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ORIGINAL ARTICLE

Periodontal clinical status, microbial profile, and expression of interleukin-1β in men under androgenic anabolic steroids abuse

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Abstract

Objectives Androgenic anabolic steroids (AAS) abuse is a serious health problem associated to several systemic complications. Here, we evaluated the periodontal clinical status, microbial profile, and expression of total protein (TP) and interleukin (IL)-1β in men using AAS.

Materials and methods Men using AAS were recruited (case group) and matched for age with men who had never used AAS (control group) but also performed physical activities. Plaque index (PI), marginal bleeding (MB), probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BoP) were evaluated. Crevicular fluid and subgingival biofilm were collected from healthy and diseased sites (PD ≥ 4 mm with CAL ≥ 1 mm and BoP) and evaluated for TP, IL-1β, and proportions of 40 bacterial species.

Results Thirty patients were included (n = 15/group). AAS consumers had significantly higher mean PD and higher percentage of diseased sites; sites with PD ≥ 4 mm or with CAL ≥ 1 mm than non-consumers. Also, AAS users showed a more dysbiotic biofilm containing lower proportions of host-compatible species and higher proportions of pathogens. IL-1β expression was statistically higher in diseased than in healthy sites only in the control group. A statistically positive correlation was detected between periodontal pathogens and IL-1β expression. The number of AAS cycles was positively associated with higher percentages of periodontal pathogens, but not with IL-1β or total protein concentrations.

Conclusions AAS intake can worsen clinical and immunological periodontal conditions and the biofilm composition in healthy sites.

Clinical relevance Dental care professionals should perform full mouth periodontal screening and schedule regular follow-up appointments for patients under AAS use.

Keywords Testosterone · Steroids · Periodontium · Periodontitis

Introduction

Testosterone is the main male sex hormone responsible for the development of primary and secondary adult sexual characteristics (e.g., muscle mass, facial hair, libido, and sperm production) and, to a lesser extent, water, and electrolyte balance [1, 2]. Synthetic steroids derived from testosterone, such as androgenic anabolic steroids (AAS) have been developed to treat men suffering from hypogonadism, late puberty, and some types of impotence [3, 4]. However, these products started to be widely used by professional or recreational athletes aiming to increase performance, gain lean muscle mass, and decrease body fat percentage [5]. The use of AAS in high doses by athletes and bodybuilders may lead to a variety of side effects, which include fluid retention, acne, hepatic

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intoxication, arterial hypertension, blood cells disorders, anemia, depression, infertility, and even heart-related conditions [6]. The adverse effects of inappropriate and abusive use of AAS vary according to the individual's age and sex, as well as dose, duration, and type of steroid. The protocol of use of AAS (e.g., dose, duration and interval between courses) varies according to the aim of the treatment and is frequently called AAS cycle [3].

It has been suggested that steroid hormones may influence the physiology of oral and periodontal tissues due to the homeostasis of anabolic and catabolic functions in the connective tissue and bone matrix [7]. These hormones and their derivatives may play a role in the progression of periodontal disease, including alterations in the composition of the microbiota and the healing process [8–13]. The periodontal biofilm is a complex structure that may change according to the host immune competence and the environmental conditions [14]. Environmental changes can generate a shift in microbiological profile by increasing microorganisms associated with the onset and progression of periodontitis (red and orange microbial complexes) and decreasing those associated with periodontal health (purple, yellow, green, and *Actinomyces* complexes) [15–17].

In humans, the use of AAS has been associated with gingival enlargement [18], higher prevalence of severe periodontitis, greater gingival inflammation, and an increased *odds ratio* to be infected with *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Candida spp*. than non-AAS users [13]. Similarly, high endogenous testosterone levels were correlated with higher prevalence and severity of periodontitis in men [19]. In animals, treatment with supraphysiological doses of testosterone has been shown to increase ligature-induced bone loss in orchiectomized male rats [20]. In vitro, it was demonstrated that high doses of testosterone increased osteoclasteogenesis directly from RAW264.7 cells, in addition to increasing RANKL/OPG ratio in murine osteoblast primary culture [20, 21]. However, to the best of our knowledge, a combined evaluation of the microbiological and immunological oral response to AAS has not been performed in humans. Among immunological features, interleukin (IL)-1β is one of the most common biomarkers that can be used as indicator of periodontal disease progression [22].

