Article:

Cheng, Z, Do, TD orcid.org/0000-0002-5668-2181, Mankia, K et al. (7 more authors) (2020) Dysbiosis in the Oral Microbiomes of anti-CCP Positive Individuals at Risk of Developing Rheumatoid Arthritis. Annals of the Rheumatic Diseases. ISSN 0003-4967

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SUPPLEMENTARY MATERIAL

Supplemental method

Patient and Public Involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research

Study population

Ethical approval was provided by National Research Ethics Service Committee Yorkshire and the Humber, Leeds West. Thirty-two asymptomatic healthy controls (HC), 48 CCP+ at-risk individuals with musculoskeletal symptoms but no clinical synovitis and 26 anti-CCP Ab positive early RA patients (within the first 3 months of disease-modifying anti-rheumatic drug, DMARD, therapy) were recruited. CCP+ at-risk individuals were recruited from the Leeds CCP cohort.[14, 15] Patients older than 18 years presenting to general practitioners or allied health professionals with new-onset, non-specific musculoskeletal symptoms but no clinical synovitis were invited to participate. Anti-CCP antibody testing was performed in Leeds using the Bioplex 2200 kit (BioRad). Those with a positive test were invited to the Leeds CCP research clinic, where recruitment to this study took place. Patients from secondary care clinics who were anti-CCP positive but without clinical synovitis were also invited to the Leeds CCP research clinic. Of 48 CCP+ at-risk individuals, 46 (96%) had no ultrasonographic evidence of synovitis suggesting the absence of clinical or subclinical joint inflammation in these subjects. Healthy control participants had no joint disease or history of inflammatory arthritis (and no affected first-degree relatives). Healthy control participants were typical of the general population with a range of socioeconomic groups represented. They included colleagues (staff members at University of Leeds), including academic staff, administrative staff and maintenance workers. Healthy control participants were also recruited from the local community (e.g. community centres). No CCP+ at-risk individuals or healthy individuals had received DMARDs. Patients with early RA were all anti-CCP-positive and within the first 3 months of DMARD therapy. Of 26 patients with early RA, 10 (38%) were DMARD naive. Patients with early RA who had commenced DMARD therapy were all receiving monotherapy with a median duration of only 2 weeks; 1 patient had commenced sulfasalazine and the remainder, methotrexate.

Sample collection

Periodontal assessments and subgingival plaque sampling was performed by three experienced dentists (V.C, A.S and A.T).[9] According to the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions, periodontally healthy sites were defined as sites with ≤3 mm probing depth and no bleeding on probing.[16] Diseased sites were those with ≥ 4 mm probing depth and ≥ 2 mm clinical attachment loss (CAL).[17] Six sites on each natural tooth were examined. Sites were selected for sample collection if accessible and where moisture contamination could be controlled. Samples were rejected if bleeding on probing was extensive at that site. Supragingival plaque was removed with cotton wool pledgets prior to sample collection. Two sterile paper points (Maillefer Pro Taper Paper Points F3; The Dental Directory, UK) were used to collect subgingival plaque from a maximum of three healthy sites and three diseased sites for each participant, avoiding bleeding, and immediately placed in a cryovial containing 0.5ml of RNA protect Bacteria Reagent (Qiagen, Germany). Cryovials were vortexed and centrifuged at 8000g for 1 minute. Supernatants were removed by pipetting and cryotubes containing pellets were stored at -80°C.

DNA extraction, library preparation and sequencing

All healthy site samples were pooled and all diseased site samples were pooled for each participant. DNA was extracted from pooled samples using the UltraClean® Microbial DNA Isolation Kit (Qiagen, Germany) and quantified by using PicoGreen ® dsDNA Reagent and Kits (Thermo Fisher Scientific, America). The DNA was sheared to 200 bp by use of a S220 Focused-ultrasonicator (Covaris, UK). According to manufacturer's instructions, either NEBNext® Ultra™ DNA Library Prep Kit for Illumina® or NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, America) was used for library construction including end preparation, adaptor ligation and PCR enrichment. AxyPrepTM Mag PCR Clean-up beads (Corning, America) were used during and after the library preparation. DNA libraries tagged with different index primers were paired-end sequenced on the Illumina Hiseq 3000 machine (Illumina, America).

Shotgun metagenomic data processing

Sequence data were uploaded to the MG-RAST metagenomics analysis pipeline (version 4.03) for quality processing and basic taxonomic analysis (project number: mgp18407, 17175, 19372, 21724, 21828).[3] Low-quality regions (bases with quality scores lower than 15), as well as reads shorter than 15 bp were discarded. Artificial replicate sequences and host specific species sequences (e.g. plant, human or mouse) were also removed. Taxonomic abundance profiles at species level were generated by annotation against the Refseq database housed within MG-RAST with the threshold of 95% identity using a representative hit strategy.[4]

Alpha diversity was computed using *phyloseq* in R. Abundance Coverage Estimator (ACE) was used as estimators of alpha diversity. Core species were identified using *microbiome* R package when species were present in at least 80% of the patients in each group. A species was included in the co-occurrence network analysis if it was present in more than 30% of samples, at an abundance of at least 0.01%. Co-occurrence networks were calculated in R scrip (Co-occurrence network analysis) and the graphs were constructed in Cytoscape. Network correlations were determined by significant pairwise using Spearsman's correlation ($P < 0.01$) and network graphs were calculated in R scrip (Co-occurrence network analysis) and visualized in Cytoscape. To explore the hubs in the network, the nodes (species) were ranked by maximal clique centrality (MCC) algorithm into the top 20 species.[5] For functional capability analysis, annotation was performed against the clusters of orthologous genes (COGs) protein database with the threshold of 95% identity.[20]

Statistical analysis

Differences between alpha diversities group-wise was measured using Kruskal-Wallis Test and the Dunn-Bonferroni correction of the P value was performed for multiple testing. The permutation test (indicspecies R-package) was used to identify taxa with significantly higher relative abundance in one of three groups. Sidak's correction was applied for multiple testing and then Wald-test for pairwise comparison. Pearson Chi-Square test was used to examine the frequency of detection. A closed testing procedure was performed for adjustment. Spearman's correlation analysis was used to identify strong ($q < -0.7$ or > 0.7) and significant (corrected $P < 0.01$) pairwise correlations in the co-occurrence network. To explore the hubs in the network, the species were ranked by maximal clique centrality (MCC) algorithm into the top 20 species.[21] *DESeq2* R-package was used to perform data normalisation and estimate between-group differences in functional capability. The P-values were corrected for multiple testing (Wald Test, Benjamini-Hochberg method for FDR adjustment).

