Apical Periodontitis as a Contributive Risk Factor for Atherosclerosis

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences - Oral Biology

University of Alberta

ABSTRACT

Background and Objectives: Atherosclerosis is a chronic inflammatory process which leads to heart disease and death. Apical periodontists (AP) is a common inflammatory condition associated with infected teeth. In a recent systematic review, we identified a positive association between AP and cardiovascular disease; however, no studies examined causality. The overall goal of our study was to determine if there is evidence for a causal relationship between AP and atherosclerosis. To investigate mechanisms and causality, we created a mouse model to determine whether AP increases the risk for atherosclerosis. Our specific aims were to determine whether there is a difference between mice with AP and mice without AP in (1) Atherosclerosis lesion burden (percentage of atherosclerotic lesion area compared to the total aortic area). (2) Levels of systemic inflammation (inflammatory cytokines in the plasma). (3) Levels of systemic oxidative stress, as measured by the levels of nitrotyrosine. **Hypothesis:** We hypothesize that mice with AP, as compared to mice without AP, will show increased atherosclerosis lesion burden, increased levels of systemic inflammation and nitrotyrosine. **Methods**: AP was induced in the pro-atherogenic low-density lipoprotein receptor knockout (LDLR KO) mouse model by exposing the dental pulp of four molars in each mouse in the AP group (Rx) (n=17). Controls received only anesthesia (n=23). Mice were fed a high-fat, Western diet to induce atherosclerosis. Plasma was collected to characterize the inflammatory profile (cytokine array) at eight and 16 weeks after AP induction. At 16 weeks, the mice were euthanized and the aortas were collected to measure the atherosclerosis lesion burden (oil red O staining). AP lesions were validated using micro-CT and histology. **Results:** There were no differences in weight gain or levels of total plasma cholesterol between the groups at 16 weeks. These data show that these risk factors of atherosclerosis will not potentially confound our

results. Despite our hypothesis that mice in the Rx group would show an increase in atherosclerosis lesion burden as compared to the sham group, we found they, in fact, developed a similar degree of atherosclerosis (lesion area in the Rx group 7.65 ± 0.47%, compared with 7.46 ± 0.44% in the sham group, p=0.77). The similar atherosclerosis lesion burden percentages in both groups are aligned with the lack of difference in pro-atherogenic cytokines. Lack of difference in both of the above can be explained by the variability in the number of periapical lesions within the Rx group, which might have resulted in local inflammation below the level of creating a systemic inflammatory difference. **Significance:** To our best knowledge, this is the first study using the LDLR KO mouse model for atherosclerosis to study the influence of AP. Although no difference was found between the groups, we recommend viewing this study as a launch pad for future studies, after addressing the challenges which are discussed in the manuscript. Since AP still has the potential to have a contributive role in the development of atherosclerosis, this area should be further explored.

PREFACE

This thesis is an original work by Yuli Berlin-Broner. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Apical Periodontitis as a Contributive Risk Factor to Atherosclerosis", AUP00001782, September 21, 2016.

Appendix 1 of this thesis has been published as Berlin-Broner Y, Febbraio M, Levin L. *Association between Apical Periodontitis and Cardiovascular Diseases: Systematic Review of the Literature*. International Endodontic Journal. International Endodontic Journal. 50(9):847-859 (2017)

Appendix 2 of this thesis has been published as Berlin-Broner Y, Febbraio M, Levin L. *Chronic Apical Periodontitis and Atherosclerosis – Is there a Link? Review of the Literature and Potential Mechanism of Linkage.* Quintessence Int. 48(7): 527-534. (2017)

I was responsible for the literature review and manuscript composition. Both Prof. Maria Febbraio and Prof. Liran Levin were involved in the planning and reviewing the manuscripts.

ACKNOWLEDGEMENTS

I would like to acknowledge the great support and assistance that was provided by my supervisors, Maria Febbraio and Liran Levin, for their dedication, patience, and availability. I am grateful for their precious time, which they have put into this research, along with my personal development during this project. It was a real honor for me to work together and learn from their scientific knowledge and human qualities.

I am truly thankful to Maria Alexiou, for being part of this study, for her intellectual and practical contributions to this research.

I am grateful to the members of my supervisory committee, Ava Chow, and Patrick Flood, for their support, advice and useful suggestions along the way of my research, and to Les Campbell for being the arm's length to review this project

I would also like to thank other members of the Department of Dentistry, for their contribution, in various aspects of this project: Pranidhi Baddam, Raisa Catunda, Danielle Clark, Daniel Graf, Konrad Lehmann, Mohammad Omar, Pallavi Parashar, Julia Piche and Umar Rakhi.

Finally, I would like to thank the funding agencies who provided support to this project: Canadian Association for Dental Research (CADR) and Network for Canadian Oral Health Research (NCOHR), University of Alberta (Motyl Graduate Studentship in Cardiac Sciences; Fund for Dentistry; Graduate Student Fellowship; Medical Sciences Graduate Program Scholarship) and Alpha Omega Foundation of Canada Research.

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CHAPTER 1: INTRODUCTION

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1.1.1 Epidemiology of cardiovascular disease

Cardiovascular disease (CVD) is the number 1 cause of death throughout the world [http://www.who.int]. In Canada, a person dies from CVD every 7 minutes despite the prevalence of lipid-lowering drugs [http://www.who.int]. An underlying cause of CVD is atherosclerosis, defined as a progressive inflammatory accumulation of lipid (plaque) involving large to medium-sized arteries (Hansson et al. 2005). Atherosclerosis can lead to CVD, heart attack and stroke [http://www.nhlbi.nih.gov]. Atherosclerosis is recognized as a chronic inflammatory disease with multiple risk factors, such as hyperlipidemia, age, gender, high blood pressure and smoking (Hansson GK 2005). However, less focus has been directed towards inflammatory processes that may further contribute to its development. Recently, increased CVD morbidity has been linked to other inflammatory diseases such as rheumatic diseases, obesity, diabetes, chronic oral diseases; these may contribute to additive or synergistic systemic inflammation/oxidative stress (Castaneda S. et al 2016).

