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Testicular microbiome in azoospermic men—first evidence of the impact of an altered microenvironment

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STUDY QUESTION: Given the relevant role of the extracellular microenvironment in regulating tissue homeostasis, is testicular bacterial microbiome (BM) associated with germ cell aplasia in idiopathic non-obstructive azoospermia (iNOA)?

SUMMARY ANSWER: A steady increase of dysbiosis was observed among testis with normal spermatogenesis vs. iNOA with positive sperm retrieval and iNOA with complete germ cell aplasia.

WHAT IS KNOWN ALREADY: Tissue-associated BM has been reported to be a biologically important extracellular microenvironment component for numerous body habitats, but not yet for the human testis.

STUDY DESIGN, SIZE, DURATION: Cross-sectional study, investigating tissue-associated BM in the testis of (i) five men with iNOA and negative sperm retrieval at microdissection testicular sperm extraction (microTESE); (ii) five men with iNOA and positive sperm retrieval at microTESE; and (iii) five normozoospermic men upon orchiectomy. Every testicular specimen was histologically classified and analyzed in terms of bacterial community.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Massive ultra-deep pyrosequencing was applied to investigate testis microbiome. Metagenome was analyzed using Quantitative Insights Into Microbial Ecology (QIIME). Tissue-associated bacterial load was quantified by digital droplet PCR.

MAIN RESULTS AND THE ROLE OF CHANCE: Normozoospermic men showed small amounts of bacteria in the testis, with *Actinobacteria*, *Bacteroidetes*, *Firmicutes Proteobacteria* as the dominating *phyla*; iNOA individuals had increased amounts of bacterial DNA ($P = 0.02$), associated with decreased taxa richness due to the lack of *Bacteroidetes* and *Proteobacteria* ($P = 2 \times 10^{-5}$). Specimens with negative sperm retrieval at microTESE depicted complete germ cell aplasia and a further decrease in terms of *Firmicutes* and *Clostridia* ($P < 0.05$), a complete lack of *Peptoniphilus asaccharolyticus*, but increased amount of *Actinobacteria*.

LIMITATIONS, REASONS FOR CAUTION: The limited number of specimens analyzed in this preliminary study deserves external validation. The paraneoplastic microenvironment could have an impact on the residential bacterial flora.

WIDER IMPLICATION OF THE FINDINGS: Human testicular microenvironment is not microbiologically sterile, containing low amounts of *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. A dysbiotic bacterial community was associated with iNOA and complete germ cell aplasia. Novel findings on testicular BM could support future translational therapies of male-factor infertility.

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Introduction

Azoospermia is defined as the absence of sperm in the semen, afflicting 1% of men. Non-obstructive azoospermia (NOA) is the most severe form of infertility, with up to 80% of NOA men classified as idiopathic in nature (Cocuzza *et al.*, 2013). Recent data suggest that male infertility leads to a higher risk of developing tumors and age-related diseases, such as cardio-vascular and metabolic disorders (Ventimiglia *et al.*, 2016). A sort of early ageing process is also observed in the testicular tissue of young infertile men, showing several similarities to the tissue of aged individuals (Yi-chao *et al.*, 2014).

A number of animal models have established the relevance of the extracellular microenvironment throughout the process of spermatogenesis, thus indicating that restoring a proper testicular niche might be of major importance in terms of germ cell production recovery (Ogawa *et al.*, 2000; Ryu *et al.*, 2006; Zhang *et al.*, 2006). The bacterial microbiome (BM) has been described as a relevant extracellular microenvironment component. In this context, gut BM has been shown to impact both local and distant organs, modulating a variety of physiological processes including the circulating levels of testosterone (Markle *et al.*, 2013), estrogens (Baker *et al.*, 2017) and the vitamin B complex (Biesalski, 2016), each of which can strongly impact spermatogenesis. Overall, a number of human body habitats have been studied, and tissue-associated BM has been described for several tissues and organs (Lloyd-Price *et al.*, 2016), but not for human testis. Therefore, we analyzed BM in the human testis using specimens from men with idiopathic NOA (iNOA) with positive or negative sperm retrieval at microdissection testicular sperm extraction (microTESE), and from normozoospermic tissue.

