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Functional and Taxonomic Dysbiosis of the Gut, Urine, and Semen Microbiomes in Male Infertility

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Abstract

Background: Little is known about the role of the genitourinary and gastrointestinal microbiota in the pathogenesis of male infertility.

Objective: To compare the taxonomic and functional profiles of the gut, semen, and urine microbiomes of infertile and fertile men.

Design, setting, and participants: We prospectively enrolled 25 men with primary idiopathic infertility and 12 healthy men with proven paternity, and we collected rectal swabs, semen samples, midstream urine specimens, and experimental controls.

Outcome measurements and statistical analysis: We performed comprehensive semen analysis, 16S rRNA sequencing for quantitative high-resolution taxonomy, and shotgun metagenomics with a median of 140 million reads per sample for functional metabolic pathway profiling.

Results and limitations: We identified a diverse semen microbiome with modest similarity to the urinary microbiome. Infertile men harbored increased seminal α -diversity and distinct β -diversity, increased seminal *Aerococcus*, and decreased rectal *Anaerococcus*. *Prevotella* abundance was inversely associated with sperm concentration, and *Pseudomonas* was directly associated with total motile sperm count. Vasectomy appeared to alter the seminal microbiome, suggesting a testicular or epididymal contribution. Anaerobes were highly over-represented in the semen of infertile men with a varicocele, but oxidative stress and leukocytospermia were associated with only subtle differences. Metagenomics data identified significant alterations in the S-adenosyl-L-methionine cycle, which may play a multifaceted role in the pathogenesis of infertility via DNA methylation, oxidative stress, and/or polyamine synthesis. *Conclusions:* This pilot study represents the first comprehensive investigation into the microbiome in male infertility. These findings provide the foundation for future investigations to explore causality and identify novel microbiome-based diagnostics and

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therapeutics for men with this complex and emotionally devastating disease.



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www.eu-acme.org/europeanurology to answer questions on-line. The EU-ACME credits will then be attributed automatically. **Patient summary:** We explored the resident populations of bacteria living in the gut, semen, and urine of infertile and fertile men. We found several important bacterial and metabolic pathway differences with the potential to aid in diagnosing and treating male infertility in the future.

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1. Introduction

Roughly half of the 50 million infertile couples suffering from infertility worldwide have difficulty conceiving due to male factors [1], and recent studies suggest that this epidemic may be expanding as sperm counts worldwide continue to fall for unclear reasons [2]. Despite extensive testing, over half of all men who undergo traditional workup will have no identifiable cause for their infertility [3]. Infection has historically thought to account for 15% of cases [4], but this figure may be misleading as <2% of all known bacterial strains can be successfully cultured and identified using traditional methods [5]. The microbiota is a term describing the unique community of all resident microbes residing within nearly every niche in the human body. The microbiome, which refers to the genetic material comprising the microbiota, plays critical roles both in normal physiological symbiosis and in pathogenic dysbiosis (bacterial maladaptation in disease). While next-generation 16S ribosomal RNA (rRNA) marker gene-based sequencing analysis provides valuable bacterial microbiome taxonomic information, shotgun metagenomics provides additional insight into the functional metabolic aberrations underpinning these taxonomic differences.

Despite the growing body of microbiome literature in general and the urinary microbiome in particular [6,7], there is a paucity of literature exploring the role of the microbiome in male infertility. Several small pilot studies have explored the male reproductive microbiome using 16S rRNA gene sequencing in semen and testicular samples with limited control populations [8]. No studies to date, however, have examined the role of the human gut in male reproductive health or explored potential mechanistic pathways using metagenomics. We hypothesized that dysbiosis of the gut and genitourinary microbiota is associated with idiopathic male infertility. To test this hypothesis, we explored the rectal, midstream urine, and semen microbiomes of infertile men and paternity-proven fertile controls. Using both 16S rRNA and shotgun metagenomics, we assessed high-resolution taxonomy and functional metabolic profiles and identified several key aberrant taxa and biochemical pathways that may play a role in the pathogenesis of male infertility [9].

2. Patients and methods

Detailed methods can be found in the Supplementary material (Methods section).

