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The features of the subgingival periodontal microbiome in chronic periodontitis patients with and without type 2 diabetes mellitus

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Abstract

Background: Since periodontitis and type 2 diabetes mellitus are complex diseases, full understanding of their pathogenesis requires knowing how a relationship of these pathologies with other disorders and environmental factors develops.

Methods: In this study, the subgingival periodontal microbiome structure of 46 subjects was investigated by 16S rRNA gene sequencing and shotgun sequencing of pooled samples. We examined 15 patients with chronic periodontitis (CP), 15 patients with chronic periodontitis associated with type 2 diabetes mellitus (CPT2DM), and 16 healthy subjects (Control).

Results: Generalized chronic periodontitis in both periodontitis groups (CP, CPT2DM) of patients was moderate (stage II). Each group had approximately equal numbers of males (22 in total) and females (24 in total) with the average age of 53.9 $\pm$ 7.3 and 54.3 $\pm$ 7.2 years, respectively. The number of overweight patients (BMI 30-34.9 kg/m2) and patients with class I-II obesity (BMI 35–45.9 kg/m2) was significantly greater in the CPT2DM group than that in the group of patients having only chronic periodontitis or in the Control group. However, no statistical significant difference in clinical indices was identified between the CP and CPT2DM groups. An analysis of the metagenomic data revealed that alpha diversity in the CPT2DM group was increased compared to that in the CP and Control groups. The microbiome biomarkers associated with experimental groups were evaluated. In both groups of patients with periodontitis, relative abundance of *Porphyromonadaceae* was increased compared to that in the Control group. The CPT2DM group was characterized by decreased relative abundance of *Streptococcaceae*/*Pasteurellaceae* and increased abundance of *Leptotrichiaceae* compared to those in the CP and Control groups. Furthermore, the CP and CPT2DM groups differed in terms of relative abundance of *Veillonellaceae* (decreased in the CPT2DM group compared to CP) and Neisseriaceae (increased in the CPT2DM group compared to CP). In addition, differences in bacterial content were identified by shotgun sequencing of pooled samples and genome-resolved metagenomics combination.

Conclusions: The results prove that there are subgingival microbiome specific features in patients with chronic periodontitis associated with type 2 diabetes mellitus.

Keywords: oral microbiome; periodontitis; type 2 diabetes mellitus; 16S rRNA gene sequencing; metagenomics

Background

The development of high-throughput sequencing technologies has led to a significant breakthrough in human microbiome research. Scientists have obtained tools to study the taxonomic and functional characteristics of the human microbiome, as well as related pathological processes. However, when using any breakthrough technologies, new findings give rise to new scientific questions that still need to be answered [1].

The human oral cavity is one of the most interesting microbial habitats [2]. More than 700 microbial species living in the oral cavity and forming unique communities have been found thus far. The composition of such communities is quite stable for a healthy oral cavity [3] and plays an important role in maintaining the dynamic ecological equilibrium with the host [4, 5]. The most inhabited part of the oral cavity is the periodontal sulcus, which may contain about 400–500 microbial species [6, 4]. Changes in the structure and biological functions of the periodontal sulcus microbiome lead to an altered state, which can be associated with the onset and progression of periodontal diseases [7], as well as other local or systemic pathological conditions [8, 9]. One of the most common associations of periodontal diseases with systemic pathology is the combination of chronic periodontitis (CP) and type 2 diabetes mellitus (T2DM) [10, 11, 12]. Changes in the composition of the oral microbiota are among the main manifestations of this combination [13, 14]. For example, the possible influence of the oral microbiota on the development of T2DM has been studied using the *Porphyromonas gingivalis*, a part of the complex of main periodontal pathogenic bacteria.

Oral administration of *P. gingivalis* to mice for experimental periodontitis induction has a significant effect on the combined expression level in the host cells of the Irs1 and Sirt1 genes, which suppress insulin sensitivity of adipocytes and other cells [15]. The pathogenetic role of *P. gingivalis* in periodontitis complicated by type 2 diabetes mellitus (CPT2DM) can also be implemented by direct stimulation of adipocytes by lipopolysaccharides (LPS). This may be the reason for the production of adipocytokines and proinflammatory cytokines and provoking of oxidative stress [16]. *P. gingivalis* LPS were also found to induce secretion of angiopoietin-like protein 2 in epithelial cells of the periodontium, which affects angiogenesis and has pro-inflammatory properties [17]. These changes can contribute to the development of systemic inflammation and are associated with lipid peroxidation, which is a component of the pathogenesis of diabetes mellitus [18]. Thus, the oral cavity microbiome (including the periodontal pathogenic bacteria) can be involved in the pathogenesis of diabetes mellitus, which makes it an expedient topic to study.

