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Effects of Statins on Multispecies Oral Biofilm Identify Simvastatin as A Drug Candidate Targeting *Porphyromonas Gingivalis*

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Abstract

Background: Statins effectively reduce risk of cardiovascular-related morbidity and mortality in patients with hyperlipidemia, hypertension or type-II diabetes. In addition to lowering cholesterol levels, several studies have attributed statins with immunomodulatory and bactericidal properties. Therefore, the aim of this study was to investigate statins' antimicrobial activity against relevant for periodontal homeostasis bacteria.

Methods: Statin effect on bacterial growth was tested using planktonic monocultures and multibacterial biofilms. The latter consisted of five microbial species (*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Actinomyces naeslundii*, *Tannerella forsythia*, *Streptococcus gordonii*) associated with dysbiosis of the oral microbiota underlying establishment and perpetuation of periodontitis.

Results: All four tested statins efficiently inhibited *P. gingivalis* growth and significantly decreased the cumulative bacterial load in developing and established biofilms. Simvastatin was most efficient and decreased *P. gingivalis* counts more than 1300-fold relative to the control.

Conclusions: These findings suggest that similar effects on bacterial composition of the dental plaque may occur in vivo in patients on statins thus leading to a shift of the oral microbiome from a dysbiotic to a more homeostatic one. Simvastatin, being highly effective against *P. gingivalis* while not affecting commensal microbiota, possesses many properties qualifying it as a potential

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adjunctive treatment for chronic periodontitis. Further studies are needed to evaluate whether similar effects on bacterial composition of the dental plaque may occur in vivo in patients on statins thus leading to a shift of the oral microflora from dysbiotic to a more homeostatic one.

Keywords

periodontitis; plaque control; antiplaque agents

Introduction

Statins inhibit hydroxymethylglutaryl coenzyme A reductase, the enzyme catalyzing the first step of cholesterol biosynthesis. By doing so, they reduce risk of cardiovascular morbidity and mortality in patients with hyperlipidemia, hypertension, or type-II diabetes.¹ Since their discovery 40 years ago,² a number of statins has been introduced to the market. Based on their origin, they are generally categorized into one of two groups. Obtained through fungal fermentation, type-1 statins such as simvastatin, lovastatin and pravastatin closely resemble their founding member, demastatin, while type-2 statins such as atorvastatin and fluvastatin are fully synthetic molecules.³

In addition to statins' cholesterol-lowering activity, several studies have demonstrated their anti-inflammatory properties.⁴ Our current understanding attributes these immunomodulatory effects to their capacity of lowering levels of intermediate metabolites in the cholesterol biosynthesis pathway.⁴ This plays a pivotal role in the regulation of cell growth, cell migration, apoptosis and cell trafficking.⁴ Decreased amount of these components inhibits post-translational prenylation of numerous proteins that function as molecular switches.⁴ In subjects at risk for cardiovascular disease, statins were also shown to down-regulate interleukin (IL)-8 and IL-6 production by epithelial cells,⁵ reduce the levels of C-reactive protein in serum⁶ and inhibit oxidized low-density lipoprotein-induced cell death.⁷ Furthermore, as statins were reported to greatly affect the process of bone regeneration via actions on mesenchymal stem cells, osteoblasts, endothelial cells, and osteoclasts, they sparked significant interest in the fields of tissue regeneration and tissue engineering.⁸

Statins have been investigated for antibacterial properties and several epidemiological studies have evaluated their effect on morbidity and mortality of patients suffering from infectious diseases.⁹ Although there is little evidence that *de-novo* administration of statins can significantly improve the outcomes of infections, they appeared to influence the course of infections indirectly. This may occur via the mechanisms affecting the rate of phagocytosis,¹⁰ stimulation of production of neutrophil extracellular traps (NETs)¹¹ or inhibition of bacterial escape from phagosomes in concert with promoting anti-inflammatory mechanisms.¹² Direct microbicidal effects of statins for numerous bacterial species were also reported.^{13,14} However, biological value of these studies is limited by the fact that antibacterial activity was assessed in planktonic cultures. This neglects the fact that *in vivo* most infections are caused by bacteria embedded and interacting within biofilms.¹⁵

Biofilm is a multilayered microbial community that adheres to solid surfaces. In such a structure the formation of physical barriers significantly enhances bacterial resistance to

antimicrobial agents.¹⁵ Additionally, in multispecies biofilms one species can bestow its protective capacity onto others. This can occur via mechanisms such as secretion of molecules counteracting therapeutic compounds and/or the sharing of resistance genes.¹⁵ Oral diseases such as caries and periodontal diseases critically depend on the capability of bacteria to form biofilm and that poses a real challenge for their treatment and prevention.¹⁶