Referecen:

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Supplemental figures

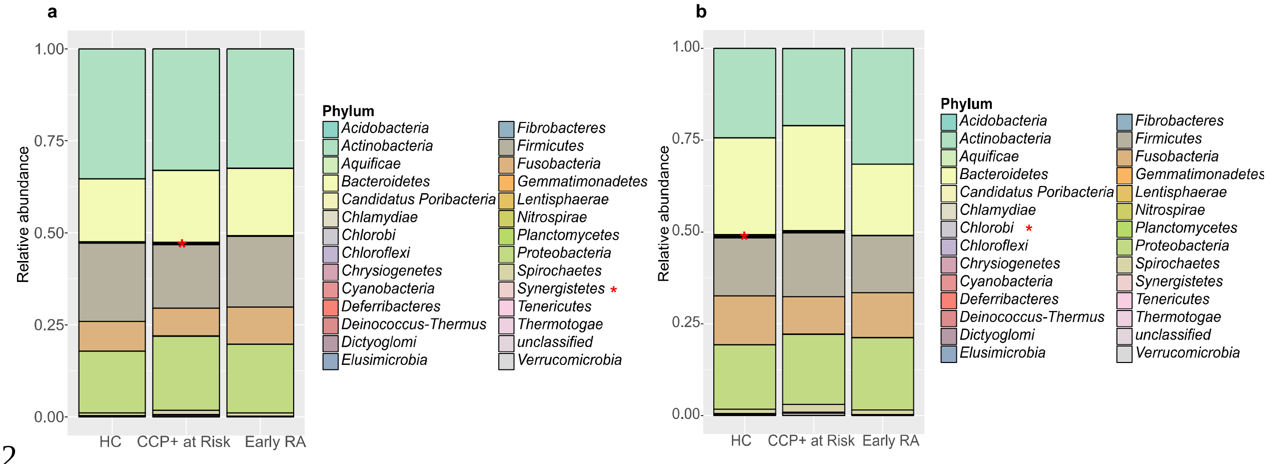

Figure S1. Phylum composition of subgingival plaque from periodontally healthy and diseased sites in healthy control (HC), CCP+ at-risk and early RA groups. The relative abundance of the oral microbiota (phylum level) was compared between different groups in (a) periodontally healthy sites and (b) diseased sites. *: corrected $P < 0.05$ (Sidak's correction).

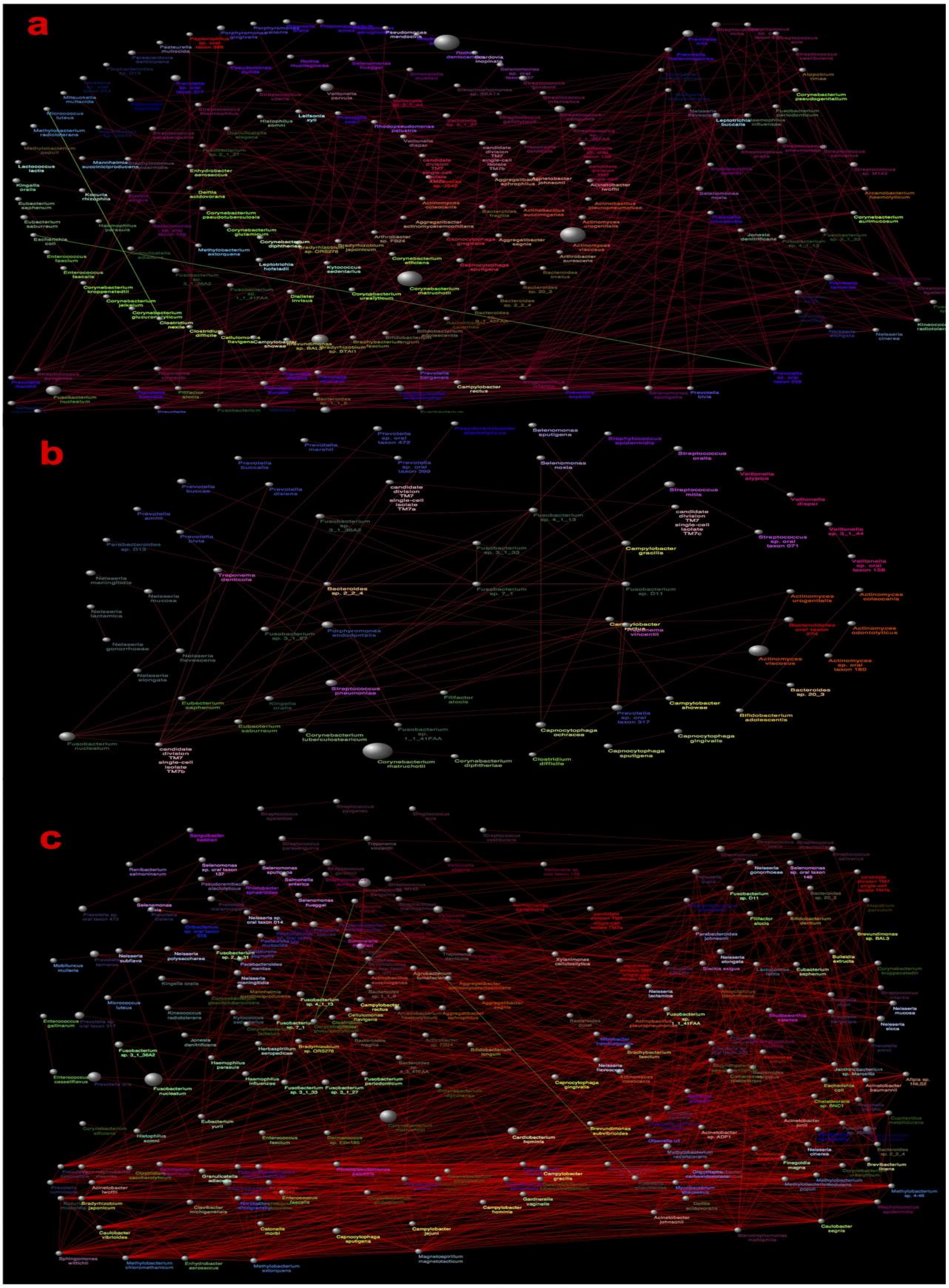

Figure S2. Co-occurrence networks in periodontally healthy site samples of subgingival plaque. Spearman's rank coefficients (q) between species were calculated pairwise in (a) healthy control (HC), (b) CCP + at-risk and (c) early RA groups. Edges represent a strong ($q > 0.7$ or $q < -0.7$) and significant (adjusted $P < 0.01$) correlation. Red edges illustrate positive correlations and green negative. The size of each node is proportional to the relative abundance of the species. The species were coloured by the genus-level taxonomy.