Thus, the present study was aimed at conducting a comparative analysis to describe more features of the subgingival microbiome in patients with CPT2DM. In our study, we used 16S rRNA gene sequencing and compositional data analysis (CoDa) to characterize the subgingival periodontal microbiome. Additionally, plague samples were pooled and characterized using shotgun sequencing and the genome-resolved metagenomics approaches.

Material and Methods

Subject population and study design

The study involved 46 subjects, which were divided into three groups (Table 1). The index group consisted of 15 patients with chronic periodontitis associated with

type 2 diabetes mellitus (CPT2DM group); the reference group consisted of 15 patients with chronic periodontitis without somatic comorbidities (CP group); and the control group consisted of 16 healthy subjects having signs of neither chronic periodontitis nor type 2 diabetes mellitus (Control group). All the subjects were followed up at the clinics of the Department of Propaedeutic Dentistry of the A.I. Evdokimov Moscow State University of Medicine and Dentistry in 2015–2018. Patients with T2DM were treated at the Department of Endocrinology and Diabetology of the same university. The patients were not receiving treatment for chronic periodontitis over the past 6 months. Figure 1 shows the schematic visualization of the experimental design.

Diagnostic and inclusion criteria

Healthy subjects were added to the study only after they had consulted a dentist and an endocrinologist. Patients were diagnosed with chronic periodontitis according to the clinical and radiological data in compliance with 2018 classification of periodontal diseases [19].

The study groups contained approximately equal numbers of non-smoking males and females aged 41–65 years. Patients with periodontitis had stage II (moderate) periodontitis with a generalized lesion, probing pocket depths of 3–4 mm, loss of bone tissue around the teeth no more than 1/3 of the root length, and virtually no tooth loss associated with periodontitis. Patients with type 2 diabetes mellitus were in remission.

The analysis of the dental status involved determining the following indices adopted in dental studies: PHP (Patient Hygiene Performance); OHI-S (Simplified Oral Hygiene Index); CAL (Clinical Attachment Level); MMI (Miller's Tooth Mobility Index); PBI (Papillary Bleeding Index); and TL (Tooth Loss). Patients were diagnosed with type 2 diabetes mellitus in accordance with the World Health Organization (WHO) diagnostic criteria 1999/2006/2011 [20] with allowance for the clinical, anamnestic, and laboratory evaluations. In all T2DM patients, disease duration ranged from 3 to 7 years; the disease course was moderately compensated; blood glucose level was below 7.8 mmol/L and glycated hemoglobin level was below 8%.

Collection and sequencing of plaque samples

The contents of the periodontal pocket in patients with chronic periodontitis (CP), chronic periodontitis with type 2 diabetes mellitus (CPT2DM) and the contents of the gingival sulcus in healthy (Control) subjects were the study material. The samples were taken in the morning on an empty stomach (between 9 am and 11 am) before the patients used a toothbrush and other hygiene products. The biological material was sampled from four spots of the periodontal pockets / sulcus at the level of the second molars [21] using sterile paper endodontic posts (# 25) [22], which were placed together in a test tube containing 0.2 mL of sterile physiological saline solution and shaken.

The samples were delivered to the laboratory and subsequently stored at -20°C. Total DNA was extracted from the collected samples using a QIAamp DNA Investigator Kit (Qiagen, Germany) in accordance with the manufacturer's protocol.

Genomic DNA content was determined on a Qubit 2.0 fluorometer (Invitrogen, USA) in accordance with the manufacturer's instructions. The enriched microbial DNA (50–100 ng) was fragmented using a Covaris S220 system (Covaris, Woburn, Massachusetts, USA). The final fragment size was determined using an Agilent 2100 bioanalyzer (Agilent, USA) in accordance with the manufacturer's instructions. Briefly, extracted DNA was amplified using standard 16S rRNA gene primers complementary to the V3–V4 region and containing 5'-illumina adapter sequences. Sequencing was carried out on a HiSeq 2500 platform (Illumina, USA) in accordance with the manufacturer's instructions.