Hence, the aim of this study was to evaluate if AAS influences periodontal clinical and microbiological parameters, as well as to assess the expression of total protein and interleukin (IL)-1β in gingival crevicular fluid.

Material and methods

This case-control study was approved by the Brazilian National Commission for Research Ethics—CONEP (1.906.729–2.443.657) and conducted in accordance with the Helsinki Declaration as revised in 2013. This was a convenience sample study. Recruitment of individuals took place from March 2017 to July 2018 at the Federal University of Paraná Dental Clinic Building (Curitiba, PR, Brazil). The authors went to gyms and bodybuilding events to promote and explain the research. We invited individuals who were taking AAS and also recruit those not taking AAS but willing to participate as controls. Interviews in the local radio and television network were also broadcasted. All included individuals signed an informed consent form.

Eligibility criteria

Inclusion criteria are as follows: case group—males aged 18 years or older under AAS treatment at the time of the interview and physical activity practitioners (at least 3 times/week for at least 3 months), with at least 20 teeth excluding third molars, and control group—same inclusion criteria as the case group, but who had never used AAS. The groups were matched according to age (± 3 years). Each control individual was included after the inclusion of their 'case' counterpart.

Exclusion criteria are as follows: smoking, ongoing dental treatment, any systemic disease that could be associated with inflammatory, and immunological or hormonal changes.

Interview and clinical examination

All individuals were interviewed for age, self-declared ethnicity, income, and educational level. Current AAS medication and the number of previous AAS cycles each individual had undergone were recorded. Plaque index [23] (PI) and marginal bleeding (i.e., bleeding in the coronal marginal gingiva after applying pressure to the lateral wall of the pocket or sulcus using a periodontal probe; MB) were measured at 4 sites per tooth, while probing depth (PD), bleeding on probing (BoP), and Clinical Attachment Level (CAL) were assessed at 6 sites per tooth. All exams were performed by a single previously trained and calibrated examiner (SVSCW, Kappa ± 1 mm = 0.97).

Microbiological samples

Subgingival samples were collected from the deepest interproximal site in the mouth presenting detectable attachment loss (CAL ≥ 1 mm) and PD ≥ 4 mm with BoP (diseased sites) and a contralateral site with no attachment loss and PD ≤ 3 mm without BoP (control sites). If the interproximal sites around the contralateral tooth did not fit those criteria a more mesial tooth was then used. Supragingival biofilm was removed from the sampling sites and the teeth were isolated with sterilized cotton roles. Subgingival biofilm samples were collected using sterilized 5-6 Gracey scaler (Hu-Friedy Mfg.

Co., IL, USA). The samples were placed in individual tubes containing 150 μL of Tris-EDTA buffer (TE) and 100 μL of a 0.5 M solution of sodium hydroxide (NaOH) was added to each tube. All samples were stored in a – 80 °C freezer until Checkerboard DNA-DNA hybridization analysis [24]. Bacterial species from the blue (*Actinomyces*), purple, yellow, and green complexes were considered to be associated with health (host compatible), while those from the orange and red complexes were considered to be associated/compatible with disease [15]. For the biofilm proportion analysis, the sum of health and disease-compatible microorganisms comprised 100%.

Total protein and IL-1β analysis

Subgingival biofilm samples were collected from the diseased and healthy sites described above. Sixty to 90 s after collecting the biofilm samples, the teeth were washed to remove any blood traces [25]. Then, four gingival crevicular fluid (GCF) sample were collected per site, using paper points. Each paper point was left in place for 10 s, with 3-min intervals between samples. Samples were stored in 200 μL of phosphate-buffered saline (PBS) containing protease inhibitor and frozen at − 80 °C until analysis. Paper points visually contaminated with blood were discarded. The tubes were vortexed for 1 min and centrifuged for 10 mins at 12,000×*g* to elute components. Samples were evaluated for the total amount of protein (pg/mL) collected in 40 s of exposition [26] using the DC Protein Assay (BioRad, Hercules, CA, USA), according to the manufacturer's instructions. IL-1β concentration was assessed using ELISA (Quantikine HS ELISA kit IL-1ß/IL-1F2, R&D Systems, Houston, TX, USA).

