THE UNIVERSITY OF MANITOBA

RELATIONSHIPS BETWEEN SALIVARY STEROIDS, CERTAIN BACTEROIDES AND GINGIVAL INFLAMMATION

BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

INDEX OF FIGURES

INDEX OF TABLES

INDEX OF APPENDIX TABLES

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ABSTRACT

Relationships between four steroids, determined by radioimmunoassay of whole saliva, and clinical and bacteriological parameters were studied in 90 subjects; males, nonpregnant females and pregnant females. Pocket depths and plaque- and bleeding scores were recorded. Anaerobically grown subgingival plaque from 9-14 subjects in each group yielded total counts (CFU), %Gram-negative organisms, %Bacteroides and %B. intermedius.

None of the clinical parameters for the pregnant women differed significantly from those of nonpregnant females, nor did these parameters show any significant correlation to progression of pregnancy. No correlations were detected between bacterial and clinical parameters in the pregnant group. There were no statistically significant differences between the CFU from the three groups, yet males had significantly higher proportions of Gram-negatives, Bacteroides and B. intermedius than females and pregnant women. Proportions of B. intermedius did not differ significantly between the two female groups, nor was there any correlation with progression of pregnancy.

While some steroids appeared to affect some clinical or bacteriological parameters in some groups, no obvious patterns, consistent with different steroid levels were detected. The results do not indicate that increased hormone levels cause more severe gingivitis in pregnant women, nor that high salivary steroid concentrations result in increased recovery of B. intermedius from subgingival plaque.

The study does not confirm earlier findings of the importance of steroids on certain constituents of the subgingival microflora.

INTRODUCTION

The knowledge of which organisms within the periodontal flora are pathogenic is still in its infancy. Several disease entities have recently been described for what has hitherto been collectively classified as periodontitis. While all of these diseases trace their origin to microbial plaque, differences in the composition of the floras associated with the various diseases are becoming evident. Currently, periodontal diseases have been placed into 5 groups.

Gingivitis, a reversible inflammation of the gingiva, may be a non-specific response, caused by increased proportions of several microbial species as a result of allogenic and autogenic successions within the plaque community as the habitat becomes more suitable to the needs of specific taxa. Conversely, specific organisms could be directly important in the etiology of gingivitis. Actinomyces spp., F. nucleatum, lactobacilli, treponemes and others have been implicated as periodontal pathogens.

It is not completely clear to what extent the various periodontal disease entities which involve tissue destruction are related. Based on preliminary studies Actinobacillus and Capnocytophaga ssp. have been associated with juvenile periodontitis (JP), and B. gingivalis, B. intermedius, F. nucleatum and several others with chronic adult periodontitis (CAP). Rapidly progressive periodontitis (RPP) bears certain similarities to both JP and CAP.

Only limited information is available on the microflora associated with acute necrotizing ulcerative gingivitis (ANUG). Various unidentified spirochetes are regularly seen in microscopic preparations, along with 'fusiforms'. The latter may be of many different taxa, including

Actinobacillus, Capnocytophaga, Fusobacterium, Bacteroides, and Leptotrichia.

Steroid hormones may affect periodontal health. Both the incidence and severity of gingivitis have been reported to increase at puberty and during other physiologic or drug-induced hormonal increases in the human body. In young menstruating females this is seen as a fluctuation in the severity of gingivitis during the normal menstrual cycle, peaking at the time of ovulation. The incidence and severity of gingival inflammation both increase as pregnancy progresses and with the use of oral contraceptives. All of the above changes in periodontal health could be related to elevated levels of steroid hormones which may locate in the gingiva.

Gingival tissues and fibroblasts from the periodontal ligament contain specific receptors for estrogen, progesterone and testosterone and enzymes metabolizing these steroids have been demonstrated in gingiva. These findings strongly suggest that gingiva and periodontal ligament are target tissues for steroids. However, histologic changes, similar to those seen in vaginal epithelium following hormonal changes, have not been described in the gingiva. This raises a question about the function of hormone receptors in the gingiva.

One mechanism whereby estrogen may influence periodontal health is via prostaglandin stimulation. Prostaglandins of the E group (PGE) induce inflammation and stimulate bone resorption, and inflamed gingiva contains greately increased concentrations of PGE2. As estrogen is known to stimulate synthesis of PGE2 in vitro it may affect periodontal health, in vivo.

Hormones may also affect periodontal health by stimulation of the subgingival microbial flora. B. intermedius has been implicated as one of the pathogens responsible for periodontal disease. This Gram negative anaerobic rod depends on other bacteria for its vitamin K requirement, as does B. melaninogenicus. Both species will grow when the female steroid hormones progesterone or estradiol are used in lieu of menadione (vitamin K1) to supplement growth media. Steroids may serve as vitamin K substitutes in vivo where increased concentrations of steroids may cause shifts in the subgingival microflora and favor the growth of some of the potentially more pathogenic organisms.

This study examines the relationships between four endogenous steroid hormones found in human saliva, certain species of oral Bacteroides, and gingival inflammation. The subject group studied included males, menstruating females and pregnant females.

LITERATURE REVIEW

Role of microorganisms in the etiology of periodontal diseases

The dento-gingival microbial flora is largely responsible for periodontal diseases. What is not clear, as of yet, are the mechanisms by which the microorganisms upset periodontal health, nor is it well understood which bacterial species constitute the primary pathogens. Bibby (1953) stated: "...almost every type of organism ever described has at one time or another been isolated from or, at least, observed in periodontal lesions." Furthermore, there is no proof that all organisms in the oral cavity have been accounted for (Socransky 1970). The correlation between dental plaque and periodontal diseases has been extensively reviewed (Socransky 1970, 1977; Kelstrup & Theilade 1974; Slots 1979, Van Palenstein Helderman 1981).

Over 300 years ago, Van Leeuwenhoek described living microorganisms in dental plaque which he termed animalcules (Tal 1980). Van Leeuwenhoek speculated on oral cleanliness, bleeding gums, stinking mouths and animalcules and apparently saw some link between bacteria and the condition of the oral tissues, but the matter was not pursued further.

The role of bacteria in the etiology of periodontal disease, therefore, remained obscure for another 282 years. Towards the end of this dark period, Bibby (1953) reviewed the existing evidence on the matter and stated: "...there is no incontrovertible evidence...that bacteria of themselves are ever primarily responsible for periodontal disease."

The classic proof for the causative role of bacteria in gingivitis comes from the work of Loe and his coworkers (1965). They showed that accumulation of bacterial plaque led to inflammation of the gingivae, whereas its removal reversed the situation. Later studies by Axelsson

and Lindhe (1974, 1981) have highlighted the value of long-term plaque control in the prevention of both caries and periodontal diseases in children and adults.

Bibby (1953) only recognized three different periodontal disease entities, i.e. acute gingivitis, marginal gingivitis and periodontitis. Recent studies have shown periodontitis to be a heterogenous group of diseases, sometimes sharing common symptoms, rather than a single disease with a spectrum of symptoms (Socransky et al. 1982a). Page et al. (1983a,b) recently described prepubertal periodontitis and rapidly progressive periodontitis as distinct clinical entities. Juvenile periodontitis (Manson & Lehner 1974), ANUG (Schaffer 1954), and chronic adult periodontitis (Moore et al. 1983) have also been identified as specific diseases. Further refinements of the present classifications of periodontal diseases are to be expected (Socransky et al. 1982a).

Problems in periodontal bacteriology

The first problem lies in the slow, chronic, cyclic nature (Goodson et al. 1982) of most periodontal diseases and in distinguishing between various disease entities (Socransky 1970; Ellison 1970; Socransky et al. 1982a,b; Page et al. 1983a,b; Moore et al. 1982a,b, 1983).

Due to ethical considerations, the irreversible destruction in periodontal disease prevents longitudinal observations of the natural course of the disease in humans. This has led to association studies in humans and longitudinal studies using animal models (Slots & Hausmann 1979; Socransky 1970; Loe 1971; Levy 1971; Jordan 1971). Socransky (1970) discussed the two greatest drawbacks of animal models; different indigenous floras prevent direct extrapolation of results and, secondly,

animal commensals interfere with the transplantation of human microorganisms. Levy (1971) concludes: "The task of translating the findings in animal studies to an understanding of human periodontal disease still confronts us."

The complex composition of plaque and its heterogeneity at different sites makes it difficult to study (Hardie & Bowden 1976). The single biggest problem is sampling (Dwyer & Socransky 1968). While older studies, which pooled plaque from many parts of the mouth, are now generally interpreted with caution (Hardie & Bowden 1976), the more recent practice of microsampling presents its own problems. Socransky (1970) reviewed the problem of sampling. He concludes that there is no 'representative' sampling method. Incomplete descriptions of bacteria in older literature and introduction of previously unknown microbial species recovered with new techniques further complicates these matters (Loesche, Hockett & Syed 1972).

Subgingival plaque is composed of two phases; a phase adherent to the tooth root which takes up about two-thirds of the volume, and a loosely adherent phase (Fine et al. 1978). Mousques, Listgarten and Stoller (1980b) addressed the effect of repeated sampling on the composition of subgingival floras. The results indicate a surprisingly little effect, but were partially obscured by improved oral cleanliness during the course of the study. Samples were taken at intervals of several days and the bacteria may have had time to repopulate the pocket. Gajewska and coworkers (1983), however, found that when sampling was repeated at short intervals, the first sample largely depleted the pockets. Previous periodontal treatment also alters the flora (Mousques, Listgarten & Phillips 1980a). The importance of proper subject selection is obvious.

Barnett, Baker and Olson (1982) recently raised a question related to the above, i.e. what is the effect of probing periodontal pockets on transmitting bacteria from site to site? Their results indicate that all bacterial forms which have been associated with periodontal disease can adhere to the periodontal probe and may thus be transplanted from periodontally involved sites to noninvolved sites. While no conclusion is reached as to the actual survival or implantation of bacteria riding on the probe between pockets, the study points out possible consequences of probing periodontal pockets in the process of selecting sites to be sampled. Transmission of bacteria may not have deleterious effects in spreading pathogens, but it is conceivable that this may lead to false results when reporting 'representative' species.

The sampling technique itself has also changed with time (Hardie & Bowden 1976). The change in sampling devices from scalers, curettes (Loesche et al. 1982; Moore et al. 1982 a, b, 1983) or paper points (van Palenstein Helderman 1975) to oxygen-free gas-flushed syringes (Newman et al. 1976; Newman & Socransky 1977) or more sophisticated devices (Gajewska et al. 1983), indicates the importance placed on proper sampling techniques. The cyclic nature of periodontal diseases (Goodson et al. 1982) also raises the question of when to sample and what changes in the flora take place with changes in disease activity.

The necessity of adhering to strictly anaerobic conditions at all steps from sampling to incubation (Gordon, Stutman & Loesche 1971) depends on the bacterial species being isolated. Loesche (1969) has shown that anaerobes can be divided into at least two categories. 'Strict anaerobes', which include many spirochetes and motile rods, do not grow on agar when oxygen pressure is higher than 0.5%. 'Moderate anaerobes',

including several species of Bacteroides, Fusobacterium nucleatum and others, not only grow on agar in the presence of 2 to 8% oxygen, but also remain fairly viable (14-103%) after being exposed to atmospheric oxygen for up to 6 hours. From 9-40% of each of 5 strict anaerobes similarly tested survived a one hour exposure to atmospheric air.

The composition of the anaerobic atmosphere and cultivation techniques are of further importance in maximizing viable counts. Gordon et al. (1971) studied the effects of varying the oxygen exposure and cultivation techniques of plaque samples on the viable count. These authors were able to cultivate from 24% to 70% of the microscopic count.

Another step in the handling of plaque samples that has recently come under scrutiny is the dispersal of bacterial aggregates. Since Williams and Eickenberg (1952) showed that ultrasonic treatment of salivary samples resulted in higher viable counts the method has been widely used. Despite the findings of Williams and Eickenberg (1952) that sonication results in a numerical shift in bacterial populations, the technique was adopted without too much study of its effects on the various microorganisms (Robrish et al. 1976). Only recently has proper attention been directed towards the deleterious effects of sonication (Leadbetter & Holt 1974; Hardie & Bowden 1976; Robrish et al. 1976; Manganiello et al. 1977).

In pure cultures, S. mutans has been found to tolerate 76 and 600 times more physical energy than B. fragilis and F. nucleatum, respectively (Robrish et al. 1976). Gram negative bacteria are much more susceptible to killing by ultrasonic dispersal than Gram positive genera. This inevitably results in both a qualitative and quantitative bias when cultivable counts are compared to microscopic counts (Leadbetter & Holt

1974). Furthermore, the medium in which the sample is dispersed and diluted is important. Dispersion in EDTA-supplemented reduced transport fluid (RTF) (Loesche et al. 1972), or in supplemented 25% Ringer's solution (Manganiello et al. 1977) yield higher viable counts than if either broth or unsupplemented RTF are used.

In view of the above, it is not surprising that older means of disrupting plaque aggregates have been revived or new ones invented. These include grinding, shaking with or without glass microspheres (Hardie & Bowden 1976) and repeated passage through pipettes or syringe needles (Williams, Pantalone & Sherris 1976). Loesche et al. (1982) found that shaking plaque samples for 20 seconds on a Vortex mixer followed by a 20 second sonication gave highest yields of gram negative bacteria but killed spirochetes.