DNA samples for shotgun sequencing were pooled and prepared by ligating the genomic DNA of the samples inside each study group taken at equimolar amounts. The amount of the mixed DNA pool was determined on a Qubit 2.0 fluorometer (Invitrogen, USA) in accordance with the manufacturer's instructions. A NEBnext Microbiome DNA enrichment kit was used for enriching the microbial genomic DNA in the mixed pools in microbial genomic DNA in accordance with the manufacturer's instructions. The libraries of paired terminal fragments were prepared in accordance with the manufacturer's guidelines using a Nebnext Ultra II DNA Library Prep Kit (New England Biolabs, USA). The libraries were indexed using NEBNext multiplex Oligos for Illumina kits (96 Index Primers) (New England Biolabs, USA). The distribution over library size and quality was assessed using a high-sensitivity DNA microarray (Agilent Technologies). Subsequent quantification of the libraries was performed using a high-sensitivity Quant-iT DNA Assay Kit (Thermo Scientific). Sequencing was conducted on a HiSeq 2500 platform (Illumina, USA) in accordance with the manufacturer's instructions using the following reagent kits: HiSeq Rapid PE Cluster Kit v2, HiSeq Rapid SBS Kit v2 (500 cycles), HiSeq Rapid PE FlowCell v2, and a 2% Phix spike in controls.

Bioinformatic and statistical analysis

The 16S rRNA gene sequencing data were processed using DADA2 pipeline [23] according to the published protocol [24]. As a result, the phyloseq [25] object was obtained, which contains an amplicon sequence variant (ASV) table, a taxonomy table, and a phylogenetic tree. The ASVs were pooled at the family level. In addition, the top eight families in relative abundance were selected for statistical analysis. The CoDa (Compositional Data Analysis) approaches, such as Aitchison distance [26, 27] and CoDa dendrogram, were used for data visualization and exploration analysis. CoDa dendrogram is a dendrogram-like graph that shows: (a) the way of grouping parts of the compositional vector; (b) the explanatory role of each sub-composition generated in the partition process; and (c) the decomposition of the total variance into balance components associated with each binary partition [28, 29]. Before constructing the CoDa dendrogram, Bayesian estimation of (non-zero) proportions was performed to remove rare taxa and substitute zeros [30]. The Songbird approach [31] implemented in QIIME2 framework [32, 33, 34, 35, 36] was used to discover biomarkers significantly discriminating the experimental groups. Wilcoxon signed rank test was used for additional statistical comparison. The GNU/R statistical environment was used for data analysis [37].

The metaWRAP pipeline was used to construct the metagenome-assembled genomes (MAGs) [38] (containing MEGAHIT [39], MetaBAT2 [40], MaxBin2 [41],

BWA [42]), with the following parameters of the resulting bins: completeness > 35%, contamination < 15%. Multiple alignment and phylogenetic tree plotting for 43 marker amino acid sequences of MAGs and Human Oral Microbiome Database (HOMD) genomes [43] was performed by CheckM means [44]. The CAT/BAT tool was used for additional taxonomic annotation of MAGs [45]. Next, the closest HOMD genomes to MAGs were found to follow the coverage of the obtained MAGs in all the pooled metagenomic samples.

Statistical analysis of the clinical data was performed using the SPSS software package version 21.

Results

Description of the demographic and clinical parameters of the experimental cohort

The demographic characteristics of the analyzed groups (Table 1) showed no significant intergroup differences in sex and age. However, based on the body mass index, the percentage of overweight patients (BMI 30-34.9 kg/m^2) and patients with class I-II obesity (BMI 35-45.9 kg/m^2) was significantly higher in the CPT2DM group than in the CP group or the Control group.

Oral health level, assessed using various dental indices, and glycosylated hemoglobin levels are summarized in Table 2. The PHP indices being indicative of the oral hygiene status showed no differences between the CP and CPT2DM groups but were significantly higher in patients with chronic periodontitis compared to those in the control group.