According to the present consensus, a small consortium of bacteria can orchestrate the pathological changes in the periodontium during initiation and progression of periodontitis.¹⁶ By invading the commensal microbial community that populates a tooth's surface, these anaerobic and Gram-negative bacteria may trigger fundamental shifts in the composition of the dental biofilm, establishing a state of pathogenic imbalance also known as dysbiosis.¹⁶

Periodical mechanical removal of subgingival bacterial plaque by scaling and root planing is still the standard treatment for chronic periodontitis.¹⁷ Antibiotics may be administered systemically as an adjunctive therapy, but such a treatment is imprecise and leads to adverse effects.¹⁸ Importantly, repeated antibiotic-based regimens also contribute to the already substantial problem of antibiotic resistance among human pathogens such as *Staphylococcus aureus* and enteropathogenic *Escherichia coli*.¹⁹

Numerous studies have reported that statins improve clinical presentation of periodontitis, including gingival bleeding and loss of bone density and teeth.^{20, 21} The majority of these studies focused on immunomodulatory and bone-regenerating properties of statins. Hence, very little is known about direct effects statins may exert on periodontal pathogens and biofilm species composition. Thus, using planktonic cultures and biofilm models, the present study aimed at characterizing and quantifying the impact of atorvastatin, fluvastatin, lovastatin and simvastatin on the established periodontopathogens, *P. gingivalis* and *Tannerella forsythia*, and oral bacteria commonly present in dental plaque such as *Fusobacterium nucleatum*, *Streptococcus gordonii*, and *Actinomyces naeslundii*. In a broader context, these data assess the potential of statins as an adjacent treatment for periodontitis.

Materials and Methods

Bacteria strains and culture conditions

P. gingivalis ATCC 33277 was cultured in 3% tryptic soy broth (TSB; SigmaAldrich, St. Louis, USA), 0.5% yeast extract (YE; Bioshop, Burlington, Canada), 0.0001% menadione (SigmaAldrich), 0.05% L-cysteine (Bioshop) and 0.001% hemin (SigmaAldrich), *F. nucleatum* ATCC 25586 in 3% TSB, 0.5% YE and 0.05% L-cysteine, *T. forsythia* ATCC 43037 in 1.85% brain-heart infusion (BHI; BD, Franklin Lakes, USA), 1% YE, 0.0001% menadione, 0.001% hemin and 0.001% N-acetylmuramic acid (NAM; SigmaAldrich), *A. naeslundii* ATCC 12104 in 1.85% BHI, 1% YE, 0.0001% menadione and 0.001% hemin, and *S. gordonii* ATCC 10558 in 3% TSB. Agar plates were supplemented with 5% defibrinated sheep's blood (PWWiU Pro Animal, Wrocław, Poland). Bacteria were cultured under anaerobic conditions (80% N₂, 10% H₂ and 10% CO₂) at 37°C.

Minimum inhibitory concentration (MIC)

The strains were inoculated into the respective broths. Bacterial cultures were then diluted to optical density (OD) of 0.1 at 600nm. 200 µl of bacterial suspensions were transferred to 96-well plates. Stock solutions of atorvastatin, fluvastatin, lovastatin and simvastatin (SigmaAldrich) were prepared in DMSO and diluted to concentrations ranging from 0.25 µg/ml to the point of solubility. After addition of statins, the 96-well plates were incubated under anaerobic conditions for a minimum of 24 hours before OD_{600nm} was measured using a microplate reader FlexStation 3 (Molecular Devices, San Jose, USA) and bacteria were plated on agar. The lowest concentration of statin exhibiting no visible growth was considered MIC.

Biofilm assay

Susceptibility of multispecies biofilms to statins was assessed according to a protocol described previously.²² Following anaerobic overnight culture, the bacteria were suspended in broth containing 1% tryptone, 1% gelatin peptone (SigmaAldrich), 0.5% YE, 0.1% glucose (BioShop), 0.5% sodium chloride, 0.1% L-arginine, 0.1% sodium pyruvate (Sigma Aldrich), 0.0003% menadione, 0.001% hemin, 0.001% NAM, 0.0001% β-nicotinamide adenine dinucleotide (NAD; SigmaAldrich), 5% defibrinated blood and added to wells pre-coated with poly-L-lysine (SigmaAldrich). Statins were added at inoculation (referred to as developing biofilm) or 24 hours after inoculation (established biofilm). For any condition, all biofilms were harvested 48 hours after the addition of statins with their corresponding untreated controls. DNA extraction was performed using 6% Chelex 100 Resin (BioRad, Hercules, USA) suspension according to the manufacturer's instruction. The concentration of DNA was determined using NanoDrop 1000 (Thermo Fisher, Waltham, USA).