1.1.2 Biological mechanisms of atherosclerosis

Atherosclerotic lesions, or atheromas, are asymmetric focal thickenings of the innermost layer of the artery, which is known as the intima (Hansson GK. 2005). The intima is in contact with blood and is lined by endothelial cells. Atheromas begin when lipid from circulating high fat activates endothelial cells. Increased blood lipids lead to changes in shear stress, and this is perceived by mechano-sensors on endothelial cells as a danger signal (Chien S. 2007).

Endothelial cells respond to these abnormalities by expressing chemo-attractants for monocytes (Sprague AH & Khalil RA. 2009). The endothelial cell surface also changes, and expresses receptors, such as a vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), to guide monocyte migration and extravasation to the site of lipid accumulation in the sub-intima (Sprague AH & Khalil RA. 2009). As a result of these signals, monocytes mature into macrophages, under the influence of monocyte colony stimulating factor (M-CSF) produced locally. The elevated plasma low-density lipoproteins (LDLs) also enter the sub-intimal space where they are subject to modification by reactive

oxygen species or enzymes expressed by the intimal cells. These macrophages express cell surface scavenger receptors, which are able to bind and endocytose sufficiently modified lipoprotein. Not only do these modifications of lipoproteins allow for the recognition by macrophage receptors, but they also represent neo-antigens that stimulate the adaptive immune system to produce antibodies (Moore KJ et al. 2013, Getz GS & Reardon CA 2015). Although macrophages can take up and degrade the lipid, this process can be overwhelmed by chronic high fat, leading to entrapment of macrophages within the sub-intima.

Increased oxidative stress in the plasma is known to accelerate the progression of atherosclerosis by interaction with lipids accumulating in the artery wall (Lubos E et al. 2008). Peroxynitrite is a potent reactive oxygen species formed from the reaction between superoxide radicals and NO. Peroxynitrite and the surrogate marker 3-nitrotyrosine have been associated with the oxidative stress found as a result of chronic inflammation in atherosclerosis, leading to endothelial dysfunction and pathological changes in the vessel wall (Lubos E et al. 2008). Oxidative stress has also been implicated in periodontal disease (Chapple IL 2007). Since AP isa local inflammatory process, similarly to periodontal disease, it can potentially influence systemic oxidative stress, and therefore accelerate atherosclerosis. Therefore, to explore mechanisms connecting AP and atherosclerosis, systemic oxidative stress can be compared between mice with and without AP.

The initial lesion of atherosclerosis is known as the fatty streak and consists almost entirely of lipid-engorged macrophages. As the disease progresses, these lesions grow, attract other immune cells and smooth muscle cells, which encapsulate the lesion. Cells die as a result of lipotoxicity, and lipid, cholesterol, and cellular debris accumulate, along with connective tissue elements, as the body attempts to heal this wound (Stary H. et al. 1995). Atherosclerosis plaques may be characterized as stable or unstable, depending upon the strength and thickness of the smooth muscle cell cap (van der Wal AC & Becker AE. 1999). Unstable plaque can rupture, spilling contents that promote blood clotting and occlusion of the vessel (van der Wal AC & Becker AE. 1999). This may result in myocardial infarction or stroke with potentially fatal consequences.

Atherosclerosis is recognized as a chronic inflammatory disease in that metabolic risk factors, such as hypercholesterolemia and hypertriglyceridemia, lead to an activated endothelium

which triggers an immune response, and this initiates, propagates and activates lesions in the arterial tree (Hansson GK. 2005, Shapiro MD & Fazio S. 2016). For more information, please refer literature reviews (Berlin-Broner et al. 2017[a, b]).

1.2 Apical periodontitis

1.2.1 Epidemiology of apical periodontitis and periodontal disease

In the oral cavity, a degree of inflammation is almost always present in the form of the two most common oral infectious inflammatory diseases, periodontal disease (gum disease) and apical periodontitis (AP) (endodontic disease). A positive epidemiological association between periodontal or 'gum' disease and atherosclerosis has been established, and there have been some supportive basic science studies investigating the mechanisms (Janket SJ et al. 2003, Blaizot A 2009). However for AP, although there is evidence for an association between it and CVD from epidemiologic studies, basic science support is lacking (Berlin-Broner Y et al. 2017[a]).

Periodontal disease is inflammation of the tissues surrounding the tooth, including the periodontal ligament, cementum, and alveolar bone. It develops as a response to (mostly) gram-negative, anaerobic bacteria, which originate in the oral cavity and accumulate on the tooth surface as dental plaque (Beck JD et al. 2000, Clark D et al. 2017). The host mounts an inflammatory response to the bacteria in plaque. Whether acute or chronic, the inflammation can eventually lead to loss of bone supporting the tooth structures. Severe periodontal disease is found in 15–20% of middle-aged (35-44 years) adults. More than 70% of adults 65 and older have moderate to severe periodontal disease [http://www.who.int].

The association between periodontal disease and CVD has been widely investigated and a positive association has been reported in a systematic review and meta-analysis of observational studies (Blaizot A. et al. 2009). The meta-analysis concluded that periodontal disease is associated with a 19% increase in the risk of future coronary heart disease (Blaizot A. et al. 2009). Furthermore, the increase in relative risk was more prominent (44%) in persons aged 65 years and older (Blaizot A. et al. 2009). Several basic science studies have shown a causative contribution of periodontal disease to atherosclerosis (Velsko IM et al. 2015, Brown PM et al. 2015).

