterial load, alpha diversity and relative abundance data, the Mann Whitney U test was employed. For analysis of association between urinary and balanopreputial data, Fisher's exact test was performed, only for patients with available microbiota data for both balanopreputial sac and urine.

RESULTS

Population characteristics

Twenty uncircumcised patients with MGLSc and 20 healthy uncircumcised males were enrolled in this study. The MGLSc patients had a median age of 37 years (range 26-73). The control group had a median age of 42 years (range 19-63).

Bacterial load

To determine whether the bacterial load in the balanopreputial sac and urine differed between MGLSc patients and controls, a qPCR targeting the 16S rRNA gene was performed (**Supplementary Figure S1, Table S2**). For the balanopreputial sac, the median bacterial load was 8.20 (range 5.34 – 9.44) log gene copies/mL for the MGLSc patients and 7.88 (range 5.57 – 9.24) log 16S rRNA gene copies/mL for the controls. The median bacterial load of the urine was 4.23 (range 0 – 6.18) log gene copies/mL for the MGLSc patients and 4.26 (range 0.98 – 7.19) log 16S rRNA gene copies/mL for the controls. No significant difference in bacterial load between both groups in either the balanopreputial sac ($p = 0.758$) or urine ($p = 0.235$) was observed.

Microbiota analysis

To determine the bacterial microbiota composition of the balanopreputial sac and urine, amplicon sequencing of the 16S rRNA gene was performed. For seven MGLSc patients and five controls, the bacterial load of the urine specimen was too low for microbiota analysis. For the remaining specimens, the alpha-diversity, beta-diversity and microbiota profiles were analysed.

The median alpha-diversity was 2.61 and 2.17 for the balanopreputial sac of MGLSc patients and the controls, respectively (**Figure 1**). For urine, it was 2.38 and 2.33 for MGLSc patients and the controls, respectively. No significant difference was observed between the MGLSc patients and the controls in either the balanopreputial sac (p = 0.201) or urine (p = 1.000).

Beta-diversity analysis of the balanopreputial sac showed a relatively high variation in microbiota composition between the MGLSc patients and between the controls (**Figure 2a**). The microbiota composition of the balanopreputial sac differed between the MGLSc patients and the controls, whereas that of the urine was more comparable between the groups (**Figure 2b**).

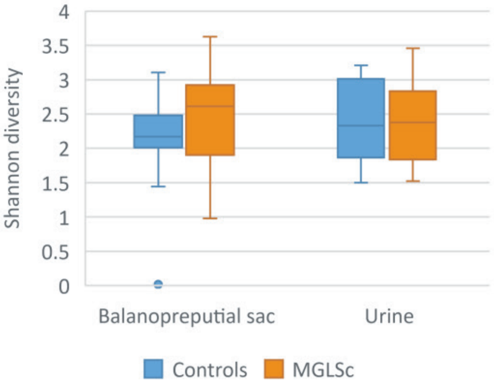

Figure 1. Alpha diversity of swabs of balanopreputial sac and urine of controls and MGLSc patients