Scanning electron microscopy

Samples were cultured on glass slides in biofilm broth. Biofilm was allowed to form for 24 hours before atorvastatin and simvastatin were added in final concentrations of 254 and 45 µg/ml, respectively. After 48 hours of further incubation, slides were washed twice with PBS, fixed using 2.5% glutaraldehyde (SigmaAldrich) in 0.1 M sodium cacodylate (SigmaAldrich), pH 7.4, for an hour at room temperature, and dehydrated in ascending concentrations of ethanol. Following critical point drying, slides were coated with gold and mounted on stubs. Imaging was performed using JSM-7400F microscope (Jeol, Akishima, Japan).

Quantitative real-time PCR

To assess the amount of each bacterium in biofilm, the quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher) with equal volumes of extracted DNA. For each of the strains the primers (Table 1) were determined based on the relevant literature^{23–25} or kindly provided by prof. Sigrun Eick of University of Bern and used at the concentration of 5 µM. qRT-PCR was performed under the following conditions: 2 minutes at 95°C, 40 cycles: 15 seconds at 95°C, 1 minute at 60°C. The quality of amplification was assessed via the melting curves evaluation. Threshold cycles (Ct) were used to calculate the amount of bacteria in

reference to DNA standards specific for each strain, purified from single-species cultures with established colony forming units (CFUs).

Statistical analysis

All data was obtained via conducting three independent experiments, each performed in duplicate. To achieve symmetric data distribution, prior to comparison of group means, the data obtained from biofilms was log¹⁰-transformed. Missing values were not substituted and excluded list-wise for analysis and display. Mean amounts of bacteria per group were compared using one-way analysis of variance (ANOVA). As a post-test Dunnett's test was computed with the respective control group serving as reference. 2-tailed *p*-values of less than 0.05 were considered significant. For symmetrical representation of positive and negative fold changes (FC), these values were log¹⁰-transformed and plotted using antilog numbering. All analyses were computed using SPSS software version 23 (IBM, Armonk, USA).

Results

Simvastatin and lovastatin exhibit strongest antibacterial activity against *P. gingivalis*

Using microdilution assays, we determined bacteriostatic concentrations (Table 2) and MICs (Table 3) of atorvastatin, fluvastatin, lovastatin and simvastatin for the most important anaerobic periodontopathogens and commensal bacteria. Out of the five-microbial species examined, with bacteriostatic concentrations of 2 µg/ml for lovastatin and 1 µg/ml for simvastatin, we found *P. gingivalis* to be highly sensitive to statins (Table 2). Simvastatin was also potent against *S. gordonii*, *A. naeslundii* and *T. forsythia*. However, absolute concentrations of the other statins required to reach bacteriostatic values for these species were in most cases considerably higher. *F. nucleatum* was the most resilient to the direct antibacterial effect of statins. In case of simvastatin we established a minimum bacteriostatic concentration value that was 20-fold higher for *F. nucleatum* when compared to the one required for *P. gingivalis* (Table 2).

In correspondence with the patterns emerging from determining minimum bacteriostatic concentrations, MIC-values were the lowest for simvastatin. With MIC of 10 µg/ml, *P. gingivalis* was the most sensitive to this statin (Table 3). In contrast and with the exception of fluvastatin and lovastatin, vitality of *F. nucleatum* appeared to be unaffected by the compounds. Similar resistance was observed in case of *S. gordonii* and *A. naeslundii*, with the only statins affecting their growth being lovastatin and simvastatin. Atorvastatin showed generally weak antibacterial activity for any of the bacterial strains tested (Table 3).

Effects of statins on biofilm formation

Oral biofilm is the crucial extrinsic factor responsible for triggering, exacerbating and perpetuating periodontitis.²⁶ In this context, it is well established that periodontal pathogens embedded in the structure of biofilm show increased tolerance to antimicrobial agents.¹⁵ Therefore we investigated the impact of statins on the five periodontitis-associated bacteria organized as a multispecies biofilm. In this process streptococci and actinomycetes species represent the initial colonizers that are commonly followed by fusobacteria playing a pivotal

role in promoting co-aggregation of anaerobic bacteria such as *T. forsythia* and *P. gingivalis*.
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Based on the bacteriostatic and MIC-values established in planktonic cultures (Table 2 & 3), we used 3 different concentrations for each statin: a bactericidal, an intermediate, and a sub-bacteriostatic. In Figures 1 and 2 these concentrations are denoted as high, intermediate and low, respectively. As the degree of biofilm organization is known to be a crucial factor when determining efficacy of antibacterial compounds, we evaluated effects of statins on developing and established biofilm.