The prevalence of AP, inflammation of the periapical tissue as a result of bacterial infection of the dental pulp, is as high as 34–61%, and it increases with age (Dugas NN et al. 2003, Jimenez- Pinzon A et al. 2004, Lopez-Lopez J et al. 2012). This is likely an underestimation due to the limited sensitivity of radiographs (Petersson A et al. 2012, Lennon et al. 2011). Frequently, AP can be detected only in later stages when the size/location of the lesion is significant (Petersson A et al. 2012, Green TL et al. 1997). Since AP is usually asymptomatic, patients may be unaware they are affected (Yu VS et al 2012). Thus, AP may be overlooked during clinical assessment of CVD, despite the fact that it is very common in the age group of patients prone to atherosclerosis (Dugas NN et al. 2003). Therefore, a study to reveal the impact of AP on systemic health is needed. Although epidemiologic evidence for association between AP and CVD exists, and new studies continue to emerge, demonstrating that this area of research draws interest within the dental research community (Berlin-Broner Y et al. 2017[a]), basic science evidence is lacking (An GK et al. 2016, Garg P & Chaman C et al.2016, Liljestrand JM et al. 2016, Berlin-Broner Y et al. 2017 [a]).

Please refer to Berlin-Broner Y et al. 2017 [a, b] to read about connections between apical periodontitis and other systemic conditions.

1.2.2 Biological mechanisms of apical periodontitis

Periapical lesions are the manifestation of the immune system aiming to prevent the spread of bacteria from the infected root canal into the surrounding bone. Formation of these lesions is associated with bone loss in the area surrounding the root apex, appearing as radiolucent periapical lesions on a radiograph. The inflammatory response and its progress or healing are commonly evaluated by the size and morphology of the lesion as shown on a radiographic image (Metzger Z 2000).

The histopathologic analysis of a diseased tissue or organ only shows structural changes of cells and extracellular matrix at the time the tissue or organ is removed. Therefore, it does not represent the complete kinetics or spectrum of disease development. Histologic classification of AP is based on types of cells participating in inflammatory responses in the periapical tissues (Hargreaves K & Berman L. 2015). Acute and chronic inflammatory responses are in fact not rigid phases of a single programmed event but two overlapping responses with partially different triggering mechanisms and programs (Majno G & Joris I 2004). Histopathological evaluation

revealed that of 256 specimens of periapical lesions, 35% were classified as periapical abscess, 50% as granuloma, and 15% as cysts (Nair PNR et al. 1996).

Traditionally, the terms asymptomatic chronic AP and periapical granuloma are used interchangeably. A granuloma is a focal area of granulomatous inflammation, which is a histologic term for a chronic inflammatory reaction. Granulomatous inflammation is characterized by the presence of activated macrophages with modified epithelioid cells in diseases such as tuberculosis, leprosy, syphilis, cryptococcosis, sarcoidosis, rheumatic fever, and foreign body granuloma (Hargreaves K & Berman L. 2015). A granuloma is relatively avascular, whereas a chronic AP is very vascular. Histologically, some but not all chronic AP lesions may show some features of granulomatous inflammation, so the terms apical granuloma and asymptomatic chronic AP should not be used interchangeably (Hargreaves K & Berman L. 2015). Two categories of cyst were found: the apical true cysts and the apical pocket cysts. The true cyst is completely enclosed by a lining epithelium, and its lumen has no communication with the root canal of the involved tooth. In contrast, the pocket cyst's lumen opens into the root canal of the involved tooth (Nair PNR et al. 1996).

The cells involved in the inflammatory process of AP include B- and T-lymphocytes, plasma cells, macrophages and polymorphonuclear leukocytes (PMNs) (Metzger Z 2000). Macrophages and lymphocytes are predominant cells in asymptomatic AP lesions (Hargreaves K & Berman L. 2015).

Please refer to Chapter 3 to read about the suggested mechanism connecting apical periodontitis with CVD.

1.3 Animal model considerations

There are established mouse models to study AP as well as atherosclerosis. However, there are no studies to date that address the relationship between AP and atherosclerosis in a single animal model (Berlin-Broner Y et al. 2017 [a]). Therefore, we created a novel mouse model, which combines the two diseases. This model allows us to investigate the relationship between AP and CVD, while avoiding other potential common confounders that exist in a human study setting, including periodontal disease, medications, and other systemic conditions.

1.3.1 Mouse models to study atherosclerosis

Animal models used for the investigation of atherosclerosis have included rabbits, pigs, and nonhuman primates, each of which has its advantages and disadvantages (Getz GS & Reardon CA et al. 2015). The currently favored species is the mouse, which is small, has a relatively short lifespan and is relatively inexpensive to maintain. By far the most important aspect with regards to the use of mice is the availability of specific gene variants, due to the relative ease of genetic manipulation (such as gene knockout) (Getz GS & Reardon CA et al. 2015).

LDLR KO and apoE KO are the two most extensively used mouse models (Getz GS & Reardon CA 2016). Both of these models are characterized by hypercholesterolemia which is a prerequisite for the development of atherosclerosis in murine models, but there are some differences. Unlike the apoE KO, LDLR KO mice do not develop atherosclerosis when fed standard rodent chow diets; and thus a high cholesterol diet is needed to provide the hyperlipidemic drive for atherogenesis (Getz GS & Reardon CA 2016).

In this project, we used LDLR KO mice (B6.129S7-Ldlr^{tm1Her}/J, strain #002207). This is a classic model and recognized in the field as reliably mirroring aspects of human disease (Zadelaar S et al. 2007). Lesions begin as fatty streaks and over time can mature and increase in complexity (Lichtman AH 1999).

Based on the differences between the two models above, one reason we used the LDLR KO model was in order to control and set the onset of atherosclerosis development at the same time point in the control and AP groups by initiation of the Western type diet (WTD). As a result, we could use older mice, without worrying that the process of atherosclerosis had already begun. Older mice were used because weight at the time of anesthesia proved to be important for survival. Additionally, the slower development and overall less lesion burden, compared to the apoE KO, would allow us to identify differences between the two groups more easily.

