MATERIALS AND METHODS

Study population of iNOA men

According to the World Health Organisation (WHO) criteria, infertility was defined as not conceiving a pregnancy after at least 12 months of unprotected intercourse regardless of whether or not a pregnancy ultimately occurred. Primary infertility was defined as when a couple had never been able to conceive. Patients with NOA underwent at least two consecutive semen analyses, both showing no sperm (World Health Organization, 2010).

Inclusion criteria were (i) patients with clinical diagnosis of iNOA; (ii) age ≤ 45 years; (iii) European-Caucasian; (iv) free of any known bacterial infections and antibiotic therapies at the time of surgery and throughout the 6 months prior to enrollment; (v) undergoing mono vs. bilateral microTESE with the same experimental conditions and (vi) complete blood test profiles in a time frame of 0–3 months before surgery. Exclusion criteria were (i) NOA associated with known testicular factors already associated with infertility (e.g. unilateral or bilateral maldescended testis; left varicocele; disturbance of erection/ejaculation); (ii) genetic abnormalities (any type); (iii) known and modifiable hypothalamic/pituitary abnormalities; (iv) previous pituitary or testicular surgery; (v) vasectomy or any other genital tract obstruction and (vi) previous testicular tumor, lymphoma, leukemia and sarcoma. As a whole, inclusion and exclusion criteria described a condition of iNOA.

INOA patients were then dichotomized according to sperm retrieval outcomes (namely, positive versus negative sperm retrieval) at microTESE, as reported (Alfano et al., 2017).

Hormonal profile

Venous blood samples were drawn from each patient between 7 and 11 am after an overnight fast at the time of surgery; hormones were measured as detailed (Alfano et al., 2017).

Tissue-associated microbiome analysis

Digital droplet PCR (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 (BIORAD, Italy). Plates were quantified in a QuantaLife droplet reader (BIORAD, Italy), and the concentrations of the targets in the samples were determined using QuantaSoft software (BIORAD, Italy). The number of 16S copies was normalized to the total ng of loaded DNA.

The human bacterial profile was analyzed at the level of phylum, class, order, family and genus by performing 16S amplicon sequencing, as reported (Cosorich et al., 2017); the V3–V5 region of the 16S rRNA gene was amplified starting from 200 ng of extracted DNA 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 16S–R1093 GTT GCG CTC GTT GCG GGA CT; and by using the following thermal cycling 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 16S V3–V5 regions using the following barcode-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 and purified with the QiaQuick Gel Extraction kit (Qiagen). Extracted amplicons were purified two times with AMPure XP beads (Beckman Coulter). Bacterial profiles were analyzed at the level of phylum, class, order, family and genus performing an emulsion-PCR followed by ultra-deep pyrosequencing of barcoded 16S rRNA gene amplicons, according to the 454 GS Junior manufacturer’s instructions (Roche). Sequences with a high-quality score and a length of >250 bp were used for the taxonomic analysis with QIIME (Quantitative Insights Into Microbial Ecology version 1.9.0) software. The operational taxonomic units (OTUs) 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. The identification of Peptoniphilus asaccharolyticus was carried out looking for and selecting the sequences of Peptoniphilus genus from the list of all the sequenced 16S RNA. Thirty sequences were selected and compared with the BLAST (http://blast.ncbi.nlm.nih.gov) sequence database: more than 95% of the analyzed sequences were identified as Peptoniphilus asaccharolyticus.

REFERENCES