The developing biofilm was considerably more susceptible to statins than the established biofilm. The patterns of growth inhibition observed for the forming biofilm were in general consistent with the results obtained in planktonic cultures (Figure 1 and Table 2). When administered at high concentrations, all statins significantly decreased the number of *P. gingivalis* (Figure 1, mean FC = -240; mean $p = 0.003$). Significant inhibition in *P. gingivalis* growth was the major factor underlying the significant decrease in total bacterial load observed (Figure 1, mean FC = -4.2; mean $p = 8.6E-5$). Except in the case of lovastatin, intermediate concentrations were already sufficient to yield statistical significance (Figure 1, mean FC = -4.0; mean $p = 0.007$). While being highly efficient against *P. gingivalis* in forming biofilm, fluvastatin and simvastatin had no significant effect on the population-sizes of *F. nucleatum* (Figure 1). This dynamic led to the respective biofilm compositions to become increasingly *F. nucleatum*-dominated as statin concentrations increased (Figure 2). In contrast, affecting 3 out of 5 strains, atorvastatin had a more generalized but rather weak inhibitory effect on several species comprised in the biofilm (Figure 1).

Effects of statins on established biofilm

As mentioned above, it is assumed that increased structural organization characteristic of the established biofilm significantly alters a compound's properties with respect to its antibacterial activity.¹³ Indeed, in our experiments we observed that developing biofilm was much more susceptible to the statins as opposed to established biofilm. Overall, statin induced change in the total abundance of the biofilm, as well as any change in the abundance of particular specie in relation to the untreated controls significantly declined when statins were applied to established biofilm (Figure 1). Notably, while any statin significantly lowered the cumulative bacterial load of the developing biofilm, for the established biofilm this capacity was retained solely, and to a lower degree, by fluvastatin, in which case the inhibition of predominantly *P. gingivalis* growth was not countered by significant proliferation of competing species (Figure 1).

With significantly increased numbers when biofilm was cultivated in the presence of 45 µg/ml of either lovastatin (FC = 4.3; $p = 0.008$) or simvastatin (FC = 7.4; $p = 0.0002$), *S. gordonii* emerged as the dominating species in these biofilms. Its frequency rose from 16% (SEM 4%) measured in untreated established biofilm to 67% (SEM 12%) and 87% (SEM 4%) as a consequence of lovastatin and simvastatin administration, respectively (Figure 2). Correspondingly, in the same samples, for lovastatin *P. gingivalis* decreased (FC = -14.5, $p = 6.6E-05$) with its frequency dropping to 19% (SEM 10%) whereas for simvastatin *P.*

gingivalis load diminished 1321-fold ($p = 3.7\text{E-}8$), coinciding with its frequency declining to 0.08% (SEM 0.04%). In comparison, *P. gingivalis* was contributing with a dominating 73% (SEM 5%) to the makeup of untreated established biofilm.

Although to a much smaller degree, numbers of *A. naeslundii* were also significantly lower when the biofilm was exposed to high concentrations of simvastatin (FC -6.6 , $p = 0.002$). Interestingly, *F. nucleatum* did not take advantage of its resistance to statins to increase its absolute numbers in established biofilm, neither was it affected by fluvastatin concentrations well above its MIC. Thus, expansion of *S. gordonii* allowed the latter to dominate established biofilm gradually in dependence of increasingly higher concentrations of any statin tested (Figure 2).

Distinct changes in the biofilm structure were observed via scanning electron microscopy (Figure 3). Even in cases of established biofilms, their exposure to statins affected their physical structure considerably. This was especially obvious when assessing biofilm conformation in the presence of simvastatin. Bacteria grew less abundantly and formed fewer layers. Similarly, when treated with atorvastatin, biofilm formation appeared not as thick as measured for control specimens.

Discussion

Despite the advances in understanding the pathogenesis of periodontitis and the promising developments towards personalized medicine in other diseases, for decades the treatment modalities for periodontitis practically did not change. Patient care still follows the algorithm: *i*) review and reinforce oral hygiene, *ii*) perform scaling and root planing, and *iii*) periodontal surgery if the disease process has not yet been halted by the preceding measures. All these procedures reduce the bacterial load in the periodontal pocket and efficient control of subgingival biofilm is a pivotal component of successful treatment and long-lasting clinical improvements.^{27,28}

Although mechanical debridement is the primary strategy, systemic or topical administration of antibiotics is well recognized as an adjunctive therapy for both aggressive and chronic periodontitis.²⁹ A number of studies reported improvements of major periodontal parameters in patients treated with azithromycin,³⁰ metronidazole³¹ or quinolones³² when compared to subjects administered with placebo.