No single medium can support growth of all microorganisms, nor are there selective or elective media available on or in which all species will grow (Socransky 1970). While selective media may facilitate recovery of strains present in low numbers (Socransky 1970) by preventing overgrowth of more numerous ones (Hardie & Bowden 1976), certain interactions between species, necessary for the survival of one or all of the participants (Kaufman et al. 1972; Mayrand & McBride 1980) could be fatally disrupted. Another disadvantage of selective media is that their selectivity is often too high, inhibiting the bacteria being sought, or too low, permitting growth of other species with similar requirements (Dwyer & Socransky 1968; Socransky 1970).

Incorporation of processed blood in culture media increases recovery of microorganisms from 72-309% in Brewer jars, but a slight decrease in counts occurs with Roll Tubes (Manganiello et al. 1977).

An often employed shortcut around time-consuming cultivation studies is microscopic study of cells from dental plaques. Morphological types of bacteria identified by dark-field microscopy correlate well with the morphology of Gram-stained samples, but poorly with cultivation results. More than a four times higher proportion of motile rods is seen in dark field microscopy as compared to anaerobic cultivation (Wilson, Woods & Ashley 1983). Dead cells are counted morphologically but not cultivated, which may account for some of the discrepancy between microscopic and viable counts. Microscopy has its value, however, in identifying organisms that have yet to be cultured.

Results from bacteriological studies are expressed and manipulated statistically in many ways but the most common quantitative expression is the percentage of the total viable count (Hardie & Bowden 1976). Relative proportions of microorganisms, however, ignore the qualitative aspects of plaque composition (van Palenstein Helderman 1981). Nonparametric analysis of the data from bacteriological studies is thought to be superior to parametric analysis of variance because of the variability in counts, interpretation of zero values, and transformation of data to a normal distribution (Socransky, Haffajee & Tanner 1983).

Microbial population shifts

Dental plaque is a complex and organized bacterial ecosystem. It is composed of metabolically interactive colonies of organisms embedded in a sparse matrix of bacterial and salivary origin (Frank & Houver 1970; Tinanoff, Gross & Brady 1976). With the exception of gingivitis, little information is available on population shifts in each periodontal disease entity in humans but some evidence is emerging from animal studies.

The oxidation reduction potential (Eh) of developing dental plaque and periodontal pockets was studied by Kenney and Ash (1969). They found significant differences in the Eh in periodontal pockets as compared to gingival sulci in both periodontally affected subjects and healthy controls, -48 mV and +73-74 mV, respectively. These changes can result from bacterial metabolism. V. parvula and streptococci, in particular, may reduce the Eh of the microenvironment and create a habitat suitable for other organisms (Kenney & Ash 1969).

The initially bacteria-free fetus is exposed to a variety of microorganisms on its short journey to infancy. However, the oral cavity appears to be sterile for a short while after birth (Hardie 1982), indicating that the oral environment is sufficiently different from the female genital tract to prevent bacterial transmission from mother to child at birth. Carlsson et al.(1970), Socransky and Manganiello (1971), Gibbons and van Houte (1975), Hardie and Bowden (1976) and Hardie (1982) have discussed acquisition of the oral flora.

The importance of bacterial attachments in the establishment of bacteria in infant mouths is evident. While S. salivarius, which has a predilection for oral mucous membranes, is invariably found after 1-2 days, S. sanguis, preferring non-shedding surfaces such as teeth, appears concomitantly with the eruption of teeth (Carlsson et al. 1970). Initially, microorganisms colonize in irregularities or on protected smooth surface areas close to the marginal gingiva, giving rise to microcolonies. Peripheral spreading of a single layer of bacteria results in coalescence with adjacent colonies and subsequent piling up of columnar microcolonies perpendicular to the tooth surface (Lie 1977).

It is well recognized that coccal-shaped organisms are the initial colonizers (Loe et al. 1965; Theilade & Theilade 1970; Eastcott & Stallard 1973; Listgarten, Mayo & Tremblay 1975; Socransky et al. 1977; Theilade, Theilade & Mikkelsen 1982) and predominate in one-day old plaque. Isolated elongated organisms, occasionally branching, may be seen (Listgarten et al. 1975). At two days the plaque becomes locally dominated by filamentous forms, growing in from the surface. These are often surrounded by pear-shaped cocci, attached to the filaments at the narrow end. The peculiar corn cob configurations are seen in 3-4 day-old supragingival plaque (Jones 1972; Listgarten, Mayo & Amsterdam 1973; Lie 1977). At one week, filamentous forms are replacing the deeper layers of coccal forms. At three weeks the plaque is predominated by spiral forms but large and small filaments are also seen (Listgarten et al. 1975).

The findings of attachments between dissimilar bacteria has led several workers to postulate the role of motile organisms in co-transporting nonmotile ones (Leadbetter & Holt 1978); the so-called 'piggy-back' hypothesis of subgingival colonization of nonmotile microorganisms (To, Sasaki & Socransky 1978).

Alexander (1971) has reviewed in detail the characteristics and the underlying factors governing microbial successions and establishment of 'climax communities'. Once a habitat becomes colonized successions usually proceed rapidly and thus the initial stages may go undetected as the pioneers are overgrown (Alexander 1971). Given time and relative stability in the environment, the climax community is established with only minor fluctuations in the population. As organisms die they are replaced by relatives and the climax community maintains itself.

Considerable increase in the amount of both supragingival and subgingival plaque is characteristic of developing gingivitis. Healthy gingiva is associated with a thin film of plaque, approximately 20 cells thick, while gingivitis is seen when the plaque reaches a thickness of 100-300 cells (Loe et al. 1965; Listgarten 1976). Gradual changes from a predominance of Gram positive cocci through additions of filamentous organisms and slender rods, vibrios and spirochetes are seen in experimental gingivitis (Loe et al. 1965). Cultural studies show a good correlation between increasing proportions of the facultative A. viscosus and A. naeslundi, and the onset of gingivitis (Williams et al. 1976). Loesche and Syed (1978) noted a significant increase in the proportion of A. israelii, at the expense of Streptococcus ssp, in initial gingivitis. A similar correlation was noted between A. viscosus and B. melaninogenicus and more severe, bleeding gingivitis. Almost identical proportions of Gram positive cocci, mainly S. mitis and S. sanguis, Gram positive rods, mainly composed of the three Actinomyces species; A. naeslundi, A. viscosus and A. israelii, and a group of Gram negative rods, have been isolated from chronic gingivitis subjects (Slots et al. 1978).

Mature plaque in gingivitis is very complex (Socransky 1970). The extreme variations have their roots in person-to-person variations and the different inflammatory stages at which samples are taken (Moore et al. 1982b; Evian, Rosenberg & Listgarten 1982). Based on extensive cultural findings, Moore and coworkers (1982b) disagree with others on the importance of Actinomyces species as pathogens in gingivitis. F. nucleatum, and species of the genera Lactobacillus, Streptococcus, Veillonella and Treponema were thought to be more likely etiological agents.

The microorganisms associated with juvenile periodontitis (JP) are difficult to identify. Significant proportions of the predominant Gram negative anaerobic rods could not be classified (Slots 1976; Newman & Socransky 1977). The Gram negative flora, almost exclusively facultative, capnophilic or anaerobic rods, comprised 63-66% of the predominant cultivable flora, as compared to 14-36% at uninvolved sites in the same subjects. Interestingly, the subgingival plaque appears to be less attached to root surfaces and present in much lower concentrations than that associated with other forms of destructive periodontal diseases, often resulting in minimal superficial inflammation (Listgarten 1976; Westergaard, Frandsen & Slots 1978; Liljenberg & Lindhe 1980).

The disease-associated microorganisms in JP appear, at present, to be Actinobacillus actinomycetemcomitans (A.a.) and Capnocytophaga spp., especially C. sputigena (Slots, Reynolds & Genco 1980). A.a.-like organisms have been found to invade the periodontal connective tissues (Saglie et al. 1982a,b). Carranza and coworkers (1983) also found Gram negative fusiforms and coccobacilli, spirochetes and Mycoplasma invading the tissues and in close association with resorbing alveolar bone in one 15 year old victim of localized juvenile periodontitis (LJP). A large number of the organisms resembled small Capnocytophaga and A.a.

Generalized JP appears to be associated with a different flora. B. gingivalis has been recovered in relatively high proportions from such patients (Loesche et al. 1981). It would not be surprising if the flora was found to resemble even more the one found in rapidly progressive periodontitis, especially in the older subjects with generalized JP.

Page and coworkers (1983a) have reported preliminary findings on 7 patients with rapidly progressive periodontitis (RPP) characterized by

extremely rapid bone loss and severe inflammation. It is not known to what extent RPP and JP are related. Some of the cases in the above study had a history of earlier JP, some became evident later in life and without the characteristic features of JP, i.e. localized angular bone loss around the first permanent molars. Still others occurred at the 'right' age for JP but without the characteristic pattern of bone loss. Based on immunological findings, B. gingivalis and A.a. are thought to be important etiological agents, while others, such as F. nucleatum along with unidentified species, are thought to be important too (Page et al. 1983a). Similar findings were seen in two cases of advanced periodontitis, one of which had a radiographic history of LJP (Tanner et al. 1979).

Evidence for tremendous heterogeneity in the microbial floras associated with 'periodontitis' has been reported although it is often quite conflicting. Recently some of the most extensive cultural studies on periodontal floras were published (Moore et al. 1982 a, b, 1983). Over 7500 isolates were characterized from 47 subjects. Comparisons between supra- and subgingival floras within individuals, as well as between variously diseased and noninvolved subjects were performed (Moore et al. 1983). Among those taxa that occurred in equal or greater numbers subgingivally in healty subjects than in patients with chronic periodontitis were all three species of Capnocytophaga and Bacterionema matruchotii. They were thus rejected as likely pathogens. Taxa which were only detected in one to four of the 22 subgingival samples and in low proportions include A.a., B. buccae, B. buccalis, B. capillosus, B. melaninogenicus and B. zoogleoformans.

When the remaining 28 taxa that predominated in the disease-associated subgingival flora were compared to those found in experimental

gingivitis (Moore et al. 1982a) the following was noted: Most taxa were also found in experimental gingivitis and thus were not introduced as a result of the disease. These included F. nucleatum, B. intermedius, B. pneumosintens, A. naeslundii, Peptostreptococcus anaerobius and Eubacterium brachy, all of which are known to be pathogens elsewhere in the body. Wolinella recta and A. israelii were also suspect pathogens. Of special interest were those taxa that occurred late in the gingivitis process, concurrent with bleeding gingivae. As this may be a transitory stage in the conversion of gingivitis to periodontitis these taxa, Eubacterium nodatum, B. intermedius and a Selenomas spp., are potential pathogens. B. gingivalis was considered to be an opportunist rather than a frank pathogen. Similarly, on the grounds of little difference between healthy and diseased sites, B. oralis and B. loeschii were rejected, while several Treponemes may be important in the pathogenesis of periodontitis. It was concluded that, while proportions may vary, etiological agents in chronic periodontitis and rapidly progressive periodontitis may be the same, the latter being an aggravated form of the former.

Few cultural studies have been carried out on acute necrotising ulcerative gingivitis (ANUG). Recently, Loesche et al. (1982) reported on the predominant cultivable flora from 11 sites in 3 ANUG patients. Samples from another 5 patients were partially identified. Spirochetes made up 30% of the microscopic counts. A significant number of organisms could not be identified. B. intermedius and Fusobacterium spp. were detected in all samples. Other frequently encountered taxa included, in descending order of frequency, A. odontolyticus, S. sanguis, Capnocytophaga spp., A. viscosus, and Veillonella. With the methods used, B. intermedius predominated, averaging 20 - 24% of the cultivable flora.

Steroids and the periodontium

Coles (1874) reported that gingival inflammation increased during pregnancy. Ziskin and coworkers (1933) implicated the possible role of hormones in gingival inflammation during pregnancy and reported that the incidence of pregnancy gingivitis was 70%. Subsequently, they introduced the term hormonal gingivitis to replace pregnancy gingivitis (Ziskin, Blackberg & Slanetz 1936). Maier and Orban (1949) reported a 55% incidence of gingivitis in pregnancy but Loe and Silness (1963) found gingivitis in all pregnant subjects.

Parfitt (1957), in a five-year longitudinal study on children, found increased incidence and severity of gingivitis coinciding with the onset of puberty. Similar changes have been observed in the periodontium during the normal menstrual cycle (Lindhe & Attstrom 1967; Lindhe & Bjorn 1967; El-Ashiry et al. 1970), and following the use of oral contraceptives (Knight & Wade 1974; Kalkwarf 1978; Pankhurst et al. 1981).

The gingivitis that results from oral contraceptive use appears to be time-related, increasing with the duration of use of birth control pills (Knight & Wade 1974; Pankhurst et al. 1981). Loe and Silness (1963), and Samant et al. (1976) noted a similar tendency of pregnancy gingivitis to increase with time. Kalkwarf (1978), however, found no correlation between duration of progestagen therapy and gingival health or plaque accumulation. He suggested that the known increase in gingival exudation in females taking oral contraceptives could have a washing-out effect on the disclosing agent used to detect plaque. Silness and Loe (1964) found no difference in the quantity or the character of oral debris between pregnant and post-partum subjects. They suggested that during pregnancy some factor, perhaps altered tissue metabolism, is

introduced which, together with plaque, may be responsible for the aggravated gingival inflammation. Their findings have been confirmed by Cohen et al. (1971). Others have reported either an increased (Samant et al. 1976), or decreased amount of plaque in pregnant women (Hugoson 1970; O'Neil 1979).