1.3.2 Mouse models to study apical periodontitis

A model of AP has already been established in rodents. The first study in the field that used a rodent model for induction of periapical lesions (PALs), was performed by Kakehashi S et al. (1965) in rats. Numerous studies have been published since then in mice (da Silva RA et al 2012, Aranha AM et al. 2013, Ma J et al. 2013, Gao B et al. 2013, Araujo-Pires AC et al. 2014,

Sousa NG et al. 2014, Wan C et al. 2014, da Silva RA et al. 2014, Oliveira KM[a] 2015, Nikolaeva EP et al. 2015, Francisconi CF et al. 2016, Hao L et al. 2015, de Oliveira KM[b] et al. 2015 and others). The classic study by Kakehashi and colleagues (Kakehashi S et al. 1965) demonstrated that pulp necrosis and periradicular inflammation developed in rats raised in conventional facilities when the pulps of teeth were exposed to oral microorganisms. However, in germ-free laboratory rats, no pulp necrosis and periradicular inflammation occurred, even when the pulps of teeth were exposed to the oral environment and packed with sterile food debris. A similar response occurs in humans. Only when bacteria were isolated from traumatized teeth with intact crowns and necrotic pulps was there radiographic evidence of periradicular bone destruction (Sundqvist G 1976).

Since the classical study of Kakehashi and colleagues (Kakehashi Set al. 1965), others adopted their protocol and used it in mice to induce PALs (Wang CY & Stashenko P 1991, Balto K et al. 2000, von Stechow D et al. 2003, Shah A et al 2018). The success rate for induction of PALs by exposure of the pulp to the oral cavity was reported to be 85% (17/20) of the drilled teeth (4 molars in each mouse) (Balto K et al. 2000, von Stechow D et al. 2003). The remaining fifteen percent of the drilled molars presented with minimal periradicular bone destruction. The authors reported that a closer examination of the corresponding histological sections revealed that the pulp exposures in these teeth were very small, resulting in delayed pulpal infection and necrosis and slowed periradicular bone destruction (Balto K et al. 2000, von Stechow D et al. 2003).

In addition to histological sections, PALs can be detected and followed radiographically, since they appear several days after pulp exposure, as a periapical radiolucency or widening of the periodontal ligament (PDL) space. As described by Akamine A et al. (1994), radiographic examination revealed an increase in the width of the PDL space at the distal root apex on the 4th day after pulp exposure in radiographs, and small but distinct periapical radiolucencies were detected on day 10. Between days 10 and 60, the size of the periapical radiolucencies increased in a time related fashion, and some specimens showed large radiolucencies which extended up to the furcation area. After 90 days, the size of the radiolucencies was stable or was slightly decreased compared with that observed at 60 days (Akamine A et al. 1994). The presence and kinetics of bone-resorbing activity in periapical lesions were studied in a rat model, where the

lesions were found to expand most rapidly between induction on day 0 and day 15 (= the "active phase"), with enlargement occurring at a slower rate thereafter (days 20 and 30 = the "chronic phase", Wang CY & Stashenko P 1991).

As mentioned above, in animal models, periapical bone resorption may be validated by histological evaluation (Sun Z et al. 2014), conventional and digital radiography (Teixeira RC et al. 2011). However, since, digital and conventional two-dimensional radiographic examinations are not entirely suitable for imaging of teeth in rats and mice, due to their small size and specific jaw anatomy (Kalatzis-Sousa NG et al. 2017), a useful tool for evaluation of PALs in mice and rats is by means of micro-computed tomography (micro-CT) (Rittling SR et al. 2010).

Histological evaluation is considered the gold standard for assessing PALs (von Stechow D et al. 2003). Nevertheless, this method is time-consuming, leads to sample destruction (Balto K et al. 2000) and is technique sensitive. Micro-CT may, therefore, be an important tool for research involving periapical bone lesions in small animals (von Stechow D et al. 2003). Despite the advantages of the micro-CT and its increasing usage in studies of AP in small rodents, in a recently published systematic review, Kalatzis-Sousa NG et al. concluded that there is no defined protocol for acquiring and analyzing micro-CT images of periapical lesions in rats and mice. Furthermore, acquisition, reconstruction, and analysis parameters are not adequately explained, which may compromise the scientific impact of the studies (Kalatzis-Sousa NG et al. 2017).

1.4 Rationale

In a systematic review, we identified a positive association between AP and cardiovascular disease, however, no studies examined causality (Berlin-Broner Y et al. 2017 [a]). Performing a longitudinal study in humans is challenging due to the complexity of the systemic conditions influencing inflammatory status and the difficulty in controlling all potential confounders along with AP. A causative contribution of periodontal disease to the development of atherosclerosis was established in a mouse model (Brown PM et al. 2015). The lack of animal studies regarding AP and atherosclerosis may be attributed to the complexity of the experimental setting, which requires microsurgical techniques and long-term follow up. Thus, there is a gap in our knowledge regarding the causality of the relationship between AP and atherosclerosis and the mechanism(s) by which they may be linked. Therefore, an animal model is essential to study the role of AP as a separate risk factor in causing atherosclerosis.

1.5 Research question, objectives and hypotheses

As background investigation to develop this research question, a systematic review of the literature (Chapter 2) and a narrative review (Chapter 3) were performed and published. The overall goal of our study was to determine if there was evidence for a causal relationship between AP and atherosclerosis. Based on the literature review, we found feasibility for using a mouse model to investigate whether AP has a contributive role in the development of atherosclerosis. In order to address this research question, we defined 3 specific aims: Aim 1. To determine whether there is a difference between mice with AP and mice without AP in atherosclerosis lesion burden (percentage of atherosclerotic lesion area out of the total aortic area). Aim 2. To determine whether there is a difference between mice with AP and mice without AP in the levels of systemic inflammation (inflammatory cytokines in the plasma). Aim 3. 3. To determine whether there is a difference between mice with AP and mice without AP in the levels of systemic oxidative stress, , as measured by the levels of nitrotyrosine. Nitrotyrosine serves as a surrogate marker of dysregulated nitrous oxide (NO) synthase at sites of inflammation.

We hypothesize that mice with AP, as compared to mice without AP, will show increased atherosclerosis lesion burden, increased levels of systemic inflammation and nitrotyrosine.