Bacteria populating the oral cavity commonly form biofilms that may not only comprise of hundreds of different taxa, but also collaborate to resist antimicrobial agents.³³ Rising numbers of antibiotic-resistant species are representing a serious problem as such resistances have recently emerged faster than the new classes of antibiotics could be developed.¹⁹ Moreover, most antibiotics for treating periodontitis are prescribed without analyzing patients' biofilm species compositions.³⁴ This might not only compromise treatment efficacy but also decimate the individual's commensal oral microbiome and further promote antibiotic-resistance.¹⁹

Due to their pleiotropic nature, statins have spurred interest with respect to their roles in disease prevention, and effects on morbidity and mortality of various medical conditions. In

this study we assessed the antimicrobial activities of fluvastatin, atorvastatin, lovastatin and simvastatin against oral bacteria most frequently found in dental plaque in the models of planktonic culture and the multi-species biofilm. The observed effects were clearly dose-dependent. The statin concentrations required for induction of significant antimicrobial activities were, however, clearly above the plasma concentration reached by atorvastatin (up to 66 ng/ml) or simvastatin (up to 34 ng/ml) when administered systemically (40 mg/day).³⁵ Thus we believe that in such situations local delivery systems for compounds such as simvastatin possess numerous advantages over systemic antibiotic treatment.

All statins tested here demonstrated their highest efficacy against *P. gingivalis* irrespective of the model studied. We believe this discovery to be highly relevant as today *P. gingivalis* is considered by many as the “keystone” pathogen of periodontitis^{16,17} due to its unsurpassed capacity to initiate a transition of a commensal oral microbiome to a state of pathogenic microbial imbalance.^{16,17} Issuing dysbiosis leads to exaggerated and perpetuated inflammatory processes underlying pathological changes in the periodontium.^{16,17} Selective elimination of *P. gingivalis* restored oral homeostasis, halted progression of periodontitis and prevented alveolar bone loss in animal models, including nonhuman primates.³⁶ In humans, difficulties to treat chronic refractory periodontitis was also associated with the presence of *P. gingivalis*. Preventing re-colonization of these specific sites by targeting *P. gingivalis* virulence factors via administration of monoclonal antibodies contributed to successful treatment of these cases.³⁷

Across all types of experiments performed here, simvastatin was the statin with the most potent antibacterial activity against *P. gingivalis*. While not having a significant impact on total bacterial counts in established biofilm, *P. gingivalis* was virtually eliminated from biofilms exposed for 48h to 45 µg/ml simvastatin. The thereby created niche was populated by the *S. gordonii*, which occurred in contrast to the relatively high susceptibility of this species observed in the planktonic cultures. Thus, we speculate that *in vivo* such a shift from *P. gingivalis* towards streptococci may rebalance the oral microbiome from a disbiotic state to a more homeostatic one.

F. nucleatum, with its ability to attach directly to salivary pellicle and create a bridgehead for further colonization, is the second key initiator species of oral biofilm.³⁸ When organized within established biofilm, its counts remained remarkably stable in the presence of statins. In contrast with *S. gordonii* however, *F. nucleatum* did not take advantage of the niche created as a consequence of the extreme sensitivity of *P. gingivalis* to statins in general and simvastatin in particular. Whereas the exact mechanisms underlying such selective antibacterial actions remain unclear, they appeared somewhat independent of bacterial cell structure. We found Gram-negative as well as Gram-positive strains to be similarly susceptible to statins irrespective of if they were cultured in planktonic or as multi-species biofilm.

In addition to their *P. gingivalis*-directed antibacterial activity, patients treated with statins could benefit from their anti-inflammatory effects. Appropriate immunomodulation could accelerate the return to homeostasis and promote tissue repair.³⁹ Although several studies, including randomized clinical trials, indicate that topical administration of statins can benefit

patients via reducing inflammation and improving bone regeneration,^{40,41} other investigations could not confirm these findings.⁴² Suggested by the data presented here, *P. gingivalis*-targeted antimicrobial activity of simvastatin may indeed represent a pivotal mechanism underlying the improvements of periodontitis observed.³⁹

Elimination of the pathogenic biofilms remains a major challenge as their resilience to antibacterial treatments renders antibiotic use difficult and, in a larger context, often questionable. For the statins tested here, to date only simvastatin effects on biofilm formed by *S. aureus*⁴³ or *Candida albicans* were tested.⁴⁴ In these models simvastatin significantly affected the development and viability of mature biofilm.^{43,44} Our study is the first to investigate four statins, prescribed to millions of individuals,¹ for their effects on major periodontopathogens as well as commensal bacteria associated with dental plaque. Statins demonstrated substantial antimicrobial activities and a remarkable specificity against *P. gingivalis* both in the planktonic culture and the developing biofilm. By more closely mimicking the situation *in vivo*, we observed a very substantial microbial shift when exposing established biofilm to concentrations of simvastatin appropriate for topical application. Governed by expansion of *S. gordonii*, such intervention led to an almost complete abrogation of *P. gingivalis*.