Lindhe and Attstrom (1967) studied changes in gingival exudation in young females during normal menstrual cycles and found significantly increased exudation on the day of ovulation when compared to the menstrual phase. The amount of gingival exudate correlated well with the estrogen level. No such fluctuations were seen in male subjects. They suggested that increased estrogen was responsible for the increased gingival fluid flow. Holm-Pedersen and Loe (1967) reported similar results, stating also that normal gingiva did not give rise to increased exudation, either during menstruation, pregnancy or post-partum. Increased gingival exudation has also been observed accompanying the intake of oral contraceptives (Lindhe & Bjorn 1967). The authors felt that this could be due to increased permeability of vessels of the sulcular plexus. Contrary to Holm-Pedersen and Loe (1967), Lindhe and Bjorn (1967) noted increased gingival exudation from clinically healthy gingiva without any corresponding increase in dental plaque.

Lindhe and coworkers (1969) found that progestagen increased gingival fluid flow in young women, especially during the menstrual phase. It is during the menstrual phase that ovarian production of estrogen and progesterone is low. Lindhe et al. (1968a) used an intracrevicular sampling method to demonstrate increased gingival exudation in gingivitis-free dogs receiving estrogen and progesterone. They showed that with an extracrevicular sampling method the influence of sex steroids could not

be demonstrated. In a following experiment they found a marked increase in gingival fluid flow from dogs with chronically inflamed gingiva when injected with estrogen or progesterone. Withdrawal of the hormone supply resulted in a rapid decrease in the exudation, indicating that steroids could affect the permeability of vessels of the sulcular plexus (Lindhe, Attstrom & Bjorn 1968b). Hugoson (1970) observed the same results in initially healthy gingiva of dogs regenerating after periodontal surgery.

Hormones could affect periodontal health through their effects on the subgingival microflora. Kornman and Loesche (1980) found a significant increase in gingivitis during the second trimester of pregnancy, and a proportional increase in the number of anaerobic bacteria, including B.intermedius. Both the gingivitis and the proportion of B.intermedius then decreased towards the termination of pregnancy. This is in agreement with the findings of Jensen and coworkers (1981) who reported a dramatic increase in Bacteroides ssp. in pregnant women and those using oral contraceptives.

Kornman and Loesche (1980) also found that the subgingival microflora took up significantly more female sex steroids during pregnancy. Steroid uptake correlated well with the proportions of B.intermedius in the plaque, which, on the other hand, showed a high correlation with plasma concentrations of female sex hormones.

Bacteroides intermedius depends on other bacteria for its vitamin K or analogue requirements as does B.melaninogenicus (Gibbons & MacDonald 1960). These two species will grow when female steroid hormones, i.e. progesterone or estradiol, are used in lieu of menadione to supplement growth media (Kornman & Loesche 1982). Despite substantial differences

in the chemical structures of steroids (Fig. 1) and vitamin K1 (Fig. 2) some steroid hormones, including estradiol and progesterone may serve as vitamin K1 substitutes. Increased concentrations of these steroids could cause shifts in the subgingival microbial ecology and favor growth of some potentially pathogenic organisms (Table 1).

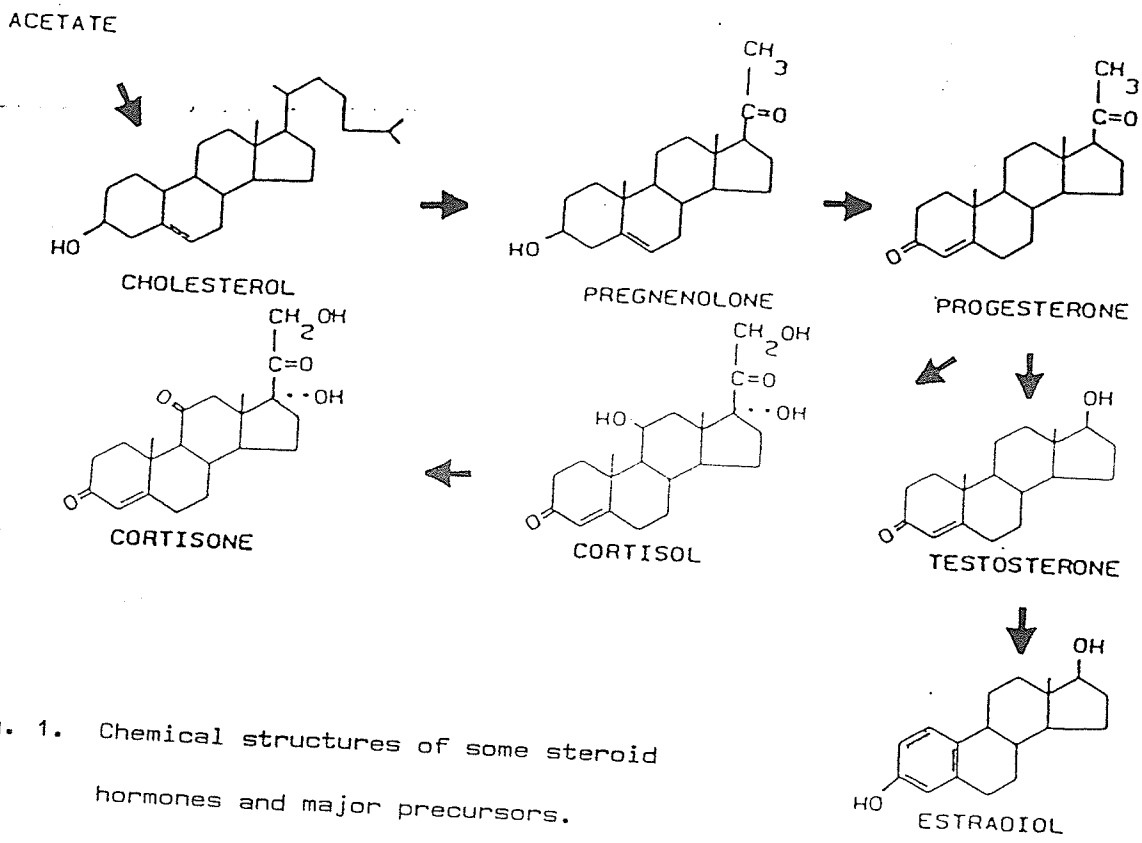

Fig. 1. Chemical structures of some steroid hormones and major precursors.

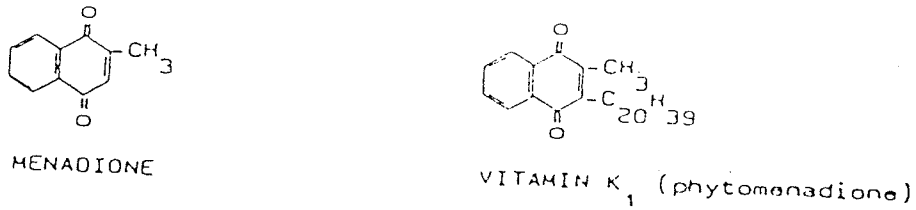

Fig. 2. Chemical structures of menadione (vitamin K_3) and vitamin K_1.

Table 1

Some potential pathogenic microorganisms in various periodontal diseases.

References	Gingivitis	Chronic Periodontitis	Rapidly Progressive Periodontitis	Juvenile Periodontitis
Savitt & Socransky (1984)	E. corrodens F. nucleatum C. gingivalis spirochetes	E. corrodens F. nucleatum spirochetes		A. a C. ochracea spirochetes
Tanner, Socransky & Goodson (1984)				B. gingivalis 'fusiform' Bacteroides spirochetes
Moore et al. (1983, 1982 a,b)	F. nucleatum Lactobacilli Streptococci Veillonella Treponemas	F. nucleatum B. intermedius A. naeslundi E. brachy W. recta A. israelii	same as chronic periodontitis	
Page et al. (1983)			B. gingivalis A. actinomycetemcomitans F. nucleatum	
Loesche et al. (1981)		B. gingivalis		
Slots et al. (1980)			C. sputigena A. actinomycetemcomitans	
Slots (1979)	F. nucleatum B. intermedius Actinomyces ssp.	B. gingivalis F. nucleatum	Bacteroides ssp. Unidentifiable anaerobes	
Syed & Loesche (1978)	B. melaninogenius A. viscosus A. israelii			

Kornman and Loesche (1980) observed a five-fold increase in the proportion of B. intermedius at the time of maximal gingival bleeding. However, a 16-fold increased proportion of black-pigmenting Bacteroides ssp. in a group of females who used birth control pills was not associated with a significant effect on gingival health (Jensen et al. 1981). In the latter study, however, gingival health was assessed according to the gingival index (Loe & Silness 1963) and, also, only the proportion of all black-pigmenting Bacteroides, collectively, was reported.

Vittek et al. (1979) evaluated the concentration of plasma hormones and metabolism of androgens in healthy and inflamed gingival tissues. Increased plasma concentration of progesterone was noted in the majority of subjects with periodontal disease. Subjects with normal gingival tissue had normal plasma levels of progesterone. The gingiva readily metabolized androgens. A significant correlation was reported between androgen metabolism in both sexes and the concentration of progesterone in plasma. The severity of periodontal disease and the metabolism of androgens also had a significant positive relationship in patients of both sexes.

Southren et al. (1978) demonstrated the existence of a receptor in human gingiva, both in males and females, that could specifically bind dihydrotestosterone (DHT). Hyperplastic gingiva, whether caused by pregnancy or was drug-induced (phenytoin), contained a significantly higher number of DHT binding sites per milligram of protein than did normal gingiva. Hence, gingiva may be a target organ for androgens. Ojanotko et al. (1980) reported increased testosterone metabolism in inflamed gingiva. Several different enzyme systems, capable of metabolizing testosterone, were found in the gingival tissue, again suggesting that the

gingiva could be a target tissue for androgens. Receptors for estrogen and progesterone have also been demonstrated in human gingiva (Vittek et al. 1982a,b).

Inflamed human gingiva metabolizes progesterone (El-Attar 1971) twice as fast as normal gingiva (El-Attar, Roth & Hugoson 1973). From the metabolites that were identified the presence of three steroid enzyme systems was revealed. This indicates that the gingiva is a target tissue for progesterone (El-Attar 1971). Inflamed human gingiva converts estrone to estradiol three times faster than normal gingiva (El-Attar & Hugoson 1974). Hyperactivity of certain enzyme systems involved in steroid metabolism, with the following accumulation of hormones, e.g. estrogen and progesterone, and their metabolites, may play a role in the etiology of periodontal diseases (El-Attar et al. 1973).

Female steroid hormones may have detrimental effects on the periodontium through their action on the synthesis of prostaglandins. Prostaglandins are mediators of inflammation and stimulate bone resorption. El-Attar (1976) found an 18 times higher PGE2 concentration in inflamed gingiva than in normal tissue. He also reported that estradiol alone, or combined with progesterone, caused a significant increase in the <u>in vitro</u> synthesis of PGE2. He suggested that "...hormonal gingivitis (e.g. during pregnancy) could be due to increased prostaglandin synthesis in gingival tissues as a result of increased levels of female sex steroids in the blood."

Not only the steroids of reproduction have been implicated in the etiology of periodontal diseases. Increased serum levels of glucocorticoids could also have a serious effect on the periodontium. In Cushing's disease patients exhibit elevated concentrations of cortisol and related

glucocorticoids. One of the prominent intraoral features of Cushing's disease is osteoporosis. Cortisone also retards wound healing by delaying the growth of granulation tissue (Shafer, Hine & Levy 1974). Blood glucose levels may rise to the extent of constituting adrenal diabetes which in turn may exhaust the insulin-producing cells and cause permanent diabetes mellitus (Guyton 1971). Corticosteroids also have a marked, but poorly understood effect on the immune response. Cupps and Fauci (1982) have reviewed the corticosteroid-mediated immunoregulation in man.

Steroid hormones in saliva

Concentrations of steroids have in the recent past been monitored almost exclusively in blood samples (Besch et al. 1984). The major disadvantages of using plasma to assess steroid concentration include a time-consuming sampling technique which requires highly skilled personnel, and repeated venipunctures which are distressing to the patients. Measurements of the biologically active, free fractions in plasma are technically very difficult, hence results are often expressed as the total steroid concentration (Riad-Fahmy et al. 1982, 1983; Besch et al. 1984; Landon et al. 1984; Truran,Read & Pearson 1984). Furthermore, increased cortisol levels of over 200% have been reported in stressful situations of either painful or anxious origin (Stahl & Dorner 1982), making results from venipuncture questionable (Landon et al. 1984).

