Introduction

Periodontal disease (PD) gradually progresses in dogs. Determining the rate, severity, and extent of this progress in dogs is difficult, for three principal reasons. First, uncovering overall PD extent and severity can be costly, imprecise and subjective. Second, research to establish PD pathogenesis rate has often compared dogs of different ages, rather than follow them through time. This cross-sectional approach cannot isolate age from other factors, such as diet, health, condition, and disease etiology. Third, a few longitudinal studies provide insight, but these were hampered by the assessment methods used. Those from last century lacked effective microbial tests. Recent work that anesthetizes dogs does so very infrequently.

There remains little data about how PD's gradual development proceeds. PD is an infectious disease. Understanding pathogenesis of an infectious disease is critical for uncovering preventative methods and developing treatments. This proposal is to use recently developed microbial tests that non-invasively assess PD status, for the purpose of determining PD pathogenesis over weeks and months.

Part 1 (pages 2-6) provides an overview of PD diagnostic criteria and PD cross-sectional and longitudinal research.

Part 2 (pages 6-9) describes the diagnostic method developed to sample a collagenase secreted into gingival fluid that closely corresponds to PD pathogenesis status. This non-invasive, relatively inexpensive method can be used to monitor PD progress incrementally.

Part 3 (pages 9-11) describes the research approach, that will result in a publishable paper about PD pathogenesis. This includes realistic scenarios about how dogs may be monitored.

The diagnostic tool has been explored by the author as a way to test a patented device, Chulite, that uses 405 nm light to reduce pathogenic bacteria that are the root cause of PD. Because there was no data about PD pathogenesis over weeks and months, the likely duration of a Chulite trial, it was necessary to find a way to do so. Since control animals demonstrate ordinary PD development, this part of the trial is itself a worthy goal.

Part 1

1(a): Assessing PD

Determining PD status across all of an adult dog's 42 teeth is difficult. They can have differing PD severity. Measures of severity vary. PD diagnoses can be based on different pocket depths, or combined presence of deep pockets and loss of attachment, or tooth mobility, or missing teeth. To corral the different criteria used to diagnose PD in dogs, Harvey et al. proposed a Total Mouth Periodontal Score for Gingivitis (TMPS-G) and a TMPS for Periodontitis (TMPS-P.) They separated these, because inflammation (i.e. gingivitis) can be present without tissue loss (i.e. PD) and tissue loss may be extensive without inflammation. To develop the TMPS indices they used cadaver dogs whose heads were defleshed, teeth removed, scanned, the images measured with an endodontic ruler.[1] While TMPS may be exhaustive, it would be exhausting, if not impossible, to carry out on live dogs. Each tooth has three roots which must be assessed. This results in scoring 120 root sites for gingivitis scores and 120 for PD, 240 total. The authors followed up by reanalyzing their data to determine if a group of sampled teeth could provide adequate proxy to represent all teeth. They concluded that restricting the scored root set to buccal sites on one side of the mouth, resulting in 20 root sites, was sufficient to achieve over 90% concordance with full mouth results. They did not discuss the problem of overlooking one or more teeth that may be especially problematic, nor the dilemma of interpreting missing teeth.[2]

Human PD research is less optimistic about using subset samples. According to a review by Beck & Löe, sampling inevitably underestimates PD prevalence (number of teeth) although it is possible it can overestimate PD extent (severity.) Use of just a quadrant of human teeth underestimates both prevalence and severity. Summary measures of pocket depth and severity scores can adjust for bias, to better compare to full mouth measures. Examination variance is difficult to adjust for, however.[3] More recent data further calls sampling into question. Eke et al. showed National Health and Nutrition Examination Survey data of PD prevalence, based on full mouth data, was over 50% greater than the prevalence determined by partial samples.[4]

1 Harvey, C.E. et al. (2008) "Scoring the full extent of periodontal disease in the dog: development of a total mouth periodontal score (TMPS) system" J. Vet. Dent.; 25(3):176-80

2 Harvey, C.E., Laster, L. & F.S. Shofer (2012) "Validation of use of subsets of teeth when applying the total mouth periodontal score (TMPS) system in dogs" J. Vet. Dent.; 29(4):222-6

3 Beck, J.D. & H. Löe (1993) "Epidemiological principles in studying periodontal diseases" Periodontol 2000; 2:34-45

4 Eke, P.I. et al. (2010) "Accuracy of NHANES periodontal examination protocols" J. Dent. Res.; 89(11):1208-13

In most studies, and in general veterinary practice, dog dental clinical examination is performed under general anesthesia. This is the best way to assess accurately the degree of PD using physical methods, including degree of root exposure. It is not safely possible to probe the gingival sulcus in unsedated dogs.[5] However it limits examination to younger animals and is not appropriate for weekly and monthly assessments.