CHAPTER 2: METHODOLOGY

2.1 Materials

- Mice: Low density receptor knock out (LDLR KO) mice, B6.129S7-Ldl rtm1Her/J, strain ,#002207. The Jackson Laboratory.
- Western type diet (WTD): Teklad Diet #88317 (21% fat, 0.2% cholesterol, no cholate). Envigo. Indianapolis, IN, USA.
- Cytokine array: Proteome ProfilerTM Array, Mouse Cytokine Array Panel A. R&D systems, INC. Catalog number (CN): ARY006. Minneapolis, MN, USA.
- Quantification of protein: PierceTM BCA Protein Assay Kit. Thermo Scientific. CN: 23227. Rockford, IL, USA.
- Cholesterol assay: Total Cholesterol Assay Kit (Colorimetric), Cell Biolabs, INC. CN: STA-384. San Diego, CA, USA.
- Decalcification: Ethylenediamine tetraacetic acid (EDTA), Disodium Salt dihydrate, S311-500, Fisher Scientific, Ottawa, ON, Canada.
- Anti-coagulation: EDTA, Dipotassium Salt Dihydrate. CN: BP119-50, Fisher Scientific, Ottawa, ON, Canada
- Anesthesia: Ketamine hydrochloride (100mg/mL), Ketaset, Pfizer Animal Health, Pfizer Canada Inc. CN: DIN 02173239- Kirkland, QC, Canada; Xylazine, provided by Health Sciences Laboratory Animal Services (HSLAS), University of Alberta.
- Analgesia: Buprenorphine hydrochloride (HCl), provided by Health Sciences Laboratory Animal Services (HSLAS), University of Alberta.
- Euthanasia: Pentobarbital Sodium, euthanyl, Bimeda-MTC, Animal Health Inc. CN: DIN 00141704, Cambridge, ON, Canada.
- Thioglycollate: Thioglycollate CN: CM0023. OXOID LTD, Basingstoke, Hampshire, England.
- Lyses of Macrophages: Dave's Lyses buffer (components by Fisher and Sigma)
- Histology sectioning and hematoxylin and eosin (H&E) staining: HistoCore histology services, Alberta Diabetes Institute, University of Alberta, Edmonton, Canada.
- Automatic tissue processor: Leica TP1020, Leica Biosystems, Concord, Ontario, Canada,
- Embedding Center: Leica EG1160, Leica Biosystems, Concord, Ontario, Canada

- Embedding wax: Histoplst LP Paraffin, CN: 22900702, Fisher Scientific, Ottawa, Ontario, Canada
- Micro-CT: Milabs U-Micro CT (Utrecht, The Netherlands)
- Software for Micro-CT analysis: Amira v5, Thermo Fisher Scientific. , Ottawa, Ontario, Canada
- Surgical Microscope for AP induction: Carl Zeiss F-125, Germany.
- Microscope for dissection of aortas: Leica S6, Leica Microsystems, Concord, Ontario. Canada,
- Upright microscope for histology: Leica DMRE, Leica Microsystems, Concord, Ontario. Canada.
- Camera for histology images: Leica MC170 HD, Concord, Ontario, Canada.,Circulating pump to circulate water through a therapeutic pad: T/Pump Classic, Model: #TP650, Gaymar, Orchard Park, NY, USA.
- Drilling: NSK MIO motor, Control unit: model NE116, CN U399; E-Type Micro-motor: model MIO-35EM, CN E236015. Contra-angle head: NSK FFB 07500210
- Sterile #1/4 burs, ISO#10 k-files, Micro surgical round mirror #4, 3mm diameter, Hu-Friedy. Chicago, IL, USA.
- Vannas scissor, Excelta, CN: 366, Buellton, Canada.,Fine tweezers, Excelta, CN: 1746177, Buellton, Canada.
- Artificial Tears (Contains glycerin 0.2% and hypromellose 0.2%), CN: DIN 0235453, atoma, Canada.
- Scanner for aortas: CanoScan, Cannon, LiDE 210, Melville, NY, USA.
- Software for atherosclerosis lesion measurement: Adobe Photoshop 5.5, Adobe Inc. San Jose, California, U.S.
- Software for statistical analysis: GraphPad Prism 5
- Oil Red O, Biological Stain, Acros Organics, CN: 1320-06-5, Fisher Scientific, Ottawa, Ontario, Canada.
- Analysis of cytokine array: Chemi Doc imaging system, Image Lab, Bio Rad software
- Cytokine array reader: BIO RAD ChemiDoc MP Imaging System.

- Microscope slides, Superfrost Plus (25X75X1.0mm), CN: 12-550-15, Fisher Scientific, Pittsburg, PA, USA.
- Formaldehyde 10%, Formaldefresh, Fisher, Fisher Scientific, Ottawa, ON, Canada.
- Phosphate-buffered saline (PBS), Fisher BioReagents CN: BP3994, Fisher Scientific, Ottawa, ON, Canada.
- Spectrophotometric microplate reader, Synergy H1, BioTek, Vermont, U.S.A.
- Miscellaneous laboratory chemicals by Fisher and Sigma

2.2 Ethics: The Animal Care and Use Committee (ACUC) of the University of Alberta approved all animal procedures (AUP00001782) prior to study initiation.

2.3 Sample size: The number of mice in each group is based on a power calculation. Results from the CVD study that used a periodontal disease model driven by *Porphyromonas gingivalis* (Pg) in LDLR KO mice showed that sham treated mice had a mean lesion area of 6% and Pg treated mice had a mean lesion area of 12%. The standard error was 3%. We expect a similar trend in the atherosclerosis studies contained herein. Therefore, in order to achieve a p value <0.05 with 90% power, the sample size will be 11 mice per group.

Because these studies involve the use of anesthesia, have a surgical arm and are very long (16 weeks), there is the potential for complications and dermatitis, such that the mice may need to be terminated early. Therefore, we added an additional 6 mice/group (n=17) to ensure that our study goals were reached.