Lipid soluble, free steroids in blood probably diffuse intracellularly through the acinar cells of salivary glands to enter the saliva. The protein-bound fraction is not sufficiently lipid-soluble to take this route, but minute amounts may escape by ultrafiltration via tight

junctions between acinar cells (Vining & McGinley 1984). Equilibrium between free steroids in blood and saliva, through the intracellular diffusion, is so rapid that, for all practical purposes, salivary concentrations of steroids are independent of the flow rate of saliva (Vining & McGinley 1984; Truran et al. 1984; Walker et al. 1984a). Following intravenous administration of cortisol in healthy adults, salivary cortisol levels reflect the changes in plasma cortisol levels within 1 minute, and peak within 3-4 minutes from injection (Walker et al. 1984a). Furthermore, steroid levels are comparable in parotid and whole saliva (Walker, Riad-Fahmy & Read 1978a).

Highly specific and sensitive radioimmunassays to measure steroids in saliva have been introduced and improved in recent years. All steroid hormones of diagnostic significance can now be assessed from samples of saliva. For comprehensive reviews of salivary steroid assays see Riad-Fahmy et al. (1982, 1983).

During the normal menstrual cycle, levels of estrogens and progesterone in saliva are low in the follicular phase, often below detection. Their levels rise sharply just before (estrogen) or at the time of ovulation (progesterone), decrease late in the luteal phase and at the onset of menses are very low again (Connor, Sanford & Howland 1982; Walker, Read & Riad-Fahmy 1978c; Donaldson, Jeffcoat & Sufi 1984).

Concentrations of total and unbound steroids gradually rise during pregnancy (Rosenthal, Slaunwhite & Sandberg 1969). Progesterone concentrations peak one to three weeks prior to parturition. Postpartum steroid concentrations in saliva are low and resemble those in the follicular phase (Connor et al. 1982).

High correlations have repeatedly been reported between concentrations of the various steroids in saliva and in matched plasma samples. Salivary hormones, which are free or non-protein bound, comprise between 1-13% of the total concentration of steroids in serum. Variations between the different steroids and, particularly, between various laboratories have yet to be explained (Besch et al. 1984; Landon et al. 1984; Walker et al. 1978a,b, 1980, 1984b).

Monitoring hormonal concentrations in saliva is cost-effective. Sampling can be done by the patient at home, samples can be stored frozen for prolonged periods of time and appear to be stable long enough at room temperature to allow mailing to laboratory. The noninvasiveness of the sampling technique ensures better patient cooperation and permits taking more frequent samples (Riad-Fahmy et al. 1982, 1983; Besch et al. 1984; Truran et al. 1984).

The purpose of this study was to try to determine whether any relationship exists between three clinical parameters, levels of four steroid hormones in whole saliva, and four bacteriological parameters, including the proportion of B. intermedius in subgingival plaque samples. The subject group studied includes males, menstruating females and pregnant females.

MATERIALS AND METHODS

Subject selection

Ninety healthy adults, 30 males and 60 females, who had not received any dental treatment for six months prior to the study, were examined. A minimum of 20 teeth, excluding third molars, were present in all subjects. Females were grouped into 2 groups, pregnant and not pregnant with normal menstrual cycles. Pregnant females were asked about their state of pregnancy. Subjects receiving any steroid therapy, including oral contraceptives, were excluded.

Clinical examination

Demographic data, including name, age, stage of pregnancy, time of last dental visit or professional cleaning, and medical history with emphasis on treatment with steroids, was aquired. The presence or absence of supragingival plaque was assessed and recorded for the four marginal aspects of each tooth by running a periodontal probe along the tooth after first gently drying the teeth with an air syringe. The results were expressed as plaque score, viz. the number of sites exhibiting plaque divided by all sites examined. Periodontal pockets were probed by a University of Michigan probe with Williams-O black markings and the depth (Van der Velden 1979) recorded to the nearest mm. Intrasulcular bleeding upon gentle probing of all interproximal sites was assessed by a modified periodontal pocket bleeding index (Van der Velden 1979), i.e. without the use of a standardized probing force of 0.75N. The results were expressed as an interproximal bleeding score, i.e. the number of sites exhibiting bleeding within 30 seconds of probing, divided by total number of sites probed.

Sampling methods: saliva

The subjects rinsed their mouth with water and submitted approximately 10 ml of whole saliva by spitting into disposable plastic test tubes. For women with normal menstrual cycles, salivary sampling was repeated on four consecutive weeks in order to calculate an average of the hormonal concentration throughout one menstrual cycle. Saliva was frozen at -20 °C until assayed for estrogen, progesterone, testosterone, and cortisol.

Sampling methods: subgingival plaque

Subgingival plaque was collected from 5 of the most periodontally involved sites in 9-14 subjects from each of the three groups. The sampled sites were selected on the basis of probed pocket depth and bleeding upon probing. Each individual's plaque samples were pooled in order to reduce the task of culturing and identifying the bacteria, and to provide a generalized observation of each subject. Sites to be sampled were isolated with cotton rolls, supragingivally scaled and polished with a tuft of cotton held by cotton pliers and gently dried. Paper points were then inserted into the depth of the pockets and left in place for 30 seconds, and immediately transferred to a vial containing 1 ml of prereduced RTF (Loesche & Syed 1973) supplemented with 0.01% laked horse blood (SR 48, Oxoid Canada) to protect the anaerobic bacteria in the sample. Samples were taken directly to the laboratory and cultured under conditions favouring Gram negative anaerobic rods as described below.

Isolation and enumeration of Bacteroides species

Immediately upon arrival to the laboratory samples were sonicated by placing the probe tip of a micro-ultrasonic cell disrupter (Kontes) in contact with the paper points for one minute at a setting of 4. Serial dilutions were made by transferring 100 µl aliquots of each sample to vials containing 0.9 ml of prereduced RTF with 0.5% laked blood, and mixed on a Vortex mixer (Scientific Industries, N.Y.) for 10 seconds. This procedure was serially repeated five times to obtain dilutions from 10^2 to 10^6. The diluted samples were then spread with a spiral plater (Spiral Systems, Ohio) onto 5% v/v sheep blood agar (BA) (Blood Agar Base No.2, C.M. 271, Oxoid Canada), supplemented with 5.0 µg/ml haemin and 0.1 µg/ml vitamin K1 (Holdeman, Cato & Moore 1977), and BA supplemented with 50 µg/ml Kanamycin (BAK). All procedures were performed on the bench but samples were not exposed to atmospheric air for more than 30-45 minutes.

The total number of colony forming units (CFU) was established from suitable BA plates after 5 days incubation at 37 °C in an anaerobic (80% nitrogen; 10% carbon dioxide; 10% hydrogen) chamber (Coy Manufacturing Co. Ann Arbor, MI). Plates with 200-300 colonies were chosen for counting. A cutting instrument was used to cut sectors of known size in the agar. Colonies within the sectors were counted and the numbers used to calculate the total counts based on multiplication factors provided with the spiral plater (Spiral Systems Inc., Ohio).

Colonies of each morphological type were selected from both media with the aid of a stereomicroscope. Their number and appearance on the media was recorded and they were transferred to 1/8 sectors of BA medium. These plates were incubated anaerobically for 3-4 days. Each

isolate was Gram's stained, and Gram negative rods and oval organisms were streaked on sectors of two BA plates each and incubated under anaerobic and aerobic atmospheric conditions for 3-4 days. Organisms which only grew anaerobically were subcultured onto a BA plate for further purification and characterization.

Identification of Bacteroides: biochemical tests

API 20A biochemical test kits (Analytab Products, Plainview, NY) were used to assess fermentation of 16 carbohydrates, hydrolysis of esculin, and production of gelatinase and indole (Moore, Sutter & Finegold 1975; Starr et al. 1973). Bacterial suspensions were made in basal medium supplied with the kits and used to inoculate 20 microtubes. Results were recorded after a 24-48 hour anaerobic incubation period at 37 ^{o}C.

Identification of Bacteroides: acid end-product analysis

Pure cultures were streaked on agar plates supplemented with 0.4% v/v laked horse blood and 0.5% w/v glucose (BAG), and incubated for 3-5 days. The agar was scraped free off bacteria with a steel blade, approximately one-fourth of the area cut into small cubes and placed into a centrifuge tube containing 1.5 ml of deionized water. Tubes were stored at -20 ^{o}C until assayed. Thawed samples were centrifuged at 4000 RPM for 20 minutes. One ml of the supernatant was recovered for acid end-product analysis by the method of Salanitro & Muirhead (1975).

Identification of Bacteroides: immunodiffusion

Suspensions of the test strains were prepared by scraping pure cultures of organisms from the agar with a sterile platinum wire loop.

Two loop-fulls were placed into 400 µl of 0.0625 M Tham (Tris) HCl buffer at pH 6.8. Bacterial aggregates were broken up by repeated passage through a sterile Pasteur pipette. The suspension was sonicated by a micro-ultrasonic cell disrupter (Kontes) for 2 minutes at a setting of 6, and frozen at -20 °C to aid the release of antigens. Agar slides for double-diffusion were made by dissolving one agar tablet (Oxoid ID agar BR 27, Oxoid, England) in 12 ml of 0.1M barbitone buffer (BR.11G, Oxoid, England) at pH 8.6 and adjusted to 50 ml with deionized water. The suspension was agitated and heated on a hot plate magnetic stirrer (Corning, PC 351) to dissolve the agar. After cooling to 50-60 °C in a water bath, the volume was readjusted to 50 ml with deionized water. Eleven ml of the agar solution were poured onto a leveled glass plate, 8.5 by 9.5 cm., and left to cool. Wells were cut in the gels with a cutting instrument, four sets of six wells surrounding central wells on each glass plate.

Rabbit antisera against a range of oral Bacteroides (Table 2) were placed in the central wells and the thawed bacterial Tham-suspensions in the peripheral wells. The glass slides were kept humid for 24-48 hours at room temperature and refrigerated for an additional 24 hours before they were examined under oblique light, at which time visible precipitin lines were manually recorded. The gels were washed in four changes of 0.85% saline at 4 °C over 24 hours. A final wash in deionized water for 2 hours removed residual sera and antigens. Sandwiching the slides in several changes of absorbent paper towels reduced the time required for drying. Completely dried glass slides were fixed for 2-3 minutes in 1% acetic acid and stained for 30 minutes in a filtered solution of 10% v/v glacial acetic acid and 90% v/v ethanol saturated with commassi blue. To

render the precipitin lines visible the slides were destained in several changes of a solution of 10% v/v glacial acetic acid, 85% v/v ethanol, and 5% v/v deionized water. Slides were then dried at 37 ^{o}C for 2 hours, or until completely dry, and photographed to provide permanent records.

Table 2

Strains and origins of Bacteroides used to produce antisera

Species	Strains	Source
B.asaccharolyticus	NCTC 9337	National Collection of Type Cultures
	BM-4	Dr. G.H.W. Bowden, clinical isolate
B.buccae	TIP 6	Dr. G.H.W. Bowden, clinical isolate
B.buccalis	HS-4	Dr. H. Shah, clinical isolate
B.corporis	ATCC 33574	American Type Culture Collection
B.denticola	Soc-1221	Dr. S. Socransky, clinical isolate
B.gingivalis	ATCC 33277	American Type Culture Collection
	W50	Dr. H. Werner, clinical isolate
	BH 18/10	Dr. G.H.W. Bowden, clinical isolate
B.intermedius	BH 18/23	Dr. G.H.W. Bowden, clinical isolate
	BH 20/30	Dr. G.H.W. Bowden, clinical isolate
B.levii	ATCC 29147	American Type Culture Collection
B.macacae	ATCC 33141	American Type Culture Collection
B.melaninogenicus	VPI 4196	Dr. J. Parker, clinical isolate
B.oris	ATCC 33575	American Type Culture Collection

Identification of Bacteroides: whole cell protein profiles

Three standard loopfuls (3 mm diameter) of cells from 3-4 day-old culture plates were placed in 300 µl of buffer in Eppendorf tubes (0.01 M EDTA, 0.15 M NaCl, 0.1 M Tris buffer) at pH 7.4, the suspension was mixed well on a Vortex mixer, then heated for 10 minutes at 60 °C.

The amount of proteins in the samples was determined by taking 50 µg of sample, adding 50 µl of 0.1N NaOH and mixing well. To this, 400 µl of deionized water were added and mixed. One hundred microliters were placed into a second test tube to which 5 ml of 1:4 solution of freshly made Bio-Rad Protein Assay Reagent (Bio-Rad, Rockville, NY) was added. This suspension was sonicated for 2 minutes and the optical density of the solution read, within 30 minutes, on a spectrophotometer (Ultrospec 4050, LKB Biochrom, Cambridge, England) at 595 nm. The results were calculated from a standard curve for bovine albumin. Wells were loaded with approximately 30 µg protein.

Samples of bacterial proteins were prepared by taking 50 µl of sample and adding 25 µl of 3 x sample buffer (15 ml glycerol, 7.5 ml 2-mercaptoethanol, 8.45 g sodium dodecyl sulphate, 8.0 ml 0.1% bromophenol blue, 0.88 g Tris buffer, with deionized water to 50 ml at pH 6.8). The resulting mixture was boiled for 10 minutes, then centrifuged for 15 minutes in an Eppendorf centrifuge. Calculated amounts of sample mixtures were placed in wells made in a 16 x 1.5 mm 12% polyacrylamide gel in a vertical electrophoresis apparatus (Hoeffer Scientific, San Francisco) and run to separate polypeptides by the method of Swindlehurst et al. (1977).