This latter reason may be the greatest constraint to uncovering PD pathogenesis. The frequency of undergoing general anesthesia has been assessed in young children. Banerjee et al. found that pediatric populations with multiple anesthesia exposures suffer neurocognitive impairment. The authors note that few studies examine anesthetic frequency.[6] Monkeys exposed to multiple anesthesia events exhibited motor reflex deficits at 1 month of age and responded to their new social environment with increased anxiety. These problems were not found in monkeys exposed to a single anesthesia event.[7]

Anesthesia carries risk. A multinational assessment of anesthetic risk in dogs found anaesthetic-related mortality was 0.69%. This study involved 405 veterinary centers in Spain, Argentina, France, the UK, the USA, Chile, Portugal and Australia.[8] Other studies in the US and UK have typically found lower mortality rates. Brodbelt et al.'s UK survey found an overall rate of 0.18% for anaesthetic-related deaths in dogs.[9] The different rates may be because Redondo et al. included post-operative mortality, rather than just perioperative. Several research studies have shown that older dogs are especially vulnerable, with anesthesia-related mortality doubling after age 9. However Shoop-Worrall et al. found it also doubles from age 3-5 to age 5-7. Mortality associated with dental work was higher than most other surgical procedures. The overall rate was 0.10–0.14%.[10]

Given a rate of 0.1%, a study of 10 dogs that used general anesthesia monthly for six months would have a cumulative mortality risk of 6%. A rate of 0.2%, with assessments biweekly, would result in a

5 Fernandes, N.A. et al. (2012) "Prevalence of periodontal disease in dogs and owners' level of awareness - a prospective clinical trial" Rev. Ceres; 59(4)
6 Banerjee, P. et al. (2019) "Association Between Anesthesia Exposure and Neurocognitive and Neuroimaging Outcomes in Long-term Survivors of Childhood Acute Lymphoblastic Leukemia" JAMA Oncol.; 5(10):1456–63
7 Raper, J. et al. (2015) "Multiple Anesthetic Exposure in Infant Monkeys Alters Emotional Reactivity to an Acute Stressor" Anesthesiology; 123:1084–92
8 Redondo, J.I. et al. (2024) "Anaesthetic mortality in dogs: A worldwide analysis and risk assessment" Vet. Rec.; 195(1):e3604
9 Brodbelt, D.C. et al. (2008) "The risk of death: the confidential enquiry into perioperative small animal fatalities" Vet. Anaesth. Analg.; 35: 365–73
10 Shoop-Worrall, S.J.W. et al. (2022) "Mortality related to general anaesthesia and sedation in dogs under UK primary veterinary care" Vet. Anaesth. Analg.; 49: 433–42

total risk of 12% over three months. These risks are excessive. Exposing animals to multiple anesthetic events, sufficient to monitor PD pathogenesis over weeks and months is not advisable.

1(b): Assessing PD progress: cross-sectional studies

To assess PD over time, many studies use cross-sectional approaches with dogs of different ages sampled in one period. Cross sectional studies have weaknesses. They cannot determine the temporal relation between outcomes and risk factors. Some studies sample subjects from a heterogeneous study population. This is sometimes called convenience sampling, and is considered prone to bias. A convenience sample is defined as sampling the most accessible individuals; a first-come, first-served sample from a heterogeneous population that maintains heterogeneity may be considered systematic.[11] Because physical examination of the teeth of many dog breeds can be difficult, many studies use research beagles. This generates a bias like that found in microbiology's reliance on *E. coli* as a model bacterium, which skews understanding how other prokaryotes function. For example, beagles do not chew their food as much as other dogs, which may alter PD progress.