Statistical analysis

Statistical analysis was performed using software (GraphPad version 7.0, La Jolla, CA, USA). In order to observe the correct pairing (± 3 years), ages were compared using Student's *t* test. All quantitative variables were compared using Student's *t* test or Mann-Whitney test, depending on the homogeneity of variances and normal distribution. Exploratory analyses were performed to observe correlation between IL-1β or total protein levels and PD, number of AAS cycles or percentage of disease-compatible complexes using Pearson's correlation test. Fisher's exact test was used for frequency comparisons. Data were presented as means and standard deviations. Statistical significance was set at $p < 5\%$.

Since this was a convenient sample, calculation of the power of the test was performed a posteriori for the primary outcome (difference between groups for mean full mouth PD). Considering the observed difference in our study of 0.3 mm between case and control groups with mean standard deviation of 0.2, fifteen patients per group represented 96% power at a significance level of 5%.

Results

Clinical findings

Fifteen men were included in the final sample of the 'case' group and matched with another 15 men who served as age-matched controls. All of the individuals in the case group reported using AAS to improve physical fitness and none reported medical prescription. Used drugs included nandrolone ($n = 6$), testosterone propionate ($n = 6$), testosterone cypionate ($n = 4$), methandrostenolone ($n = 3$), testosterone enanthate ($n = 2$), Drostanolone propionate ($n = 1$), boldenone undecylenate ($n = 1$), trenbolone acetate ($n = 1$), and anastrozole (not an AAS but an aromatase inhibitor; $n = 1$). As inferred from the data, some individuals used protocols of drug combination. It was observed that all these medications were not prescribed by a medical professional. The mean age of all participants was 29.1 ± 4.55 years, ranging from 23 to 40 years. The majority of participants were white (96.7%) and had completed high school (60% in the AAS and 73.3% in the control group). Average income was US$1083.64. No statistically significant differences were observed between groups for those parameters ($p > 0.05$; Table 1).

PI, MB, and BoP were higher in the AAS group, but the difference was statistically significant only for MB ($p = 0.04$). Shallow sites (PD 1–3 mm) were more prevalent in the control group ($p = 0.001$), while the percentage of diseased sites, sites with PD ≥ 4 mm, and sites with detectable attachment loss were significantly higher in the AAS group ($p = 0.0005$, $p = 0.001$, and $p = 0.003$, respectively). The AAS group also presented significantly higher full mouth mean PD ($p = 0.004$; Table 2).

Microbiological findings

Healthy sites

The mean proportions of some host-compatible microorganisms were lower in healthy sites of the AAS group in comparison with the control group, such as *Streptococcus gordonii* and *Streptococcus intermedius* ($p = 0.01$). *Fusobacterium nucleatum vincentii* and *Propionibacterium acnes* were also significantly reduced ($p = 0.001$ and $p = 0.03$, respectively). Disease-compatible microorganisms *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* were increased in AAS group, although the difference was not statistically significant ($p > 0.05$; Fig. 1a). Also, the total mean proportion of host-compatible bacterial species in healthy sites was significantly lower in AAS users when compared to the

Table 1 Demographic and socioeconomic data of patients in the case group (AAS) and control group (non-AAS)

Variable	AAS (n = 15)	Control (n = 15)	p value	Test
Age (years)	30.1 ± 4.4	28.1 ± 4.6	0.3_{NS}	t test
Race/ethnicity (%, n)				
White	92.5 (n = 14)	100 (n = 15)	0.5_{NS}	Fisher
Non-White	7.5 (n = 1)	0 (n = 0)		
Income (USD)	1,071 ± 923.2	1,087.5 ± 333.7	0.2_{NS}	Mann-Whitney
Educational level (%)				
High School	60% (n = 9)	73.3% (n = 11)	0.7_{NS}	Fisher
College/University	40% (n = 6)	26.6% (n = 4)		

NS not statistically significant

control group (25.1% and 43.1%, respectively; $p = 0.01$) and the disease-compatible bacterial species were significantly higher (74.9% and 56.9%, respectively; $p = 0.03$) (Fig. 1b).