Resulting polyacrylamide gels were fixed for 16 hours in 10% v/v acetic acid in 45% v/v methanol, stained for 2 hours in 0.25% w/v

Comassie blue (0.25 g w/v in 100 ml 10% acetic in 45% methanol), destained in 10% v/v acetic acid in 45% v/v methanol, and stored in 7% v/v acetic acid until photographed for permanent recording.

Storage of isolates

Pure cultures were suspended in filter sterilized FD broth at pH 7.4 (1.0 g Tryptone (Difco), 0.5 g Yeast extract (Difco), 0.1 g Glucose (Fisher), 0.1 g L-Cysteine HCl (Sigma), 100 ml deionized water) supplemented with 1% fetal bovine serum (Oxoid). Two hundred microliters were pipetted into sterile glass ampoules plugged with sterile cotton. After centrifuging, the samples were freeze-dried overnight in vacuum and subsequently sealed by drawing the glass tubes to a closure with the aid of a gas torch. Representative strains from this study are thus available for future more detailed analysis such as isoenzyme determination (Shah & Williams 1982) and Western Blotting (Bowden & Nolette 1984).

Hormone assay procedures: general considerations

Hormone levels in saliva were measured by using commercially available radioimmunoassay (RIA) kits for assessment of plasma or serum concentrations of the four steroids. All the kits employ the I125 labelling of antigens to quantitate existing amounts in a given sample. Standard curves were produced by serially diluting respective steroids in a serum-free buffer. The sensitivity, or minimal detectable levels, of each assay are reported as 95% of initial binding, B/Bo.

Detailed methodology involved in using RIA for the measurement of testosterone (Landman et al. 1976), progesterone (Walker, Read & Riad-Fahmy 1979), estradiol (Riad-Fahmy et al. 1983), and cortisol (Walker et

al. 1978a) in saliva has been described. Minor modifications which were made to each kit in this study are described below.

Testosterone assay

I125 Testosterone kit (Radioassay Systems Laboratories, Inc., Carson, CA) was used in the analysis. Eight ml of ether were used to extract 0.6 ml of saliva. The organic phase was separated by freezing and the supernatant was decanted. The organic phase was dried completely under gaseous nitrogen, and the volume reconstituted with 0.6 ml of diluent buffer and 0.5 ml was used for the assay. The standards were diluted 1:10 in buffer and used at a volume of 0.5 ml. From then on, the assay procedure was followed. The sensitivity of the assay was 8.6 pg/ml. Inter- and intra-assay coefficients of variation were 21.4% and 4.5%, respectively.

Progesterone assay

Progesterone Coat-A-Count(R) kit was used (Diagnostic Products Corp., Los Angeles, CA). 0.6 ml of saliva was extracted with 8 ml of ether, separated and dryed as described above. Sample volume was reconstituted with 0.6 ml of diluent buffer and 0.5 ml transferred to coated assay tubes. The standards were diluted 1:5 in buffer and used at a volume of 0.5 ml. Sensitivity was 14.5 pg/ml. Inter- and intra-assay coefficients of variation were 15.9% and 12.2%, respectively.

Cortisol assay

Cortisol Coat-A-Count(R) solid phase I125 radioimmunoassays (Diagnostic Products Corp., Los Angeles, CA) was used according to the

instructions supplied with the kit, except for incubation which was 90 minutes instead of 45 minutes. The standards were diluted 1:20 in buffer as sample volumes were increased 20-fold from those suggested. Inter- and intra-assay coefficients of variation were 18.1% and 3.3%, respectively, and sensitivity 0.25 ng/ml.

Estradiol assay

I125- Estradiol Direct Radioimmunoassay Kit ER-155 (EIR, Wurlingen, Switzerland) was used according to instructions supplied with the kit except for the following modifications. For males and non-pregnant females one ml of saliva was used, for pregnant women 0.1 ml of saliva in 0.9 ml of deionized water. Samples were extracted with 8 ml of ether and the organic phase was separated by freezing. The supernatant was decanted and the organic phase dried with gaseous nitrogen. The samples were then washed twice with ether, dried with nitrogen and reconstituted with 0.25 ml of the buffer supplied with the kit. Inter- and intra-assay coefficients of variation were 23.4% and 4.8%, respectively, and sensitivity 0.20 pg/ml.

Data analysis

The significance of differences between means of variables for the three main groups as well as the three subgroups was tested by Duncan's new multiple range test (Duncan 1955). Pearson's correlation coefficient was used in the simple correlations between pairs of variables. Multiple regression analysis was used, where applicable to test effects of single variables from each group of parameters on variables in other groups. Statistical significance was established at 95% confidence level.

RESULTS

Clinical parameters

The means and standard errors of the means of the clinical parameters (age, pocket depth, plaque- and bleeding scores) are summarized in Table 3. The subgroups of subjects (Mb,Fb,Pb) from which bacteria were analysed are tabulated separately. Corresponding profiles for each individual subject are listed by the three main groups in appendix Tables 1A to 3A. For males, the age ranged from 20 to 64 years, females from 22 to 39 years, and pregnant women from 19 to 40 years. The mean pocket depths for the males had a range from 2.57 to 6.82 mm, while much smaller ranges were detected in females and pregnant females, 2.38 to 3.94 mm, and 2.12 to 3.84 mm, respectively. In all groups plaque score ranged from near zero to close to 100%. Bleeding score, similarly, had an upper limit of 100% in all groups, with a lower limit of 5% for pregnant females, and 15% and 39% for males and females, respectively.

Only one subject presented with near-healthy periodontal tissues, as assessed by the methods of this study. The majority of the subjects exhibited generalized marginal gingivitis and/or adult periodontitis. All males had one or more periodontal pockets of at least 5 mm depth, while three females (10%) and six pregnant women (20%) had no pockets deeper than 4 mm (Tables 1A to 3A). Male subjects, as a group, were statistically older and had significantly deeper periodontal pockets than either of the two female groups (Tables 3 & 6).

There were no significant differences between mean bleeding scores for the three groups, but the males had a mean plaque score which was significantly higher than the plaque score of females.

Pocket depth increased somewhat as pregnancy progressed, and mean plaque and bleeding scores for pregnant women peaked during the second trimester (data in Table 3A). At this time the scores were slightly higher than those for the non-pregnant females and approached the corresponding scores for the male group (Table 3). None of the three clinical parameters for pregnant women, however, showed a significant correlation with the progression of pregnancy.

Steroid hormone levels in whole saliva

The means and the standard errors of the means of the steroid hormone levels in whole saliva are shown in Table 4. Individual concentrations of the four steroids for each of the ninety subjects are listed, by group, in appendix Tables 1A to 3A. The three groups had significantly different mean testosterone levels, with males exhibiting the highest and females the lowest concentrations (Table 6). Pregnant females had concentrations of the three other steroids, progesterone, estradiol and cortisol, which were significantly higher than those from both males and females. The means of three of the four salivary steroid concentrations in the three subgroups were similar to those from the parent groups. The exception was cortisol, for which there were no significant differences between the means for the three groups.

Progesterone and cortisol levels were significantly correlated with weeks of pregnancy in both the main group and subgroup of pregnant women. In the main group of pregnant women estradiol levels were also related to the progression of pregnancy (Tables 9A to 10A).

Statistically significant positive correlations were found between progesterone and cortisol levels in all female and pregnant groups

(Tables 7A to 10A), and between estradiol and cortisol in the male (Table 5A) and pregnant (Table 9A) main groups. Only the pregnant main group showed a significant positive correlation between concentrations of salivary estradiol and progesterone (Table 9A).

A significant negative correlation was seen between age and testosterone levels in the male and pregnant main groups and female subgroup.

Bacteriological parameters

The means and standard errors of the means of the four bacterial parameters are tabulated for each subgroup in Table 5. Individual bacterial profiles for each of the 37 subjects, from whom plaque samples were analysed, are listed, by group, in appendix Table 4A.

The mean numbers of colony forming units within the samples were not significantly different for the three subgroups (Table 6). The male subgroup, however, had significantly higher percentages of Gram-negative organisms, Bacteroides as a group, and B. intermedius. There were no statistically significant differences between the female and pregnant subgroups for any of the means of bacterial parameters.

Correlations between hormonal and clinical parameters

Simple correlation coefficients between pairs of the variables studied are listed, by group, in appendix Tables 5A to 10A. Significant positive relationships, at a 95% level of confidence, were detected between testosterone concentrations and both the pocket depth and the plaque score in the female subgroup. Progesterone levels correlated positively with pocket depth in the pregnant subgroup, and with plaque score in the male group. Statistically significant negative

correlations were noted between testosterone concentrations and plaque score in the male subgroup; between testosterone levels and the bleeding scores of both the male subgroup and the female group; between cortisol concentrations and the bleeding score of pregnant women; and between estradiol levels and the bleeding score of the female group.

Significant results of the multiple regression analysis of the effects of hormonal and bacteriological parameters on clinical parameters, and of the hormonal on the bacteriologicalal, for the three main groups and/or the three subgroups, are depicted in Table 7.

Multiple regression analysis showed significant negative effects of testosterone on pocket depth in the male main group, and on plaque score in the male subgroup. Testosterone had a positive effect on pocket depth in the female subgroup, and on plaque score in both female groups, and a negative effect on bleeding score in the female main group. Progesterone had a significant positive effecte on the pocket depths of both pregnant groups and the male group as well as the plaque score of males. Cortisol negatively affected both pocket depth and plaque scores in both pregnant groups. Cortisol also negatively affected the bleeding score in the pregnant main group. Significant positive effects of estradiol were noted on pocket depth in the pregnant subgroup. Estradiol also had a positive effect on plaque score in the male subgroup and a negative effect on the bleeding scores of both pregnant groups.

Correlations between bacteriological and hormonal parameters

Simple correlations (Tables 6A,8A,10A) revealed significant positive relationships between the number of colony forming units and both progesterone and cortisol concentrations in the females. Gram-negative

organisms were positively related to progesterone and estradiol in males, and negatively related to progesterone in females. Percentage of Bacteroides was positively related to progesterone in males and cortisol in pregnant females. Percentage of B. intermedius showed a positive relationship with progesterone and estradiol levels in males, and to cortisol in pregnant women. However, a more powerful multiple regression analysis (Table 7) showed that only the number of colony forming units in the plaque samples had a positive correlation with cortisol in males and females. Table 8 summarizes results of Tables 7 and 5A through 10A.

Correlations between bacteriological and clinical parameters

Significant positive correlations were detected between the number of colony forming units in plaque samples and the pocket depth in the female subgroup (Table 8A), and between the percentage of Gram-negative organisms and plaque score for the subgroups of both males and females (Tables 6A and 8A). In the male subgroup there were positive relationships between the percentages of Bacteroides as well as B. intermedius, and pocket depth. In the female subgroup the percentage of Bacteroides in the plaque samples was positively related to plaque score. The male subgroup exhibited a significant negative correlation between the number of colony forming units and plaque score.

DISCUSSION

Clinical parameters

Subjects who participated in this study were selected from patients referred to the Postgraduate Periodontal Clinic at the University of Manitoba. As well, others were recruited from the support staff of other departments in the Faculty of Dentistry. These were mostly nonpregnant females. Others, mostly pregnant women, came from the nursing and medical staff of the Health Sciences Centre in Winnipeg. Availability of subjects, facilities and manpower dictated subject selection. Therefore, it was not feasible to try and match the subjects, according to age, socioeconomical status and extent or distribution of periodontal destruction. Further randomization of the population sample was not attempted.

Simple correlation showed a weak, yet statistically significant positive relationship between age and pocket depth in the male main group. This relationship, however, was not detected in the male subgroup or in any of the other four groups. It is possible that this could represent a chance finding in the male group or, conversely, that the males did, indeed, differ from the females in this respect. If the latter is true, it may reflect the fact that more male subjects apparently came from the lower socioeconimical groups than females and pregnant women. The relatively low number of subjects in the male subgroup may account for the lack of statistical significance between pocket depth and age in this group.

A statistically significant positive relationship was noted between age and plaque score in both male groups. The only other group to show such a correlation was the female subgroup, where it was negative. This apparent discrepancy may partly be contributed to the fact that the

subjects generally knew beforehand what would be involved in the clinical examination. The women, therefore, may have been more concerned about their oral appearance than the males. That there were no differences in the mean bleeding scores for the groups, despite differences in age, pocket depth and, especially plaque score, suggests that the apparently superior oral hygiene of the women had not been long-lasting.

Not only do levels of certain endogenous steroid hormones increase during pregnancy, but these levels are also sustained for a period of several months. Pregnancy is therefore a reversible physiologic state which is ideally suited for the study of the effects of prolonged increase in hormone concentrations on parameters of gingival health. In the present study, none of the clinical parameters for pregnant women differed significantly from those of the nonpregnant females, nor did those parameters show any significant correlation to the progression of pregnancy. These findings contradict the findings of Loe and Silness (1963) and, to some extent, those of Lindhe and Attstrom (1967), Knight and Wade (1974), Pankhurst et al. (1981) and Kalkwarf (1978) who have reported correlations between levels of either endogenous or exogenous steroid hormones, or their duration of use, and clinical parameters.

Steroid hormone levels in whole saliva

The results of the present study generally agree with other studies of the levels of salivary steroids (Riad-Fahmy et al. 1982).