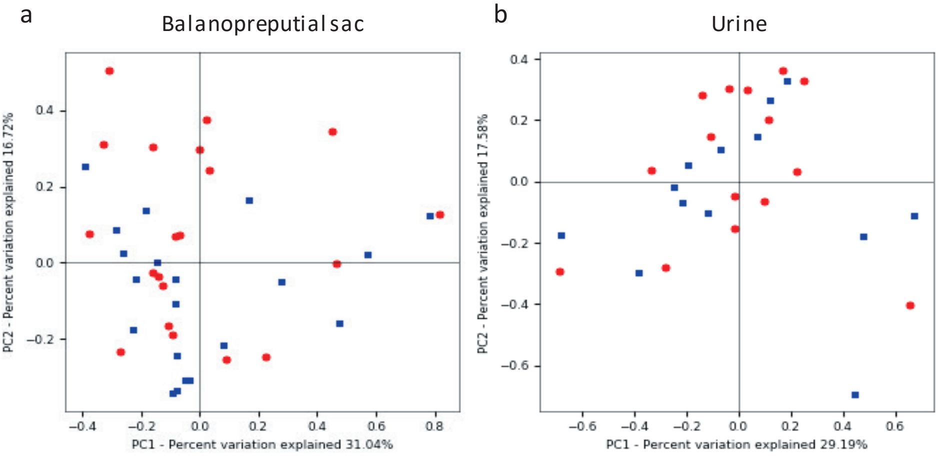

Figure 2. Unifrag weighted PCOA plots of (**a**) balanopreputial sac and (**b**) urine of healthy controls and MGLSc patients. The blue squares are the healthy controls and the red dots are the MGLSc patients

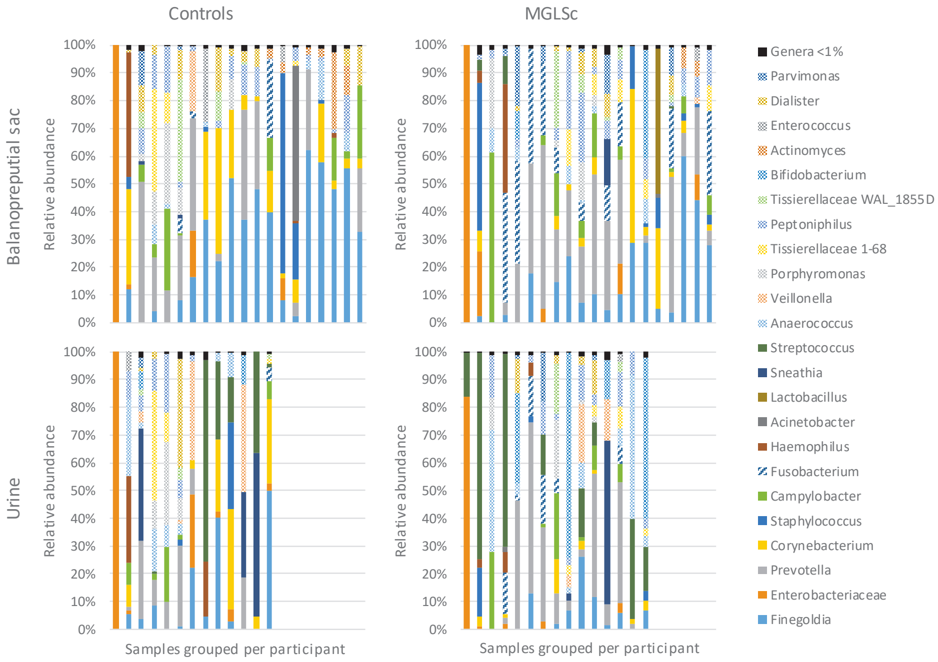

Figure 3. Microbiota composition of the balanopreputial sac and urine in healthy controls and MGLSc at genus level

Analysis of the microbiota profiles of both balanopreputial sac and urine indicated differences between the groups (**Figure 3, Supplementary Table S3, S4**). In the balanopreputial sac, the genus *Finegoldia* was present in 17 (85%) of both the MGLSc patients and controls, but the median relative abundance of 9% (range 0% – 60%) in MGLSc patients was lower than in controls (28%, range 0% – 62%) (**Supplementary Table S5**). Furthermore, the presence and/or relative abundance of the genera *Fusobacterium* and *Prevotella* was higher in patients with MGLSc. *Fusobacterium* spp. were present in 10 (50%) of the patients, while it was detected in 3 (15%) of the controls; its median relative abundance was higher in MGLSc patients (4% [range 0% – 41%] vs 0% [range 0% – 28%]). *Prevotella* was present in 15 (75%) of the patients with MGLSc and 11 (55%) of the controls; its median relative abundance was higher in MGLSc patients (20% [range 0% – 59%] than in controls (4% [range 0% – 51%]).