Materials and Methods

Ethical approval

Data collection followed the principles outlined in the Declaration of Helsinki; all patients signed an informed consent agreeing to supply their own anonymous information and tissue specimens for future studies. The study was approved by the IRCCS Ospedale San Raffaele Ethical Committee (Protocol URI001-2010).

Study population of iNOA men

Male-factor infertility and azoospermia were defined according to WHO (World Health Organization, 2010) criteria. INOA patients were dichotomized as positive or negative for sperm retrieval according to microTESE outcomes (Alfano *et al.*, 2017). Further details are available in the Supplementary Information Materials and Methods.

Tissues with normal germline maturation

Non-neoplastic specimens were collected from the testis of five men submitted to unilateral orchiectomy for non-metastatic seminoma. Non-neoplastic tissue was obtained from the most distant area from the tumor, and identified as normal for germline cell maturation according to a thorough histological analysis. For each neoplastic patient, a preoperative semen cryopreservation was available, along with a detailed semen analysis excluding azoospermia.

Tissue collection

Testicular specimens were collected using sterile procedures, placed in a sterile saline solution in the operating room, and processed for storage under sterile conditions within 30 minutes. Testicular tissue from iNOA men was stored in a freezing solution (10% dimethylsulfoxide (DMSO)/90% fetal bovine serum (FBS)) and non-neoplastic tissue in optimal cutting temperature (OPT) compound both procedures were performed in sterile conditions, using single-use materials and equipment. All recruited individuals were free of bacterial infections and antibiotic therapies throughout the 6 months prior to surgery. Testicular specimens were histologically classified according to the Johnsen's score (Johnsen, 1970).

Tissue-associated microbiome analysis

The QIAamp DNA FFPE Tissue Kit (Qiagen, Italy) was used to isolate DNA from the testis and from PC3 cell lines that had developed in the presence of antibiotics (used as the negative control for the quantification of 16 S copies). DNA from a mucosal swab was used as a positive control, using a swab stabilization buffer and buccal-prep DNA isolation kit from Isohelix (Kent, UK). Testis parenchyma, PC3 cells and mucosal swabs were processed in parallel. To avoid exogenous contamination, samples were processed under a sterile hood, using sterile and single-use materials and avoiding the use of the cryostat. Total DNA was amplified for the quantification of 16 S copies, using 16 S probes for pan-bacteria (code Ba04230899_s1, (Thermofisher, Italy)) and digital droplet PCR (ddPCR) according to the manufacturer's instructions. Briefly ddPCR was performed in duplicate in a final volume of 20 µl. Up to 20,000 monodispersed droplets for each sample were prepared using the QuantaLife droplet generator. Plates were quantified in a QuantaLife droplet reader, and the concentrations of the targets in the samples were determined using QuantaSoft software. The number of 16 S copies was normalized to the total ng of loaded DNA. The freezing solution and OCT used for tissue storage tested negative for the quantification of DNA, and were not processed by ddPCR.

The human BM was analyzed up to the level of *genus* by performing amplifications of the V3–V5 region of 16 S rRNA by nested PCR using the FastStart High Fidelity PCR System (Roche, Basel, Switzerland) with the following nested PCR protocol. The outer amplification was performed with the following primers: 16S-F8 AGA GTT TGA TCC TGG CTC AG and