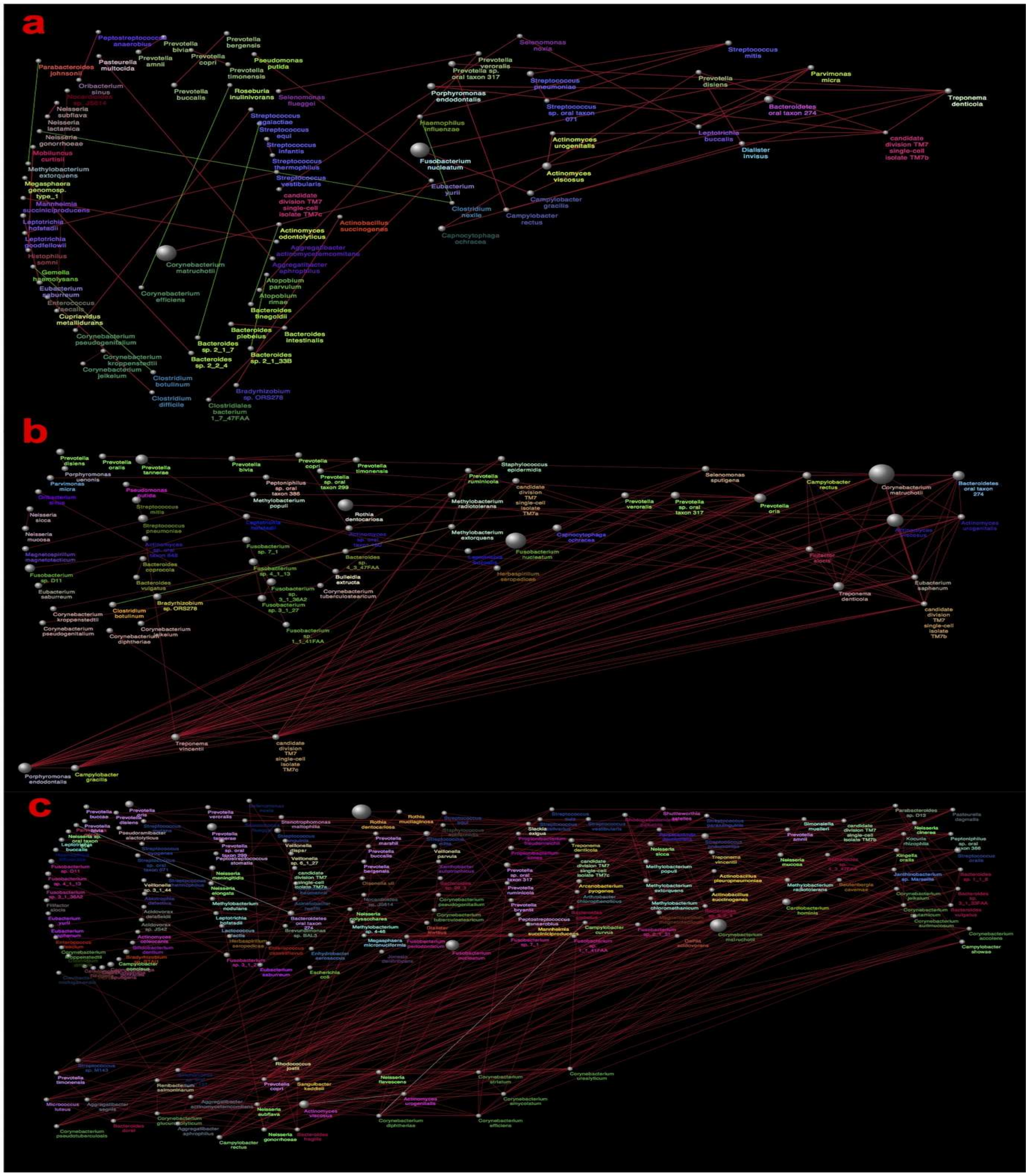

Figure S3. Co-occurrence networks in periodontally disease site samples of subgingival plaque. Spearman's rank coefficients (q) between species were calculated pairwise in (a) healthy control (HC), (b) CCP + at-risk and (c) early RA groups. Edges represent a strong ($q > 0.7$ or $q < -0.7$) and significant (adjusted $P < 0.01$) correlation. Red edges illustrate positive correlations and green negative. The size of each node is proportional to the relative abundance of the species. The species were coloured by the genus-level taxonomy.

Supplemental tables

Table S1: Baseline characteristics of study subjects according to RA status. Groups were balanced for age, sex and smoking status. CCP+ at-risk, anti-CCP positive at-risk individuals; Early RA, early rheumatoid arthritis; DMARDs, synthetic disease-modifying anti-rheumatic drugs (included methotrexate, sulfasalazine and hydroxychloroquine).

	Healthy Controls (n=32)	**CCP+ at-risk (n=48)**	**Early RA (n=26)**
Age: mean (SD)	49.4 (15.3)	51.9 (11.4)	54.4 (16.7)
Female: n (%)	19 (59)	31 (65)	14 (54)
Current smoker: n (%)	6 (19)	12 (25)	4 (15)
Ex-smoker: n (%)	12 (38)	19 (40)	13 (50)
Current DMARDs: n (%)	0 (0)	0 (0)	16 (62)

Groups were balanced for age, sex, and smoking status.

Table S2. List of core species (> 80% prevalence) in healthy control (HC), CCP+ at-risk and early RA groups in samples from periodontally healthy sites

Group	**n**	**Species**
HC, CCP+ at Risk, Early RA	81	*Neisseria flavescens*
		Propionibacterium acnes
		Campylobacter showae
		Prevotella marshii
		Rhodopseudomonas palustris
		Prevotella tannerae
		Prevotella sp. oral taxon 472
		Atopobium rimae
		Streptococcus sanguinis
		Selenomonas sp. oral taxon 149
		Bradyrhizobium sp. ORS278
		Mannheimia succiniciproducens
		Capnocytophaga sputigena
		Streptococcus anginosus

Actinomyces odontolyticus
Corynebacterium glutamicum
Neisseria sicca
Fusobacterium sp. 3_1_36A2
Fusobacterium sp. 4_1_13
Paracoccus denitrificans
Veillonella sp. 3_1_44
Prevotella sp. oral taxon 317
Methylobacterium radiotolerans
Methylobacterium extorquens
Fusobacterium sp. 3_1_33
Abiotrophia defectiva
Sanguibacter keddieii
Rothia mucilaginosa
Streptococcus sp. oral taxon 071
Neisseria meningitidis
Rothia dentocariosa
Prevotella disiens
Corynebacterium matruchotii
Prevotella oris
Neisseria elongata
Streptococcus parasanguinis
Bacteroides fragilis
Fusobacterium sp. D11
Megasphaera micronuciformis
Selenomonas sp. oral taxon 137
Rhodobacter sphaeroides
Capnocytophaga ochracea
Streptococcus thermophilus
Leptotrichia hofstadii
Oribacterium sp. oral taxon 078
Eubacterium saburreum
Selenomonas flueggei
Streptococcus pneumoniae
Bifidobacterium longum
Veillonella atypica
Haemophilus influenzae
Acidovorax sp. JS42
Streptococcus oralis
Prevotella veroralis