The CAL indices, which characterize the state of periodontal pockets, were significantly higher in both chronic periodontitis groups (CP and CPT2DM) in the absence of changes in other indicators of dental status regardless of the association with type 2 diabetes mellitus. The level of glycosylated hemoglobin (HbA1c) was expected to be higher in the CPT2DM group, while the blood glucose level was approximately the same in different groups.

Overall, no significant differences between the two experimental groups were observed for most studied dental indices. The revealed differences between the groups of chronic periodontitis were related only to pathogenetically significant signs due to the presence of T2DM. This allows one to identify significant characteristics of the subgingival microbiota associated with periodontitis and type 2 diabetes.

Characteristic of the subgingival periodontal microbiota based on 16S rRNA gene sequencing of the collected samples

The identification of microbiome biodiversity in each clinical group involved two phases. During the first phase, 16S rRNA gene fragments were sequenced to evaluate the abundance of various bacterial families in plaque samples. Data analysis was performed using DADA2 [23] and phyloseq [25] packages for GNU/R. After quality filtering, the 16S rRNA gene sequencing data contained on average 50,060 *pm* 15,902 paired reads per sample. The summary sequencing statistics are presented in Supplementary Table S1. Statistical intergroup differences in community richness (alpha diversity) were identified using the Chao1, Shannon and Simpson indices. These indices take count of the identified species and their abundance in the microbial community (see Figure 2).

An increase in alpha diversity was revealed in the CPT2DM group compared to the Control group using the Shannon index (Wilcoxon rank sum test with FDR correction for multiple testing $p < 0.05$), while the Simpson index showed an increase in statistical significance in the CPT2DM group compared to both the Control and CD groups (Wilcoxon rank sum test with FDR correction for multiple testing $p < 0.05$). However, no significant intergroup differences in the Chao1 index were revealed.

An analysis of the 16S rRNA gene sequencing data revealed 26 bacterial families (Supplementary Table S2) in the samples from all clinical groups. The observed family distribution is shown in Figure 3A. The eight most abundant bacterial families were selected for further data analysis. The NMDS bidimensional visualization is presented in Figure 3B. The taxonomic data revealed no clustering by groups. The balance dendrogram (CoDa dendrogram) was used to construct the model of taxonomic differences between the experimental groups. This approach allows one to identify specific balances (the ratio between taxonomic abundances) involved in distinction between the metagenome groups [28, 29]. This model describes the intensity of taxonomic reshapes when moving the metagenomes profiles from "healthy state" to periodontal "disease state" (see Figure 3C).

According to the CoDa dendrogram main balance, the CPT2DM group was associated with increased relative abundance of four bacterial families such as *Leptotrichiaceae*, *Prevotellaceae*, *Fusobacteriaceae*, and *Porphyromonadaceae*, while the Control group was characterized by increasing relative abundance of *Streptococcaceae*, *Veillonellaceae*, *Neisseriaceae*, and *Pasteurellaceae*. The CP group occupies a boundary position between the CPT2DM and Control groups.

The Songbird approach was used for statistical validation of the balance dendrogram (see Figure 4). The primary output from Songbird is a file containing differentials. These describe the log-fold change in features with respect to certain field(s) in sample metadata. The most important aspect of these differentials is rankings, which are obtained by sorting a column of differentials from the lowest to the highest ones. These rankings give information on the relative associations of features with a given covariate [31]. In both groups of periodontitis, the relative abundance of *Porphyromonadaceae* was increased compared to that in the Control group. The CPT2DM group was characterized by a decrease in relative abundance of *Streptococcaceae/Pasteurellaceae* and an increase in *Leptotrichiaceae* compared to the CP and Control groups. Furthermore, the CP and CPT2DM groups differed in terms of relative abundance of *Veillonellaceae* (decreased in the CPT2DM group compared to CP) and *Neisseriaceae* (increased in the CPT2DM group compared to CP).