In a cross-sectional report of PD progression, Kortegaard et al. studied 98 beagles, from 1 to 6 years of age. This was the entire population of Danish research dogs. The authors proposed that because these animals were a single breed, housed in similar conditions, and given similar diets, they avoided inter-breed and condition differences found in convenience samples. But the research dogs are a convenience sample, being individuals from a specifically homogeneous population, not a random or systematic sample. Dogs received a complete full-mouth examination. A finding of "pocket depth >4 mm" was found in 47% of 1 year olds, 44% of 2 year olds, and 81% of 3 years and older. "Teeth with mobility" followed a similar pattern, reaching 91% after age 3. Minor attachment loss increased by 3 times from year 1 to 2, but 33% after. The authors conclude that PD increases steadily with age. They cautioned that a major burden was carried by relatively few dogs, with most dogs having fewer than 10 teeth affected. They suggest that PD cannot be detected as it progresses in young dogs.[12]

11 Wang, X. & Z. Cheng (2020) "Cross-Sectional Studies: Strengths, Weaknesses, and Recommendations" Chest.; 158(1S):S65-S71
12 Kortegaard, H.E., Eriksen, T. & V. Baelum (2008) "Periodontal disease in research beagle dogs – an epidemiological study" J. Small Anim. Practice; 49, 610–6

An earlier cross-sectional study by Lindhe et al., of 74 research beagles between 1 and 12 years, found attachment loss severity increased significantly at age 6 to 7. This was considerably later than what Kortegaard et al. found in their beagles. Lindhe et al. also found that PD was not very associated with increased pocket depth, unlike Kortegaard et al.. But Lindhe et al. also suggested it may be difficult to detect the disease progress.[13] Isogai et al. studied stray and pet dogs in Japan (mongrels) and found that between 1.5 and 5 years of age, gingivitis increased its presence from about 1/4 to 3/4 of dogs. Only at age 5 did the number of remaining teeth significantly reduce, similarly in both strays and pets. Whereas Kortegaard et al. reported that PD was present in maxilla teeth much more than mandible teeth, Isogai et al. found PD impacts were similar in these two areas.[14]

Assuming some congruence in estimates of teeth mobility and attachment loss, these cross-sectional studies vary in the age at which PD develops. Incipient disease may be detected at any age, or between 1 and 2 years, or 1.5 and 5. A majority of studied dogs may have at least some PD at age 3, age 5, or age 6-7. It is not particularly informative to conclude that PD pathogenesis extends over a few or quite a few years. Cross-sectional studies cannot provide insight into disease related factors that cause PD to worsen more or less rapidly.

1(c): Assessing PD progress: longitudinal studies

50 years ago a number of researchers pioneered longitudinal tests of dog PD, meaning they studied animals repeatedly over time. Unfortunately their tools were not able to capture change over weeks and months. Lindhe, Hamp & Löe produced a fascinating longitudinal study. Treatment dogs got daily brushing, control dogs did not. Dogs were examined once a week for a month, then every two months for a year, and at 18 months. The authors inserted amalgam fillings in tooth surfaces just above the gingival margin, to aid in determining gingival margin change. Further, dog gingival fluid was removed with film, and leukocyte (white blood cells of the immune system) counts were made by microscopy.

Increase in gingival leukocyte scores was greatest, in the control animals, in the first three weeks of the test. The immune cells then plateaued for the next 18 months. Only a slight recession of the gingival

13 Sorenson, W.P., Löe, H. & S.P. Ramfjord (1980) "Periodontal disease in the beagle dog. A cross sectional clinical study" J. Period. Res.; 15, 380-89

14 Isogai, H. et al. (1989) "Epidemiological study on periodontal diseases and some other dental disorders in dogs" Nihon Juigaku Zasshi.;51(6):1151-62

margin was noticed, using the amalgam markers. Fiber attachment loss in control animals was detected at around 8 months, reduced bone density was noticed only at month 18. Attachment loss (at 18 months) averaged about 0.9 mm in control animals. While this study showed that PD progressed from earlier gingivitas, the measures used were not sophisticated enough to measure change over weeks and months.[15] The study continued for three more years, with annual exams, reported on in a further article. Attachment loss increased to 2 mm in year two, and reached almost 3 mm by year four. Pocket depth also increased. Difference between treatment and control groups became very significant. However there was no blinding of groups. Treatment dogs received twice daily meticulous tooth cleaning, leading to significantly different life experiences between the groups over the four year period. Increased attention reduces stress and improves well-being.[16] In 2019 Wallis et al. reported a longitudinal study that also compared dogs, Yorkshire terriers, with and without tooth brushing. Dental status was assessed under anesthesia, every 10 to 12 months in some dogs. Teeth were classified as PD or not. Once dogs were diagnosed with PD, they were removed from the study. This did not consider PD etiology. Instead, average time to PD was determined, for different tooth types. Canine teeth reached PD threshold fastest, in all tooth types but palatal/lingual, at around 47 weeks (~4 years) on average. Average PD increased from 25.5% at 37 weeks (9 months) to 48.3% at 78 weeks (20 months.) PD did not statistically differ between tooth brushing groups, and this was dropped from the analysis.[17]

