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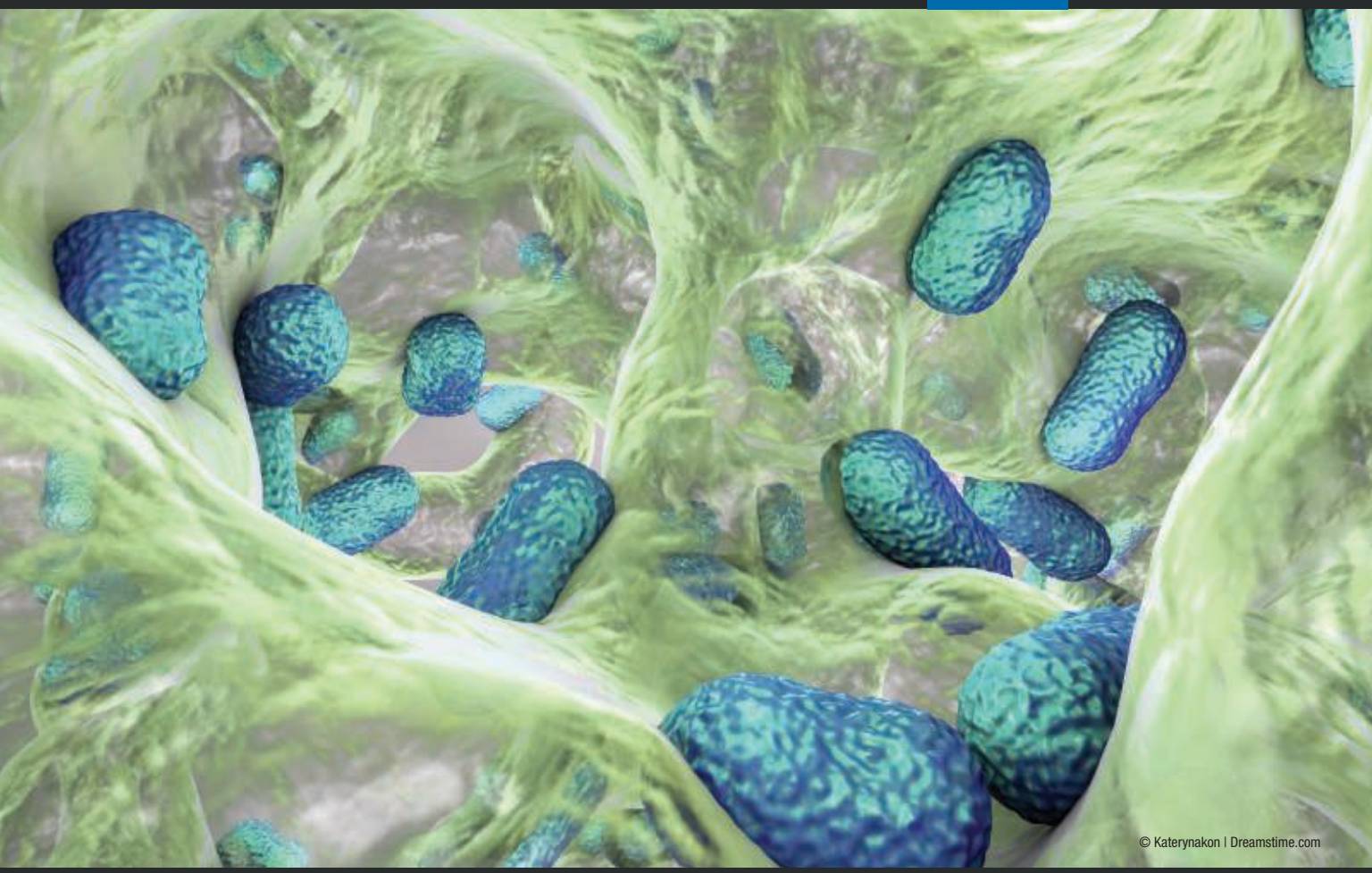

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Contemporary approaches to biofilm management in the 21st century's oral health crisis

A peer-reviewed article written by Lisa Dowst-Mayo, MHA, BSDH, RDH

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Contemporary approaches to biofilm management in the 21st century's oral health crisis

Abstract

Annual gross domestic product dollars spent on oral care in the US continues to decrease. Concurrently, prevalence rates of periodontal disease and the percentage of dentally uninsured adults is increasing. This public health crisis puts added pressure on dental practitioners to treat patients in oral and systemic dysbiosis. This course will discuss current research trends in biofilm management that utilize the most current technology available on the market. In order to emphasize the relationship between oral and systemic disease states, the oral microbiome states of symbiosis and dysbiosis will be presented along with the role genetics plays in the management of oral health.