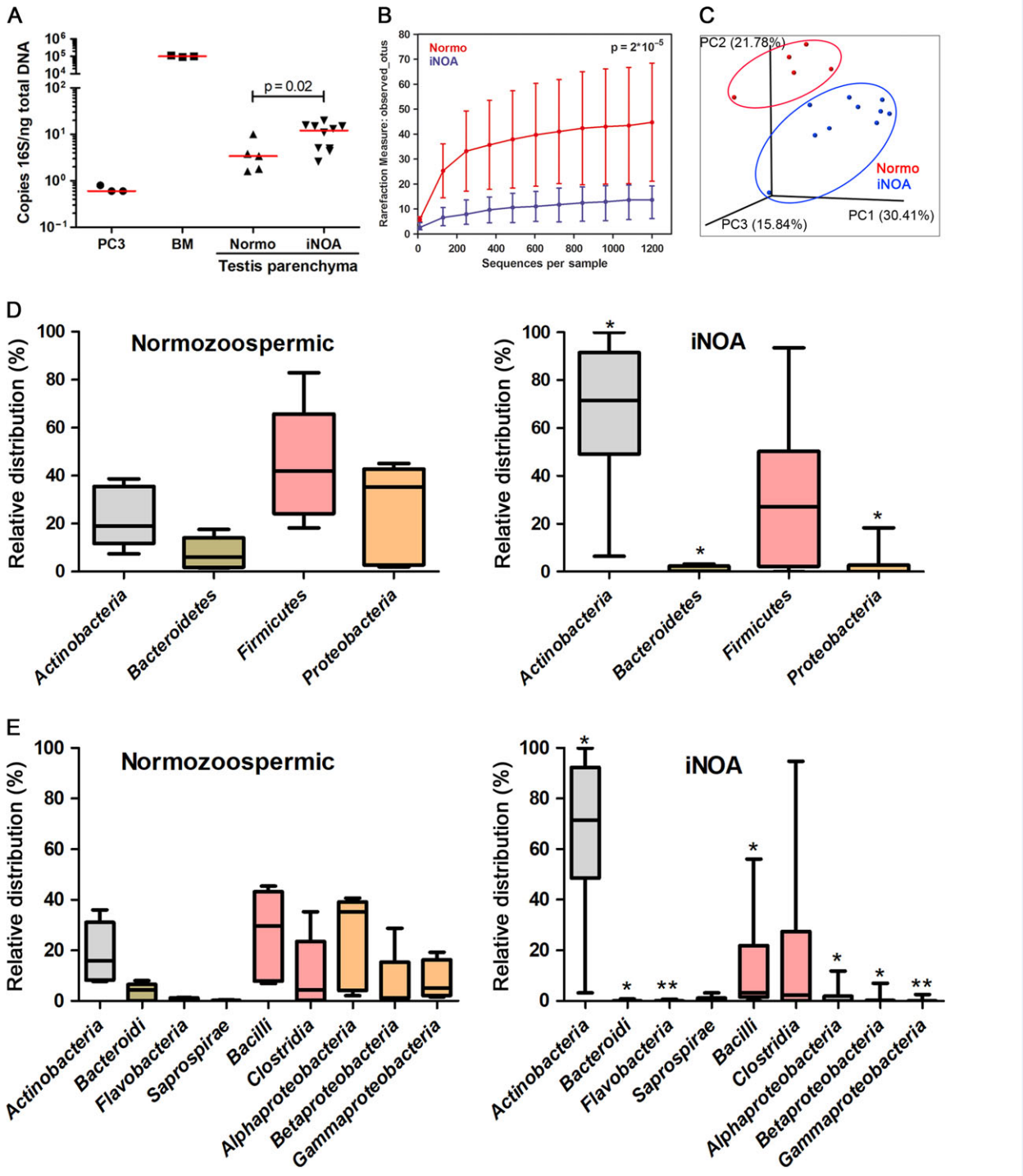

Figure 1 BM community in the normozoospermic and iNOA human testis parenchyma. Quantification of the 16 S copies/ng in (i) 200 ng of total DNA from the PC3 cell line and 0.1 ng of total DNA from human buccal mucosa (BM), used as negative and positive controls, respectively and (ii) 200 ng of total DNA from normozoospermic and iNOA testis parenchyma (**A**). The microbial community in normozoospermic and iNOA testis parenchyma was analyzed for alpha and beta diversity. Diversity within samples (α-diversity) was estimated using >1200 sequences per sample (**B**, statistical significance by two-tail unpaired and non-parametric *T*-test). Diversity between samples (β-diversity) was evaluated by principal component analysis and represented by weighted variance (**C**). The relative distribution of taxonomic rank at the level of *phyla* (**D**) and class (**E**) for the BM of normozoospermic and iNOA testis parenchyma. Statistical significance was estimated by two-tail Mann–Whitney test and considered significant at a *P*-value <0.05 (represented by asterisks in panels D and E, when comparing normozoospermic vs. iNOA testis parenchyma).