Diseased sites

AAS users harbored significantly higher proportions of disease-associated bacteria (67.3%) than non-users (50.1%; $p = 0.049$). This difference was particularly noted for the increased proportions of *T. forsythia* in AAS users, although that finding was not statistically significant (Fig. 1a, b).

Patient's microbial profile

Two individual host-compatible species, *S. gordonii* and *S. intermedius* were significantly higher in the control group than in patients using AAS ($p = 0.01$). Accordingly, the total mean proportions of health compatible bacterial species were significantly higher in non-AAS users (48.3% and 25.8%, respectively; $p = 0.001$). Moreover, the species compatible with disease were found in significantly higher total mean proportion in patients using AAS than in those that did not use: 74.2% and 51.7%, respectively ($p = 0.002$). Disease-associated bacteria *Fusobacterium periodonticum*, *T. forsythia*, and *T. denticola* were possibly influencing those results, although those particular differences were not statistically significant ($p > 0.05$; Fig. 1a, b).

Immunological findings

Total protein concentration was similar between AAS and control groups in healthy and diseased sites. Diseased sites presented a non-significantly higher protein concentration in both AAS (≅ 50%) and control (≅ 38%) groups ($p > 0.05$) in comparison with healthy sites. IL-1β expression in diseased sites was significantly higher than in healthy sites in the control group (≅ 2.4×; $p = 0.03$), but not in the AAS group (Fig. 2).

Association findings

There was a significant correlation between PD and both IL-1β (Fig. 3a) and total protein (Fig. 3b) levels in the control group ($r = 0.62$ and 0.44; $p = 0.0008$ and $p = 0.03$,

Table 2 Mean (SD) clinical parameters of patients in the case group (AAS) and control group (non-AAS)

Variable	AAS (n = 15)	Control (n = 15)	p value	Test
Plaque Index (%)	44.3 ± 25.3	28.7 ± 18.2	0.06_{NS}	t test
Marginal Bleeding (%)	12.1 ± 6.8	7.9 ± 3.6	0.044	t test
Bleeding on Probing (BoP; %)	23.7 ± 8.1	18.9 ± 7.7	0.11_{NS}	t test
Probing Depth (PD; mm)	2.6 ± 0.3	2.3 ± 0.1	0.004	t test
PD ≤ 3 mm (% sites; # individuals)	88.4 ± 10.5 (n = 15)	98 ± 1.8 (n = 15)	0.001	Mann-Whitney
PD ≥ 4 (% sites; # individuals)	11.6 ± 10.5 (n = 14)	2 ± 1.8 (n = 11)	0.001	Mann-Whitney
PD ≥ 5 (% sites; # individuals)	0.6 ± 1.2 (n = 6)	0.2 ± 0.3 (n = 4)	0.06_{NS}	Mann-Whitney
PD ≥ 6 (% sites; # individuals)	0.1 ± 0.2 (n = 2)	0.04 ± 0.2 (n = 1)	0.74_{NS}	Mann-Whitney
PD ≥ 4 mm, with BoP and detectable CAL (% sites; # individuals)	3.2 ± 2.7 (n = 14)	0.7 ± 0.8 (n = 11)	0.0005	Mann-Whitney
Clinical Attachment Level (CAL; mm)	0.1 ± 0.08	0.06 ± 0.06	0.17_{NS}	Mann-Whitney
Any attachment loss (CAL ≥ 1 mm; % sites)	5.2 ± 2.9	2.7 ± 1.9	0.003	t test