Additionally, due to possible variability in success of induction of PALs after pulp exposure, we planned to validate, via micro-CT scan, the presence of PALs at an early time point of four week, and to adjust the number of mice in the treatment (Rx) group based on our findings (for example, adding more mice to the Rx group in order to have at least n=11 mice with four PALs at the 16 week time point). For this purpose, of PALs evaluation at four weeks, we planned to use six Rx mice (n=6) and to compare them with sham mice (n=6) at the same time point. Finally, we also included five baseline mice, as controls. Experimental groups are shown in Figure 2.1.

We began with male mice and depending on our findings, we may extend our work to female mice at a later time (in the Pg study, both sexes were similarly affected).

2.4 Experimental overview and time line

At time 0, we planned to begin with two cohorts of mice to go on a WTD (total n=46), one to a four weeks' time point (6 mice per group, sham or Rx, total n=12) and one to the end-point of 16 weeks (17 mice per group, sham or Rx, total n=34).

At time 0, all the mice (n=46) were anesthetized. At this point, four molars were drilled to expose the pulp in the Rx group mice (n=23), resulting in 96 drilled molars in total and no teeth were drilled in the sham group mice (n=23).

Right after the surgery or sham anesthesia, and twice daily in the next 48 hours, all mice received analgesia. Also, all mice had their normal chow diet replaced into WTD, on which they remained for four (n=12) or 16 weeks (n=34).

After four weeks, the cohorts of the PALs validation groups, Rx (n=6) and sham (n=6) were euthanized and the heads were collected. After eight weeks, blood was collected from the 16 weeks cohort mice, Rx (n=17) and sham (n=17), for analysis of plasma cytokines. At the end-point (16 weeks), the Rx (n=17) and sham (n=17) mice, were euthanized and samples were collected, including blood, aorta, and heads. A summary of the experimental timeline can be found in Figure 2.1.

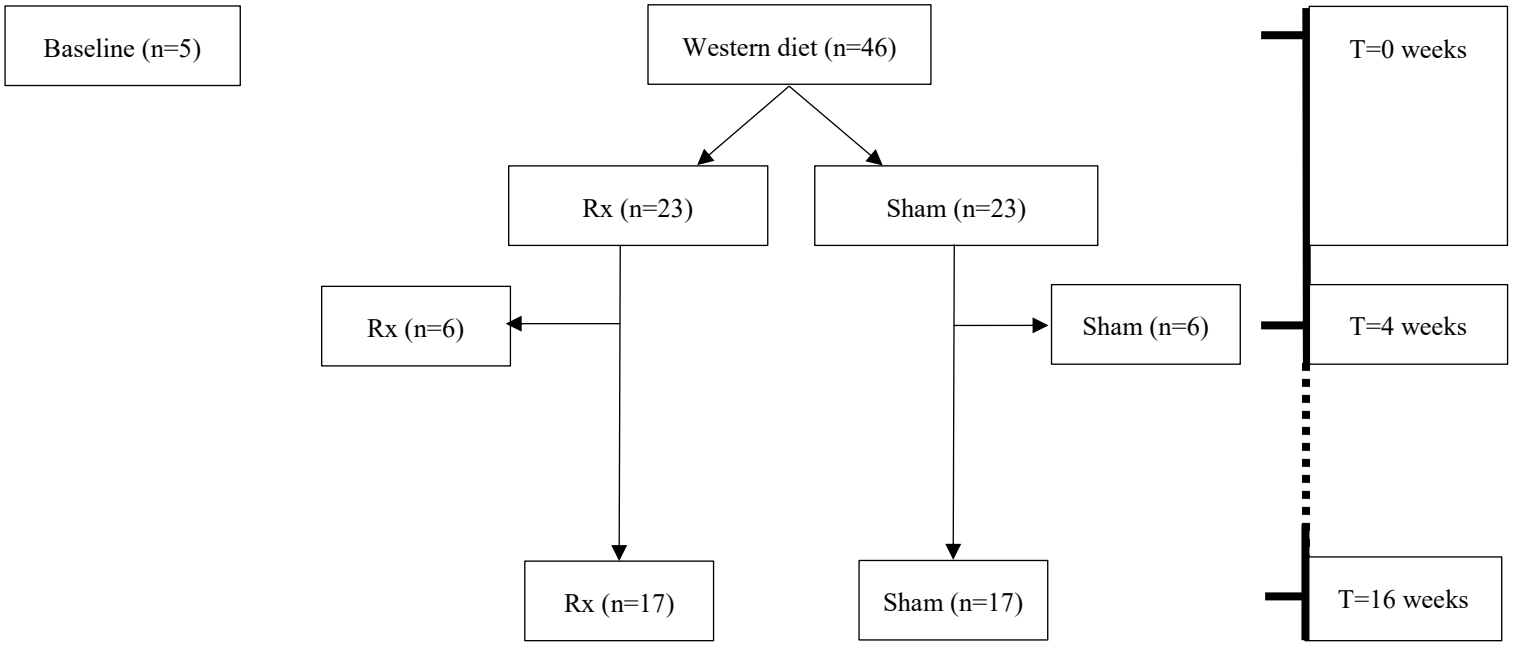


Figure 2.1: Experimental timeline. At time 0 (T=0 weeks) 46 mice in total started WTD, right after surgery/sham anesthesia, Rx group (n=23), sham (n=23). After four weeks (T=4 weeks), six mice from each (Rx/sham) were planned to be euthanized, in order to evaluate success of PALs induction via micro-CT. After 16 weeks (T=16 weeks), the remaining 17 from each group (Rx/sham) were planned to be euthanized. Additionally, 5 mice were used as baseline. They were not placed on WTD and were euthanized at baseline (T=0 weeks).

2.5 Mice: This project utilized 12-14-week-old LDLR KO male mice. This age was chosen based on our preliminary results and cadaver studies because the mice are large enough to allow anesthesia and induction of AP, as will be described below in 2.6 'apical periodontitis induction' section. Mice were kept in the same environmental conditions (temperature, humidity, and light-dark cycles).