A genome-resolved metagenomic analysis of shotgun sequencing data of the pooled samples

At the next stage, the DNA samples within each group were pooled and sequenced using the shotgun technology. The bacterial genomes were restored from the metagenomic data using genome-resolved metagenomic approaches based on the metagenomic assembly and clustering of contigs through the metagenomic binning procedure (see Materials and Methods). As a result, 26 MAGs were assembled for all

the metagenomic samples with selected quality parameters (completeness > 35%, contamination < 15%). Six MAGs were obtained for the Control group; 9 and 11 MAGs, for the CP and CPT2DM groups, respectively. The binning statistics are shown in Supplementary Table S3.

The analysis of MAGs involved two steps. First, taxonomic annotation of MAG sequences was obtained using the CAT/BAT tool (see Supplementary Table S3). To verify the taxonomic annotation, a unified multiple alignment and a phylogenetic tree (also using the CheckM tool) were constructed using MAGs sequences and expanded Human Oral Microbiome Database (eHOMD) bacterial genomes [43]. On the tree, the MAGs nearest neighbors were determined and used for constructing the combined phylogenetic tree (Figure 5).

According to this analysis, *Haemophilus* spp., *Veilonella* spp., and *Neisseria* spp. bacteria are common in all the groups. The sample of the Control group is characterized by the presence of unique MAGs close to such bacteria as *Streptococcus sanguinis*, *Fusobacterium nucleatum*, and *Prevotella pleuritis*. The bacteria close to *Lautopia mirabilis*, *Prevotella loescheii*, and *Prevotella nigrescens* were present in the CP MAG group. Bacteria close to *Alloprevotella* sp. HMT 473, *Corynebacterium matruchotii*, *Prevotella intermedia*, *Porphyromonas pasteri*, and *Saccharibacteria* (TM7) [G-1] HMT-952 were present in the CPT2DM MAG group only. Meanwhile, MAGs close to *Porphyromonas gingivalis* and *Bacteroidales* [G-2] HMT-274, as well as *Treponema* spp. (while maintaining species differences), are common for the CP and CPT2DM groups. Thus, the taxonomic representation in healthy subjects differs from that in both groups of patients with periodontitis. *Bacteroidetes* and *Spirochaetes* spp. MAGs were found in both groups of periodontitis patients, whereas unique MAGs can also be distinguished between these groups.

Discussion

Chronic periodontitis (CP) and type 2 diabetes mellitus (T2DM) are widespread multifactorial diseases. These pathologies are interconnected [46]. T2DM is a major factor in the development of periodontitis, while periodontitis severity can affect glycemic control and complications in patients with diabetes (impaired tissue repair ability being among the reasons for that) [46, 47]. Therefore, treatment of periodontitis is considered an important component of diabetes therapy [48, 49, 50]. The consortia of oral bacteria form fairly stable communities [51]. Even in healthy people, the microbial composition of different parts of the oral cavity has its individual characteristics [3].

However, it is still not exactly clear how the diabetic state affects the composition of the community of oral microorganisms and how exactly they modify it. The Griffen et al. [52] described 25 taxa including six bacterial genera (among them *Neisseria*, *Streptococcus*, *Haemophilus*, *Pseudomonas*) whose relative abundance in the oral microbiota was different for patients with T2DM compared to that in the control groups. According to Matsha et al. [13] and Kistler et al. [53], changes in oral microbiota in patients with T2DM and periodontitis depended both on the glycemic status and the stage of periodontal disease. However, the question which of these factors had the main impact still remains unsolved. In addition, Wolcott et al. [54] have arrived at conclusion that the observed changes in the subgingival microbiome

associated with T2DM and periodontitis are potentially caused by metabolic and immune dysregulation of the host.

In this paper, we identified the differences in the structure of the subgingival microbiota between groups of healthy subjects (Control group), patients with chronic periodontitis (CP group), and patients with chronic periodontitis associated with type 2 diabetes (CPT2DM group), which can be considered potential microbial biomarkers of these conditions. The demographic characteristics of the analyzed groups (except for BMI) show no significant intergroup differences. The percentage of overweight patients (BMI 30-34.9 kg/m^2) and patients with class I-II obesity (BMI 35-45.9 kg/m^2) was significantly higher in the CPT2DM group compared to that in the CP group or the Control group. This is a significant limitation to the study conclusions because the oral microbiome can be changed in obese people regardless of the glycemic status [55]. No statistically significant differences were observed between the main dental indices in the CP and CPT2DM groups. However, the level of glycosylated hemoglobin (HbA1c) was expectedly higher in the CPT2DM group, while the blood glucose level was approximately the same in different groups.