The microbiota profiles of the urine specimens differed between the participants of both groups and from the skin microbiota (**Supplementary Table S6**). However, there was an association between the presence of several genera, including *Fusobacterium*, in the balanopreputial sac and urine in patients with MGLSc (**Supplementary Table S7**). These data suggest an association between the microbiota of the urine and that of the balanopreputial sac.

DISCUSSION

To our knowledge this represents the first combined investigation of the composition of the microbiota of both the balanopreputial sac and urine in MGLSc. The presented data suggests that dysbiosis of the balanopreputial sac microbiota could be involved in MGLSc. The relative abundance of *Finegoldia* spp. was decreased in patients with MGLSc while the relative abundance of *Fusobacterium* spp. was higher in patients with MGLSc. *Fusobacterium* spp. exhibit properties and associations that could be relevant to the defining characteristics of MGLSc.

Recent advances in culture-independent microbial detection technology have shed light on the important role of the genus *Fusobacterium*, in particular *F. nucleatum*, in inflammatory diseases such as periodonitis and inflammatory bowel disease (21). *In vitro* and *In vivo* studies of the specific pro-inflammatory properties of *Fusobacterium* spp. indicate potential relevance in LSc; *F. nucleatum* upregulates the anti-microbial peptides hBD-2 and S100A7 that are also upregulated in LSc (22-25). *F. nucleatum* also upregulates several cytokines, including IL-1, IL-4, IL-6, CCL5, CXCL10, CXCL11 and TNF-α that are upregulated in LSc (17, 18, 21-23, 26-28).

LSc is a fibrosing disease (1). The cause of fibrogenesis remains unexplained by previously proposed aetiological factors, and the mechanism leading to aberrant fibrogenesis in LSc has not been the focus of specific investigation. Studies in other fibrotic diseases such as systemic sclerosis and idiopathic pulmonary fibrosis implicate perturbations in the Wnt/β-catenin (29, 30) and mitogen activated protein kinase (MAPK) signalling pathways, including the subfamilies JNK and p38 MAPK (31, 32). These pathways are involved in regulation of extracellular matrix deposition and degradation and collagen gene expression, and induction of these pathways can cause fibrosis (31-35). Whether dysbiosis could play a role in aberrant fibrogenesis in LSc remains unknown. Several *Fusobacterium* spp. have the capacity to activate the Wnt/β-catenin and MAPK signaling pathways, including JNK and p38 MAPK (36, 37). *In vitro* studies of keratinocyte lines have shown that *Fusobacterium* spp., upregulate secretion of matrix metalloproteinases (MMPs), including MMP-1, MMP-2 and MMP-9, in keratinocytes (23, 26, 38) that are also upregulated in LSc (18, 39, 40). The pathway of fibrogenesis in LSc, and any involvement of the microbiota therein, require further investigation.

MGLSc and human papillomavirus represent the two main risk factors for penile carcinoma (41, 42). However, little is known of the molecular mechanisms involved in progression from MGLSc to penile carcinoma, and the role of dysbiosis has not been investigated in this context. The role of dysbiosis in carcinogenesis has demonstrated in other cancers, including oral squamous cell carcinoma and colorectal carcinoma; studies have highlighted the important role of *F. nucleatum* in particular (13, 37, 43-47). Studies in murine models and *In vitro* studies indicate that *F. nucleatum*-mediated carcinogenesis is effected via upregulation of Wnt/β-catenin signaling and STAT3 signaling (37, 41, 48). Upregulation of Wnt/β-catenin signaling is observed in penile carcinoma (49). These observations suggest that *Fusobacterium* spp. deserve specific focus in future studies of the link between MGLSc and penile carcinoma.

Circumcision has a dramatic impact on the composition of the penile microbiota, significantly reducing the abundance of anaerobes, particularly *Fusobacterium* spp. (15, 50). The changes in microbiota composition following circumcision could explain the curative and preventative effect of circumcision in MGLSc.