2.6 Apical periodontitis induction: Mice were randomly assigned to one of two groups: Rx or sham. In the Rx group, apical periodontitis was induced; sham group mice served as controls. Just before the surgery, mice were weighed and the amount of ketamine and xylazine to be administered was calculated. The mice were anesthetized by intraperitoneal (IP) injection of ketamine (80mg/kg) and xylazine (12mg/kg). The surgery or sham procedures were performed on a water circulating heat pad (37^{o}C), in order to prevent a drop in the body temperatures of the mice during the period under anesthesia. 'Artificial Tears' (glycerin 0.2% and hypromellose 0.2%), in the form of eye drops, were applied every 5 minutes to keep the eyes moisturized while the eyelid reflexes are not active due to the anesthesia, to avoid damage to the eyes.

The pulps of the first molars (4 total) in each mouse were exposed to the oral environment. Due to the small size of the teeth (1.5-2mm occlusal surface), the drilling procedure, to expose the pulp, was performed under a surgical microscope (Carl Zeiss F-125, Germany), with the help of a microsurgical mirror round mirror #4, 3mm diameter (Hu-Friedy. Chicago, USA.) (Figure 2.2). The procedure was performed by one operator, who performed the drilling, and another operator, who performed the retraction of soft tissues, and monitored the mice during anesthesia. The drilling was performed with a dental round bur #¼ (0.5mm), on a slow-speed dental hand piece (NSK MIO motor) to create a cavity, equivalent to the diameter of the #¼ round dental bur, 0.5mm diameter (Figure 2.3). Sham group mice were anesthetized only. Pulp exposures were verified with a small instrument (endodontic hand file #10 k-file), which was inserted into the exposure site, as demonstrated in Figure 2.3(C). This ensured that we created a route for infection from the oral cavity to the pulp, which will eventually result in apical periodontitis.

The exposed pulps were left open to the oral environment; therefore, food, debris, and contaminating microorganisms could become impacted into the pulpal tissues. This is a well-established technique for AP induction (Kakehashi S et al. 1965, Wang CY& Stashenko P 1991).

Figure 2.2 (A-C): Instruments used for PALs induction. (A) Microsurgical round mirror #4, 3mm diameter, which was used for the drilling procedure (black circle), placed next to a dental mirror. (B) Round dental bur #¼, 0.5mm diameter, that was used for drilling, in order to expose the pulp (C) Number ¼ round dental bur during the drilling process in a right mandibular first molar (yellow circle).

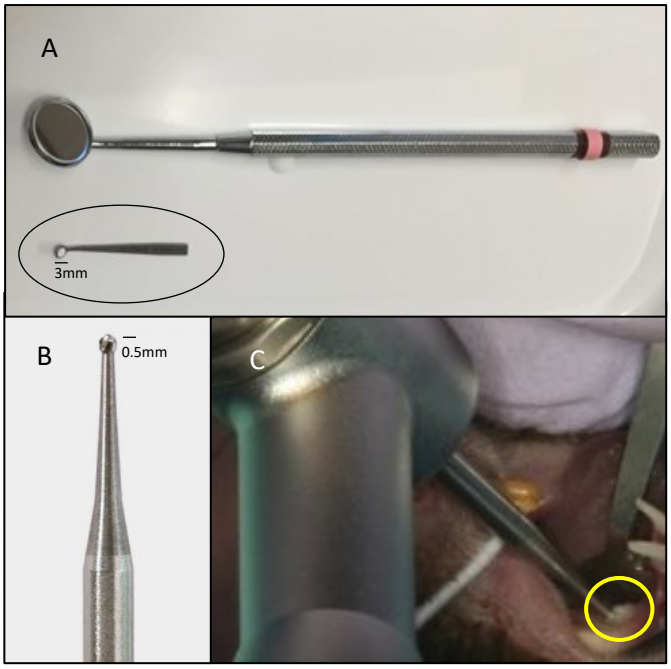
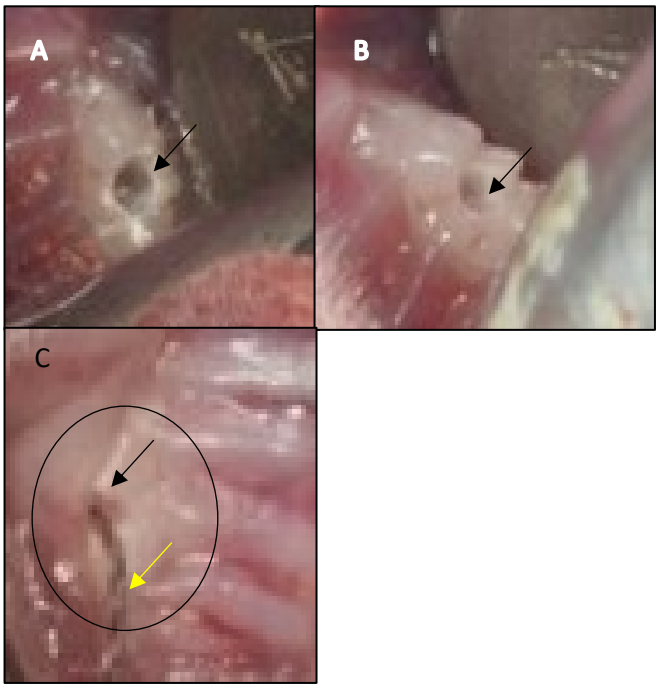


Figure 2.3 (A-C): Pulp exposure and verification in the first molars. The cavities created were approximately the size of a #¼ round bur, 0.5mm (black arrows). (A) Left maxillary first molar (black arrow pointing to the drill cavity). (B) Right mandibular first molar (black arrow pointing to the drill cavity). (C) Right maxillary first molar, showing a #10 K-file (yellow arrow) inserted to the drilled cavity (black arrow), in order to verify pulp exposure, confirming a connection between the oral cavities to the pulp.