Wallis et al.'s study was limited to annual assessments by the use of anesthesia. Lindhe et al.'s study was limited in focusing on leukocytes. These cells, also called neutrophils, may be progenitors of matrix metalloproteinase-8 (MMP-8) that forms the basis for this proposed study. Neutrophils were found to plateau early in PD pathogenesis in Lindhe et al.; contemporary research that finds a steady increase of MMP-8 during PD development can be explained by the fact that a stable population of neutrophils continues to produce an aggregate amount of MMP-8.

15 Lindhe, J., Hamp, S.-E. & H. Löe (1973) "Experimental periodontitis in the beagle dog" J. Period. Res.; 8, 1-10

16 Lindhe, J., Hamp, S.-E. & H. Löe (1975) "Plaque induced periodontal disease in beagle dogs. A 4-year clinical, roentgenographical and histometrical study" J. Period. Res.;10(5):243-55

17 Wallis et al. (2019) "A longitudinal assessment of periodontaldisease in Yorkshire terriers" BMC Veter. Res.; 15:207

Part 2

2: Biomarker analysis of PD

Clinical and radiological diagnosis of PD have been used in human dentistry for decades. Clinicians can only detect and measure PD after clinical manifestations using these methods. Biomarkers were introduced to provide earlier diagnosis, standardize PD staging, customize treatment, and improve patient compliance.[18] Since bacteria initiate and drive PD, their analysis might be considered the optimal diagnostic method. But bacterial populations are extraordinarily complex, with putative species interacting in massively multidimensional dynamics.[19] Bik et al. found each one of 10 research subjects with good oral health exhibited a distinct population of bacterial species. They found no clustering of oral microbial communities based on gender, age, or ethnicity. Each person's mouth harbored a unique community of bacterial species.[20] Further, the overall structure of the oral microbiome may not be significantly affected by disease status.[21] Interindividual diversity, microbial heterogeneity, and uncertain effects of disease on microbiomes undermines the use bacterial species analysis in PD diagnostics.

However immune system molecules provide a constrained population of biomarkers closely associated with PD progress. The junctional epithelium in the gingival sulcus seals off periodontal tissues from the oral environment. PD begins when conditions like inflammation damage the epithelium. Interstitial fluid between cells leaks into the sulcus, forming gingival crevicular fluid (GCF.) Infectious PD pathogen concentrations also increase, their proteinases triggering more inflammation. Inflammatory eukocytes, monocytes, and T-cells aggregate. As junctional epithelium is lost, plasma cells and macrophages infiltrate, causing further inflammation. Immune response molecules gather, interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-a), prostaglandin E2 (PGE2), and matrix metalloproteinases (MMPs), especially MMP-8.[22]

18 Carinci, F., Romanos, G.E. & L. Scapoli (2019) "Molecular tools for preventing and improving diagnosis of peri-implant diseases" Periodontol 2000;81(1):41-7

19 Di Stefano, M. et al. (2022) "Impact of Oral Microbiome in Periodontal Health and Periodontitis: A Critical Review on Prevention and Treatment" Int. J. Mol. Sci.; 23(9):5142

20 Bik, E.M. et al. (2010) "Bacterial diversity in the oral cavity of 10 healthy individuals" ISME J.;4(8):962-74

21 Wang, K. et al. (2016) "Preliminary analysis of salivary microbiome and their potential roles in oral lichen planus" Sci. Rep.; 6:22943

22 Leppilahti, J.M. et al. (2011) "Oral rinse MMP-8 point-of-care immuno test identifies patients with strong periodontal inflammatory burden" Oral. Dis.; 17(1):115-22

MMP-8 helps disintegrate and process collagens in wound healing, tissue remodeling, and malignant tissue. But MMP-8 is also responsible for much of the tissue destruction in PD. Pathologically elevated concentration of active MMP-8 (aMMP-8) has been demonstrated to distinguish healthy tissue from gingivitis and periodontitis. aMMP-8 levels, determined from saliva containing GCF, are consistently and closely related to the degree of PD progress.[23]