Educational objectives

At the conclusion of this course, the dental provider will be able to do the following:

1. Develop a better understanding of health statistics in the United States as they pertain to dental services
2. Differentiate between symbiosis and dysbiosis and relate those concepts to the etiology of periodontal diseases
3. Identify the potential role genetics play in the predisposition and management of patients' periodontal disease status
4. Utilize the most current dental technology available on the market for biofilm management to promote oral symbiosis

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Introduction

The Centers for Disease Control and Prevention (CDC) estimates that 47.2% of adult Americans have periodontal disease.[1] That is almost one out of every two Americans over the age of 30. When you couple this statistic with the fact that only 45–57% of adult Americans ages 18–64 have dental insurance, it is unequivocally apparent we are facing a public health crisis in this country with regard to periodontal disease prevalence and control.[2] Of the percentage of adults with dental coverage, 14–29% did not utilize their benefits in 2018.[2] It's no wonder 47.2% of American adults suffer with periodontal disease.

In 2018, the United States spent 17% of GDP dollars on health services with only 4% on dental expenditures.[3] Americans spent more on prescription drugs with 10% GDP dollars than they did on their oral health.[3] In 2013, GDP dollars spent on dental services was 7%.[4] This sharp decline of 3% GDP for dental services between 2013 to 2018, coupled with the prevalence of periodontal disease, demonstrates a growing need for outcry from the dental community for the public's health and wellness.

Symbiosis and dysbiosis

When Americans lack dental insurance, their access to routine oral care becomes limited, and then disease rates inevitably rise. When the oral cavity trends toward disease, so does the patient's systemic health. Systemically, the human microbiome is composed of our genetic material, environmental influences, and microbiota (bacteria, fungi, protozoa, and viruses).[5] The composition of microbiota varies throughout the body. The most diverse microbiomes reside in the gut and oral cavity. When the microbiota and the human microbiome live in harmony with each other, this is termed symbiosis.[5] If there are any disruptions in this state of harmony, either from external or internal factors, then dysbiosis results and patients' health is adversely affected.[5]

When in a symbiotic state, the human microbiome can assist in regulating the immune system and provides a balance between pro-inflammatory and anti-inflammatory processes. When periodontal disease is present, the equilibrium of the oral ecosystem is disrupted, and the mouth enters dysbiosis.[5] It is during this time that the body is unable to regulate inflammatory responses efficiently and allows disease-promoting bacteria to populate the soft and hard tissues.[5] Dysbiosis in the mouth can occur due to many factors, such as tobacco use, poor oral hygiene, stress, saliva flow or composition change, alteration in diet, systemic diseases, genetics, or medications. Any dysbiosis in the oral cavity will eventually lead to dysbiosis systemically.

When active periodontal disease is identified in the oral cavity, the practitioner knows for certain at least two things: (1) the patient's oral microbiome is in dysbiosis and (2) plaque biofilm is present in quantities that have caused the dysbiosis. The best clinical service dental providers can render to their patients in this situation is effective biofilm removal. When biofilm insult is reduced, the symbiotic balance in immune inflammatory control—and the balance of pathogenic versus beneficial microbiota—will return. These internal changes will assist the patient in recovering from a state of dysbiosis back into a symbiotic relationship within his or her oral microbiome and subsequent improved systemic health.

Periodontal disease etiology

Periodontal diseases affect the epithelium, connective tissues, cementum, and underlying bony structures of the periodontium.[6] Plaque biofilm is the etiological agent of periodontal diseases, and when coupled with contributing factors, managing its progression becomes challenging to dental practitioners.[6,7] As biofilm matures, it creates an environment of dysbiosis through the initiation of the immune system's inflammatory response.