In conclusion, the results presented here provide the basis to hypothesize that, via selective targeting of *P. gingivalis*, simvastatin may lead to a significant improvement of chronic periodontitis. We believe that simvastatin should be explored further as a prototype compound when developing alternatives to antibiotics use. The compound possesses many qualities required for an adjacent treatment option for chronic periodontitis, where its anti-inflammatory properties could prove invaluable.

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References

1. Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *The Lancet* 2005;366:1267–1278.
2. Endo A, Kuroda M, Tanzawa K. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. *FEBS Lett* 1976;72:323–326. [PubMed: 16386050]
3. Ulivieri C, Baldari CT. Statins: from cholesterol-lowering drugs to novel immunomodulators for the treatment of Th17-mediated autoimmune diseases. *Pharmacol Res* 2014;88:41–52. [PubMed: 24657239]
4. Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* 2006;6:358–370. [PubMed: 16639429]
5. Ascer E, Bertolami MC, Venturinelli ML, et al. Atorvastatin reduces proinflammatory markers in hypercholesterolemic patients. *Atherosclerosis* 2004;177:161–166. [PubMed: 15488879]

6. van de Ree MA, Huisman MV, Princen HM, Meinders AE, Kluft C, Group DA-S. Strong decrease of high sensitivity C-reactive protein with high-dose atorvastatin in patients with type 2 diabetes mellitus. *Atherosclerosis* 2003;166:129–135. [PubMed: 12482559]
7. Chu W, Guan L, Huang D, Ren Y, Zhou Y. Lovastatin exerts protective effects on endothelial cells via upregulation of PTK2B. *Exp Ther Med* 2016;12:1741–1749. [PubMed: 27602089]
8. Shah SR, Werlang CA, Kasper FK, Mikos AG. Novel applications of statins for bone regeneration. *Natl Sci Rev* 2015;2:85–99. [PubMed: 26543666]
9. Bjorkhem-Bergman L, Bergman P, Andersson J, Lindh JD. Statin treatment and mortality in bacterial infections - a systematic review and meta-analysis. *PLoS One* 2010;5:e10702. [PubMed: 20502712]
10. Tanaka N, Abe-Dohmae S, Iwamoto N, Fitzgerald ML, Yokoyama S. HMG-CoA reductase inhibitors enhance phagocytosis by upregulating ATP-binding cassette transporter A7. *Atherosclerosis* 2011;217:407–414. [PubMed: 21762915]
11. Chow OA, von Kockritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL. Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe* 2010;8:445–454. [PubMed: 21075355]
12. Parihar SP, Guler R, Lang DM, Suzuki H, Marais AD, Brombacher F. Simvastatin enhances protection against *Listeria monocytogenes* infection in mice by counteracting Listeria-induced phagosomal escape. *PLoS One* 2013;8:e75490. [PubMed: 24086542]
13. Masadeh M, Mhaidat N, Alzoubi K, Al-Azzam S, Alnasser Z. Antibacterial activity of statins: a comparative study of atorvastatin, simvastatin, and rosuvastatin. *Ann Clin Microbiol Antimicrob* 2012;11:13. [PubMed: 22564676]
14. Bergman P, Linde C, Putsep K, Pohanka A, Normark S, Henriques-Normark B. Studies on the antibacterial effects of statins - *in vitro* and *in vivo*. *PLoS One* 2011;6:e24394. [PubMed: 21912631]
15. de la Fuente-Nunez C, Reffuveille F, Fernandez L, Hancock RE. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* 2013;16:580–589. [PubMed: 23880136]
16. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol* 2015;15:30–44. [PubMed: 25534621]
17. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol* 2010;8:481–490. [PubMed: 20514045]
18. Harvey JD. Periodontal Microbiology. *Dent Clin North Am* 2017;61:253–69. [PubMed: 28317565]
19. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015;13:42–51. [PubMed: 25435309]
20. Meisel P, Kroemer HK, Nauck M, Holtfreter B, Kocher T. Tooth loss, periodontitis, and statins in a population-based follow-up study. *J Periodontol* 2014;85:e160–168. [PubMed: 24304227]
21. Cunha-Cruz J, Saver B, Maupome G, Hujoel PP. Statin use and tooth loss in chronic periodontitis patients. *J Periodontol* 2006;77:1061–1066. [PubMed: 16734582]
22. Schmidt KE, Auschill TM, Heumann C et al. Influence of different instrumentation modalities on the surface characteristics and biofilm formation on dental implant neck, *in vitro*. *Clin Oral Implants Res* 2016;28:483–490. [PubMed: 27000771]
23. Dalwai F, Spratt DA, Pratten J. Use of quantitative PCR and culture methods to characterize ecological flux in bacterial biofilms. *J Clin Microbiol.* 2007;45(9):3072–6. [PubMed: 17596351]
24. Conrads G, Gharbia SE, Gulabivala K, Lampert F, Shah HN. The use of a 16s rDNA directed PCR for the detection of endodontopathogenic bacteria. *J Endod.* 1997;23(7):433–8. [PubMed: 9587296]
25. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol.* 1996;11(4):266–73. [PubMed: 9002880]
26. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *The Lancet* 2005;366:1809–1820.
27. Apatzidou DA, Kinane DF. Nonsurgical mechanical treatment strategies for periodontal disease. *Dent Clin North Am* 2010;54:1–12. [PubMed: 20103469]