2.1. Patient recruitment

This study was performed under institutional review board approval. Sexually active men aged 18–55 yr and presenting for primary infertility or paternity-proven controls were screened for eligibility based upon exclusion criteria listed in Supplementary Table 1.

2.2. Biological specimen collection

All samples were collected using the aseptic technique and immediately snap frozen to -80 °C. Rectal swabs were inserted at least 2 cm beyond the anal verge for collection. Urine samples were collected as midstream voided urine specimens. Semen samples were collected via masturbation after 2–7 d of sexual abstinence and without lubrication or saliva.

2.3. Semen analysis

Semen analysis was performed by our certified institutional andrology laboratory. Following liquefaction, $200 \,\mu$ l aliquots were sterilely aliquoted for further microbiome analysis, and the remaining sample was used for basic semen analysis and to measure oxidation-reduction potential (ORP) as previously described [10].

2.4. DNA extraction

DNA from all three source tissues was extracted in an identical protocol with the exception of semen samples, which underwent a host DNA depletion step first (QIAamp Microbiome Kit; Qiagen, Germantown, MD, USA). Beadbeating was performed for all samples using the MP Biomedical Fastprep-24 5 G (MP Biomedicals, Solon, OH, USA) for two cycles with a 5-min pause. Samples were then extracted using lot-matched Qiagen PowerFecal Pro kits according to the manufacturer's instructions. Numerous negative and positive controls were included and are described further in the Supplementary material.

2.5. Analysis of 16S rRNA gene sequencing

The 16S rRNA gene region V3-V4 index polymerase chain reaction was performed according to the published Illumina MiSeq 16S sample preparation guide (Illumina, Forest City, CA, USA) with minor modifications [11]. Illumina Nextera library preparation was performed according to manufacturer specifications and sequenced in a paired-end fashion using the Illumina MiSeq platform. Raw data were demultiplexed using *QIIME1.9.1*, processed using Divisive Amplicon Denoising Algorithm 2 (DADA2), and analyzed using *microbiomeSeq* and *phyloseq* in R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). The output was processed using DAtest to correct for covariates. Two group analyses were performed using the STAMP package with White's nonparametric *t* test.

Shotgun metagenomic library preparation was performed using Nextera DNA Flex library preparation chemistry (Illumina) with 100 ng input. Sample libraries were validated and sequenced on the NovaSeq 6000 platform (Illumina) with paired-end 150-bp chemistry on an S4 flow cell.

2.7. Shotgun metagenomics

Nesoni pipeline was used to quality filter the raw demultiplexed reads, and host reads were removed using BBmap aligner. Taxonomic characterization and functional characterization of trimmed and decontaminated reads were performed using the MetaPhlAn and HUMAnN2 pipelines, respectively.

2.8. Generalized linear models

Generalized linear models were implemented in the glm package in R (R foundation for Statistical Computing) using Poisson regression and "log" link. The correlation between microbial taxa abundance and clinical variables was evaluated using the ANOVA.glm function.

2.9. Network analysis

Co-occurrence patterns were analyzed between features/taxa in 16S rRNA amplicon data using *microbiomeSeq* and converted into graph objects and plotted using *igraph* and *ggraph* packages.

2.10. Statistical analysis

Clinical data were maintained in an encrypted database and aggregate values were reported as median (IQR). The α value was set a priori to 0.05. All *p* values were adjusted for multiple comparisons according to the Benjamini and Hochberg method to control false discovery rate.

3. Results

After applying rigorous exclusion criteria (Supplementary Table 1), we recruited 25 men with primary idiopathic infertility and 12 control men with recently proven paternity with a median interval of 2 mo between childbirth and study enrollment (Supplementary Fig. 1). All men had female partners and were sexually active. Baseline demo-

Table 1 - Study demographics and semen analysis parameters for infertile and fertile men