It is worthwhile discussing the role dental calculus plays in the pathogenesis of periodontal disease. It is easy for dental calculus to become the focus during hygiene procedures because its removal takes significantly longer than that of plaque biofilm; however, dental practitioners need to remember that calculus is not the etiological agent and keep their focus on biofilm removal to improve patient outcomes. Dental calculus is not a completely benign substance in the mouth, and its accumulation can contribute to the pathogenesis of periodontal diseases, so effective removal is still needed.

Dental calculus creates a roughened surface to which plaque biofilm adheres, and its nonmineralized portions appear microscopically as channels that contain bacteria and other debris.[6,8] Dental calculus formation begins with the mineralization of plaque. As it matures, it will contain both organic components (carbohydrates, lipids, proteins) and inorganic components (calcium phosphate, calcium carbonate, magnesium, sodium, potassium, fluoride, zinc, strontium, hydroxyapatite, octacalcium phosphate, magnesium whitlockite, brushite).[6,9] Removing the irritant of calculus during hygiene procedures will assist in decreasing plaque biofilm concentrations in the mouth, thus promoting a symbiotic relationship between oral tissues and the oral environment.

Genetic predisposition to periodontal disease

Exploration into the genetic origins of periodontal disease has been going on for decades. There is evidence to support that genes and gene polymorphisms (such as an allele that occurs in at least 1% of the population) play a role in the predisposition and progression of periodontal diseases.[7] Discovering genetic links to any disease leads to improved therapies, intervention strategies, and diagnostic criteria, so discovering specific allele variants (alternative form of a gene that is located at a specific position on a chromosome) that are associated with periodontal diseases can lead to improved clinical interventions and recommendations by dental providers.[7]

Genetic factors influence the inflammatory and immune responses in patients and can dictate their responses to changes in the oral microbiome.[7] People respond to antigens in ways predicted by their genes. For example, people with asthma will respond to an allergen via IgE hypersensitivity reactions that result in bronchial swelling, inflammation, and airway obstruction, while those without asthma may only suffer with a runny nose or cough.[10] The same holds true for patient responses to biofilm insults in the oral microbiome. Some may progress to periodontal disease faster and more aggressively than others, or they may be more resistant to traditional therapy approaches.

Genetic alterations/mutations at a single gene locus can change a patient's susceptibility to periodontitis. For example, mutation of the SOS1 gene has been identified in individuals with hereditary gingival fibromatosis.[11] The SOS1 gene determines whether cells grow, divide, or differentiate, and the only clinical manifestation of mutated SOS1 appears in the periodontium.[11] When treating periodontal disease clinically, providers need to remember that they may be battling a genetic response, and those genes may be altering a patient's response to rendered therapy.

AGGRESSIVE PERIODONTITIS GENETIC LINKS

A landmark study conducted by Boughman et al. in 1986 identified an autosomal-dominant form of localized aggressive periodontitis in one family.[12] This study showed statistically significant results that this form of periodontal disease had a link to a single gene locus on chromosome 4. Other studies have shown a significant association exists between generalized aggressive periodontitis and alleles associated with rheumatoid arthritis (DRB1).[7]

CHRONIC PERIODONTITIS GENETIC LINKS

Investigation into the genes that encode for interleukin-1 (IL-1) has shown an ability to detect a person's susceptibility to periodontitis; however, this genotype has limitations in specific racial and ethnic groups where its presence is absent.[7] Interleukin is a pro-inflammatory cytokine associated with immune and healing responses and is an inflammatory mediator commonly found in periodontal diseases.[6,7] Cytokines are small, secreted proteins released by cells. Interleukin is released by a leukocyte and acts on other leukocytes throughout the body.[13] The polymorphism genotype is one of several involved in the genetic risk for chronic periodontitis, and its presence confirms a person's risk for chronic periodontitis.[7]

The reality right now is we are unsure why some patients are more susceptible to aggressive or chronic periodontitis. While genetic testing is being conducted globally, it is important for clinicians to remember that periodontal disease is a multifactorial disease process involving systemic disease modifiers (metabolic disorders, cardiac diseases), environmental factors (smoking), and modifiable factors (oral hygiene, microbial phenotypes). Prevention of the disease is paramount and can be complicated by a person's genetic composition. If prevention fails, then controlling the progression of the disease becomes paramount in maintaining oral and systemic health.