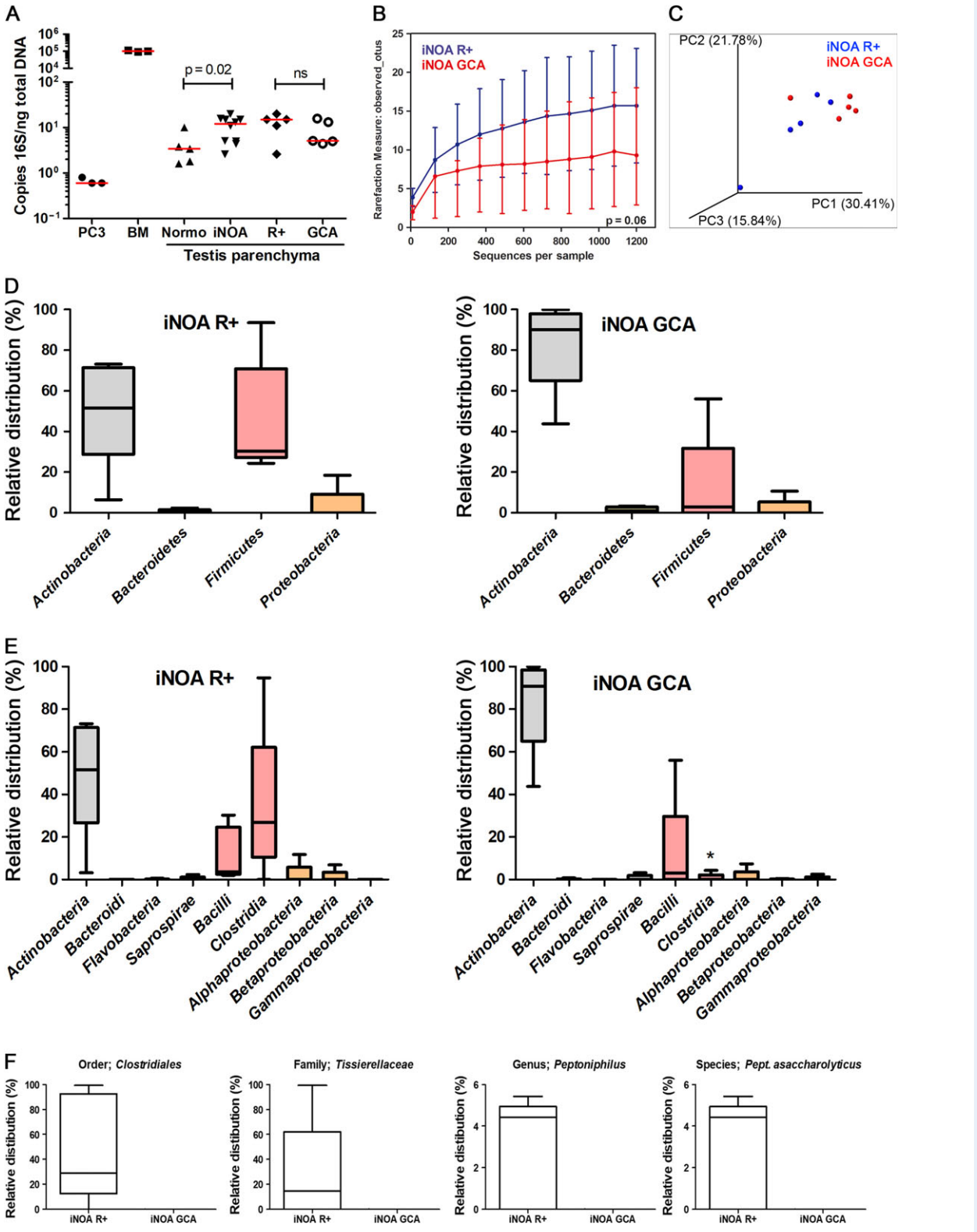

Figure 2 BM community in the iNOA human testis parenchyma classified as sperm retrieval positive or germ cell aplasia. Quantification of the 16 S copies/ng of loaded DNA in the (i) PC3 cell line and human buccal mucosa, BM, used as negative and positive controls, respectively, (ii) normozoospermic and iNOA testis parenchyma and (iii) the iNOA testis parenchyma according to positive sperm retrieval (R+) or complete germ cell aplasia (GCA) (**A**). The microbial community in iNOA-R+ and iNOA-GCA testis parenchyma was analyzed for alpha and beta diversity. Diversity within samples (α-diversity) was estimated using >1200 sequences per sample (**B**, statistical significance by two-tail unpaired and non-parametric *T*-test). Diversity between samples (β-diversity) was evaluated by principal component analysis and represented by weighted variance (**C**). The relative

16S-R1093 GTT GCG CTC GTT GCG GGA CT; and by using the following thermal cycler profile: 95°C for 3 min, 15 cycles of 94°C/30′, 55°C/45′ and 72°C/1 min, 72°C for 8 min and stored at 4°C. A second nested amplification step was performed to amplify the 16 S V3–5 region using barcoded sample-specific primers: 16S-F331 ACT CCT ACG GGA GGC AGC and 16S-R920 CCG TCA ATT CMT TTG AGT TT. The FastStart High Fidelity PCR System and the following cycling conditions were used: 95°C for 3 min, 35 cycles of 95°C/30′, 55°C/45′ and 72°C/1 min, 72°C/8 min and then stored at 4°C until usage. Amplicons were loaded on 1.5% agarose gel, extracted with the QiaQuick Gel Extraction kit (Qiagen) and purified twice with AMPure XP beads (Beckman Coulter, Italy). Then an emulsion-PCR was performed, followed by an ultra-deep pyrosequencing of barcoded 16 S rRNA gene amplicons on the 454-GS Junior platform (Roche, CT, USA). Sequences with a high-quality score and a length of >250 bp were used for the taxonomic analysis with QIIME version 1.9.0 software. The OTUs (operational taxonomic units) were identified using the UCLUST clustering method. Taxonomy was assigned using the RDP Classifier. Diversity within samples (α-diversity) was estimated using > 1200 sequences per sample and the 'observed-otus' parameter. Diversity between samples (β-diversity) was evaluated using the phylogeny-based weighted Unifrac distance matrices and represented by weighted variance (further details available in Supplementary Information Materials and Methods).