Alpha diversity was increased in the CPT2DM group compared to that in the Control and CP groups. However, the CP and Control groups did not differ in terms of this characteristic. High bacterial richness in the oral microbiome is significantly associated with poor oral health, including the presence of decayed teeth, periodontitis, and poor oral hygiene [56]. Earlier, the richness of the subgingival community was not found to increase in patients with periodontitis [52, 57]. The presence of bleeding was not associated with different alpha diversities in patients with periodontitis. However, bleeding sites showed higher total bacterial load [57].

In addition, the beta diversity differences between the experimental groups were discovered. First, an ecological model based on the principles of compositional data (CoDa) analysis describing the shifts from the healthy state to the CPT2DM state was constructed. The obtained data allowed us to distinguish two different "microbiota states" associated with the Control and CPT2DM groups. The "healthy state" included bacterial families such as *Streptococcaceae*, *Veillonellaceae*, *Neisseriaceae*, and *Pasteurellaceae*, while *Leptotrichiaceae*, *Prevotellaceae*, *Fusobacteriaceae*, and *Porphyromonadaceae* formed the "disease state". Interestingly, the CP group occupies a boundary position between the CPT2DM and Control groups. The "disease state" is formed by bacterial families including a wide range of bacteria overrepresented in periodontitis compared to healthy controls [58, 59, 60, 61, 62, 63], while the "healthy state" is characterized by formation of the oral microbiota commonly present in healthy subjects [58, 59, 63, 60, 61].

Second, statistically significant biomarkers distinguishing experimental groups have been identified. Both periodontitis groups were associated with an increase in relative abundance of *Porphyromonadaceae* compared to healthy controls. However, the CDT2DM group was characterized by a decrease in relative abundance of *Streptococcaceae/Pasteurellaceae* and an increase in relative abundance of *Leptotrichiaceae* compared to the CP and Control groups. Furthermore, the CP and CPT2DM groups differed in terms of relative abundance of *Veillonellaceae* (decreased in the CPT2DM group compared to CP) and *Neisseriaceae* (increased in

the CPT2DM group compared to CP). The obtained data are partially consistent with the above ecological model. It was previously shown that the content of pathogenic species was higher in T2DM, both complicated and uncomplicated by periodontitis, compared with the non-diabetic controls [64].

Additionally, the genome-resolved metagenomic methods were used to analyze pooled metagenomic samples. This set of computational techniques allows one to reconstruct bacterial genomes from the metagenomic data (metagenome-assembled genomes, MAGs). According to the results, *Haemophilus* spp., *Veilonella* spp., and *Neisseria* spp. MAGs were common in all groups. The main part of *Bacteroidetes* and all the identified *Spirochaetes* MAGs were found in both groups of periodontitis patients, whereas unique MAGs can also be distinguished between these groups. These findings are consistent with the results of previous studies obtained using 16S rRNA gene sequencing of subgingival bacterial communities [52]. The bacteria close to *P. gingivalis* and *Bacteroidales* [G-2] bacteria HMT-274, as well as *Treponema medium* (while maintaining species differences), were common for both periodontitis groups. *P. gingivalis* are strongly associated with periodontal disease. We would also like to mention the differences in the revealed *Treponema* species between the CP and CPT2DM groups, which may be due to the nature of coaggregation, clinical manifestations of periodontitis, or specific characteristics of the environment.

The differences in the content of *Prevotella* spp. are particularly interesting. Bacteria close to *Prevotella intermedia* were detected only in the pooled metagenomic sample of the CPT2DM group, while *Prevotella nigrescens* and *Prevotella loescheii* were detected only in the CP group. The association of these bacteria with disease severity was revealed. *P. intermedia* is associated with more severe forms of periodontitis, while *P. nigrescens* is associated with mild to moderate disease [65]. Another distinctive feature of the CPT2DM group was the presence of unique MAGs, including *Actinobacteria* (*Corynebacterium matruchotii*) and Candidatus *Saccharibacteria* (TM7) [G-1] HMT-952. *Corynebacterium matruchotii* was implicated in nucleation of oral microbial consortia leading to biofilm formation [66]. The role of TM7x bacteria in the oral microbiome has not been elucidated yet. It is worth noting that the pooled metagenomes do not have sufficient representativeness to talk about any significant differences in the experimental groups. However, the findings are consistent and supplement the conclusions drawn using the results obtained by 16S rRNA gene sequencing analysis.