28. Becker W, Becker BE, Caffesse R et al. A longitudinal study comparing scaling, osseous surgery, and modified Widman procedures: results after 5 years. *J Periodontol* 2001;72:1675–1684. [PubMed: 11811503]
29. Herrera D, Alonso B, Leon R, Roldan S, Sanz M. Antimicrobial therapy in periodontitis: the use of systemic antimicrobials against the subgingival biofilm. *J Clin Periodontol* 2008;35:45–66. [PubMed: 18724841]
30. Haas AN, de Castro GD, Moreno T et al. Azithromycin as an adjunctive treatment of aggressive periodontitis: 12-months randomized clinical trial. *J Clin Periodontol* 2008;35:696–704. [PubMed: 18631245]
31. Sgolastra F, Gatto R, Petrucci A, Monaco A. Effectiveness of systemic amoxicillin/metronidazole as adjunctive therapy to scaling and root planing in the treatment of chronic periodontitis: a systematic review and meta-analysis. *J Periodontol* 2012;83:1257–1269. [PubMed: 22220767]
32. Fujikawa K, Chiba M, Tanaka M, Sato K. *In vitro* antibacterial activity of DX-619, a novel des-fluoro(6) quinolone. *Antimicrob Agents Chemother* 2005;49:3040–3045. [PubMed: 15980395]
33. Abusleme L, Dupuy AK, Dutzan N et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J* 2013;7:1016–1025. [PubMed: 23303375]
34. Ellen RP, McCulloch CA. Evidence versus empiricism: rational use of systemic antimicrobial agents for treatment of periodontitis. *Periodontol* 2000 1996;10:29–44. [PubMed: 9567936]
35. Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacol Ther* 1999;84:413–428. [PubMed: 10665838]
36. Persson GR, Engel D, Whitney C, Darveau R, Weinberg A, Brunsvold M. Immunization against *Porphyromonas gingivalis* inhibits progression of experimental periodontitis in nonhuman primates. *Infect Immun* 1994;62:1026–1031. [PubMed: 8112836]
37. Booth V, Lehner T. Characterization of the *Porphyromonas gingivalis* antigen recognized by a monoclonal antibody which prevents colonization by the organism. *J Periodontol Res* 1997;32:54–60. [PubMed: 9085243]
38. Kolenbrander PE, Palmer RJ Jr., Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 2010;8:471–480. [PubMed: 20514044]
39. Lindy O, Suomalainen K, Makela M, Lindy S. Statin use is associated with fewer periodontal lesions: A retrospective study. *BMC Oral Health* 2008;8:16. [PubMed: 18482446]
40. Pradeep AR, Kumari M, Rao NS, Martande SS, Naik SB. Clinical efficacy of subgingivally delivered 1.2% atorvastatin in chronic periodontitis: a randomized controlled clinical trial. *J Periodontol* 2013;84:871–879. [PubMed: 23030241]
41. Seto H, Ohba H, Tokunaga K, Hama H, Horibe M, Nagata T. Topical administration of simvastatin recovers alveolar bone loss in rats. *J Periodontol Res* 2008;43:261–267. [PubMed: 18447852]
42. Saver BG, Hujoel PP, Cunha-Cruz J, Maupome G. Are statins associated with decreased tooth loss in chronic periodontitis? *J Clin Periodontol* 2007;34:214–219. [PubMed: 17257156]
43. Graziano TS, Cuzzullin MC, Franco GC et al. Statins and Antimicrobial Effects: Simvastatin as a Potential Drug against *Staphylococcus aureus* Biofilm. *PLoS One* 2015;10:e0128098. [PubMed: 26020797]
44. Liu G, Vellucci VF, Kyc S, Hostetter MK. Simvastatin inhibits *Candida albicans* biofilm *in vitro*. *Pediatr Res* 2009;66:600–604. [PubMed: 19707174]

Key findings:

simvastatin is able to decrease the amount of *Porphyromonas gingivalis* present in a multispecies biofilm by more than a 1000-fold when compared to control.

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Figure 1. Effects of statins on the growth of periodontitis-associated pathogens and dental plaque bacteria when organized as either developing or established multispecies biofilm.