HC, Early RA	225	*Veillonella parvula*
		Prevotella melaninogenica
		Streptococcus mitis
		Neisseria gonorrhoeae
		Delftia acidovorans
		Magnetospirillum magnetotacticum
		Veillonella sp. 6_1_27
		Fusobacterium nucleatum
		Aggregatibacter segnis
		Aggregatibacter aphrophilus
		Streptococcus sp. M143
		Prevotella buccae
		Pseudomonas fluorescens
		Veillonella sp. oral taxon 158
		Prevotella oralis
		Cardiobacterium hominis
		Veillonella dispar
		Fusobacterium sp. 7_1
		Aggregatibacter actinomycetemcomitans
		Bradyrhizobium sp. BTAi1
		Neisseria lactamica
		Streptococcus sp. 2_1_36FAA
		Neisseria subflava
		Neisseria mucosa
		Prevotella timonensis
		Fusobacterium sp. 3_1_27
		Clostridium botulinum
		Paludibacter propionicigenes
		Bacteroides uniformis
		Fusobacterium sp. 2_1_31
		Actinomyces sp. oral taxon 848
		Mesorhizobium loti
		Eubacterium rectale
		Mobiluncus curtisii
		Neisseria polysaccharea
		Eikenella corrodens
		Nocardiopsis dassonvillei
		candidate division TM7 single-cell isolate TM7c
		Clostridium nexile

Ralstonia solanacearum
Corynebacterium accolens
Corynebacterium kroppenstedtii
Treponema denticola
Leeuwenhoekiella blandensis
Granulicatella elegans
Gemella haemolysans
Enterococcus faecium
Parvimonas micra
Fusobacterium periodonticum
Shuttleworthia satelles
Kocuria rhizophila
Bacteroides sp. 1_1_6
Porphyromonas endodontalis
Enterococcus faecalis
Methylibium petroleiphilum
Nitrobacter winogradskyi
Neisseria sp. oral taxon 014
Janibacter sp. HTCC2649
Finegoldia magna
Rhodococcus jostii
Pseudoramibacter alactolyticus
Methylobacterium sp. 4-46
Mycobacterium vanbaalenii
Olsenella uli
Clostridium bolteae
Methylobacterium populi
Streptococcus salivarius
Arthrobacter sp. FB24
Kytococcus sedentarius
Mitsuokella multacida
Corynebacterium jeikeium
Acinetobacter lwoffii
Brevundimonas sp. BAL3
Streptococcus gallolyticus
Streptococcus gordonii
Zunongwangia profunda
Prevotella copri
Peptostreptococcus anaerobius
Pseudomonas mendocina

Corynebacterium glucuronolyticum
Saccharopolyspora erythraea
Actinosynnema mirum
Dialister invisus
Escherichia coli
Rhizobium leguminosarum
Pseudomonas putida
Peptoniphilus sp. oral taxon 386
Campylobacter rectus
Methylobacterium chloromethanicum
Corynebacterium striatum
Bacteroidetes oral taxon 274
Campylobacter fetus
Bifidobacterium dentium
Mycobacterium avium
Porphyromonas gingivalis
Collinsella aerofaciens
Clostridium difficile
Streptococcus agalactiae
Treponema vincentii
Oribacterium sinus
marine actinobacterium PHSC20C1
Streptosporangium roseum
Thermobifida fusca
Arthrobacter aurescens
Porphyromonas asaccharolytica
Afipia sp. 1NLS2
Capnocytophaga gingivalis
Nocardia farcinica
Streptococcus pyogenes
Filifactor alocis
Streptococcus mutans
Clavibacter michiganensis
Brachybacterium faecium
Corynebacterium pseudogenitalium
Catonella morbi
Chryseobacterium gleum
Campylobacter hominis
Bacteroides thetaiotaomicron
Propionibacterium freudenreichii

Lactococcus lactis
Klebsiella pneumoniae
Haemophilus parasuis
Streptococcus suis
Nitrobacter sp. Nb-311A
Oligotropha carboxidovorans
Xanthobacter autotrophicus
Kribbella flavida
Mycobacterium abscessus
Bacteroides sp. 4_3_47FAA
Cellulomonas flavigena
Croceibacter atlanticus
Bacteroides intestinalis
Prevotella bryantii
Rhodococcus erythropolis
Bacteroides vulgatus
Mycobacterium tuberculosis
Kineococcus radiotolerans
Bacteroides ovatus
Listeria monocytogenes
Saccharomonospora viridis
Campylobacter concisus
Eubacterium saphenum
Arthrobacter chlorophenolicus
Mycobacterium sp. KMS
Jonesia denitrificans
Nocardioides sp. JS614
Beutenbergia cavernae
Sphingomonas wittichii
Stenotrophomonas sp. SKA14
Corynebacterium aurimucosum
Corynebacterium diphtheriae
Acidovorax citrulli
Bacteroides sp. 2_2_4
Rhodococcus equi
Bacteroides sp. 20_3
Parabacteroides sp. D13
Campylobacter jejuni
Corynebacterium lipophiloflavum
Methylobacterium nodulans

Prevotella buccalis
Granulicatella adiacens
Pasteurella multocida
Pseudomonas aeruginosa
Acinetobacter johnsonii
Streptococcus uberis
Enhydrobacter aerosaccus
Burkholderia pseudomallei
Xylanimonas cellulosilytica
Porphyromonas uenonis
Bulleidia extructa
Enterococcus casseliflavus
Stenotrophomonas maltophilia
Slackia exigua
Nakamurella multipartita
Salinispora tropica
Bacteroides dorei
Leifsonia xyli
Streptococcus vestibularis
Campylobacter curvus
Campylobacter gracilis
Renibacterium salmoninarum
Bacteroides plebeius
Maribacter sp. HTCC2170
Gordonia bronchialis
Ralstonia pickettii
Acidovorax delafieldii
Leptotrichia buccalis
Leptotrichia goodfellowii
Gramella forsetii
Geodermatophilus obscurus
Actinomyces sp. oral taxon 180
Xanthomonas campestris
Agrobacterium tumefaciens
Actinomyces urogenitalis
Actinomyces coleocanis
Robiginitalea biformata
Neisseria cinerea
Staphylococcus epidermidis
Selenomonas sputigena

Corynebacterium efficiens
Streptococcus infantarius
Histophilus somni
Flavobacterium johnsoniae
Nitrobacter hamburgensis
Streptococcus infantis
Cupriavidus metallidurans
Atopobium parvulum
candidate division TM7 single-cell isolate TM7a
Streptococcus equi
Fusobacterium sp. 1_1_41FAA
Cryptobacterium curtum
Corynebacterium urealyticum
Corynebacterium amycolatum
Mobiluncus mulieris
Gardnerella vaginalis
Corynebacterium tuberculostearicum
Janthinobacterium sp. Marseille
Mycobacterium smegmatis
Parabacteroides merdae
Actinobacillus succinogenes
Actinomyces viscosus
Bacillus halodurans
Eubacterium yurii
Selenomonas noxia
Brevibacterium mcbrellneri
Caulobacter vibrioides
Kingella oralis
Prevotella sp. oral taxon 299
Prevotella amnii
Bifidobacterium adolescentis
Pasteurella dagmatis
Prevotella ruminicola
Salmonella enterica
Acinetobacter baumannii
Comamonas testosteroni
Dermacoccus sp. Ellin185
Herminiimonas arsenicoxydans
Micrococcus luteus