Conclusions

The taxonomic composition of the subgingival microbiome clearly differentiates between the healthy and the disease states, as well as the possible transition to chronic inflammation associated with T2DM. Interestingly, this process is accompanied by increased biodiversity. The identified biomarkers of clinical conditions may be useful in the future for developing test systems for clinical applications.

Availability of data and materials

The analytical scripts and the obtained data are available at https://github.com/RCPCM-GCB/CPT2DM_project. Raw metagenomic data are also deposited at the NCBI Sequence Read Archives under the BioProjects accession number PRJNA664107.

List of abbreviations
CP: clinical periodontitis. **T2DM**: type 2 diabetes mellitus. **CPT2DM**: clinical periodontitis with type 2 diabetes mellitus. **IDF**: International Diabetes Federation. **CoDa**: compositional data. **GRM**: genome-resolved metagenomics. **MAG**: metagenome-assembled genome. **NMDS**: non-metric multidimensional scaling.**PHP**: patient hygiene performance. **OHI-S**: simplified oral hygiene index. **CAL**: clinical attachment level. **MMI**: Miller's tooth mobility index. **TL**: tooth loss. **PBI**: papillary bleeding index.

Author's contributions
BI - performed statistical analysis of clinical data, contributed to the research ideas and the interpretation of the obtained results, wrote the manuscript. **OE** - analyzed 16S rRNA gene sequencing and shotgun sequencing data, partially wrote the manuscript, and performed interpretation of the results. **VM**, **KK** - performed 16 rRNA gene and shotgun sequencing of collected samples. **KA** - contributed to manuscript preparation and interpretation of the results. **BE** - performed clinical observations and sample collection. **OL**, **LY** - contributed to manuscript preparation and interpretation of the results. **SI** - contributed to clinical data analysis and manuscript preparation. **TV** - contributed to study design and interpretation of the results. **MA** - partially performed the study design. **GA** - contributed to data analysis, study design, developing the general line of result interpretation, manuscript preparation, and project management. **IE** - contributed to data analysis and study design, was involved in result interpretation and manuscript preparation. **AS** - contributed to the research ideas, the general study design, was head of the project.

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Ethics declarations
Ethics approval and consent to participate
The study was approved by the Inter-university Ethical Committee of Moscow (Protocol No. 07/16, Jul 14, 2016). The participation was voluntary for each person involved in this study. Before the start of the study, each patient signed an informed consent.

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no competing interests.

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Figure 1 Schematic visualization of the experimental design.

Figure 2 Alpha diversity distribution between the study groups. (A) The Chao1 index. **(B)** The Shannon index. **(C)** The Simpson index. The data were analyzed by Wilcoxon rank sum test with FDR correction for multiple testing. The median value, the interquartile range and standard deviation are indicated.

Figure 3 The major bacterial genera present in the subgingival periodontal microbiota of control and CP / CPT2DM patient groups. (A) Columns correspond to the samples; the group is denoted with a top color bar. Hierarchical clustering was performed using the Euclidean distance and complete linkage. Logarithmic transformation of read counts was performed using pseudo counts. The top eight families according to their relative abundance were selected for further analysis. **(B)** Non-metric multidimensional scaling biplot of taxonomic profiles (family level) of patients' plaque samples using 16S rRNA gene sequencing and Aitchison distance. Bacterial families referring to the samples collected from healthy subjects, CP and CPT2DM patients are shown in red, blue and green, respectively. **(C)** The CoDa dendrogram shows an ecological model of differences between the experimental groups. Decomposition of total variance by balances between groups of genera is shown using vertical bars (red bars denote the Control group; blue, the CP group; and green, the CPT2DM group). The mean balances are shown using anchoring points of vertical bars. The red bars denote the "healthy" state balance, while the blue area denotes the "disease" state balance.