NS not statistically significant, # number

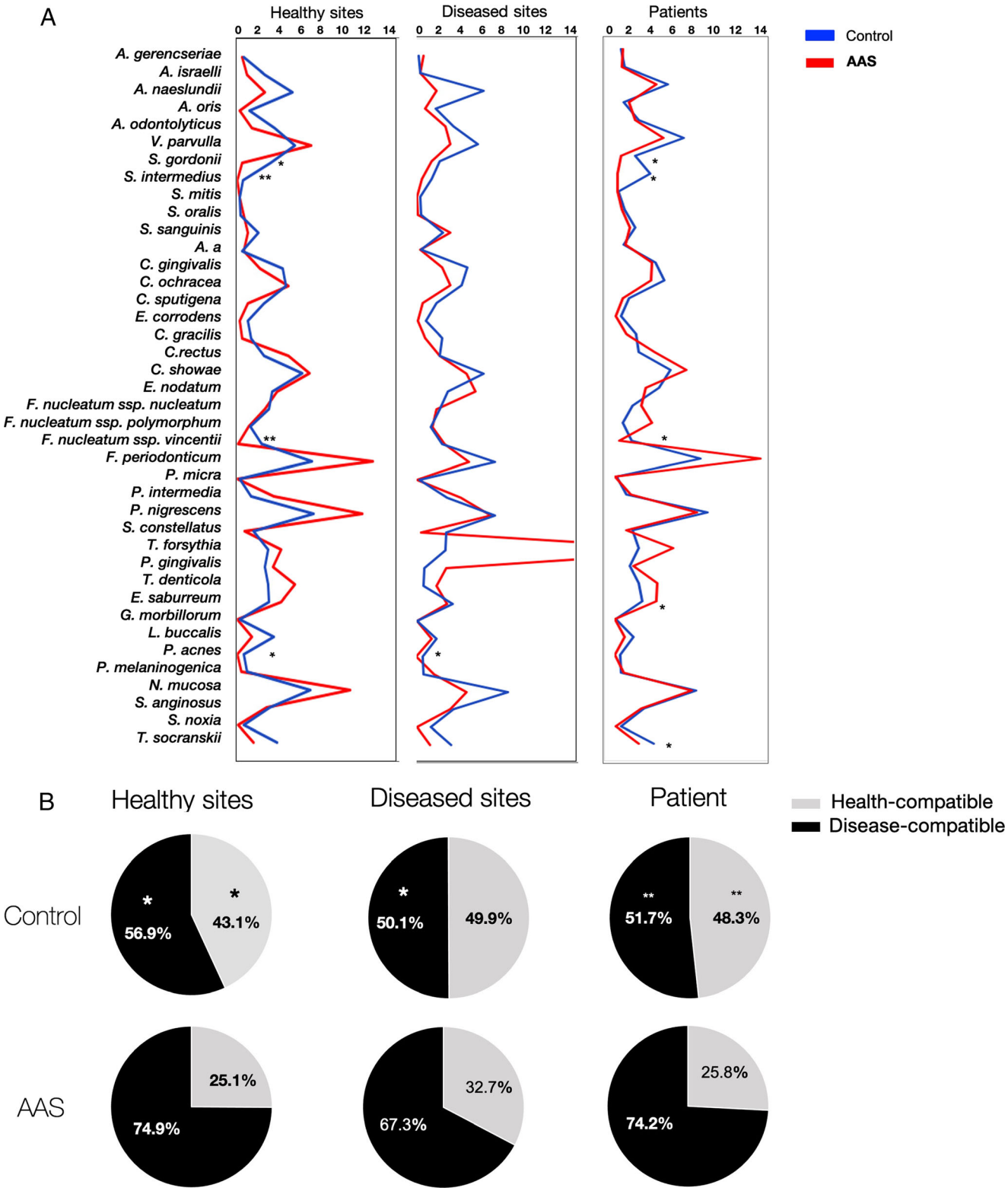

Fig. 1 Mean proportions (%) of **a** individual species or **b** health and disease-associated microorganisms in healthy, diseased, or combined sites of individuals from the control and AAS groups. **b** The blue (*Actinomyces*), purple, yellow, and green complexes were considered to be associated with health (in gray color), while orange and red complexes were considered to be associated with disease (in black color) [15], and the sum of those complexes comprised 100%. Asterisk indicates statistically significant difference between control and AAS ($p < 0.05$). Double asterisk statistically significant difference between control and AAS ($p < 0.01$)

respectively), but not in the AAS group (r = 0.04 and 0.31, respectively; $p > 0.05$). The number of AAS cycles was not significantly correlated with IL-1β (r = 0.28; $p > 0.05$; Fig. 4a), but there was a statistically significant correlation with the percentage of disease-compatible microorganisms (r = 0.62; p = 0.02; Fig. 4b).