A meta-analysis of nine research articles drawn from 120 potential articles, selected by Cochrane Group filtering, assessed the diagnostic accuracy of GCF molecular biomarkers. Only 4 of 36 biomarkers, all enzymes, were statistically linked to PD diagnosis in the articles. Of these 4, the meta-analysis found aMMP-8 was most tightly correlated with estimating PD progress. MMP-8 predictive specificity was 0.92; sensitivity 0.77. Positive predictive values ranged between 81.8%–94.7%.[24]

Given concerns about sampling, limitations of repeated anesthesia, and variation in determining bleeding on probing and other physical measurements, MMP-8 provides a non-invasive, non-subjective, total mouth sample whose levels differentiate PD stages. As bone loss, pocket depth, and bleeding on probing increases, levels of aMMP-8 increase. Longitudinal studies show that aMMP-8 assays predict PD progress, attachment loss, and treatment effects.[25] aMMP-8 assays have a stronger association with subclinical PD than bleeding on probing. Patients who improve oral health habits have decreased aMMP-8 levels, while plaque and bleeding levels may be hard to detect.[26]

MMP-8 tests are mostly used for human patient chair-side diagnosis. But MMP-8 tests offer a relatively low-cost method to monitor PD progress over weeks and months. No research has yet determined this for individuals in a longitudinal study, as Lindhe, Hamp & Löe tried with dogs using leukocyte neutrophils. MMP-8 in tissues is primarily derived from degranulating neutrophils, although non-neutrophil cells also express MMP-8.[27] Polymorphonuclear leukocytes, with multi-lobed nucleus, release only a fraction of their content of MMP-8 when activated by proinflammatory mediators. The

23 Lähteenmäki, H. et al. (2022) "Active MMP-8 point-of-care (PoC)/chairside enzyme-test as an adjunctive tool for early and real-time diagnosis of peri-implantitis" Clin. Exp. Dent. Res.; 8(2):485-96

24 Arias-Bujanda N. et al. (2019) "Accuracy of single molecular biomarkers in gingival crevicular fluid for the diagnosis of periodontitis: A systematic review and meta-analysis" J. Clin. Periodontol.; 46:1166–82

25 Sorsa, T. et al. (2020) "Active MMP-8 (aMMP-8) as a grading and staging biomarker in the periodontitis classification" Diagnostics (Basel); 10, 61

26 Raivisto, T. et al. (2019) "Active Matrix Metalloproteinase-8 Chair Side Mouth Rinse Test, Health Behaviour and Oral Health in Finnish Adolescent Cohort" J. Clin. Diagn. Res.; 14(1):35-9

27 Kuula, H. et al. (2009) "Local and Systemic Responses in Matrix Metalloproteinase 8-Deficient Mice during Porphyromonas gingivalis-Induced Periodontitis" Infect. Immun.; 77(2)

activation of neutrophils by other immune system molecules steadily but modestly increases MMP-8 expression as well.[28] Given neutrophils turn over daily, a stable population of neutrophils continues to generate constantly growing levels of MMP-8 over time.[29] The resulting MMP-8s then aggregate. At normal body temperature (around 37°C) MMPs are considered structurally and functionally stable.[30] Indeed, it has as been shown that MMPs are stable over very long time frames. This was analyzed for longitudinal trials investigating disease progression. MMP samples from plasma were stored at -80°C for nine and 12 years. After this time, the levels were compared to the initial baseline levels. There was no statistical difference between baseline and nine or 12 year results.[31]

The accumulation of MMP-8 in sampled saliva (containing GCF), measured as a concentration, closely associates with degree of PD pathogenesis.

Part 3

3(a): Test Approach

Tests of MMP-8 use Elisa or 'Immuno Quantitative'-Elisa for sample analysis. Well plated materials cost $500 to $600 for 40 samples. The processing of these costs $1,000 per 50 samples. As I bear costs, it is important that test costs are minimized.

Dogs may have unpredictable durations at a housing facility. But samples of dog saliva containing GCF can be stored, in appropriate conditions, for months. Sampling involves basic tools like foam sticks and storage jars. Samples will be stored, at very low temperature, for the time needed to obtain samples for at least 8 dogs, each for at least 3 months. Duration is the time needed to show PD progression. If this involves sampling 25 dogs, because 17 drop out before sufficient time, the cost is only sampling tools. Only after samples are obtained for sufficient dogs over sufficient time to demonstrate PD progress will the samples be processed.