HC	35	*Bradyrhizobium japonicum*
		Peptostreptococcus stomatis
		Prevotella bergensis
		Clostridium saccharolyticum
		Parascardovia denticolens
		Arcanobacterium haemolyticum
		Roseburia inulinivorans
		Herbaspirillum seropedicae
		Riemerella anatipestifer
		Corynebacterium pseudotuberculosis
		Thermobispora bispora
		Parabacteroides johnsonii
		Prevotella bivia
		Brevibacterium linens
		Bacillus cellulosilyticus
		Polaromonas naphthalenivorans
		Mycobacterium marinum
		Bacillus clausii
		Simonsiella muelleri
		Bacteroides coprophilus
		Yersinia pestis
		Alkaliphilus metalliredigens
		Rhodobacter capsulatus
		Acidaminococcus fermentans
		Achromobacter piechaudii
		Intrasporangium calvum
		Opitutus terrae
		Psychrobacter sp. PRwf-1
		Scardovia inopinata
		Actinobacillus pleuropneumoniae
		Dinoroseobacter shibae
		Xanthomonas oryzae
		Laribacter hongkongensis
		Desulfitobacterium hafniense
		Acidothermus cellulolyticus
		Streptomyces avermitilis
		Clostridiales genomosp. BVAB3
		Flavobacteriaceae bacterium 3519-10
		Actinobacillus minor
		Stackebrandtia nassauensis

		Thermomonospora curvata
		Roseobacter denitrificans
		Fusobacterium sp. 3_1_5R
		Mannheimia haemolytica
		Bifidobacterium animalis
		Streptomyces sp. Mg1
		Bacillus cereus
		Frankia sp. CcI3
		Opitutaceae bacterium TAV2
Early RA	79	*Slackia heliotrinireducens*
		Rhizobium etli
		Frankia alni
		Peptoniphilus harei
		Streptomyces lividans
		Aeromonas hydrophila
		Albidiferax ferrireducens
		candidate division TM7 single-cell isolate TM7b
		Ketogulonicigenium vulgare
		Thiobacillus denitrificans
		Acidovorax avenae
		Brevundimonas subvibrioides
		Clostridium hathewayi
		Clostridium phytofermentans
		Arthrobacter arilaitensis
		Enterococcus gallinarum
		Treponema pallidum
		Aeromonas salmonicida
		Sphingopyxis alaskensis
		Thiomonas intermedia
		Anaerococcus lactolyticus
		Caulobacter segnis
		Sphingobium japonicum
		Variovorax paradoxus
		Exiguobacterium sp. AT1b
		Fusobacterium ulcerans
		Sphingobacterium spiritivorum
		Verminephrobacter eiseniae
		Holdemania filiformis
		Xanthomonas axonopodis

Clostridiales bacterium 1_7_47FAA
Chromobacterium violaceum
Dechloromonas aromatica
Dokdonia donghaensis
Clostridium sp. M62/1
Staphylococcus aureus
Sinorhizobium meliloti
Enterobacter sp. 638
Caulobacter sp. K31
Polaromonas sp. JS666
Catenulispora acidiphila
Leptothrix cholodnii
Achromobacter xylosoxidans
Xylella fastidiosa
Ochrobactrum anthropi
Flavobacterium psychrophilum
Streptococcus dysgalactiae
Pseudomonas stutzeri
Bacteroides sp. 2_1_16
Enterobacter cloacae
Mycobacterium parascrofulaceum
Parvibaculum lavamentivorans
Jannaschia sp. CCS1
Alicycliphilus denitrificans
Lactobacillus iners
Haemophilus ducreyi
Atopobium vaginae
Serratia proteamaculans
Chelativorans sp. BNC1
Bacteroides eggerthii
Streptococcus pseudoporcinus
Bacteroides sp. 2_1_7
Acinetobacter sp. ADP1
Peptoniphilus lacrimalis
Photobacterium profundum
Staphylococcus haemolyticus
Clostridium asparagiforme
Streptomyces sp. ACT-1
Mycobacterium gilvum
Burkholderia ambifaria

		Megasphaera genomosp. type_1
		Bacteroides stercoris
		Shewanella sp. ANA-3
		Eubacterium eligens
		Bacteroides sp. D2
		Alistipes putredinis
		Bacillus licheniformis
		Azorhizobium caulinodans
		Streptococcus downei

Table S3. List of core species (> 80% prevalence) in healthy control (HC), CCP+ at-risk and early RA groups in samples from periodontally diseased sites

Group	n	Species
HC, CCP+ at Risk, Early RA	42	*Fusobacterium sp. 2_1_31*
		Propionibacterium acnes
		Campylobacter showae
		Prevotella tannerae
		Prevotella sp. oral taxon 472
		Streptococcus sanguinis
		Bacteroides sp. 1_1_6
		Selenomonas sp. oral taxon 149
		Capnocytophaga sputigena
		Fusobacterium sp. 3_1_36A2
		Fusobacterium sp. 4_1_13
		Veillonella sp. 3_1_44
		Fusobacterium sp. 3_1_33
		Abiotrophia defectiva
		Streptococcus sp. oral taxon 071
		Neisseria meningitidis
		Rothia dentocariosa
		Corynebacterium matruchotii
		Prevotella oris
		Neisseria elongata
		Streptococcus parasanguinis
		Bacteroides fragilis
		Fusobacterium sp. D11
		Capnocytophaga gingivalis
		Selenomonas sp. oral taxon 137
		Capnocytophaga ochracea
		Streptococcus pneumoniae

		Haemophilus influenzae
		Streptococcus oralis
		Veillonella parvula
		Prevotella melaninogenica
		Streptococcus mitis
		Veillonella sp. 6_1_27
		Fusobacterium nucleatum
		Streptococcus sp. M143
		Prevotella buccae
		Veillonella dispar
		Fusobacterium sp. 7_1
		Aggregatibacter actinomycetemcomitans
		Bradyrhizobium sp. BTAi1
		Streptococcus sp. 2_1_36FAA
		Fusobacterium sp. 3_1_27
HC, Early RA	49	*Neisseria flavescens*
		Mobiluncus curtisii
		Eikenella corrodens
		Prevotella marshii
		Rhodopseudomonas palustris
		Atopobium rimae
		Neisseria sp. oral taxon 014
		Mannheimia succiniciproducens
		Corynebacterium glutamicum
		Mitsuokella multacida
		Neisseria sicca
		Streptococcus gordonii
		Prevotella sp. oral taxon 317
		Escherichia coli
		Rothia mucilaginosa
		Prevotella disiens
		Porphyromonas asaccharolytica
		Megasphaera micronuciformis
		Streptococcus mutans
		Clavibacter michiganensis
		Leptotrichia hofstadii
		Acidovorax sp. JS42
		Prevotella veroralis
		Bacteroides ovatus
		Bacteroides sp. 2_2_4