Technology for biofilm management

Because twenty-first-century research has turned up astonishing information about the genetic relationship to the human microbiome and the oral biome's interplay with systemic health, it is now time to turn to dentistry's available technology designed to promote a symbiotic state through biofilm management. Since oral biofilms are resistant to chemical control, they must be controlled through mechanical disruption. Dental ultrasonics, air polishers, lasers, and ozone are examples of twenty-first-century adjunctive technology available to practitioners to combat the dysbiosis oral biofilm can cause as a component to a preventive and nonsurgical periodontal therapy protocol.

Even though clinical trials do not always demonstrate statistically significant differences in treatment outcomes for traditional nonsurgical therapies versus traditional therapies plus adjunctive technology, it does not mean this technology should be ignored. Dental ultrasonics, air polishers, lasers, and ozone are becoming widely used in the private practice sector and are taught in many dental and dental hygiene programs throughout the country. Keeping in mind the poor statistics for dental insurance and GDP dollars spent in the United States on oral health—coupled with periodontal disease prevalence rates, the potential genetic links that complicate care, and the ever-evolving field of microbiome influences in the human body—it is prudent for dental professionals to stay abreast of technology that has been developed in response to these issues.

ULTRASONICS

Ultrasound has been used in medicine and dentistry for many years. Medicine has utilized ultrasound, which is defined as anything above the frequency of audible sounds, for therapeutic and detection purposes (imaging).[14] Medical therapeutic uses include physical therapy (treatment of tendinitis, bursitis, and muscle strains), gall and kidney stone lithotripsy (shock waves that break up stones), uterine fibroid ablation, cataract removal, transdermal drug delivery, and bone fracture healing, to name a few.[14]

Ultrasound induces effects through heating and nonthermal mechanisms, such as cavitation.[14] The ultrasonic vibrations produced through ultrasound energy created in dentistry's piezoelectric and magnetostrictive machines create microscopic bubbles that collapse (cavitation) and produce intense shock waves that alter cell walls of bacteria.[15,16] If cell walls are altered, the bacteria will lyse, thus decreasing the levels of pathogens in the oral cavity to assist in reestablishing a symbiotic state.

Multiple studies have demonstrated that dental ultrasonics have the ability to effectively remove adherent biofilms, whether through direct or noncontact modalities, even at a site distant from the position of the working end against the tooth (acoustic microstreaming).[15–17] With multiple tip designs, shapes, and diameters, dental clinicians now have the ability to access even the most difficult of root morphologies. According to the 2002 American Academy of Periodontology (AAP) position paper, "Due to instrument width, furcations may be more accessible using ultrasonic or sonic scalers than manual scalers."[18] Ultrasonics also result in less root damage than traditional hand instruments as demonstrated in multiple studies over the past two decades.[15,18–20] According to the AAP position paper from 2000, "Ultrasonic scalers used at medium power seem to produce less root surface damage than hand or sonic scalers."[18] Traditional approaches to biofilm removal with hand instruments do not offer the same benefits that ultrasonic devices can deliver through cavitation and acoustic microstreaming.

AIR POLISHERS

Air polishing devices in dentistry vary by manufacturer. Some devices are intended for supragingival use and others for subgingival use. Air polishing devices have the ability to disrupt biofilm and remove tooth stains with their generation of a slurry of pressurized air, powder, and water. Typically, supragingival powder options are sodium bicarbonate (antacid), aluminum

trihydroxide, calcium carbonate, and calcium sodium phosphosilicate (bioactive glass), while subgingival options are glycine (amino acid) and erythritol (sugar alcohol).[16,21] Air polishers have been shown to reduce oral biofilm but will not remove dental calculus.