Involvement of the urethra in MGLSc appears to support the aetiological role of urine (1), however the specific property of urine has not been identified (1, 6); the strong association identified between the balanopreputial sac and urine microbiota composition may account for the link. While this study represents the only study to date of both the balanopreputial microbiota and urinary microbiota, Cohen et al. investigated the urinary microbiota of men with urethral MGLSc and demonstrated a unique microbiota profile in MGLSc compared with controls, with enrichment of the orders Bacillales, Bacteroidales and Pasteurellales (51). They did not report any differences in the relative abundance of the order Fusobacteriales. The variable findings may relate to methodology in specimen collection; while Cohen et al. analysed mid-stream urine, first void urine was analysed herein, and was selected on the basis that urethral involvement in MGLSc typically develops distally and progresses proximally (52). Notably, *Borrelia* spp., previously proposed as an aetiological factor in MGLSc (2), was not identified in any specimen in this study.

The notion that dysbiosis may be involved in the aetiopathogenesis of MGLSc is a novel one. This study, with a low number of subjects, is essentially pilot in nature; as such, no firm conclusions can be drawn. Other limitations include the lack of longitudinal data preclude determination of whether balanopreputial dysbiosis represents the cause or consequence of the disease. The diagnosis of MGLSc was based on clinical features and histopathology was not sought to confirm the diagnosis. There has been historical discussion regarding the role of biopsy in the diagnosis of LSc; the literature indicates that histology may be non-specific and falsely negative, and reliance on histology can lead to delayed diagnosis and progression of the disease (53, 54). For these reasons, histopathology was not sought in this study. Identification of bacteria at the genus level also represents a limitation, since virulence factor expression can vary between species or even strains (8). Finally, the clinical phenotype arising from dysbiosis is likely to depend upon factors such as genetic predisposition (8), but host factors were not analysed herein. Further studies with increased numbers of participants are required to confirm the results and investigate the molecular role, if any, that dysbiosis may play in MGLSc.

CONCLUSION

This study suggests that dysbiosis of the balanopreputial sac microbiota may play a role in the aetiopathogenesis of MGLSc. Further studies are required to determine if dysbiosis could form the 'missing link' between occlusion, urinary exposure, epithelial susceptibility, inflammation, fibrosis and carcinogenesis.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Emily McGrath (Royal Devon & Exeter Hospital) for her early support.

FUNDING

This project received funding from the European Academy of Dermatology and Venereology and the Skin Treatment and Research Trust.

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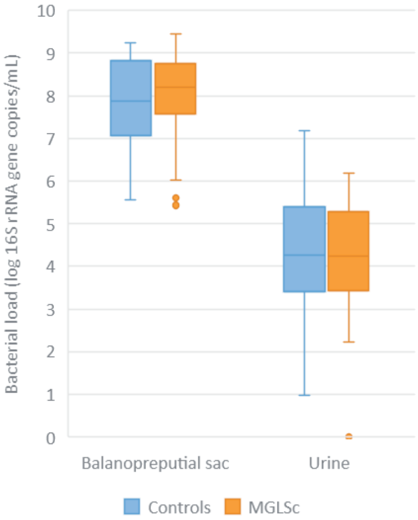
SUPPLEMENTAL APPENDIX

Supplementary Figure S1. Bacterial load of balanopreputial sac and urine in healthy controls and MGLSc patients



Supplementary Table S2. (**a**) Bacterial load of balanopreputial swabs; (**b**) Bacterial load of first catch urine

a.

Controls		**MGLSc**	
SampleID	16S copies/mL	Sample ID	16S copies/mL
1	5.91E+08	5	3.78E+07
2	8.60E+08	6	1.48E+08
4	4.95E+06	8	2.70E+05
7	1.70E+09	10	5.94E+08
9	4.11E+07	14	1.05E+06
11	6.36E+07	15	4.05E+07
12	1.09E+09	18	5.54E+08
13	6.54E+05	19	4.10E+08
16	1.81E+08	20	1.60E+08
17	3.73E+05	21	6.53E+07
22	2.28E+07	23	1.41E+07
26	8.76E+06	24	5.58E+08
27	9.04E+07	29	6.35E+08
28	2.03E+08	30	1.07E+08
32	6.31E+07	31	1.09E+09
33	1.63E+07	35	1.60E+08
34	1.01E+07	36	2.26E+08
37	3.03E+08	41	2.78E+09
38	1.74E+09	43	2.53E+08
42	6.98E+08	44	3.98E+05

b.