		Neisseria gonorrhoeae
		Bacteroides sp. 20_3
		Parabacteroides sp. D13
		Prevotella buccalis
		Granulicatella adiacens
		Pseudomonas aeruginosa
		Atopobium vaginae
		Aggregatibacter aphrophilus
		Slackia exigua
		Bacteroides dorei
		Pseudomonas fluorescens
		Prevotella oralis
		Cardiobacterium hominis
		Selenomonas sputigena
		Histophilus somni
		Fusobacterium sp. 1_1_41FAA
		Neisseria subflava
		Parabacteroides merdae
		Neisseria mucosa
		Kingella oralis
		Prevotella sp. oral taxon 299
		Bradyrhizobium japonicum
		Parabacteroides johnsonii
		Prevotella bivia
CCP+ at Risk, Early RA	9	*Streptococcus anginosus*
		Actinomyces odontolyticus
		Dialister invisus
		Porphyromonas gingivalis
		Oribacterium sp. oral taxon 078
		Eubacterium saburreum
		Selenomonas flueggei
		Corynebacterium aurimucosum
		Neisseria lactamica
HC	6	*Shuttleworthia satelles*
		Faecalibacterium prausnitzii
		Dechloromonas aromatica
		Achromobacter xylosoxidans
		Bacteroides plebeius
		Parascardovia denticolens
CCP+ at Risk	2	*Bifidobacterium dentium*

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		Eubacterium yurii
Early RA	190	*Paludibacter propionicigenes*
		Actinomyces sp. oral taxon 848
		Neisseria polysaccharea
		candidate division TM7 single-cell isolate TM7c
		Ralstonia solanacearum
		Corynebacterium accolens
		Corynebacterium kroppenstedtii
		Treponema denticola
		Streptomyces lividans
		Granulicatella elegans
		Gemella haemolysans
		Enterococcus faecium
		Albidiferax ferrireducens
		Parvimonas micra
		Fusobacterium periodonticum
		Kocuria rhizophila
		Porphyromonas endodontalis
		candidate division TM7 single-cell isolate TM7b
		Nitrobacter winogradskyi
		Janibacter sp. HTCC2649
		Bacteroides sp. 3_1_33FAA
		Finegoldia magna
		Acidovorax avenae
		Rhodococcus jostii
		Pseudoramibacter alactolyticus
		Methylobacterium sp. 4-46
		Olsenella uli
		Clostridium hathewayi
		Eggerthella lenta
		Arthrobacter arilaitensis
		Methylobacterium populi
		Streptococcus salivarius
		Arthrobacter sp. FB24
		Campylobacter lari
		Corynebacterium jeikeium
		Brevundimonas sp. BAL3
		Acinetobacter lwoffii
		Streptococcus gallolyticus
		Dichelobacter nodosus

Intrasporangium calvum
Sphingopyxis alaskensis
Paracoccus denitrificans
Zunongwangia profunda
Prevotella copri
Peptostreptococcus anaerobius
Pseudomonas mendocina
Methylobacterium radiotolerans
Saccharopolyspora erythraea
Actinosynnema mirum
Methylobacterium extorquens
Sanguibacter keddieii
Corynebacterium genitalium
Pseudomonas putida
Campylobacter rectus
Methylobacterium chloromethanicum
Bacteroidetes oral taxon 274
Campylobacter fetus
Actinobacillus pleuropneumoniae
Verminephrobacter eiseniae
Mycobacterium avium
Clostridium difficile
Streptococcus agalactiae
Treponema vincentii
Oribacterium sinus
marine actinobacterium PHSC20C1
Streptomyces avermitilis
Clostridium sp. M62/1
Anaerococcus vaginalis
Arthrobacter aurescens
Staphylococcus aureus
Nocardia farcinica
Streptococcus pyogenes
Filifactor alocis
Streptococcus thermophilus
Caulobacter sp. K31
Brachybacterium faecium
Corynebacterium pseudogenitalium
Bifidobacterium longum
Polaromonas sp. JS666

Veillonella atypica
Campylobacter hominis
Propionibacterium freudenreichii
Lactococcus lactis
Klebsiella pneumoniae
Haemophilus parasuis
Streptococcus suis
Xanthobacter autotrophicus
Kribbella flavida
Mycobacterium abscessus
Catenulispora acidiphila
Bacteroides sp. 4_3_47FAA
Cellulomonas flavigena
Prevotella bryantii
Bacteroides vulgatus
Mycobacterium tuberculosis
Kineococcus radiotolerans
Listeria monocytogenes
Campylobacter concisus
Flavobacterium psychrophilum
Streptococcus dysgalactiae
Tsukamurella paurometabola
Arthrobacter chlorophenolicus
Bacteroides sp. 2_1_16
Pseudomonas stutzeri
Jonesia denitrificans
Nocardioides sp. JS614
Beutenbergia cavernae
Sphingomonas wittichii
Stenotrophomonas sp. SKA14
Corynebacterium diphtheriae
Acidovorax citrulli
Rhodococcus equi
Delftia acidovorans
Campylobacter jejuni
Magnetospirillum magnetotacticum
Methylobacterium nodulans
Pasteurella multocida
Acinetobacter johnsonii
Streptococcus uberis

Enhydrobacter aerosaccus
Burkholderia pseudomallei
Aggregatibacter segnis
Xylanimonas cellulosilytica
Porphyromonas uenonis
Bulleidia extructa
Enterococcus casseliflavus
Streptococcus pseudoporcinus
Stenotrophomonas maltophilia
Acinetobacter sp. ADP1
Nakamurella multipartita
Leifsonia xyli
Streptococcus vestibularis
Campylobacter curvus
Campylobacter gracilis
Renibacterium salmoninarum
Azoarcus sp. BH72
Maribacter sp. HTCC2170
Gordonia bronchialis
Veillonella sp. oral taxon 158
Acidovorax delafieldii
Leptotrichia buccalis
Leptotrichia goodfellowii
Gramella forsetii
Mycobacterium gilvum
Geodermatophilus obscurus
Actinomyces sp. oral taxon 180
Agrobacterium tumefaciens
Actinomyces urogenitalis
Actinomyces coleocanis
Robiginitalea biformata
Neisseria cinerea
Staphylococcus epidermidis
Bacteroides sp. D2
Corynebacterium efficiens
Flavobacterium johnsoniae
Streptococcus infantis
Atopobium parvulum
candidate division TM7 single-cell isolate TM7a
Roseburia intestinalis

	Streptococcus equi	
	Cryptobacterium curtum	
	Corynebacterium amycolatum	
	Corynebacterium urealyticum	
	Mobiluncus mulieris	
	Micromonospora aurantiaca	
	Corynebacterium tuberculostearicum	
	Bacteroides cellulosilyticus	
	Janthinobacterium sp. Marseille	
	Mycobacterium smegmatis	
	Actinomyces viscosus	
	Actinobacillus succinogenes	
	Selenomonas noxia	
	Caulobacter vibrioides	
	Prevotella timonensis	
	Prevotella amnii	
	Pasteurella dagmatis	
	Prevotella ruminicola	
	Acinetobacter baumannii	
	Comamonas testosteroni	
	Fusobacterium gonidiaformans	
	Dermacoccus sp. Ellin185	
	Micrococcus luteus	
	Peptostreptococcus stomatis	
	Prevotella bergensis	
	Clostridium saccharolyticum	
	Arcanobacterium haemolyticum	
	Herbaspirillum seropedicae	
	Riemerella anatipestifer	
	Corynebacterium pseudotuberculosis	
	Brevibacterium linens	

Table S4. Core bacterial species with significantly higher relative abundance in the healthy control (HC), CCP+ at-risk, and early RA groups in periodontally healthy and diseased site samples of subgingival plaque.