In a randomized clinical trial published in 2014 in the *Journal of Clinical Periodontology*, 50 previously treated periodontal patients with 457 pockets over 4 mm were monitored for 12 months. The control group received ultrasonic debridement, whereas the experimental group received subgingival erythritol with 0.3% chlorhexidine powder. At the 12-month evaluation, there were no statistically significant differences found in either group for pocket depths or microbial counts.[22]

Subgingival powders have been under investigation in recent years for their benefits in peri-implant mucositis management. In a randomized clinical trial published in the *Journal of Clinical Periodontology* in 2015, 37 patients with peri-implant mucositis were randomly assigned to treatment with either glycine powder or ultrasonic debridement and were monitored for 12 months. Both groups had statistically significant improvements in pocket depths, bleeding upon probing, and mean plaque scores, but there were no statistically significant differences between the groups.[23]

When compared to traditional rubber cup polishing, air polishing has been shown to be more effective at biofilm removal with less abrasiveness because it eliminates the clinician's choice in abrasive prophy pastes and amount of pressure applied with the handpiece.[21] The two most commonly used prophy paste abrasives are calcium carbonate and flour of pumice, both of which have a Mohs hardness rating of 5–7.[24,25] Air polish powders have a Mohs hardness rating of 2–3.[25] With smaller particle size, coupled with a lower Mohs rating, air polish powders are less abrasive to hard tissues and superior in biofilm reduction as compared to traditional rubber cup polishing.

LASERS

Soft-tissue dental lasers have been on the market since the 1980s, but have gained popularity in dentistry in the last two decades for their cutting and noncutting abilities.[26,27] Dental lasers of varying wavelengths have the ability to alter the microbial concentrations in the oral microbiome through either a cutting mode function or noncutting/noncontact function. Lasers have a bactericidal effect at their target site due to multiple mechanisms of action such as thermal effects and cellular alterations.[27,28] Many periodontal pathogens are readily deactivated at temperatures of 50˚C, and dental lasers produce thermal effects well beyond that threshold.[28] Through use of the laser in a noncutting mode for sulcular debridement, pockets can be decontaminated.[28] Some lasers advertise that their wavelength can remove dental calculus as well as reduce microorganisms.[27,28] There are many other uses for dental lasers, but this course is specifically focused on oral microbiome and biofilm alterations.

A systematic review and meta-analysis published in 2015 by the *Journal of the American Dental Association* evaluated scaling and root planing results compared to scaling and root planing with adjunctive aids, including the diode laser (wavelength 660–980).[29] The authors were a panel of experts chosen by the American Dental Association Council on Scientific Affairs, and they "conducted a search of PubMed (MEDLINE) and Embase for randomized controlled trials of SRP with or without the use of adjuncts with clinical attachment level (CAL) outcomes in trials at least 6 months in duration." The authors also assessed individual study bias using the Cochrane risk-of-bias tool. The authors concluded with a moderate level of certainty that the diode laser using photodynamic therapy improved clinical attachment levels (CAL) when compared to scaling and root planing alone.

Photodynamic therapy (PDT) is used in both medicine and dentistry. PDT does not use a laser in a cutting mode, and—as with most dental lasers—it utilizes laser wavelengths in the infrared or near-infrared section of the light spectrum. It was first introduced for the treatment of cancer in medicine, as it uses autography, a method of cell catabolism, and leads to the destruction of abnormal cells.[30] PDT in dentistry is used for its wound-healing and antibacterial effects, which are great benefits when treating patients with oral dysbiosis.[30]

OZONE

Ozone used in health care is also known as triatomic oxygen or trioxygen. Its usefulness in medicine and dentistry was first discovered in the late 1800s.[31] Medical grade ozone is a mixture of pure ozone and pure oxygen (O_2) in the ratio of 0.05–5% ozone to 95–99.95% O_2.[31] Ozone in dentistry can be used as a gas, dissolved in water, or used in ozonated oils.[31] Ozonated water is a powerful disinfectant that has antimicrobial properties, among other uses. The antimicrobial effect is a result of ozone's action on altering cell organelle function. It damages cell cytoplasmic membranes through the breakage of dual bonds and can modify intracellular proteins.[31] Ozone works destructively against gram-positive and gram-negative bacteria, fungi, and viruses found in the oral cavity.[31] It also alters the byproducts produced by microorganisms as well as necrotic debris.[32]

In a randomized, controlled clinical trial published in the *Journal of Periodontology Research* in 2015, 45 patients with chronic periodontitis were split into two groups, with one receiving scaling and root planing followed by irrigation with ozonated water and the other receiving scaling and root planing followed by irrigation of distilled water.[33] Both groups of patients demonstrated statistically significant improvements in plaque index, gingival index, bleeding on probing, and pocket depth. Neither group had statistically significant results different from the other. Similar results were noted in another randomized controlled clinical trial published in 2019 in the *Journal of Applied Oral Science*.[34] In this study, there were also no statistically significant changes in the scaling-and-root-planing-alone group versus scaling and root planing followed by water ozone application.