Controls		**MGLSc**	
SampleID	16S copies/mL	Sample ID	16S copies/mL
1	1.38E+05	5	1.71E+02
2	1.85E+04	6	4.58E+03
4	1.06E+03	8	5.24E+02
7	2.89E+06	10	1.18E+04
9	1.30E+04	14	2.48E+04
11	6.70E+04	15	3.55E+03
12	1.39E+06	18	1.51E+06
13	3.05E+05	19	4.49E+04
16	5.50E+02	20	4.99E+02
17	9.61E+00	21	1.25E+05
22	6.00E+03	23	0.00E+00
26	2.29E+03	24	2.54E+03
27	1.25E+06	29	4.05E+05
28	1.79E+04	30	1.07E+04
32	6.06E+03	31	2.16E+05
33	1.83E+04	35	2.49E+05
34	9.30E+01	36	1.44E+05
37	3.59E+03	41	1.39E+06
38	7.04E+04	43	1.34E+05
42	1.55E+07	44	1.15E+04

Supplementary Table S3. Number of bacterial genera found in each group

	Balanopreputial sac	Urine	Total	Present in both samples
Control	20	20	22	18
MGLSc	23	21	23	21
Total	23	21	23	21
Present in both groups	21	20	22	18

Supplementary Table S4. Bacterial genera found in each of the four groups (balanopreputial sac in healthy controls, balanopreputial sac in MGLSc, urine in healthy controls, urine in MGLSc)

Genus	Controls		MGLSc	
	Balanopreputial sac	**Urine**	**Balanopreputial sac**	**Urine**
Acinetobacter	+	-	+	+
Actinomyces	+	-	+	-
Anaerococcus	+	+	+	+
Bifidobacterium	-	+	+	+
Campylobacter	+	+	+	+
Corynebacterium	+	+	+	+
Dialister	+	+	+	+
Enterobacteriaceae	+	+	+	+
Enterococcus	+	+	+	+
Finegoldia	+	+	+	+
Fusobacterium	+	+	+	+
Haemophilus	+	+	+	+
Lactobacillus	-	-	+	-
Parvimonas	+	+	+	+
Peptoniphilus	+	+	+	+
Porphyromonas	+	+	+	+
Prevotella	+	+	+	+
Sneathia	+	+	+	+
Staphylococcus	+	+	+	+
Streptococcus	-	+	+	+
Tissierellaceae 1-68	+	+	+	+
Tissierellaceae WAL_1855D	+	+	+	+
Veillonella	+	+	+	+

Supplementary Table S5. Balanopreputial sac: prevalences and median relative abundances of bacteria in healthy controls and MGLSc patients