Cohort characteristics	Infertile men (<i>n</i> =25)	Fertile men (<i>n</i> = 12)
Age (yr)	34 (30–36)	30 (30–34)
Body mass index (kg/m ²)	29.6 (24.9-37.0)	25.3 (22.8-27.3)
Race		
White	19/25 (76)	9/12 (75)
Black	5/25 (20)	0
Asian	1/25 (4)	3/12 (25)
Smoking status		
Never smoker	15/24 (63)	11/12 (92)
Ex-smoker	5/24 (21)	1/12 (8.3)
Current smoker	4/24 (17)	0
Alcohol use		
None	7/24 (29)	3/12 (25)
Social	16/24 (67)	8/12 (67)
Regular	1/24 (4.2)	1/12 (8.3)
Diet		
Regular	19/25 (76)	10/12 (83)
Carbohydrate controlled	1/25 (4)	0
Vegetarian/vegan	3/25 (12)	2/12 (17)
Low carbohydrate/ketogenic	2/25 (8)	0
Circumcised	16/25 (64)	9/12 (75)
Semen analysis		
Semen volume (ml)	2.4 (1.7-3.7)	4.1 (3.8-5.5)
Semen pH	7.6 (7.5–8.0)	7.6 (7.3–7.6)
Abnormal viscosity	16/25 (64)	3/10 (30)
Sperm concentration (million/ml)	36 (3.7-74)	81 (68-87)
% Motile sperm	47 (36–66)	61 (53-71)
Total sperm count (million)	57 (14.6–228)	351 (240-446)
Total motile sperm count (million)	34 (3.1-107)	211 (139-280)
% Normal morphology (Kruger strict criteria)	4 (2-7)	8 (6-12)
ORP (mV/10 ⁶ sperm/ml)	1.5 (0.5–6.8)	0.6 (0.3-0.8)
Round cells (million/ml)	1.9 (0.7-3.4)	0.5 (0.2-1.5)
Leukocytospermia	10/24 (42)	1/10 (10)
WHO concentration category		
Normozoospermia (>15 million/ml)	17/25 (68)	9/10 (90)
Oligospermia (<15 million/ml)	5/25 (20)	1/10 (10)
Azoospermia (0 million/ml)	3/25 (12)	0

IQR = interquartile range; ORP = oxidation-reduction potential; WHO = World Health Organization. Data are presented as n (%) or median (IQR).

graphics and dietary habits (Table 1) demonstrated reasonably well-matched groups with no major differences except body mass index (BMI), which has a known association with infertility [12]. A standardized infertility history, physical examination, and partner history were then collected by a reproductive urologist (Supplementary Tables 2 and 3). Laboratory evaluation in the infertile group revealed normal median hormonal values (Supplementary Table 4). All participants were asymptomatic from a genitourinary standpoint, and none had a urinalysis or culture suspicious for urinary tract infection via traditional tools. As expected, traditional semen analysis showed significantly impaired semen quality in the infertile men (Table 1).

As little is known about the interplay between the gut and genitourinary microbiomes, we first characterized the male rectal, midstream voided urine, and semen microbiomes in men irrespective of fertility status (Fig. 1). The 16S rRNA gene analysis with a median of 59 769 reads per sample uncovered Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria as the four predominant phyla (Fig. 1A). A total of 5394 unique amplicon sequence variants



Fig. 1 – Semen, urine, and rectal microbiomes in men of reproductive age. (A) Bar plot demonstrating relative abundance of taxa stratified by sample source. Genera are color coded by phylum; n=27 for each sample source. (B) Venn diagram demonstrating the distribution of taxa present according to sample source. (C) α -Diversity (Shannon index, left) and β -diversity (right) plots for both 16S (top) and shotgun metagenomics (bottom). * p < 0.05. **** p < 0.001. (D) Pairwise comparison between urine (yellow) and semen (blue) samples demonstrating differences in specific ASVs shown as mean proportion (%) and difference in mean proportions (%) with 95% confidence intervals; p values are shown after correcting for negative controls, covariates, and multiple comparisons. ASV = amplicon sequence variant.