In a publication from 2013 in *Photomedical Laser Surgery*, an erbium laser was compared to topical gaseous ozone as an adjunct to scaling and root planing.[35] This study demonstrated statistically significant differences in attachment gain and probe depth in favor of the dental laser; however, there were no statistically significant differences in microbiological parameters.

Conclusion

As we learn more about oral and systemic links to health and disease through the

interplay of the human microbiome, dental practitioners have a call to action to stay abreast of changing recommendations, technology, and treatment modalities. The goal of any treatment is to maintain symbiotic states to ward off or control disease. The public health crisis of periodontal disease prevalence, coupled with the lack of dental insurance and GDP dollars spent on oral care, adds a new dimension to the field of dentistry. Providers need to think outside the box of traditional approaches to biofilm management and look to incorporate new technology into their practices given the twenty-first-century oral-health crisis that exists in America.

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AUTHOR DISCLOSURE

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LISA DOWST-MAYO, MHA, BSDH, RDH, graduated magma cum laude with a bachelor's degree in dental hygiene and a master's degree in health-care administration. She is a clinical educator for Dentsply Sirona. She is a former dental hygiene program director and full-time professor. She has published more than 40 peer-reviewed continuing education courses and articles since 2006. She can be contacted through her website at lisamayordh.com.

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QUESTIONS

1. What is the prevalence of American adults with periodontal disease?
   A. 35%
   B. 47.2%
   C. 52%
   D. 72%

2. What percentage of adult Americans age 18–64 have dental insurance?
   A. 45–57%
   B. 50–62%
   C. 55–65%
   D. 65–75%

3. What percentage of gross domestic product (GDP) dollars was spent on dental services in 2018?
   A. 2%
   B. 4%
   C. 7%
   D. 10%

4. What percentage of GDP dollars was spent on dental services in 2013?
   A. 2%
   B. 4%
   C. 7%
   D. 10%

5. What percentage of GDP dollars was spent on prescription drug services in 2018?
   A. 4%
   B. 7%
   C. 10%
   D. 17%

6. Which of the following can compromise the human microbiome and lead to dysbiosis?
   A. Genetic material
   B. Bacteria
   C. Fungi
   D. All of the above

7. What term is used when the microbiota and the human microbiome live in harmony with each other?
   A. Symbiosis
   B. Dysbiosis
   C. Microbiome
   D. Genetics

8. Which of the following can contribute to dysbiosis in the oral cavity?
   A. Tobacco use
   B. Poor oral hygiene
   C. Change in saliva flow or composition
   D. All of the above

9. Which of the following is the etiological agent of periodontal disease?
   A. Plaque biofilm
   B. Dental calculus
   C. Smoking
   D. Type II diabetes

10. Which of the following is an inorganic component of dental calculus?
    A. Carbohydrate
    B. Lipid
    C. Protein
    D. Sodium

11. Which of the following is true regarding dental calculus?
    A. Contains organic and inorganic components
    B. Contains both mineralized and nonmineralized portions
    C. Is the sole etiological agent of periodontal disease
    D. Both A and B

12. What is the term for an allele that occurs in at least 1% of the population?
    A. Gene
    B. Genetics
    C. Gene polymorphism
    D. Chromosome

13. In the 1986 study by Boughman et al., which chromosome was shown to have a genetic link to aggressive periodontal disease?
    A. Chromosome 2
    B. Chromosome 4
    C. Chromosome 7
    D. Chromosome 22

14. Which cells release the cytokine interleukin-1?
    A. Erythrocytes
    B. Leukocytes
    C. T-cells
    D. Platelets

15. Which technology produces anything above the frequency of audible sounds and is used for therapeutic and detection purposes in medicine and dentistry?
    A. Ultrasound
    B. Air polisher
    C. Laser
    D. Ozone