Discussion

Following our observation of increased prevalence and severity of periodontitis in men with higher endogenous levels of testosterone [19], here, we investigated the periodontal status of AAS users, focusing on the microbial profile, expression of the inflammatory marker IL-1β, and total protein. Our results demonstrated that PD and MB may be increased in individuals under AAS treatment. Also, AAS users had a microbial profile and expression of IL-1β in healthy sites that were comparable to those observed in periodontitis sites.

Some authors have reported higher percentages of sites with CAL ≥ 3 mm and also gingival enlargement in AAS users [13, 18]. In the present study, AAS users demonstrated higher number of sites with detectable attachment loss and also higher percentages of sites with PD ≥ 4 mm ($p < 0.05$). We did not evaluate gingival enlargement. Clinical

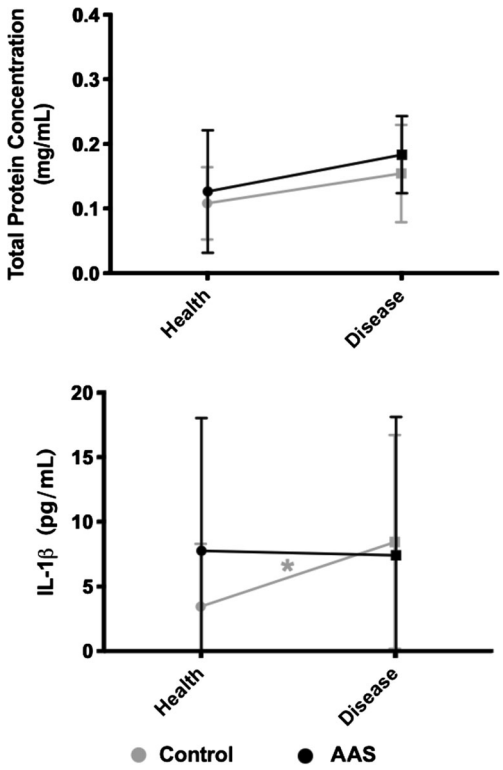

Fig. 2 Concentration of total protein and IL-1β in healthy and diseased sites in the control and AAS groups. Asterisk indicates statistically significant difference between health and disease in the control group (Student's t test; $p < 0.05$)

periodontal parameters suggest only a mild periodontitis, when present, in both groups. This was expected due to the sociodemographic characteristics of the included sample as observed in Table 1—i.e., relatively good educational level, socioeconomic status, and low mean age. That also helps explain some of the limited differences observed between healthy and diseased sites in immunological and microbiological analyses.

No statistically significant difference was observed between the two groups for PI, although there was a clear tendency for higher PI values in the case group. Our results are in-between previous studies that showed either no statistically significant difference [18] or significantly higher PI in AAS users [13]. Marginal bleeding was significantly increased in AAS subjects, while BoP was non-significantly higher in AAS than in control group. Although androgen effects on blood vessels are poorly documented to help explain that finding [27], a previous preclinical study from our group demonstrated that testosterone significantly increased the relative presence of blood vessels in the gingival tissue in orchiectomized male rats when compared to sham-controls [28].

No statistically significant differences were observed between groups for total protein levels in the gingival crevicular fluid analysis. However, the AAS group presented higher levels of IL-1β in diseased and in healthy sites while the control subjects had statistically lower levels of this cytokine in healthy sites than in diseased sites. This finding suggests that AAS users express higher amounts of IL-1β even in periodontally healthy conditions, possibly predisposing those individuals to periodontal disease. Previous studies have suggested that both total protein and IL-1β levels were higher in patients who had periodontal disease when compared to their controls [29, 30]. Periodontitis is characterized by an overproduction of innate immune cytokines, such as IL-1β, IL-6, and TNF [31]. In addition, IL-1β is associated with the progression of periodontal disease and alveolar bone resorption [32]. Here, IL-1β was assessed in a time-standardized manner according to previous reports cited in the literature [26, 33–35].

Our exploratory statistical analyses showed no significant correlation between PD and IL-1β or total protein levels in AAS users, reinforcing our hypothesis that healthy sites of AAS are more susceptible to immunological changes. Additionally, there was no significant correlation between the number of AAS cycles and IL-1β or total protein levels, suggesting that periodontal immunological changes may begin after the first use.