Figure 4 The Songbird analysis results. X-axis denotes the effect size; the Y-axis denotes the bacterial families. Bacterial families with increased abundance in healthy subjects or CP and CPT2DM patients are shown in pink and violet, respectively. The statistically significant decrease is shown in red; the statistically significant increase is shown in blue.

Figure 5 Phylogenetic analysis of the metagenome-assembled genomes. The phylogenetic tree is based on 43 marker proteins obtained from 26 MAG sequences and related eHOMD genomes [43]. The MAG groups are shown in different colors (red for the Control group, blue and green for CP and CPT2DM, respectively). The colored areas also denote the bacterial phyla.

Table 1 Demographic and clinical parameters of patients with chronic periodontitis associated and not associated with type 2 diabetes mellitus and healthy donors.

Parameters/Groups	CPT2DM (n=15)	CP (n=15)	Healthy (n=16)	One-way ANOVA
Sex Male	7/47%	7/47%	8/50%	F=0.08, p=0.79
Sex Female	8/53%	8/53%	8/50%	
Age Male	57.8±6.3	55.7±9.6	48.2±5.7	F=2.74, p=0.14
Age Female	57.9±6.4	56.2±8.6	48.9±6.7	
BMI* 25.0 - 29.9 kg/m^2	4/27%*	12/80%	10/63%	F=5.942, p=0.019*
BMI* 30.0 - 34.9 kg/m^2	5/33%*	3/20%	5/31%	
BMI* 35.0 - 45.9 kg/m^2	6/46%*	0/0%	1/6%	

BMI* – body mass index; significantly differing population distribution according to BMI was observed in patients with CPT2DM compared to healthy subjects and patients with CP only (*$p < 0.05$).

Supplementary Files

Supplementary Table 1 — Sequencing quality control data.

Supplementary Table 2 — Taxonomic composition of 16S rRNA gene sequencing samples at family level (reads counts).

Supplementary Table 3 — Binning statistic and bins taxonomic annotation.

Table 2 Comparison of study groups using dental status indices and the glycosylated hemoglobin level.

Variable	CPT2DM (n=15)	CP (n=15)	Healthy (n=16)	p-value
PHP	0.7 (0; 2.1)	1.3 (0.6; 2.1)	0.2 (0; 0.7)	$p_{1-2} = 0.23$ $p_{1-3} = 0.09$ $p_{2-3} < 0.01$
OHI-S	1.4 (0.4; 4.5)	1.4 (0.4; 3.3)	1.8 (0.5; 3.1)	$p_{1-2} = 0.72$ $p_{1-3} = 0.63$ $p_{2-3} = 0.96$
CAL	3.9 (3.0; 4.5)	3.8 (3.4; 4.3)	0.8 (0; 1.1)	$p_{1-2} = 0.70$ $p_{1-3} < 0.01$* $p_{2-3} < 0.01$*
MMI	0.8 (0; 2.0)	0.5 (0; 2.0)	0 (0; 1.0)	$p_{1-2} = 0.41$ $p_{1-3} = 0.06$ $p_{2-3} = 0.12$
TL	0 (0; 0)	0.05 (0; 1.0)	0 (0; 0)	$p_{1-2} = 0.70$ $p_{1-3} = 1.00$ $p_{2-4} = 0.87$
PBI	0.34 (0; 0.90)	0.30 (0.10; 0.90)	0.38 (0; 0.84)	$p_{1-2} = 0.70$ $p_{1-3} = 0.58$ $p_{2-3} = 0.87$
Glucose (mmol/L)	5.7 (5.0; 7.8)	5.6 (5.1; 6.1)	5.4 (4.4; 6.6)	$p_{1-2} = 0.75$ $p_{1-3} = 0.67$ $p_{2-3} = 0.59$
Glycosylated hemoglobin (HbA1c)	7.5 (6.5; 8.0)	4.5 (3.0; 6.0)	3.5 (3.0; 5.0)	$p_{1-2} < 0.01$* $p_{1-3} < 0.01$* $p_{2-3} = 0.07$

*PHP: patient hygiene performance. OHI-S: simplified oral hygiene Index. PMA: papillary-marginal-Alveolar index. PBI: papillary bleeding Index. CPITN: vommunity periodontal index of treatment needs.