Statistically significant differences between testosterone levels of the groups were noted (Table 6). While males had the highest levels, pregnant women had a significantly higher mean testosterone concentration than females. This difference, however, while obviously related to

pregnancy, was not related to its progression (Table 9A).

A negative correlation between age and testosterone levels in males, as noted in this study, is well known (Read & Walker 1984). This negative relationship was also detected in the female subgroup and in the main group of pregnant women.

Estrogens stimulate hepatic synthesis of cortisol-binding globulin (CBG) (Landon et al. 1984), and, thus, the total plasma concentration of cortisol increases considerably (Peters et al. 1984). Reports of unbound cortisol levels are, however, conflicting, likely due to differing methodologies used in assessments of the 'free' steroid (Rosenthal et al. 1969; Peters et al. 1984). Recently, salivary concentrations of cortisol have been shown to reflect quite accurately the plasma 'free' concentration (Landon et al. 1984; Walker et al. 1984a). Peters et al. (1984) found no statistically significant differences between salivary cortisol levels from groups of normal subjects and females taking oral contraceptives or in the third trimester of pregnancy, despite significantly higher plasma total cortisol concentrations in the two latter groups. In the present study, however, salivary cortisol levels were significantly higher in pregnant women than in the other groups and showed a statistically significant positive correlation to progression of pregnancy (Tables 6 & 9A). While the numbers of subjects in each group are comparable in the two studies, the time of sampling and the number of samples from each subject may account for the differing results. Rosenthal et al. (1969) reported a threefold increase in unbound plasma cortisol concentration during pregnancy. Total and unbound plasma progesterone levels were also increased significantly. Our findings of a statistically significant correlation between salivary progesterone levels and

progression of pregnancy, and a significantly higher progesterone concentration in pregnant women compared to nonpregnant females and men, agree well with Rosenthal et al. (1969). Progesterone has been found to exhibit increasing affinity for "transcortin" (CBG) in pregnancy, increasingly displacing cortisol from its inactive CBG-bound form (Rosenthal et al. 1969). The circulating concentration of CBG is very limited, and a significant increase in cortisol secretion would easily exceed the capacity of CBG and excessive cortisol would bind to albumin with much lower affinity, resulting in an increase in 'free' plasma and salivary cortisol concentrations (Landon et al. 1984).

Bacteriological parameters

In the present study, pooled subgingival plaque samples for limited bacteriological analysis, were obtained from 37 subjects. This sample size correlates favorably with many earlier studies. More extensive cultural studies on the oral microflora, in terms of number of isolates, not number of subjects, have only recently begun (Moore et al. 1982a,b, 1983). Such research requires extensive manpower, available only at a handful of the largest microbiological centres.

Established methods were used to identify the various Bacteroides species that were being looked for in this study. The general description by Holdeman and Moore (1974) of Bacteroides as non-spore forming Gram-negative, obligately anaerobic, non-motile uniform or pleomorphic rods or oval coccobacilli was adhered to. Gas liquid chromatography was used not to identify organisms to the species level, but rather to confirm the genus of the corresponding organism. Detection of large amounts of acids other than acetic or succinic, or both, would suggest

that the organism was not an oral Bacteroides (Holdeman, Cato & Moore 1977). B. gingivalis, however, is an exception, as this organism produces significant amounts of butyric and phenylacetic acids (Coykendall, Kaczmarek & Slots 1980).

Serological and fermentation tests revealed clusters of isolates within B. intermedius in which two very distinct groups of characters were detected. Additional clusters of B. intermedius strains emerged when results of the above tests were supplemented with protein profiles (polyacrylamide gel electrophoresis) of the organisms in question. Due to the time required for completion of such elaborate studies and the scant findings of such studies published so far, subdivision of B. intermedius has not been included in the data analyses in the present study. It is, however, obvious, that this species, as presently categorized (Holdeman et al. 1977), represents a group of related, yet distinctly different strains (Moore et al. 1982 a, b, 1983). This aspect is presently being explored further with clinical isolates from our study being compared to established reference strains.

Reports of actual values of the recovery of various organisms differ tremendously from laboratory to laboratory (Slots 1982). More than 250 bacterial species have been detected in association with periodontal diseases (Slots 1982). It is likely that another 50 to 150 have yet to be encountered (Socransky et al. 1982b). The lack of universally effective methods for cultivation of most of those organisms (Kornman et al. 1984) means that differences in results from different studies are to be expected.

Moore, Ranney and Holdeman (1982c) and Moore et al. (1983) found B. intermedius to be the most prevalent of the saccahrolytic pigmenting

Bacteroides in subgingival plaque. Collectively, two distinct strains of this organism represented 3.5% to 4.5% of the total flora at variously diseased sites in their studies. B. intermedius (Table 5) averaged 4.3% of the total anaerobic flora in the present study and agrees well with the results of Moore et al. (1982c). Our results also support the observations of Moore et al. (1983) in that more than one distinct strain within B. intermedius was recognized.

B. intermedius constituted 1.7% of the total flora in pregnant women (Table 5), with 1.6% and 1.85% in the second and third trimesters, respectively (can be calculated from Tables 3A & 4A), in the present study. This agrees well with the overall recovery rate of B. intermedius by Kornmamn and Loesche (1980) but does not confirm a significant difference in the proportion of this organism during the second trimester of pregnancy, when compared to the third trimester.

The weighed mean proportion of Bacteroides which were characterized as B. intermedius was 47%, ranging from 42.6% for females to 75.7% for males (Table 5). While B. intermedius was the most prevalent of all species of Bacteroides wich were analysed in our study when the subject sample was treated as a whole, this was not true for all subjects individually (Table 4A).

Moore and colleagues (1982c, 1983) detected B. intermedius in 26% to 50% of samples. The organisms were rarely recovered from healthy sites or from sites wich were categorized as having developed experimental gingivitis. Our plaque samples were pooled from the five most diseased sites, assessed by probing depth and bleeding upon probing. Furthermore, almost all of our subjects presented with at least some diseased sites. This may explain why, in the present study, B.

intermedius was discovered in 95% of the subjects, with 54% of the samples yielding the organism in more than trace proportions (1% or more, Moore et al. 1982b) of the total anaerobic flora.

The number of colony forming units within subgingival plaque samples from the three groups, as assessed by the methods of the present study, did not show statistically significant differences. Samples from males, however, had percentages of Gram-negative organisms, Bacteroides species, and B. intermedius which were significantly higher than those recovered from either females or pregnant women. While the means of all four bacteriological parameters for the pregnant group were the lowest of the three groups, they did not differ statistically from those of other females. Our results confirm the findings of Kornman and Loesche (1980) of a nonsignificant difference in both the total bacterial count and proportion of Gram-negative anaerobes between females and pregnant women (Tables 5,6).

The percentage of B. intermedius showed, as could be expected, a strong positive relationship to the collective percentage of Bacteroides in all groups. The percentage of Bacteroides in samples from males and females, similarly, showed a strong positive correlation to the proportion of Gram-negative organisms in the samples. Such a relationship was conspicuosly absent for the pregnant group. Surprisingly, none of the proportions of organisms showed any correlation to the total number of organisms wich could be recovered under the anaerobic conditions of this study. The complexity and variability of the subgingival microflora may be such (Socransky et al. 1982b, Moore et al. 1982a,b, 1983) that a much larger sample size would be required before correlations of this nature would become detectable with present-day methods. Increased specificity

and sensitivity of existing taxonomic testing procedures, and conception of new technology to deal with the variable and, apparently, constantly changing composition of plaque organisms are neccessary steps towards our full understanding of subgingival microbial ecology and, through this, the control of periodontal diseases.

Correlations between hormonal and clinical parameters

Simple correlations between hormonal and clinical parameters showed very randomized relationships between pairs of the seven parameters studied (Tables 5A to 10A). While some clinical parameters appeared to have statistically significant positive correlations to some steroids for some of the groups, the same pairs often exhibited no relationship, or a negative one, in other subject groups. Furthermore, with the introduction of the more powerful multiple regression analysis many of these apparent correlations disappeared, while others sometimes emerged (Table 8). No obvious patterns were thus detected for these correlations which were consistent with the various steroid concentrations in the three groups. O'Neil (1979) studied plasma concentrations of progesterone and estradiol in pregnancy and their correlation to gingival inflammation. He reported poor correlations between these parameters, similar to those noted in the present study.

Vittek and coworkers (1984) have reported on the salivary steroid concentrations of two groups of subjects, males and cycling females, which resemble two of the three groups in the present study. Cycling females, however, submitted saliva samples in the follicular phase only, and not throughout a whole cycle as in our study. Furthermore, some of their 17 cycling females presented with hirsutism and deep voice whereas

none of the 30 non-pregnant females in our study showed these male-like characteristics. Subject selection or geographical population differences may be responsible for this discrepancy. In the above study, increased concentrations of progesterone were related to periodontitis in both subject groups. In the present study, progesterone appeared to significantly affect pocket depth and plaque score of males, had no effect on clinical parameters of females, and affected pocket depth only, in the pregnant group. No relationship was detected between progesterone levels and bleeding upon probing in our groups, either by simple correlation or by multiple regression (Tables 7 and 5A to 10A).

Vittek et al. (1984) found significantly higher concentrations of salivary testosterone in female periodontitis patients when compared to healthy females. Males showed no such relationship. In the present study, testosterone had a statistically significant negative effect on all three clinical parameters in one or the other of the two male groups with either of the two statistical analyses used (Table 8). However, minimal consistency was noted between the two groups of males studied and between the two statistical methods used to analyse the data (Table 8). Our study does, however, confirm, to some extent, the findings of Vittek et al. (1984) for the female group. Increased testosterone concentrations, in our study, affected pocket depth and plaque score in this group. On the other hand, there was a negative relationship between bleeding score and testosterone in the female main group (Table 8). No correlations could be established between testosterone and any of the three clinical parameters in our pregnant groups.

While Vittek et al. (1984) noted significantly lower estradiol concentrations in both their periodontitis groups, the present study can only confirm lower estradiol concentration as related to the bleeding score of the female group. Estradiol was positively related to plaque score in the male subgroup and to pocket depth in the pregnant subgroup.

Correlations between bacteriological and hormonal parameters

All studies of this nature, including the present one, must assume, to a certain degree, that some, presently unknown relationship between concentrations of steroid hormones in saliva and gingival crevicular fluid exists. Given the relationships of various other components of these two body fluids that are known to exist (Cimasoni 1983), and well known correlations between concentrations of steroid hormones in saliva and plasma (reviewed by Landon & Mahmod 1984), such assumptions appear valid. However, until verified, these remain assumptions. A pilot study is presently being undertaken in order to establish the feasibility of collecting samples of gingival crevicular fluid, from isolated periodontal pockets, samples that are large enough to allow steroids to be detected and quantitated by radioimmunoassays. The results could then be compared to steroid concentrations in matched samples of whole saliva. Sampling and analysis of subgingival plaque from the very same pockets may give some indications as to the relationships between steroid levels and certain bacteriological parameters. Finally, correlations between subgingival hormonal concentrations and selected clinical parameters could be studied.

Very little information is available on the correlation between steroid hormones and the subgingival microbial flora (Kornman & Loesche

1980). One study has shown steroids to be inhibitory to Gram-positive organisms *in vitro* (Yotis 1967). If the Gram-negative sector of the subgingival microflora is indeed responsible for the onset or aggravation of periodontal diseases then a possible inhibition of Gram-positives may be a factor in the etiology of the diseases. However, the reverse is entirely possible. Suppression of the Gram-positive sector may disrupt essential interactions between bacteria to the disadvantage of the Gram-negative organisms.

In the present study, percentage recovery of Gram-negative organisms, *Bacteroides* and *B. intermedius* showed positive correlations to progesterone in males. Gram-negatives and *B. intermedius* were also positively related to estradiol in males. No other bacteriological parameters showed any correlation to estradiol in any of the groups. Testosterone had no effect on any bacteriological parameters. In females, the total count correlated positively with progesterone and cortisol, but the proportion of Gram-negatives showed a negative relationship with progesterone levels. The percentages of *Bacteroides* and *B. intermedius* appeared to correlate positively with cortisol levels in pregnant women.

Interpretation of the above findings is difficult. There is no apparent pattern wich runs through all the groups. While progesterone, to some extent, appears to influence all four bacteriological parameters in the two groups with the lower progesterone levels, viz males and females, it has no such effects on those parameters in pregnant females where progesterone concentrations are much higher (Table 5). It would be expected that any effects of this steroid would be exaggerated in this group. A possible explanation of this apparent discrepancy is that progesterone in low concentrations may stimulate the organisms of

interest in this study. However, at higher concentrations, such as those found in pregnant women, stimulation by progesterone may reach a plateau or it may even reach a point where the concentrations become inhibitory to bacteria. Kornman and Loesche (1982) have reported an inhibitory effect of increased estradiol concentrations on B. intermedius. However, progesterone, at increased concentrations, did not inhibit the organism. A study is being undertaken to examine the effects of various concentrations of 4 steroids on the growth rate of several B. intermedius strains and reference organisms.