Biofilm was inoculated to the wells of the 24-well plate and statins were added at low, intermediate and high concentration: atorvastatin (atorva) at 25 µg/ml, 125 µg/ml, 256 µg/ml, fluvastatin (fluva) at 16 µg/ml, 125 µg/ml, 256 µg/ml, lovastatin (lova) at 4 µg/ml, 20 µg/ml, 45 µg/ml and simvastatin (simva) at 1 µg/ml, 20 µg/ml, 45 µg/ml at two different timepoints: 0h for developing, and 24h for established biofilm. Following 48h incubation, the biofilm was collected, bacterial DNA was extracted, and biofilm abundance and species load were evaluated via qPCR. Changes in group means yielding statistical significance (ANOVA followed by Dunnett's post-test) are denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. P -values 2-tailed and corrected for multiple group comparison. Error bars indicate standard error of the mean (SEM).

Figure 2. Effects of statins on the frequency of individual periodontitis-associated pathogens and dental plaque bacteria when organized as either developing or established multispecies biofilm. Biofilm was inoculated to the wells of the 24-well plate and statins were added at low, intermediate and high concentration: atorvastatin (atorva) at 25 µg/ml, 125 µg/ml, 256 µg/ml, fluvastatin (fluva) at 16 µg/ml, 125 µg/ml, 256 µg/ml, lovastatin (lova) at 4 µg/ml, 20 µg/ml, 45 µg/ml and simvastatin (simva) at 1 µg/ml, 20 µg/ml, 45 µg/ml at two different timepoints: 0h for developing, and 24h for established biofilm. Following 48h incubation, the biofilm was collected, bacterial DNA was extracted, and species composition was evaluated via qPCR. Total bacterial load per condition was considered 100%, and the bacterial species composition was divided into two sections: 0–100% for the dominant bacteria constituting the biofilm, and 0–0.75% for the remaining species.

Figure 3. Representative scanning electron microscopy images of the established biofilm. Biofilm was allowed to establish for 24h, and then treated with atorvastatin at the final concentration of 256 µg/ml or simvastatin at 45 µg/ml or untreated (control). After 48h of incubation, the biofilm was fixed and prepared for imaging. The images were taken under the magnification of 1500x or 3500x.

Table 1.

Nucleotide sequences of the species-specific PCR primers.

	Nucleotide sequence
<i>Porphyromonas gingivalis</i>	5'- AGGCAGCTTGCCATACTGCG 3'- ACTGTTAGCAACTACCGATGT
<i>Fusobacterium nucleatum</i>	5'- AGAGTTTGATCCTGGCTCAG 3'- GTCATCGTGCACACAGAATTGCTG
<i>Tannerella forsythia</i>	5'- GCGTATGTAACCTGCCCCGA 3'- TGCTTCAGTGTGAGTTATACCT
<i>Actinomyces naeslundii</i>	5'- CTCCTACGGGAGGCAGCAG 3'- CACCCACAAACGAGGCAG
<i>Streptococcus gordonii</i>	5'- GCACTTGCAAAACACCCTGAA 3'- ACGAGTTGTTGCTGCAGTTG

Table 2.

Bacteriostatic concentration values for statins when assessed in single-species cultures.

	Atorvastatin	Fluvastatin	Lovastatin	Simvastatin
<i>Porphyromonas gingivalis</i>	25 µg/ml	16 µg/ml	2 µg/ml	1 µg/ml
<i>Fusobacterium nucleatum</i>	200 µg/ml	32 µg/ml	20 µg/ml	20 µg/ml
<i>Tannerella forsythia</i>	32 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml
<i>Actinomyces naeslundii</i>	25 µg/ml	32 µg/ml	16 µg/ml	6 µg/ml
<i>Streptococcus gordonii</i>	64 µg/ml	25 µg/ml	16 µg/ml	8 µg/ml

Table 3.

MIC-values for statins when assessed in single-species cultures.

	Atorvastatin	Fluvastatin	Lovastatin	Simvastatin
<i>Porphyromonas gingivalis</i>	285 µg/ml	245 µg/ml	30 µg/ml	10 µg/ml
<i>Fusobacterium nucleatum</i>	n/e	185 µg/ml	64 µg/ml	n/e
<i>Tannerella forsythia</i>	275 µg/ml	190 µg/ml	40 µg/ml	40 µg/ml
<i>Actinomyces naeslundii</i>	n/e	n/e	60 µg/ml	40 µg/ml
<i>Streptococcus gordonii</i>	n/e	n/e	64 µg/ml	35 µg/ml

n/e - statin exhibited no bactericidal effect at maximum used concentration (atorvastatin 400 µg/ml, fluvastatin 400 µg/ml, lovastatin 64 µg/ml, simvastatin 45 µg/ml).