(ASVs) were identified. Of these, 728 ASVs (65% of seminal ASVs and 13% of all ASVs) were unique to semen, and 522 ASVs (55% of urinary ASVs and 10% of all ASVs) were unique to urine (Fig. 1B). A total of 124 taxa (2.3%) were common to all three sources (Supplementary Table 5). Rectal samples exhibited increased α -diversity and distinct β -diversity compared with semen and urine samples, which demonstrated only subtle differences in β -diversity

when compared with each other (Fig. 1C). Shotgun metagenomics with a median of 140 million reads per sample recapitulated these findings and supported comparable analyses between these approaches (Fig. 1C). The differences between sample sources were quantified in a pairwise fashion, and both semen and urine contained increased abundance of *Gardnerella* and *Corynebacterium* compared with the rectum (Supplementary Fig. 2). Relative



Fig. 2 – Rectal and semen microbiome dysbiosis is associated with infertility and impaired semen parameters. (A) α -Diversity (Shannon index, left), β -diversity (middle), and differentially expressed ASVs (right) in rectal samples for fertile (orange, n = 12) versus infertile (aqua, n = 25) men. (B) Parallel analysis for semen samples. (C) Generalized linear regression models for selected 16S genera and key clinical characteristics. Statistically significant deviations are noted as colored bands, with green for inverse relationships and blue for direct relationships. ASV= amplicon sequence variant; BMI=body mass index. * p < 0.05.

to urine, semen demonstrated decreased *Veillonella* and *Prevotella* and increased *Pseudomonas, Pseudoxanthomonas,* and *Acidovorax* (Fig. 1D). The seminal microbiome was compared before and after uncomplicated antibiotic-free vasectomy in two men, and the relative abundance of both *Collinsella* and *Staphylococcus* decreased significantly (Supplementary Fig. 3). Given that the skin microbiome normalizes within 12 h following surgical preparation [13], this finding suggests a testicular or epididymal contribution to the seminal microbiome.

Next, we investigated the differences in the microbiome between fertile and infertile men (Fig. 2). We first examined potential confounding variables such as circumcision status and identified increased Finegoldia in uncircumcised men (Supplementary Fig. 4). After correcting for this and other covariates including BMI, infertile men displayed significantly increased α -diversity in semen samples and distinct β-diversity in both rectal and seminal samples (Fig. 2A and 2B). The rectum of infertile men harbored decreased abundance of Anaerococcus and increased abundance of Lachnospiraceae, Collinsella, and Coprococcus (Fig. 2A). Conversely, urine from infertile men contained increased Anaerococcus (Supplementary Fig. 5). Semen samples of infertile men contained decreased Collinsella and increased Aerococcus (Fig. 2B). Network analysis using predator-prey dynamics demonstrated a simplistic interaction network with weak interactions between limited numbers of taxa (Supplementary Fig. 6).

To better understand the direct relationship between specific taxa and individual clinical characteristics, we performed a generalized linear regression model analysis and identified a statistically significant inverse association between *Aerococcus* abundance and both leukocytospermia and semen viscosity (Fig. 2C). *Prevotella* abundance was directly associated with BMI and inversely associated with semen concentration. Finally, *Pseudomonas* abundance was directly associated with total motile sperm count but inversely proportional to semen pH (Fig. 2C).

To correlate the taxonomic differences with mechanistic underpinnings, we performed a functional pathway analysis using shotgun metagenomic data with the HUMAnN2 pipeline [14] and identified multiple differentially expressed pathways (Fig. 3A). The most differentially expressed pathway between fertile and infertile was the S-adenosyl-L-methionine (SAM) cycle (Fig. 3B), a finding that was confirmed in a subanalysis of semen and urine samples independently (data not shown). To minimize the possibility of unrecognized secondary infertility, we performed a sensitivity analysis using the more stringent cutoff of proven paternity within 1 yr (n = 10 men) and confirmed this finding (p = 0.001).

Next, we explored the microbiome in infertile men with or without leukocytospermia and found no differences in either α - or β -diversity (Supplementary Fig. 7A). Leukocytospermic men exhibited significantly higher proportions of several genera including rectal *Collinsella*, and metage-



Fig. 3 – Functional microbiome analysis reveals significant metabolic alterations in infertile men. (A) Heat map and cluster analysis for samples from fertile (orange, *n* = 12) and infertile (aqua, *n* = 25) men showing differentially expressed MetaCyc pathways via HUMAnN2. (B) Quantitative analysis showing differentially expressed pathways between fertile and infertile men.

nomics suggested increased seminal thiamine synthesis and adenine/adenosine salvage (Supplementary Fig. 7B). We analyzed the seminal microbiome in a representative patient before and after empiric doxycycline treatment for leukocytospermia [15] and noted depletion of *Acidovorax* and enrichment of *Gardnerella* and *Anaerococcus*, among others (Supplementary Fig. 8). Motivated by increased pathological oxidative stress in 30–80% of infertile men [16] and particularly those with leukocytospermia, we also correlated the seminal microbiome with elevated seminal ORP using an established cutoff of 1.34 mV/10⁶ sperm/ml [17] and identified only modest differences in three taxa including *Serratia* (Supplementary Fig. 9).