The microbiological analysis showed that AAS users had a more dysbiotic microbial profile than non-users. In general, individuals from AAS group harbored lower proportions of host-compatible bacterial species and higher proportions of periodontal pathogens than the control group. The proportions of several host-compatible taxa including *S. gordonii*, *S. intermedius*, and *Actinomyces* species were reduced in AAS

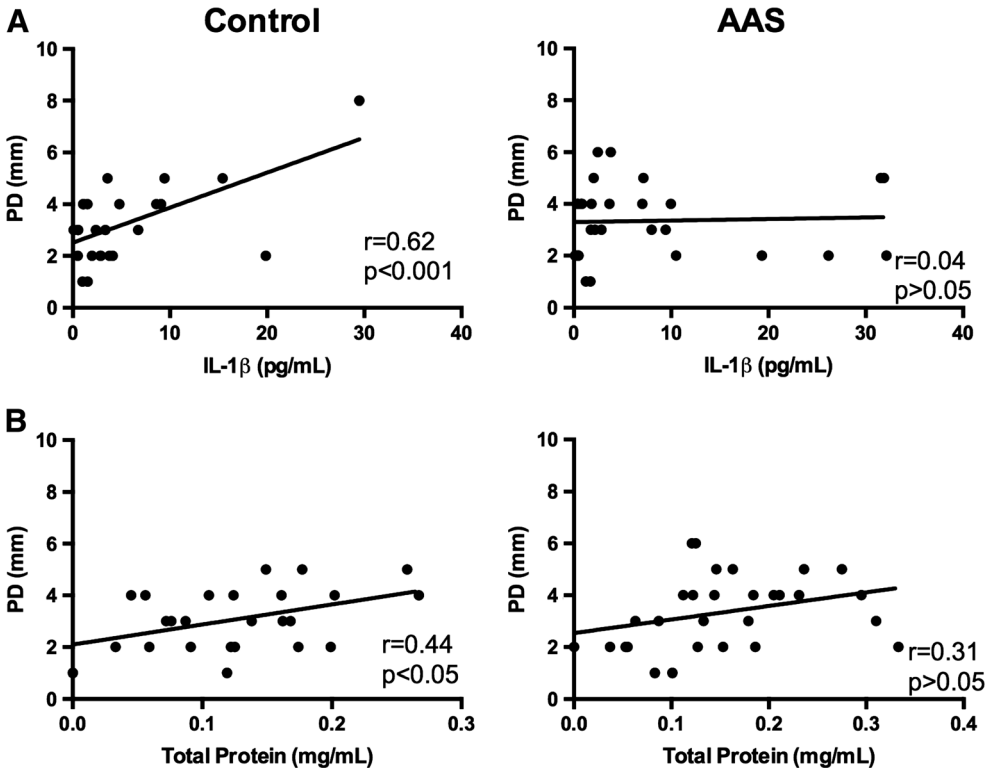

Fig. 3 Correlation between PD (mm) and **a** IL-1β or **b** total protein levels in the control and AAS groups

users in comparison with non-users. Conversely, several periodontal pathogens were elevated in AAS users in comparison with non-users, although most of these differences were not statistically significant. Diseased sites of AAS users harbored significantly higher proportions of disease-associated microorganisms than AAS non-users. Interestingly, the percentage of the disease-associated complexes was significantly correlated with the number of AAS cycles, suggesting that AAS exposure may produce a favorable environment for the growth of those microorganisms. A single previous study using culture identified that men under AAS use were more likely to be infected with *P. intermedia*, *A. actinomycetemcomitans* and *Candida spp.*, *Candida parapsilosis*, and *Candida tropicalis*, than non-users [13]. The present study observed that both *P. intermedia* and *A. actinomycetemcomitans*, although present in our population, did not differ significantly between the two groups.