Genus	Prevalence n (%)			Median relative abundance % (Range)		
	Control (n=20)	MGLSc (n=20)	*p*	Control	Urine	*p*
Acinetobacter	1 (5)	1 (5)	1.000	0 (0-56)	0 (0-1)	0.989
Actinomyces	6 (30)	2 (10)	0.235	0 (0-28)	0 (0-8)	0.314
Anaerococcus	6 (30)	7 (35)	1.000	0 (0-14)	0 (0-9)	0.738
Bifidobacterium	0 (0)	2 (10)	0.487	0 (0-0)	0 (0-39)	0.602
Campylobacter	8 (40)	9 (45)	1.000	0 (0-30)	0 (0-61)	0.799
Corynebacterium	13 (65)	11 (55)	0.748	3 (0-45)	2 (0-55)	0.314
Dialister	10 (50)	7 (35)	0.523	0 (0-16)	0 (0-8)	0.289
Enterobacteriaceae	4 (20)	5 (25)	1.000	0 (0-100)	0 (0-100)	0.758
Enterococcus	2 (10)	1 (5)	1.000	0 (0-27)	0 (0-5)	0.779
Finegoldia	17 (85)	17 (85)	1.000	28 (0-62)	9 (0-60)	0.086
Fusobacterium	**3 (15)**	**10 (50)**	**0.041**	**0 (0-28)**	**4 (0-41)**	**0.033**
Haemophilus	3 (15)	2 (10)	1.000	0 (0-45)	0 (0-39)	0.820
Lactobacillus	0 (0)	1 (5)	1.000	0 (0-0)	0 (0-52)	0.799
Parvimonas	2 (10)	6 (30)	0.235	0 (0-12)	0 (0-22)	0.265
Peptoniphilus	15 (75)	15 (75)	1.000	2 (0-17)	3 (0-28)	0.968
Porphyromonas	5 (25)	4 (20)	1.000	0 (0-29)	0 (0-25)	0.779
Prevotella	11 (55)	15 (75)	0.320	4 (0-51)	20 (0-59)	0.265
Sneathia	2 (10)	1 (5)	1.000	0 (0-2)	0 (0-17)	0.820
Staphylococcus	5 (25)	7 (35)	0.731	0 (0-72)	0 (0-54)	0.640
Streptococcus	0 (0)	3 (15)	0.231	0 (0-0)	0 (0-10)	0.429
Tissierellaceae 1-68	3 (15)	5 (25)	0.695	0 (0-37)	0 (0-13)	0.620
Tissierellaceae WAL_1855D	4 (20	5 (25)	1.000	0 (0-37)	0 (0-28)	0.841
Veillonella	1 (5)	1 (5)	1.000	0 (0-22)	0 (0-2)	0.989

Supplementary Table S6. Urine - prevalences and median relative abundances of bacteria of healthy controls and MGLSc patients

Genus	**Prevalence n (%)**			**Median relative abundance % (Range)**		
	Control (n=13)	**MGLSc (n=15)**	***p***	**Control**	**Urine**	***p***
Acinetobacter	0 (0)	1 (7)	1.000	0 (0-0)	0 (0-2)	0.786
Actinomyces	0 (0)	0 (0)	-	0 (0-0)	0 (0-0)	1.000
Anaerococcus	7 (54)	7 (47)	1.000	2 (0-28)	0 (0-52)	0.856
Bifidobacterium	2 (15)	4 (27)	0.655	0 (0-11)	0 (0-74)	0.496
Campylobacter	5 (38)	6 (40)	1.000	0 (0-20)	0 (0-28)	0.856
Corynebacterium	6 (46)	7 (47)	1.000	0 (0-36)	0 (0-12)	0.496
Dialister	3 (23)	4 (27)	1.000	0 (0-39)	0 (0-12)	0.856
Enterobacteriaceae	6 (46)	5 (33)	0.700	0 (0-100)	0 (0-84)	0.496
Enterococcus	1 (8)	1 (7)	1.000	0 (0-7)	0 (0-2)	0.964
Finegoldia	9 (69)	8 (53)	0.460	4 (0-50)	1 (0-26)	0.467
Fusobacterium	1 (8)	6 (40)	0.084	0 (0-5)	0 (0-18)	0.130
Haemophilus	2 (15)	3 (20)	1.000	0 (0-31)	0 (0-7)	0.964
Lactobacillus	0 (0)	0 (0)	-	0 (0-0)	0 (0-0)	1.000
Parvimonas	1 (8)	3 (20)	0.600	0 (0-3)	0 (0-17)	0.618
Peptoniphilus	6 (46)	10 (67)	0.445	0 (0-21)	3 (0-16)	0.440
Porphyromonas	3 (23)	4 (27)	1.000	0 (0-30)	0 (0-21)	0.964
Prevotella	7 (54)	11 (73)	0.433	1 (0-29)	3 (0-61)	0.274
Sneathia	3 (23)	2 (13)	0.639	0 (0-59)	0 (0-59)	0.650
Staphylococcus	2 (15)	3 (20)	1.000	0 (0-31)	0 (0-18)	0.856
Streptococcus	6 (46)	8 (53)	1.000	0 (0-73)	8 (0-74)	0.650
Tissierellaceae 1-68	3 (23)	4 (27)	1.000	0 (0-40)	0 (0-8)	0.964
Tissierellaceae WAL_1855D	3 (23)	2 (13)	0.639	0 (0-4)	0 (0-18)	0.751
Veillonella	4 (31)	3 (20)	0.670	0 (0-39)	0 (0-21)	0.650