The dogs with samples analyzed will have been sampled biweekly over 3 or more months. Each of

28 Owen, C.A. et al. (2004) "Membrane-Bound Matrix Metalloproteinase-8 on Activated Polymorphonuclear Cells Is a Potent, Tissue Inhibitor of Metalloproteinase-Resistant Collagenase and Serpinase1" J. Immunol.; 172(12): 7791–803

29 Sender, R. & R. Milo (2021) "The distribution of cellular turnover in the human body" Nat. Med.; 27(1):45-8

30 Decaneto, E. et al. (2015) "Pressure and Temperature Effects on the Activity and Structure of the Catalytic Domain of Human MT1-MMP." Biophys. J.;109(11):2371-81

31 Jonsson, A. et al. (2018) "Stability of matrix metalloproteinase-9 as biological marker in colorectal cancer" Med. Oncol.; 35(4):50

these analyzed samples will be drawn twice per sampling event. This results in 96 total samples, or $1,000 in test plates and $2,000 of processing. This is something I can sustain.

3(b): Test Protocol

Dogs tested in veterinary care facility, or in a shelter facility, may represent different breeds and sizes.

Dog saliva contains GCF, diluted. Obtaining saliva samples close to an infection site increases GCF concentrations. A foam stick can be pressed along a dog's gum to absorb saliva.

Several 'dog DNA' kits provide oral swab systems, which are basically sponges on a stick, and a tube with stabilizing fluid. The used sponge is inserted in the tube, and returned to the testing service.

The 'dog DNA' kits advise rubbing the inside of a dog's cheek for 20 seconds. This may be considered a time frame for saliva sampling as well. However human saliva sampling systems advise keeping a cotton ball in one's mouth for 30-60 seconds.

I developed a 'GCF Sampler' which is a hollow ball that holds a foam pad. The Sampler has openings on it, where the foam is exposed. A dog holding the Sampler in its mouth has the foam close to tooth and gum surfaces. This promotes the absorption of saliva, and avoids the difficulty of trying to press something into a dog's mouth. The Sampler prototype is plastic, a version shown in Fig. 1. It may be useful to have it on a stick, so a dog cannot swallow the Sampler.

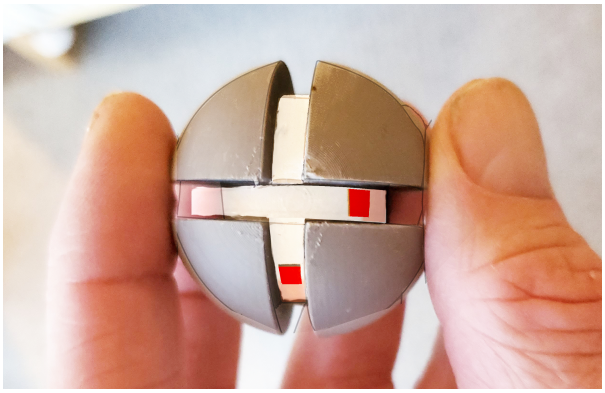

Fig. 1 Sampler

Dogs cannot have food for an hour before testing, and must not have shared a toy with another dog. The Sampler may be treated with a flavor. Individual dogs will be sampled twice per sampling event. Each dog is sampled every 14 days, or more frequently. Dogs need not be on the same schedule. Because sampling success increases with dog comfort, it may take time for a sampling to occur.

Collected saliva samples are eluted into solution, centrifuged, and frozen. Storage at very low temperatures may be carried out at a dedicated facility.

Once a dog has been tested for sufficient time to possibly reveal PD progress, it would be an optimal strategy for a veterinarian to carry out a physical dental exam. This permits a comparison of observed dental conditions with biomarker results.

3(c): Test Result

The goal of the test is to prepare an article for publication in a veterinary journal. Demonstrating the change in PD biomarkers over weeks and months illuminates how PD develops.

Uncovering the detailed pathogenesis of PD, an infectious disease that is endemic in people as well as dogs, will lead to better understanding of its etiology. Results can be used to define PD stages, and may prompt similar research in humans and other mammals. That is possible because MMP-8 is highly conserved over mammalian evolution.

Each dog in this study serves as a model defined by age, sex, condition, breed, and personality. I intend to follow this study with a trial of Chulites, a dog dental device that uses 405 nm light to inactivate PD pathogens. Each trial dog will be matched to a control dog from this study.

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