Finally, a varicocele is the dilation of the pampiniform plexus of veins and is associated with infertility through poorly understood mechanisms [18]. To test the hypothesis that a varicocele is associated with differences in the seminal microbiome, we compared the semen samples of infertile men with or without a clinical varicocele (Fig. 4) and identified significant differences in numerous anaerobic genera including Bacteroides and Peptoniphilus (Fig. 4A). Next, we correlated these taxonomic differences with functional pathway differences including amino acid synthesis (Supplementary Fig. 10). In stark contrast to the simplistic network plot for infertile men in general (Supplementary Fig. 6), a network analysis for infertile men with a varicocele revealed rich complex interactions between numerous families (Fig. 4B). We repeated this analysis in the fertile cohort with a history of varicocele and found no statistically significant differences in functional pathway expression (data not shown).

4. Discussion

In this study, we explored the relationship between the genitourinary and gastrointestinal microbiomes and male reproduction (Fig. 5). Consistent with prior literature, we identified a rich microbiome from voided urine [6,19] and semen [20–23]. Congruent with prior work comparing the gut, vaginal, and urinary microbiomes in females [7], we found that the male gut hosts a markedly different microbiome from those found in urine and semen, which appear relatively similar to each other (likely due in part to a significant urethral contribution). Pseudomonas. Pseudoxanthomonas, and Acidovorax (members of the phylum Proteobacteria) were over-represented in semen, suggesting unique contributions from upstream anatomic locations such as the seminal vesicle, prostate, and/or testis. Further supporting the presence of a testicular/epididymal microbiome was the finding that Collinsella (phylum Actinobacteria) and Staphylococcus (phylum Firmicutes) were both depleted in semen following vasectomy (Supplementary Fig. 3). Despite lacking the resolution to identify specific genera, a previous study also identified these phyla in the testis of azoospermic men [24]. Together, these data support the presence of an intrinsic testicular microbiome that may play a role in mammalian spermatogenesis. While the gut and urinary microbiomes may play an independent

role in male infertility, these samples cannot act as diagnostic surrogates for the seminal microbiome.

Next, we explored the taxonomic differences between the microbiomes of infertile men and healthy fertile controls and found significant differences in global diversity. In agreement with prior work [20] and consistent with its emerging status as a uropathogen [25], we identified increased *Aerococcus* in the semen of infertile men. We also complemented prior studies [23,26] suggesting an inverse association with *Prevotella* and semen quality. Notably, we also observed an unexpected direct association with *Pseudomonas* and total motile sperm count, which may warrant further investigation. Interestingly, we found only modest taxonomic differences in men with elevated oxidative stress or leukocytospermia.

To better understand the potential mechanistic underpinnings behind these associations, we performed shotgun metagenomics and identified the SAM cycle as strongly over-represented in both the urine and the semen of infertile men (Fig. 3). SAM is a ubiquitous metabolite with well-established roles in methylation, oxidative stress, and aminopropylation [27]. First, as the principal cellular methyl donor, SAM is critical for the maintenance of the highly unique methylation patterns seen in spermatozoa, and aberrations in sperm DNA methylation are strongly associated with male infertility [28]. Second, SAM is a potent modulator of oxidative stress via conversion to the antioxidant glutathione, which improves sperm motility in infertile men [29]. Third, SAM modulates the synthesis of polyamines such as spermidine via the aminopropylation pathway, which plays a key role in spermatogenesis and motility [30]. While it remains to be proven whether one or all of these mechanisms play a role in microbiomemediated male infertility, these data are nevertheless provocative and further studies are planned.

Finally, the reason why subfertility develops in only a subset of men with a varicocele remains controversial [18]. To this end, we identified a shift to anaerobes only in infertile men with a varicocele. This raises an intriguing mechanistic hypothesis that subfertility in some men with varicocele may develop due to underlying changes in the microbiome (or perhaps even vice versa).