16. Which of the following is an example of a medical use of ultrasound technology?
    A. Gall and kidney stone lithotripsy
    B. Tendinitis
    C. Transdermal drug delivery
    D. All of the above

ONLINE COMPLETION

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QUESTIONS

17. Which term is used to define a mechanism of action of ultrasonic technology in which microscopic bubbles are created and collapse, producing intense shock waves that alter the cell walls of bacteria?
    A. Cavitation
    B. Acoustic microstreaming
    C. Amplitude
    D. Frequency

18. Which term defines ultrasound's ability to affect a site distant from the position of the working end?
    A. Cavitation
    B. Acoustic microstreaming
    C. Amplitude
    D. Frequency

19. Which of the following devices produces a slurry of pressurized air, powder, and water to disrupt biofilm and remove tooth stains?
    A. Ultrasonic
    B. Air polisher
    C. Laser
    D. Ozone

20. Which of the following air polishing powders can be used subgingivally?
    A. Sodium bicarbonate
    B. Aluminum trihydroxide
    C. Glycine
    D. Calcium carbonate

21. What is glycine?
    A. Amino acid
    B. Sugar alcohol
    C. Antacid
    D. Bioactive glass particle

22. In the 2014 publication of the *Journal of Clinical Periodontology*, with what chemical was erythritol mixed when comparing ultrasonic debridement to subgingival powder debridement?
    A. Calcium carbonate
    B. Chlorhexidine
    C. Silica
    D. Pumice

23. What is the Mohs range for air polishing powders used in dentistry?
    A. 2–3
    B. 4–6
    C. 5–7
    D. 7–8

24. Which technology uses varying wavelengths in or near the infrared zone of the electromagnetic light spectrum to alter microbial concentrations in the oral microbiome through either cutting or noncutting functions and harnesses its thermal effects to denature pathogens?
    A. Ultrasonic
    B. Air polisher
    C. Laser
    D. Ozone

25. At what temperature in degrees Celsius are periodontal pathogens deactivated?
    A. 20°
    B. 40°
    C. 50°
    D. 90°

26. Where in the electromagnetic light spectrum do most dental lasers fall?
    A. Visible
    B. Infrared
    C. Gamma
    D. X-ray

27. Which of the following procedures can be completed with laser photodynamic therapy?
    A. Wound healing
    B. Bacterial reduction
    C. Frenectomy
    D. Both A and B

28. Which technology has an antimicrobial action on cells by damaging their cytoplasmic membranes and altering intracellular proteins to adversely affect organelle function?
    A. Ultrasonic
    B. Air polish
    C. Lasers
    D. Ozone

29. What is the ratio of pure ozone to pure oxygen in medical grade ozone?
    A. 0.05–5% : 95–99.95%
    B. 1–5% : 90–95%
    C. 5–10% : 90–95%
    D. 5–10% : 95–99.5%

30. Which of the following forms of ozone can be used in dentistry?
    A. Gas
    B. Dissolved water
    C. Ozonated oil
    D. All of the above

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ANSWER SHEET

Contemporary approaches to biofilm management in the 21st century's oral health crisis

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Educational Objectives

1. Develop a better understanding of health statistics in the United States as they pertain to dental services
2. Differentiate between symbiosis and dysbiosis and relate those concepts to the etiology of periodontal diseases
3. Identify the potential role genetics plays in the predisposition and management of patients' periodontal disease status
4. Utilize the most current dental technology available on the market for biofilm management to promote oral symbiosis

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1. Were the individual course objectives met?

   Objective #1: Yes No Objective #3: Yes No

   Objective #2: Yes No Objective #4: Yes No

Please evaluate this course by responding to the following statements, using a scale of Excellent = 5 to Poor = 0.

2. To what extent were the course objectives accomplished overall?	5	4	3	2	1	0
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5.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		20.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
6.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		21.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
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8.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		23.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
9.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		24.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
10.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		25.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
11.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		26.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
12.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		27.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
13.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		28.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
14.	Ⓐ	Ⓑ	Ⓒ	Ⓓ		29.	Ⓐ	Ⓑ	Ⓒ	Ⓓ
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