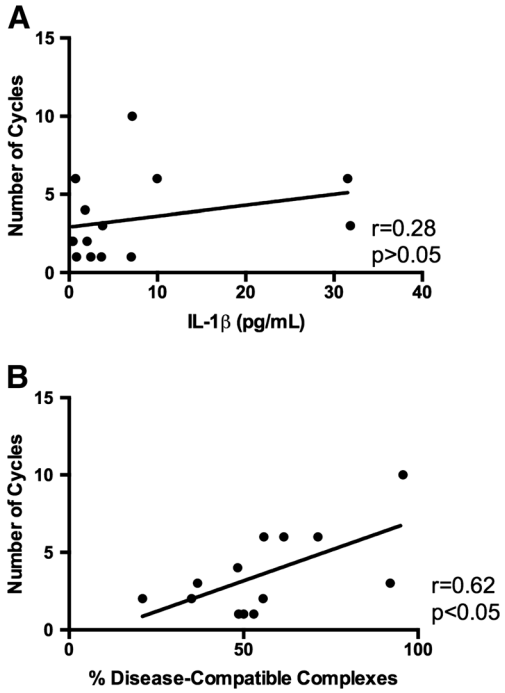

Fig. 4 Correlation between the number of AAS cycles and **a** expression of IL-1β or **b** microorganisms compatible with disease (orange and red complexes) in AAS users

Another interesting information was that even the clinically healthy sites of AAS consumers had a more pathogenic microbial profile than healthy sites of non-AAS individuals. They harbored significantly lower proportions of beneficial species than non-users and significantly higher proportion of disease-compatible microorganisms, suggesting a dysbiotic biofilm [17]. This could indicate an initial shift in microbiological profile in AAS users towards disease.

The individuals included in this study used 9 different types of drugs. They all used AAS, and only one of them also associated with Anastrozole, a class of drugs called aromatase inhibitors that is not considered an AAS but indirectly affects testosterone. Anastrozole minimizes the conversion of testosterone to estradiol allowing higher levels of testosterone in the body. None of the participants reported any adverse effects form the use of these drugs, and 50% of the subjects in the

AAS group reported that sometimes they noticed gingival bleeding.

The main strength of this study is to be the first one to conduct a comprehensive evaluation on the effects of AAS in the periodontal tissues, including clinical, microbiological, and immunological aspects. However, this study presented some limitations, such as the small sample size of 15 volunteers per group. Selecting volunteers for this type of study is a challenge as many men do not assume taking AAS because abuse of anabolic substances is a serious public health problem (and commercializing or selling without proper prescription is a crime in many countries, including Brazil). However, the power calculation a posteriori indicated power of 96% for the primary outcome mean full mouth PD. In addition, blinding was not possible in this study due to evident differences in body structures between the two groups. It is also important to notice that sampling sequence may, in theory, affect the results of either microbial amount and composition or the quality of GCF. Here, we used a previously described protocol [25, 36] that standardizes time after biofilm collection (60–90 s) and removes blood traces by washing. Additionally, visual inspection was performed to ensure the absence of blood contamination in the samples. All included groups were analyzed (and compared) using this same sampling sequence. Collection of samples from the same site was deemed to be important for a better comparison of microbiological and immunological aspects, since those findings may differ in different sites within the mouth.

Other confounding factors, such as diet and nutrition, time and length of physical activity, and testosterone levels—which have not been assessed due to financial limitations—may have influenced the results. Other controlled prospective studies with larger sample sizes should be conducted to evaluate if there is a real cause/effect relationship between the use of AAS and periodontitis.

In conclusion, the results of this study indicate that AAS intake can negatively impact the periodontal health, suggesting that dental care professionals should perform full mouth periodontal screening and schedule regular follow-up appointments for patients under AAS use.

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Author contribution All authors have made substantial contributions to conception and design of the study. SVSCW, HMV, TBCC, and JDPC worked on the acquisition and analysis of data. LCS, DMPS, MF, GSS, and JPS supervised all steps and worked on the analysis and interpretation of data. SVSCW, HMV, and JPS drafted the work and the other authors critically revised for intellectual content. All authors approved the final version and agree to be accountable for all aspects of the work.

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Compliance with ethical standards

Conflict of Interest SVSCW declares that she has no conflict of interest. HMV declares that he has no conflict of interest. TBCC declares that she has no conflict of interest. JDPC declares that he has no conflict of interest. LCS declares that he has no conflict of interest. DMPS declares that she has no conflict of interest. MF declares that she has no conflict of interest. GMSS declares that she has no conflict of interest. JPS declares that he has no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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