Group	Species	Corrected *P*
	Healthy site	

HC	*Leptotrichia buccalis*	0.015
	Veillonella parvula	0.030
CCP+ at Risk	*Arthrobacter arilaitensis*	0.047
	Arthrobacter aurescens	0.021
	Arthrobacter chlorophenolicus	0.036
	Bacillus cereus	0.012
	Bacillus licheniformis	0.044
	Bifidobacterium longum	0.030
	Catenulispora acidiphila	0.003
	Corynebacterium glutamicum	0.012
	Gardnerella vaginalis	0.033
	Gordonia bronchialis	0.003
	Mannheimia succiniciproducens	0.033
	Mobiluncus curtisii	0.021
	Mycobacterium sp. KMS	0.021
	Nocardiopsis dassonvillei	0.012
	Porphyromonas gingivalis	0.044
	Rhodococcus erythropolis	0.015
	Roseobacter denitrificans	0.024
	Streptomyces avermitilis	0.047
	Streptosporangium roseum	0.009
	Variovorax paradoxus	0.012
	Verminephrobacter eiseniae	0.041
Early RA	*Acinetobacter baumannii*	0.006
	Acinetobacter johnsonii	0.038
	Acinetobacter lwoffii	0.044
	Alistipes putredinis	0.003
	Cardiobacterium hominis	0.012
	Caulobacter segnis	0.030
	Clostridium phytofermentans	0.044
	Enhydrobacter aerosaccus	0.044
	Enterococcus casseliflavus	0.038
	Methylobacterium extorquens	0.015
	Methylobacterium nodulans	0.027
	Methylobacterium populi	0.006
	Methylobacterium radiotolerans	0.021
	Pseudomonas stutzeri	0.009
	Shewanella sp. ANA-3	0.006
	Sphingopyxis alaskensis	0.027
	Thiomonas intermedia	0.009

	Xanthobacter autotrophicus	0.044
	Xanthomonas campestris	0.015
	Diseased site	
HC	*Arthrobacter chlorophenolicus*	0.033
	Bacteroides sp. 2_2_4	0.033
	Clostridium hathewayi	0.018
	Dechloromonas aromatica	0.047
	Fusobacterium sp. 7_1	0.012
	Haemophilus parasuis	0.021
	Prevotella buccalis	0.027
	Slackia exigua	0.036
CCP+ at Risk	*Prevotella melaninogenica*	0.003
	Klebsiella pneumoniae	0.003
	Clostridium saccharolyticum	0.012
	Eubacterium yurii	0.012
	Porphyromonas gingivalis	0.030
	Prevotella disiens	0.033
	Herbaspirillum seropedicae	0.036
	Corynebacterium tuberculostearicum	0.038
	Streptococcus pseudoporcinus	0.038
	Prevotella bryantii	0.041
Early RA	*Capnocytophaga gingivalis*	0.003
	Cardiobacterium hominis	0.003
	Eikenella corrodens	0.003
	Neisseria gonorrhoeae	0.041
	Neisseria mucosa	0.006
	Neisseria sicca	0.009
	Neisseria subflava	0.033
	Streptococcus mitis	0.038
	Streptococcus oralis	0.003
	Streptococcus pneumoniae	0.009
	Streptococcus sanguinis	0.003
	Streptococcus sp. M143	0.006

Core species with significantly higher relative abundance in one of three groups were identified by using the permutation test (one-sided *signassoc* function, R). Sidak's correction was applied for multiple testing

Table S5. Topological properties of co-occurrence networks of species in the healthy control (HC), CCP+ at-risk, and early RA groups in periodontally healthy and diseased site samples of subgingival plaque.

Group	E [a] (positive%)	N [b]	Density [c]	AD [d]
Heathy site				
HC	347 (99.4)	173	2.01	4.01
CCP+ at-Risk	83 (100)	65	1.27	2.55
Early RA	1024 (99.7)	221	4.63	12.34
Diseased site				
HC	57 (86.0)	78	0.62	1.46
CCP+ at-Risk	139 (99.3)	66	2.11	4.21
Early RA	365 (100)	169	2.16	4.32

[a] E, number of edges;

[b] N, number of nodes;

[c] Density, ratio of edges to nodes;

[d] AD, average node degree;

Table S6. Functional units that were significantly over-represented in the CCP+ at-risk group or early RA group compared with the HC group in periodontally healthy site samples (adjusted *P* < 0.05, Wald test, FDR adjusted).

COG	Effect size	SE	Adjusted *P*-value	Lower 95%CI	Upper 95%CI
CCP+ at Risk *VS.* HC					
FOG: Transposase and inactivated derivatives	4.28	0.78	0.000	2.76	5.81
Glycyl-tRNA synthetase (class II)	2.39	0.49	0.001	1.43	3.35

Alpha-L-fucosidase	1.76	0.44	0.026	0.90	2.63
Beta-galactosidase/beta-glucuronidase	1.76	0.44	0.026	0.90	2.62
Predicted membrane-associated, metal-dependent hydrolase	2.36	0.60	0.026	1.19	3.53
Translation initiation factor 1 (eIF-1/SUI1) and related proteins	2.22	0.54	0.026	1.16	3.28
6-phosphogluconolactonase/Glucosamine-6-phosphate isomerase/deaminase	1.12	0.30	0.038	0.53	1.70
Glycine cleavage system H protein (lipoate-binding)	1.34	0.36	0.038	0.64	2.04
Malic enzyme	1.17	0.31	0.038	0.56	1.79
Predicted phosphatase/phosphohexomutase	1.33	0.36	0.038	0.63	2.03
Transposase and inactivated derivatives, IS5 family	1.55	0.41	0.038	0.74	2.35
Peptide chain release factor 1 (eRF1)	2.53	0.68	0.039	1.19	3.87
Serine/threonine protein phosphatase 2A, regulatory subunit	1.55	0.42	0.043	0.72	2.38
Component of SCF ubiquitin ligase and anaphase-promoting complex	2.03	0.56	0.047	0.92	3.13
Predicted ATPase of the PP-loop superfamily implicated in cell cycle control	0.92	0.26	0.047	0.41	1.43
Predicted glycosyltransferases	1.87	0.53	0.047	0.84	2.90
Selenocysteine lyase	1.00	0.28	0.047	0.45	1.55
Early RA *VS.* HC					
ATP-dependent 26S proteasome regulatory subunit	2.36	0.57	0.042	1.24	3.48
Component of SCF ubiquitin ligase and anaphase-promoting complex	2.75	0.64	0.042	1.49	4.01
Cysteine synthase	1.18	0.30	0.042	0.60	1.76
DNA helicase TIP49, TBP-interacting protein	2.27	0.58	0.045	1.13	3.41
Serine/threonine protein phosphatase 2A, regulatory subunit	1.95	0.49	0.042	1.00	2.91

Table S7. Functional units that were significantly under- or over-represented in the early RA group compared with the healthy control (HC) group and CCP+ at-risk group in periodontally diseased site samples (adjusted *P* < 0.05, Wald test, FDR adjusted).