4.1. Limitations

This is a single-institutional study with a small but wellcharacterized cohort with limited ethnic diversity, and larger multi-institutional longitudinal studies are needed to minimize the risk of overfitting and to validate these results. Urine specimens were obtained via midstream collection and likely represent a composite of urethral, bladder, and metal components. Despite controlling for BMI and other clinical covariates, it remains to be further clarified whether the dysbiosis associated with spermatogenesis merely representsa "canary in the coal mine" for overall health, or whether the differences demonstrated here are truly causal and specific to reproductive health. Further studies (including metaculturomics or gnotobiotics) will be neces-



Fig. 4 – Presence of a varicocele alters the seminal microbiome. (A) ASVs showing differentially prevalent genera in semen samples from infertile men with (dark green, *n*=9) or without (light green, *n*=16) varicocele. (B) Network analysis plot for men with varicocele with positive (cooperative) correlations noted in light blue and negative (competitive) correlations noted in dark blue. Node size represents relative abundance, and line width between two points is proportional to the strength of the correlation. ASV = amplicon sequence variant.



Fig. 5 – Microbiome in male infertility. Schematic representing taxonomic and functional changes in the genitourinary and gastrointestinal microbiomes in this study as they relate to male fertility status.

sary to validate mechanistic pathways and identify candidate therapeutic targets prior to clinical translation.

5. Conclusions

In summary, we explored the genitourinary and gastrointestinal microbiomes of the infertile male and identified a number of significant differences that may interact with spermatogenesis. To our knowledge, this study represents the first published report of the role of the human gut microbiome in male infertility, the first use of metagenomics to assess functional profiles in the male reproductive microbiome, and the first evidence exploring the microbiome in the presence of a varicocele. Our hope is that this work forms the basis for future studies investigating the potential diagnostic and therapeutic opportunities for the microbiota in male infertility and men's health.

Author contributions: Scott D. Lundy had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Lundy, Sabanegh, Vij, Eng.

Acquisition of data: Lundy, Parekh, Panner Selvam, Gupta, Vij, Agarwal. Analysis and interpretation of data: Lundy, Sangwan, Vij, Eng.

Drafting of the manuscript: Lundy, Sangwan.

Critical revision of the manuscript for important intellectual content: Lundy,

Sangwan, Parekh, Panner Selvam, Vij, Eng.

Statistical analysis: Lundy, Sangwan. Obtaining funding: Lundy, Vij, Eng.

Administrative technical on materi

Administrative, technical, or material support: McCaffrey, Bessoff, Vala. Supervision: Sabanegh, Vij, Eng.

Other: None.

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CRediT authorship contribution statement

Scott D. Lundy: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. Naseer Sangwan: Methodology, Software, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. Neel V. Parekh: Investigation, Writing - review & editing. Manesh K. Panner Selvam: Investigation, Writing - review & editing. Sajal Gupta: Investigation. Peter McCaffrey: Resources. Kovi Bessoff: Resources. Ayin Vala: Resources. Ashok Agarwal: Project administration. Edmund S. Sabanegh: Conceptualization, Supervision, Project administration. Sarah C. Vij: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. Charis Eng: Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j. eururo.2021.01.014.

References

- Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol 2015;13:37.
- [2] Levine H, Jørgensen N, Martino-Andrade A, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update 2017;23:646–59.
- [3] Punab M, Poolamets O, Paju P, et al. Causes of male infertility: A 9year prospective monocentre study on 1737 patients with reduced total sperm counts. Hum Reprod 2017;32:18–31.
- [4] Jung JH, Kim MH, Kim J, et al. Treatment of leukocytospermia in male infertility: a systematic review. World J Mens Health 2016;34:165–72.
- [5] Wade W. Unculturable bacteria—the uncharacterized organisms that cause oral infections. J R Soc Med 2002;95:81–3.
- [6] Bajic P, Van Kuiken ME, Burge BK, et al. Male bladder microbiome relates to lower urinary tract symptoms. Eur Urol Focus 2020;6:376–82.
- [7] Thomas-White K, Forster SC, Kumar N, et al. Culturing of female bladder bacteria reveals an interconnected urogenital microbiota. Nat Commun 2018;9:1557.
- [8] Altmäe S, Franasiak JM, Mändar R. The seminal microbiome in health and disease. Nat Rev Urol 2019;16:703–21.
- [9] Knight R, Vrbanac A, Taylor BC, et al. Best practices for analysing microbiomes. Nat Rev Microbiol 2018;16:410–22.
- [10] Agarwal A, Arafa M, Chandrakumar R, Majzoub A, AlSaid S, Elbardisi H. A multicenter study to evaluate oxidative stress by oxidation– reduction potential, a reliable and reproducible method. Andrology 2017;5:939–45.
- [11] Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41:e1.