Supplementary Table S7. Association between the balanopreputial sac and urine bacterial microbiota

Genus	Control (n = 13)			MGLSc (n = 15)		
	Urine+ when Balanopreputial sac-, ratio (%)	Urine+ when Balanopreputial sac+, ratio (%)	*p*	Urine+ when Balanopreputial sac-, ratio (%)	Urine+ when Balanopreputial sac+, ratio (%)	*p*
Acinetobacter	0/13 (0)	0/0 (0)	-	1/15 (7)	0/0 (0)	-
Actinomyces	0/12 (0)	0/1 (0)	-	0/15 (0)	0/0 (0)	-
Anaerococcus	5/10 (50)	2/3 (67)	1.000	**2/9 (22)**	**5/6 (83)**	**0.041**
Bifidobacterium	2/13 (15)	0/0 (0)	-	2/13 (15)	2/2 (100)	0.057
Campylobacter	**1/8 (13)**	**4/5 (80)**	**0.032**	**0/9 (0)**	**6/6 (100)**	**<0.001**
Corynebacterium	1/6 (17)	5/7 (71)	0.103	**1/8 (13)**	**6/7 (86)**	**0.010**
Dialister	0/5 (0)	3/8 (38)	0.231	**0/9 (0)**	**4/6 (67)**	**0.011**
Enterobacteriaceae	3/10 (30)	3/3 (100)	0.070	**1/11 (9)**	**4/4 (100)**	**0.004**
Enterococcus	1/12 (8)	0/1 (0)	1.000	1/15 (7)	0/0 (0)	-
Finegoldia	1/3 (33)	8/10 (80)	0.203	0/3 (0)	8/12 (67)	0.077
Fusobacterium	0/10 (0)	1/3 (33)	0.231	**0/7 (0)**	**6/8 (75)**	**0.007**
Haemophilus	1/12 (8)	1/1 (100)	0.154	**1/13 (8)**	**2/2 (100)**	**0.029**
Lactobacillus	0/13 (0)	0/0 (0)	-	0/15 (0)	0/0 (0)	-
Parvimonas	0/11 (0)	1/2 (50)	0.154	1/10 (10)	2/5 (40)	0.242
Peptoniphilus	1/3 (33)	5/10 (50)	1.000	3/4 (75)	7/11 (64)	1.000
Porphyromonas	**0/8 (0)**	**3/5 (60)**	**0.035**	**0/11 (0)**	**4/4 (100)**	**0.001**
Prevotella	1/5 (20)	6/8 (75)	0.103	**1/4 (25)**	**10/11 (91)**	**0.033**
Sneathia	2/11 (18)	1/2 (50)	0.423	1/14 (7)	1/1 (100)	0.133
Staphylococcus	2/11 (18)	0/2 (0)	1.000	1/12 (8)	2/3 (67)	0.081
Streptococcus	6/13 (46)	0/0 (0)	-	6/13 (46)	2/2 (100)	0.267
Tissierellaceae 1-68	**1/11 (9)**	**2/2 (100)**	**0.038**	**0/11 (0)**	**4/4 (100)**	**0.001**
Tissierellaceae WAL_1855D	1/9 (11)	2/4 (50)	0.203	0/11 (0)	2/4 (50)	0.057
Veillonella	3/12 (25)	1/1 (100)	0.308	3/15 (20)	0/0 (0)	-