COG	Effect Size	SE	Adjusted *P*-value	Lower 95%CI	Upper 95%CI

Eearly RA *VS*. HC					
Putative phage replication protein RstA	-5.71	1.18	0.003	-8.03	-3.39
Fe2+ transport system protein B	-3.92	0.85	0.004	-5.59	-2.24
SufE protein probably involved in Fe-S center assembly	3.89	1.10	0.042	-6.04	-1.74
dGTP triphosphohydrolase	-3.52	0.96	0.030	-5.39	-1.64
N-Dimethylarginine dimethylaminohydrolase	-3.00	0.82	0.030	-4.60	-1.40
Pyruvate:ferredoxin oxidoreductase and related 2-oxoacid:ferredoxin oxidoreductases, beta subunit	-2.70	0.68	0.020	-4.03	-1.37
Zn-dependent oligopeptidases	-2.67	0.72	0.029	-4.08	-1.27
Pyruvate:ferredoxin oxidoreductase and related 2-oxoacid:ferredoxin oxidoreductases, alpha subunit	-2.62	0.74	0.042	-4.07	-1.17
Soluble lytic murein transglycosylase and related regulatory proteins (some contain LysM/invasin domains)	-2.42	0.59	0.019	-3.57	-1.27
Long-chain acyl-CoA synthetases (AMP-forming)	-2.33	0.62	0.029	-3.55	-1.11
ATP-dependent Lon protease, bacterial type	-1.47	0.41	0.042	-2.28	-0.66
Ribosomal protein S19E (S16A)	4.21	1.06	0.020	2.12	6.29
TATA-box binding protein (TBP), component of TFIID and TFIIIB	3.55	0.88	0.020	1.83	5.28
Uncharacterized protein involved in cell differentiation/sexual development	3.47	0.87	0.020	1.76	5.19
ATP-dependent 26S proteasome regulatory subunit	2.77	0.61	0.004	1.57	3.96
U5 snRNP spliceosome subunit	2.60	0.70	0.029	1.23	3.97
DNA helicase TIP49, TBP-interacting protein	2.59	0.69	0.029	1.23	3.95
Small nuclear ribonucleoprotein (snRNP) homolog	2.38	0.65	0.032	1.10	3.65
20S proteasome, alpha and beta subunits	2.12	0.55	0.025	1.04	3.19
Citrate synthase	1.67	0.44	0.029	0.81	2.54
Early RA *VS.* CCP+ at Risk					
Fe2+ transport system protein B	-3.78	0.74	0.000	-5.23	-2.34
Ketopantoate hydroxymethyltransferase	-3.48	0.84	0.006	-5.13	-1.83
Na+-transporting NADH:ubiquinone oxidoreductase, subunit NqrD	-3.28	0.97	0.044	-5.17	-1.38
Glycogen debranching enzyme	-3.22	0.74	0.003	-4.66	-1.77
SufE protein probably involved in Fe-S center assembly	-3.18	0.96	0.049	-5.07	-1.29
Indolepyruvate ferredoxin oxidoreductase, alpha and beta subunits	-3.09	0.70	0.003	-4.46	-1.71
Outer membrane cobalamin receptor protein	-2.93	0.89	0.049	-4.67	-1.20
Cytochrome bd-type quinol oxidase, subunit 1	-2.88	0.78	0.018	-4.40	-1.36

Uncharacterized protein involved in exopolysaccharide biosynthesis	-2.74	0.82	0.049	-4.35	-1.12
Methylmalonyl-CoA mutase, N-terminal domain/subunit	-2.70	0.58	0.002	-3.84	-1.56
Pyruvate:ferredoxin oxidoreductase and related 2-oxoacid:ferredoxin oxidoreductases, beta subunit	-2.69	0.59	0.002	-3.85	-1.54
Glycyl-tRNA synthetase (class II)	-2.60	0.75	0.037	-4.07	-1.13
Predicted UDP-glucose 6-dehydrogenase	-2.56	0.66	0.011	-3.84	-1.27
Lysophospholipase L1 and related esterases	-2.523	0.639	0.011	-3.78	-1.27
Methylase of chemotaxis methyl-accepting proteins	-2.433	0.738	0.049	-3.88	-0.99
Cobalamin biosynthesis protein CobN and related Mg-chelatases	-2.331	0.707	0.049	-3.72	-0.94
Zn-dependent oligopeptidases	-2.316	0.617	0.016	-3.53	-1.11
Putative silver efflux pump	-2.297	0.589	0.011	-3.45	-1.14
DNA repair proteins	-2.279	0.636	0.026	-3.53	-1.03
DNA polymerase III, delta subunit	-2.110	0.620	0.044	-3.32	-0.90
NaMN:DMB phosphoribosyltransferase	-2.105	0.536	0.011	-3.16	-1.05
Phosphoserine aminotransferase	-1.954	0.563	0.037	-3.06	-0.85
Histidinol-phosphate/aromatic aminotransferase and cobyric acid decarboxylase	-1.511	0.447	0.044	-2.39	-0.63
2-polyprenylphenol hydroxylase and related flavodoxin oxidoreductases	-1.460	0.439	0.049	-2.32	-0.60
Fe-S cluster protector protein	4.007	0.956	0.005	2.13	5.88
ATP-dependent 26S proteasome regulatory subunit	3.234	0.522	0.000	2.21	4.26
Ribosomal protein S19E (S16A)	3.207	0.871	0.019	1.50	4.91
Dihydroorotase	3.142	0.697	0.002	1.78	4.51
Uncharacterized protein involved in cell differentiation/sexual development	3.052	0.723	0.005	1.63	4.47
TATA-box binding protein (TBP), component of TFIID and TFIIIB	2.907	0.730	0.010	1.48	4.34
Arginine methyltransferase-interacting protein, contains RING Zn-finger	2.706	0.706	0.013	1.32	4.09
20S proteasome, alpha and beta subunits	2.210	0.465	0.001	1.30	3.12
DNA helicase TIP49, TBP-interacting protein	2.202	0.583	0.016	1.06	3.34
Septin family protein	2.182	0.513	0.005	1.18	3.19
Aconitase B	1.950	0.575	0.044	0.82	3.08
Arginine/lysine/ornithine decarboxylases	1.789	0.444	0.009	0.92	2.66
Predicted GTPases (dynamin-related)	1.680	0.461	0.021	0.78	2.58
Ubiquitin	1.622	0.471	0.040	0.70	2.55
Citrate synthase	1.405	0.374	0.016	0.67	2.14

	Argininosuccinate synthase	1.189	0.357	0.049	0.49	1.89