Statistical analyses

Continuous variables were expressed as medians and interquartile ranges (IQR). For the analysis of the microbiome profile, chimeric sequences (identified using ChimeraSlayer software, Broad Institute, USA) and sequences shorter than 250 bp were excluded, whereas bacterial sequences covering >0.98 of the genome were included in the analysis. Two-tail unpaired tests were used. All statistical tests and enrichments were considered significant at *P*-value <0.05.

RESULTS

Clinical and histopathological characteristics

Age at surgery, serum total testosterone (tT) and LH levels did not differ among groups; conversely, iNOA men had higher FSH levels, suggestive of primary testicular failure. Histology showed intact spermatogenesis in the non-neoplastic specimens, but disrupted spermatogenesis in iNOA men, down to complete germ cell aplasia in negative sperm retrievals. (Supplementary Table S1, Supplementary Figure S1).

Testicular microbiome and dysbiosis among groups

Tissues with normal germline maturation showed detectable amounts of 16 S DNA (median; 3.4; IQR; 1.7-7 copies/ng of loaded DNA), over the background set up using the PC3 cell line (0.6; 0.6-0.8) (Figure 1A). An increased amount of 16 S DNA was found within testicular specimens from iNOA men (12.1; 5–15) (Figure 1A). Taxonomic diversity of the BM profiling showed significant differences among samples (α-diversity, Figure 1B) and BM clusterization within the testicular specimens from normozoospermic and iNOA men (β-diversity, Figure 1C). *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were *phyla* associated with a normal germline; conversely, only *Actinobacteria* and *Firmicutes* were retrieved in iNOA men (Figure 1D). A decreased richness in terms of classes was also observed in iNOA men compared to normozoospermic individuals (Figure 1E).