Correlations between bacteriological and clinical parameters

Evidence associating microbial organisms with the etiology of periodontal diseases is abundant (Loe & Silness 1963; Slots 1979). Results on the impact of specific bacterial species on the various periodontal disease entities recognized so far are, however, very conflicting. Undoubtedly, future improved refinements in the diagnosis of periodontal disease entities and in distinguishing between active and inactive phases of the disease (Goodson et al. 1982) will pave the way for better recognition of the role of certain species or groups of organisms in the etiology of periodontal diseases (Dzink et al. 1985).

In the present study the plaque score showed no significant correlation with the total number of bacteria recovered (CFU) in females and pregnant women (Tables 8A, 10A), and a statistically significant negative correlation in males (Table 6A). A recent report by Kho, Smales and Hardie (1985) showed no significant correlation between the amount of supragingival plaque and the bacterial community of deep periodontal pockets. This indicates that there may not be a strong relationship

between the two spatially separated floras. Bacterial counts in gingival fluid appear to be independent of the supragingival plaque mass (Cimasoni 1983). It is not clear what portion of such counts represent organisms that are washed out with the gingival fluid from the depths of the pockets and what portion is dislodged from the marginal plaque.

The proportion of Gram-negative organisms in the plaque samples correlated positively with the plaque scores of females and males (Tables 6A,8A). No statistically significant correlations were noted between bacteriological and clinical parameters in the pregnant group (Table 10A). Pocket depth in males and plaque score in females had statistically significant positive correlations with the percentage of Bacteroides in subgingival plaque (Tables 6A,8A). Only the mean pocket depth of males showed a significant, positive, correlation with the proportion of B. intermedius (Table 6A). Sawitt and Socransky (1984) found significant positive correlations between three gingival parameters, gingival index, plaque index and pocket depth, and the proportion of B. intermedius. Their results, however, were compiled from several disease entities. A statistically significant correlation between sites of destructive periodontal disease and the proportion of B. intermedius was not noted (Sawitt & Socransky 1984). The confusion surrounding the role of B. intermedius in the etiology of periodontal diseases becomes evident when the above findings are compared to those of Ozink et al. (1985) who found proportions of this organism to be increased only in active sites, and Moore et al. (1982a,b,c,1983) who did not recover the organism from sites of periodontal health or experimental gingivitis.

Conclusion

Within the limitations of this study, the results do not indicate that increased concentrations of steroid hormones in saliva cause more severe gingivitis in pregnant women, nor that high levels of salivary steroid hormones result in an increased recovery of B. intermedius from subgingival plaque.

Our study does not confirm earlier findings of the potential importance of steroid hormones on certain constituents of the subgingival microflora, possibly detrimental to the gingival health. Conceivably, sectors of the subgingival microflora that may need vitamin K1 for their survival, may have all their vitamin requirements fulfilled, irrespective of steroid levels in the crevicular fluid. Physiologic variations in steroid concentrations would thus not be expected to affect the composition of the subgingival ecology. This is not to say that steroid hormones may not be important in the etiology of periodontal diseases. Several other mechanisms may, indeed, be involved in the effect of steroids on periodontal health, such as effects on the gingival circulation, metabolism, prostaglandins, and immunological pathways.

Further studies

Further studies on the relationships between steroid hormones on one hand, and determinants of gingival inflammation and bacteriological parameters, on the other, are neccessary. One such study, involving a longitudinal monitoring of a group of pregnant women is being planned. Thirty to fifty pregnant women would be examined every six to eight weeks during their pregnancy and at six, twelve and twenty-four weeks post-partum. At those times, samples of subgingival plaque and whole

saliva will be collected and selectively analysed for their respective bacteriological and hormonal contents, and determinants of gingival inflammation would be recorded. Such a study is based on the assumption that correlations between steroid levels in saliva and crevicular fluid will be established. To this end, we are planning a pilot study in which samples of crevicular fluid will be collected and analysed for steroid contents. Finally, it must be assumed that the apparent ability of certain species of Bacteroides, B. intermedius in particular, to utilize some steroid hormones in lieu of their vitamin K requirements (Kornman & Loesche 1980, 1982), will be confirmed. A study to determine the ability of certain Bacteroides species, including some of our own clinical isolates as well as selected reference strains, to grow in broths where steroids have been supplemented in lieu of vitamin K, is now under way. The results may show that some specific strains of B. intermedius, and not others, may be able to thrive in a hypothetical environment devoid of vitamin K, provided they have access to certain steroid hormones. Strains with such adaptability may correlate better with gingival inflammation than B. intermedius as a group. Current refinements of the identification and speciation of B. intermedius, and advanced immunological studies on this organism could make results of the proposed study more meaningful.

Table 3

Demographic and clinical parameters: all groups (Means ± SEM).

Groups		n	Age (years)	Pocket Depth (mm)	Plaque Score	Bleeding Score	Pregnancy Duration (weeks)
Males	M	30	37.1±10.5	3.61±1.01	0.68±0.25	0.81±0.22	
	Mb	9	40.7± 9.2	4.48±1.13	0.80±0.33	0.88±0.23	
Females	F	30	30.7± 4.4	2.99±0.43	0.48±0.22	0.78±0.19	
	Fb	14	30.5± 5.0	3.09±0.49	0.51±0.15	0.82±0.17	
Pregnant Females	P	30	28.8± 4.6	2.98±0.44	0.52±0.29	0.71±0.27	24.4±8.2
	Pb	14	28.2± 4.6	3.10±0.36	0.58±0.30	0.84±0.16	26.1±7.5

M,F,P All Males, Females, Pregnant Females.
Mb,Fb,Pb Subgroup(s) of Males, Females, Pregnant Females from whom bacterial samples were analysed.

Table 4

Steroid hormone levels in whole saliva: all groups (Means ± SEM).

Groups		n	Testosterone (pg/ml)	Progesterone (pg/ml)	Cortisol (ng/ml)	Estradiol (pg/ml)
Males	M	30	59.5 ± 25	31.6 ± 27	1.36 ± 0.8	0.62 ± 0.24
	Mb	9	61.1 ± 26	43.1 ± 37	1.61 ± 1.1	0.72 ± 0.18
Females	F	30	16.5 ± 4	64.7 ± 24	1.25 ± 0.9	1.16 ± 0.33
	Fb	14	16.1 ± 3	68.3 ± 27	1.63 ± 1.2	1.18 ± 0.27
Pregnant Females	P	30	32.5 ± 16	573.0 ±395	2.17 ± 0.9	35.65 ±22.32
	Pb	14	36.0 ± 19	652.2 ±442	2.18 ± 0.8	36.29 ±25.75

M,F,P All Males, Females, Pregnant Females.
Mb,Fb,Pb Subgroup(s) of Males, Females, Pregnant Females from whom bacterial samples were analysed.

Table 5

Bacteriological parameters: all groups (Means ± SEM).

Groups		n	Total CFU(a) x10	% Gram-negatives	% Bacteroides	% B. intermedius
Males	Mb	9	127.8 ±184.7	25.4 ±18.4	14.4 ±13.2	10.9 ±10.4
Females	Fb	14	96.6 ±173.4	13.2 ±13.8	6.1 ± 7.6	2.6 ± 2.9
Pregnant Females	Pb	14	52.7 ± 90.0	7.0 ± 7.6	3.7 ± 4.6	1.7 ± 2.9

(a) Colony forming units on anaerobically cultured blood agar plates.
Mb,Fb,Pb Subgroup(s) of Males, Females, Pregnant Females from whom bacterial samples were analysed.

Table 6

Significant differences (a) of the means of the twelve variables for the six groups(b), assessed by multiple comparisons (c).

Variable	Main Groups			Subgroups		
1. Age	P	F	M	Pb	Fb	Mb
2. Pocket Depth	P	F	M	Pb	Fb	Mb
3. Plaque Score	F	P	M	Fb	Pb	Mb
4. Bleeding Score	P	F	M	Fb	Pb	Mb
5. Testosterone	F	P	M	Fb	Pb	Mb
6. Progesterone	M	F	P	Mb	Fb	Pb
7. Cortisol	F	M	P	Mb	Fb	Pb
8. Estradiol	M	F	P	Mb	Fb	Pb
9. CFU $x10^6$				Pb	Fb	Mb
10. % Gram-negatives				Pb	Fb	Mb
11. % Bacteroides				Pb	Fb	Mb
12. % B. intermedius				Pb	Fb	Mb

(a) Means are arranged in an ascending order from the left. The means of underlined groups are not significantly different at the 95% level of confidence.

(b) M,F,P, all males, females, pregnant females, n=30; Mb, Fb, Pb, males (n=9) females (n=14), pregnant females (n=14) from whom bacterial samples were analysed.

(c) Duncan's New Multiple Range Test (D.B. Duncan, "Multiple range and multiple F tests," Biometrics, 11:1-42, 1955.

Table 7

Means of hormonal parameters which showed significant partial regression coefficients (a) on clinical and bacteriological parameters: all groups (b)

Parameters	Group	(Means)	Hormonal Parameters: Testosterone	Progesterone	Cortisol	Estradiol
Clinical						
Pocket Depth	M	(3.61)	* (59.5)	* (31.6)		
	Fb	(3.09)	* (16.1)			
	P	(2.98)		* (573.0)	* (2.17)	
	Pb	(3.10)		* (652.2)	** (2.18)	* (36.29)
Plaque Score	M	(0.68)		* (31.6)		
	Mb	(0.80)	* (61.1)			* (0.72)
	F	(0.48)	* (16.5)			
	Fb	(0.51)	* (16.1)			
	P	(0.52)			** (2,17)	
	Pb	(0.58)			*	
Bleeding Score	F	(0.78)	* (16.5)			** (1.16)
	Fb	(0.82)				* (1.18)
	P	(0.71)			** (2.17)	
Bacterial						
CFU(c) $\times 10^6$	Mb	(127.8)			* (1.61)	
	Fb	(96.6)			** (1.63)	

(a) Parameters and groups with no significant relationships were excluded from the table.

(b) M,F,P all males, nonpregnant females, pregnant females; Mb,Fb,Pb subgroups of males (n=9), nonpregnant females (n=14), pregnant females (n=14)) from whom bacterial samples were analysed.

(c) CFU, colony forming units in plaque samples.

*, $p < 0.05$, **, $P < 0.01$.

Table 8

Comparisons of relationships between salivary hormone levels and clinical and bacteriological parameters; simple correlation vs multiple regression

		Clinical Parameters			Bacteriological Parameters			
Group	Analysis	PD	PS	BS	CFU	%GN	%B	%BI
M :	SC (a)		P					
	MR (b)	-T,P	P					
Mb:	SC		-T	-T		P,E	P	P,E
	MR		-T,E		C			
F :	SC			-T,-E				
	MR		T	-T,-E				
Fb:	SC	T	T		P,C	-P		
	MR	T	T	-E	C			
P :	SC			-C				
	MR	P,-C	-C	-C				
Pb:	SC	P					C	C
	MR	P,-C,E	-C					

a), Simple correlation. b), Multiple regression analysis.
T = testosterone, P = progesterone, C = cortisol, E = estradiol
-T,-P,-C,-E, = negative relationships.

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Table 1A

Demographic and clinical profiles and mean saliva steroid concentrations males:

Subject No.	ID.	Age (yrs)	Mean Pocket Depth (mm)	PS (a)	BS (b)	Mean Steroid Concentrations: Testosterone (pg/ml)	Progesterone (pg/ml)	Cortisol (ng/ml)	Estradiol (pg/ml)
3.	RR	46	6.82	1.00	1.00	23.1	115.7	1.20	0.79
54.	JM	45	5.77	0.64	1.00	50.1	29.3	1.87	0.65
5.	MB	46	5.11	1.00	1.00	46.1	21.0	1.41	0.76
26.	MR	50	5.07	0.89	1.00	77.6	14.1	0.98	0.60
1.	RK	38	4.99	1.00	1.00	77.7	48.4	3.35	1.12
4.	ZB	46	4.07	1.00	1.00	30.4	91.5	1.10	0.73
14.	DM	33	3.98	0.92	0.89	64.5	14.6	2.34	0.48
43.	SA	41	3.90	0.52	1.00	58.6	19.7	3.54	0.61
24.	RE	39	3.85	0.73	0.60	38.3	18.3	0.58	0.27
13.	MM	31	3.84	0.82	0.96	59.3	13.4	0.97	0.39
42.	CL	45	3.66	1.00	1.00	61.2	41.9	1.18	0.80
15.	RH	28	3.51	0.62	0.92	27.2	13.4	0.15	0.33
44.	SD	34	3.50	0.74	0.57	68.6	18.1	0.82	0.56
28.	BK	44	3.27	0.79	0.44	35.3	27.7	0.61	0.69
25.	MF	20	3.24	0.04	0.39	106.8	17.6	0.87	0.54
11.	TM	53	3.21	0.43	0.15	30.4	15.2	0.53	0.33
12.	AD	64	3.19	0.64	0.80	49.8	13.9	2.41	1.15
39.	DG	34	3.19	0.50	0.92	48.9	17.7	1.76	0.33
19.	BH	35	3.18	0.66	0.89	49.9	17.9	0.88	0.44
33.	BEH	44	3.03	0.58	0.96	43.0	16.4	1.58	1.06
50.	JH	32	3.00	0.72	1.00	72.6	61.8	0.68	0.58
9.	JT	34	2.97	0.86	0.64	70.1	13.9	0.94	0.51
51.	ThS	26	2.93	0.56	0.89	63.3	57.8	1.85	0.83
53.	GO	21	2.92	0.45	0.63	88.2	33.0	2.47	0.93
38.	JB	21	2.83	0.58	0.91	48.4	18.8	1.42	0.53
18.	AS	24	2.79	0.57	0.93	68.8	16.3	0.77	0.33
35.	RP	33	2.70	0.02	0.63	68.9	19.9	0.78	0.69
22.	LJS	32	2.69	0.55	0.88	64.9	17.0	1.43	0.42
46.	GHB	48	2.65	0.66	0.56	42.7	25.7	1.24	0.53
52.	BG	27	2.57	0.82	0.83	149.7	97.4	1.10	0.62

a) PS = Plaque Score. b) BS = Bleeding Score

Table 2A

Demographic and clinical profiles and mean saliva steroid concentrations non-pregnant females:

Subject No.	ID.	Age (yrs)	Mean Pocket Depth (mm)	PS (a)	BS (b)	Mean Steroid Concentrations: Testosterone (pg/ml)	Progesterone (pg/ml)	Cortisol (ng/ml)	Estradiol (pg/ml)
81.	LS	32	3.94	0.72	0.92	16.8	130.3	5.06	1.30
7.	HKD	30	3.83	0.81	1.00	17.0	38.1	0.84	0.56
67.	OTh	28	3.67	0.53	0.96	20.1	40.2	0.94	0.90
79.	DW	31	3.49	0.50	1.00	17.4	67.1	2.77	1.20
69.	DA	25	3.40	0.82	0.57	18.1	34.6	0.70	1.47
72.	CC	32	3.37	0.60	0.88	18.5	46.8	1.39	1.30
80.	RP	25	3.33	0.49	0.73	19.8	90.4	2.71	1.36
32.	NN	31	3.26	0.37	1.00	17.0	80.9	0.51	1.04
48.	FH	36	3.23	0.66	0.66	13.5	46.2	0.79	1.57
64.	CG	35	3.21	0.31	0.43	22.3	76.1	0.68	1.97
30.	IL	30	3.13	0.47	0.93	16.8	73.4	0.70	1.17
57.	LG	30	3.11	0.87	0.48	22.9	57.7	0.61	1.47
78.	FD	33	3.07	0.37	0.89	10.9	72.6	0.84	1.24
55.	DM	32	3.03	0.69	0.81	15.5	74.3	0.49	1.30
66.	GE	25	2.91	0.19	0.86	13.2	116.9	0.38	0.72
40.	UK	38	2.90	0.33	0.88	16.9	40.2	0.65	1.08
36.	LH	39	2.89	0.39	0.86	10.8	65.1	1.20	0.76
31.	BG	28	2.86	0.59	0.89	13.8	47.9	0.87	1.04
75.	TM	28	2.83	0.31	1.00	14.3	52.3	0.70	1.17
83.	GB	31	2.82	0.77	0.39	29.5	73.4	2.34	0.87
68.	JM	29	2.77	0.55	0.86	14.1	100.6	1.35	1.30
29.	MS	34	2.74	0.38	0.96	14.1	65.5	0.94	0.89
45.	SN	24	2.67	0.45	0.78	11.8	50.2	0.98	0.96
34.	PPa	31	2.55	0.03	0.56	18.2	53.4	0.95	1.72
8.	ABA	33	2.53	0.50	0.93	16.0	45.0	1.00	0.67
41.	CS	29	2.47	0.63	0.60	15.4	82.6	0.89	0.99
37.	PPh	36	2.42	0.24	0.42	29.4	51.4	1.03	1.19
23.	FBS	26	2.40	0.04	0.71	17.6	41.5	1.56	1.23
27.	EB	22	2.39	0.57	0.78	19.3	45.6	15.32	0.73
77.	PF	39	2.38	0.32	0.63	11.5	81.8	2.37	1.70

a) PS = Plaque Score. b) BS = Bleeding Score

Table 3A

Demographic and clinical profiles and mean saliva steroid concentrations pregnant females:

Subject No.	ID.	Weeks Pregn.	Age (yrs)	Mean Pocket Depth (mm)	PS (a)	BS (b)	Mean Steroid Concentrations: Testosterone (pg/ml)	Progesterone (pg/ml)	Cortisol (ng/ml)	Estradiol (pg/ml)
70.	DL	36	28	3.84	0.96	1.00	35.3	1342.8	2.23	53.47
91.	HD	24	32	3.76	0.83	1.00	36.7	784.4	2.44	55.23
16.	EH	16	40	3.56	0.60	1.00	9.7	163.6	0.71	10.76
89.	JK	39	24	3.56	0.82	0.56	59.2	1573.8	3.68	81.12
74.	CW	16	28	3.53	1.00	1.00	26.4	287.3	0.68	19.72
82.	SD	15	27	3.41	0.63	1.00	29.2	16.7	1.10	5.18
61.	HD	23	34	3.33	0.45	0.95	16.3	248.5	1.75	37.13
60.	GS	27	34	3.21	0.08	0.75	35.8	1305.3	2.64	no sample
88.	SM	26	27	3.14	0.90	0.93	44.9	510.7	2.49	56.82
87.	TM	37	27	3.12	0.65	0.50	21.9	784.4	2.46	48.13
56.	JR	33	31	3.09	0.06	0.26	23.6	866.1	2.87	53.03
49.	BW	33	29	3.07	0.84	0.93	26.2	745.9	2.25	60.56
90.	TD	25	19	3.06	0.64	0.82	58.3	221.2	2.16	30.39
59.	GC	20	26	3.03	0.88	0.86	62.8	636.5	2.14	81.35
86.	IL	22	31	3.03	0.71	0.75	47.9	348.2	1.59	14.48
20.	JM	27	30	2.99	0.46	0.61	17.7	506.0	3.93	20.52
65.	EJ	33	26	2.93	0.46	1.00	28.4	515.6	3.04	46.86
58.	AP	12	29	2.87	0.45	0.62	26.8	240.3	0.84	18.23
62.	LM	18	22	2.86	0.26	0.75	16.4	278.5	1.30	30.33
2.	SG	22	35	2.80	0.05	0.82	28.7	598.6	2.50	30.64
71.	BK	17	26	2.76	0.61	0.86	17.6	220.9	1.08	9.21
10.	IT	22	32	2.74	0.50	0.93	30.1	594.9	2.28	6.01
85.	CC	34	21	2.71	0.32	0.84	70.3	756.6	2.99	36.21
6.	LH	32	36	2.71	0.20	0.51	12.7	511.6	2.69	35.38
76.	SS	10	26	2.59	0.16	0.39	25.6	141.5	1.41	7.02
73.	DC	26	32	2.58	0.61	0.64	15.4	296.8	1.15	33.35
63.	HG	7	25	2.40	0.14	0.05	38.6	213.6	3.46	10.39
21.	EG	30	27	2.24	0.66	0.36	19.8	540.5	2.06	22.58
84.	JR	24	27	2.23	0.64	0.44	41.4	599.6	1.92	29.06
17.	LR	26	33	2.12	0.10	0.25	55.8	1338.5	3.37	75.31

a) PS = Plaque Score. b) BS = Bleeding Score

Table 4A

Bacterial profiles: all groups.

Subject No. ID.	CFU $\times 10^6$	% Gram-negatives	% Bacteroides	% *B. intermedius*
Males:				
1. RK	18.5	47.6	28.37	26.08
3. RR	1.7	37.1	32.65	28.20
4. ZB	0.6	53.3	31.44	12.15
5. MB	118.0	34.8	15.48	14.30
25. MF	311.0	1.3	1.02	1.02
26. MR	110.0	19.5	10.84	9.30
42. CL	44.6	7.3	2.21	0.30
43. SA	543.0	14.0	3.32	2.95
44. SD	2.7	14.0	4.31	3.86
Females:				
27. EB	37.0	3.1	1.57	0.94
30. IL	0.5	9.4	8.25	8.25
31. BG	0.4	26.0	4.34	4.06
32. NN	3.2	2.4	0.63	0.63
36. LH	0.3	10.4	7.65	1.53
37. PP	0.2	9.4	2.68	0.67
67. OT	28.5	37.9	10.87	7.43
68. JM	12.5	0.4	0.19	0.01
69. DA	11.6	42.2	27.56	6.03
72. CC	17.4	20.1	5.75	4.02
77. PF	14.7	4.0	0.34	0.14
79. DW	459.0	1.3	1.04	0.17
80. RP	301.0	1.1	0.10	0.04
81. LS	466.0	16.7	14.13	2.14
Pregnant:				
6. LH	9.8	2.1	1.05	1.05
10. IT	3.8	3.6	0.24	0.24
59. GC	59.9	3.1	2.06	0.72
60. GS	0.5	12.6	6.30	2.73
61. HD	39.0	0.7	0.57	0.54
62. LM	2.5	0.8	0.28	0.25
65. EJ	41.8	0.3	0.09	0.03
70. DL	77.9	6.5	1.30	0.67
71. BK	26.9	12.6	4.08	0.00
74. CW	41.0	25.1	14.54	9.36
85. CC	23.7	4.1	3.31	1.00
86. IL	354.0	0.3	0.17	0.09
88. SM	53.5	18.1	12.13	7.46
89. JK	4.0	8.1	5.40	0.00

Table 5A

Significant (a) simple correlation coefficients for pairs of eight of the variables studied: males (b)

Variables	No.	1	2	3	4	5	6	7	8
Age	1.	100(c)							
Pocket Depth	2.	40	100						
Plaque Score	3.	36	51	100					
Bleeding Score	4.		40	48	100				
Testosterone	5.	-49				100			
Progesterone	6.			39			100		
Cortisol	7.							100	
Estradiol	8.							55	100

(a) significant at a 95% level of confidence.
(b) All males; n=30, (c) Decimals omitted.

Table 6A

Significant (a) simple correlation coefficients for pairs of the eleven significant variables studied: males (b)

Variables	No.	1	2	3	4	5	6	8	9	10	11	12
Age	1.	100(c)										
Pocket Depth	2.		100									
Plaque Score	3.	85		100								
Bleeding Score	4.	90		79	100							
Testosterone	5.	-71		-68	-65	100						
Progesterone	6.					-74	100					
Estradiol	8.							100				
Total CFU	9.			-70					100			
% Gram-negatives	10.			67			64	65		100		
% Bacteroides	11.		71				82			94	100	
% *B. intermedius*	12.		87				65	71		79	89	100

(a) significant at a 95% level of confidence.
(b) Subgroup of males from which bacteria were analysed; n=9
(c) Decimals omitted.
Variables with no significant correlations with other variables were omitted from the table

Table 7A

Significant (a) simple correlation coefficients for pairs of the seven significant variables studied: females (b)

Variables	No.	2	3	4	5	6	7	8
Pocket Depth	2.	100(c)						
Plaque Score	3.	50	100					
Bleeding Score	4.			100				
Testosterone	5.			-37	100			
Progesterone	6.					100		
Cortisol	7.					49	100	
Estradiol	8.			-50				100

(a) significant at a 95% level of confidence.
(b) All females; n=30, (c) Decimals omitted.
Variables with no significant correlations with other variables were omitted from the table

Table 8A

Significant (a) simple correlation coefficients for pairs of the eleven significant variables studied: females (b)

Variables	No.	1	2	3	4	5	6	7	9	10	11	12
Age	1.	100(c)										
Pocket Depth	2.		100									
Plaque Score	3.	-61	56	100								
Bleeding Score	4.		53		100							
Testosterone	5.	-79	59	54		100						
Progesterone	6.						100					
Cortisol	7.						72	100				
Total CFU	9.		57				55	87	100			
% Gram-negatives	10.			58			-52			100		
% Bacteroides	11.			69						82	100	
% *B. intermedius*	12.									75	64	100

(a) significant at a 95% level of confidence.
(b) Subgroup of females from which bacteria were analysed; n=14
(c) Decimals omitted.
Variables with no significant correlations with other variables were omitted from the table

Table 9A

Significant (a) simple correlation coefficients for pairs of the nine variables studied: pregnant females (b)

Variables	No.	1	2	3	4	5	6	7	8	13
Age	1.	100(c)								
Pocket Depth	2.		100							
Plaque Score	3.		51	100						
Bleeding Score	4.		65	53	100					
Testosterone	5.	-47				100				
Progesterone	6.					44	100			
Cortisol	7.				-38	38	51	100		
Estradiol	8.					48	76	50	100	
Weeks Pregnant	13.						70	54	63	100

(a) significant at a 95% level of confidence.
(b) All pregnant females; n=30, (c) Decimals omitted.

Table 10A

Significant (a) simple correlation coefficients for pairs of the seven significant variables studied: pregnant females (b)

Variables	No.	2	3	6	7	11	12	13
Pocket Depth	2.	100(c)						
Plaque Score	3.	59	100					
Progesterone	6.	52		100				
Cortisol	7.			68	100			
% Bacteroides	11.				96	100		
% *B. intermedius*	12.				85	93	100	
Weeks Pregnant	13.			73	88			100

(a) significant at a 95% level of confidence
(b) Subgroup from which bacteria were analysed; n=14.
(c) Decimals omitted.
Variables with no significant correlations with other variables were omitted from the table.