- [12] Ohwaki K, Endo F, Yano E. Relationship between body mass index and infertility in healthy male Japanese workers: a pilot study. Andrologia 2009;41:100–4.
- [13] SanMiguel AJ, Meisel JS, Horwinski J, Zheng Q, Bradley CW, Grice EA. Antiseptic agents elicit short-term, personalized, and body sitespecific shifts in resident skin bacterial communities. J Invest Dermatol 2018;138:2234–43.
- [14] Franzosa EA, McIver LJ, Rahnavard G, et al. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods 2018;15:962–8.
- [15] Hamada A, Agarwal A, Sharma R, French DB, Ragheb A, Sabanegh ES. Empirical treatment of low-level leukocytospermia with doxycycline in male infertility patients. Urology 2011;78:1320–5.
- [16] Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. Nat Rev Urol 2017;14:470–85.
- [17] Agarwal A, Parekh N, Selvam MKP, et al. Male oxidative stress infertility (MOSI): proposed terminology and clinical practice guidelines for management of idiopathic male infertility. World J Mens Heal 2019;37:296–312.
- [18] Lundy SD, Sabanegh ES. Varicocele management for infertility and pain: a systematic review. Arab J Urol 2018;16:157–70.
- [19] Nelson DE, Dong Q, van der Pol B, et al. Bacterial communities of the coronal sulcus and distal urethra of adolescent males. PLoS One 2012;7:1–9.
- [20] Monteiro C, Marques PI, Cavadas B, et al. Characterization of microbiota in male infertility cases uncovers differences in seminal hyperviscosity and oligoasthenoteratozoospermia possibly correlated with increased prevalence of infectious bacteria. Am J Reprod Immunol 2018;79:e12838.
- [21] Hou D, Zhou X, Zhong X, et al. Microbiota of the seminal fluid from healthy and infertile men. Fertil Steril 2013;100, 1261–1269.e3.

- [22] Chen H, Luo T, Chen T, Wang G. Seminal bacterial composition in patients with obstructive and non-obstructive azoospermia. Exp Ther Med 2018;15:2884–90.
- [23] Baud D, Pattaroni C, Vulliemoz N, Castella V, Marsland BJ, Stojanov M. Sperm microbiota and its impact on semen parameters. Front Microbiol 2019;10:234.
- [24] Alfano M, Ferrarese R, Locatelli I, et al. Testicular microbiome in azoospermic men—first evidence of the impact of an altered microenvironment. Hum Reprod 2018;33:1212–7.
- [25] Pearce MM, Hilt EE, Rosenfeld AB, et al. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. MBio 2014;5:e01283–14.
- [26] Weng SL, Chiu CM, Lin FM, et al. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. PLoS One 2014;9:e110152.
- [27] Bottiglieri T. S-adenosyl-L-methionine (SAMe): from the bench to the bedside—molecular basis of a pleiotrophic molecule. Am J Clin Nutr 2002;76, 1151S–7S.
- [28] Marques CJ, Costa P, Vaz B, et al. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. Mol Hum Reprod 2008;14:67–73.
- [29] Lenzi A, Culasso F, Gandini L, Lombardo F, Dondero F. Placebocontrolled, double-blind, cross-over trial of glutathione therapy in male infertility. Hum Reprod 1993;8:1657–62.
- [30] Engel KM, Baumann S, Rolle-Kampczyk U, Schiller J, von Bergen M, Grunewald S. Metabolomic profiling reveals correlations between spermiogram parameters and the metabolites present in human spermatozoa and seminal plasma. PLoS One 2019;14:e0211679.