No differences were observed between positive and negative sperm retrievals in terms of 16 S DNA amounts (Figure 2A). Similarly, no differences in α and β diversities were found between the two iNOA groups (Figure 2B and C). Testicular tissues from both types of iNOA men were similarly enriched with *Actinobacteria* and *Firmicutes* at the *phylum* level; *Actinobacteria* and *Firmicutes* dominated in the tissue of positive sperm retrievals whereas *Actinobacteria* was the predominant taxa in negative sperm retrievals (Figure 2D). At the class level, tissues from positive sperm retrievals presented *Actinobacteria*, *Bacilli* and *Clostridia*; conversely, a significant reduction of *Clostridia* was observed in negative sperm retrievals, where *Actinobacteria* (Figure 2E) dominated and *Peptonyphilus asaccharolyticus* was undetectable (Figure 2F).

Discussion

These analyses show for the first time that the human testis is not microbiologically sterile, present novel findings about testicular tissue-associated BM, and exclude any potential contribution of bacteria communities present in the downstream tissues and/or in other organs along the urogenital tract. Testis tissues with normal spermatogenesis were characterized with *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* as the dominating *phyla*. Conversely, an increased amount of bacteria was found in the testis of iNOA men, with a predominance of *Actinobacteria* and *Firmicutes*; these two *phyla* equally dominated the tissues of positive sperm retrievals, whereas decreased bacterial richness and diversity was observed in the specimens from men with complete germline cell aplasia, where BM was dominated by the *phylum* of *Actinobacteria*. Complete germ cell aplasia was also characterized by the absence of *Clostridia*. These findings are of potential clinical relevance since two genera of the class of *Clostridia* (i.e. *Anaerococcus* and *Peptoniphilus*) have been described to be associated with human sperm motility (Hou *et al.*, 2013) and morphology (Weng *et al.*, 2014). Therefore, future characterizations of semen *Clostridia* could represent a reliable clinical marker for predicting germ cell aplasia in the real-life setting.

The same four *phyla* observed in the normozoospermic testes have been previously reported to dominate the human gut, although with different relative abundances (Shin *et al.*, 2015). Indeed, gut BM drastically differs across the ageing process, with both a reduction in terms of biodiversity and of percentage representation of *Firmicutes* among the elderly compared to younger male population, this correlating with signs of frailty, comorbidity and inflammation (Biagi *et al.*, 2010; Mariat *et al.*, 2009). Of interest, BM modifications documented in iNOA men were similar to those previously reported in the gut of elderly

distribution of taxonomic rank at the level of *phyla* (**D**) and class (**E**) for the BM of iNOA-R+ and iNOA-GCA testis parenchyma. The relative distribution of the *Clostridia* taxa down to the species *Peptoniphilus asaccharolyticus* (**F**). Statistical significance was estimated by two-tail Mann–Whitney test and considered significant at a *P*-value <0.05 (represented by asterisks in panel E, when comparing iNOA-R+ vs. iNOA-GCA).

individuals, thus providing further evidence of an early ageing phenotype of NOA men even at the testicular level.

Although this is the first study to outline that the testis holds tissue-associated commensal bacteria, we recognize that virtually normal testicular specimens came from non-neoplastic areas of tumoral testes and we cannot exclude the influence of the neoplastic microenvironment over the residential bacterial flora. Second, the BM amount and diversity within the entire testicular pulp of iNOA men could have been underestimated as the tissue was harvested upon microTESE, thus after the selection of testicular areas with the most dilated tubules. Therefore, larger studies are needed to confirm our preliminary findings that the testicular tissue is not microbiologically sterile and that the testes of iNOA men contain a more dysbiotic bacterial community.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

M.A. and A.S. conceived and designed the study; M.A., F.R., I.L., E.V., S.I., P.G., D.C., L.P., F.C., P.V., M.N. and A.S. retrieved specimens, performed experiments and analyzed the data; M.A., I.L., S.I. and A.S performed management of clinical data; M.A. and A.S. wrote the first draft of the manuscript; M.A., F.C., M.N., M.C., F. M. and A.S. revised the intellectual content of the manuscript; all authors contributed to data interpretation and reviewed the final version of the manuscript.

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Conflict of interest

The authors declared no conflict of interest.

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