After the surgery, mice were carefully monitored until awake in a cage on a clean paper towel also on a heating pad. Buprenorphine-HCl (0.03mg/kg), an opioid analgesic agent, was given by subcutaneous (SC) injection twice per day for 2 days post procedure in both the Rx and sham groups. The first dose was given immediately upon waking.

All mice were weighed before surgery and then every other day for 2 weeks post-surgery and then weekly until euthanasia (up to 16 weeks). Weight was used as a surrogate sign of distress, and included in a post-surgical monitoring sheet. Animals in the Rx group were expected to eat, gain weight, and behave in a similar manner to controls. Previous work showed that mice were not seriously compromised by the procedure (Wang CY & Stashenko P et al. 1991).

AP lesions (PALs) were expected to increase in size significantly in the first 3 weeks after pulp exposure and could grow larger as the study progressed, but at a slower pace (Wang CY & & Stashenko P et al. 1991). Therefore, in both time points (four and 16 weeks) PALs are expected to be detected.

2.7 Western diet: WTD (21% fat, 0.2% cholesterol, no cholate) was initiated simultaneously with the pulp exposure or sham procedure (T=0) and continued for up to 16 weeks (T=16W), as shown in Figure 2.1. This duration is ~4 weeks longer than the typical timeframe used by CVD researchers to investigate atherosclerosis lesions in LDLR KO mice (Lichtman AH 1999) and is based on our previous experience in the Edmonton facility in studies using the periodontal disease model. We found that at 12 weeks the amount of lesion was less than 5%. At this low level, it is difficult to detect differences. At 16 weeks, the atherosclerosis lesion burden was about 10% and significant differences could be detected between the groups.

2.8 Blood collection: Following overnight fasting (after an overnight fasting, since levels of LDL are influenced by food consumption), blood was collected at two-time points: mid-term (8 weeks) and endpoint (16 weeks).

2.8.1 Tail blood collection: At mid-term (8 weeks), blood was collected by tail bleeding from the 16 week' cohort. Briefly, each mouse was placed in a plastic restrainer, which allows access to the tail, while the rest of the body is held in a tube that allows free but restricted movement. While manually holding the tail, slightly pulled back, about 1mm of the end of the tail was cut

carefully by a razor blade to allow bleeding. Then, blood was collected in K_2EDTA, (5mM/L K_2EDTA final concentration) up to 80µL final volume.

2.8.2 Blood collection by cardiac puncture: Blood collection by a cardiac puncture: At the end point (16 weeks), following euthanasia by an overdose of pentobarbital (200mg/kg IP), blood was collected from each mouse, by cardiac puncture. Briefly, the abdomen of each mouse was soaked with 70% alcohol and then a small incision was made along the midline with sterile scissors. The abdominal skin was manually retracted to expose the intact peritoneal wall. Then, a small incision was made along the midline of the peritoneal wall, exposing the internal organs.

While the heart was still beating, the right ventricle was identified and a 25G needle was inserted just into the ventricular space. Blood was collected, in K_2EDTA (K_2EDTA 5mM/L final concentration) by applying negative pressure. In case the blood flow interfered, the needle was slightly pulled back, turned a little and gently reinserted back to the ventricle space, followed by continuous negative pressure to collect more blood. The procedure continued, until the maximal amount of blood was retrieved (about 800µL per mouse). The collected blood was immediately centrifuged (5 minutes, 21^{o}C at 1,100G and only plasma was kept. The plasma was stored at -20^{o}C for further analysis (see 4.9 'plasma cholesterol levels' 4.10 'plasma cytokine profiling' below).

2.9 Plasma cholesterol levels: Plasma cholesterol levels were measured using the Total Cholesterol Assay Kit (Colorimetric) by Cell Biolabs, Inc., according to the manufacturer's instructions. This kit measures the amount of total cholesterol present in plasma in a 96-well microtiter plate format. Sample cholesterol concentrations are determined by comparison with a known cholesterol standard (with a known concentration of cholesterol). Samples and standards are incubated for 45 minutes and then read with a standard 96-well colorimetric plate reader.

The assay is based on the enzyme-driven reaction that quantifies both cholesterol esters and free cholesterol. Cholesterol esters are hydrolyzed via cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase into the ketone cholest-4-en-3-one plus hydrogen peroxide. The hydrogen peroxide is then detected with a highly specific colorimetric probe. Horseradish peroxidase catalyzes the reaction between the probe and hydrogen peroxide, which bind in a 1:1 ratio.

Briefly, post overnight starvation the plasma that was collected at 16 weeks and stored at -20ºC, as was described in details above, in section 2.8 'Blood collection', was thawed at room temperature. An individual sample of each mouse's plasma was used. Each samples 3µl was used and diluted 1:300. Into each individual well, 50µl of the diluted plasma were pipetted, in duplicates. There were 39 samples in total (Rx n=17 and sham n=22), loaded in duplicates (two wells, with 50µl of diluted plasma in each, 78 wells in total were loaded with plasma samples).

Six cholesterol standards were prepared according to the manufacturer instructions. Briefly, we used the cholesterol standard provided in the kit and made the following dilutions: 9.65, 4.82, 2.41, 1.2, 0.6 and 0mg/dL. One well was a 'blank' and loaded with diluent only, in duplicate. Identically to the plasma samples, 50µL of each standard was loaded each well, in duplicates, with 14 well loaded with six standards, one blank in duplicates. Overall, 92 of the 96 wells plates were loaded with 50µL sample/standard each.

Next, 50µL of the Cholesterol Reaction Reagent was added to each well and mixed thoroughly. Then the plate wells were covered to protect the reaction from light and incubate the plate for 45 minutes at 37ºC. The plate was read with a spectrophotometric microplate reader (Synergy H1, BioTek, Vermont, U.S.A) at 540nm wavelength and the concentration of cholesterol within samples was calculated by comparing the sample absorbance values to the cholesterol standard curve.

2.10 Plasma cytokine profiling: Plasma that was collected at 8 and 16 weeks and stored at -20ºC, as was described in details above, in section 2.8 'Blood collection', was thawed at room temperature. We compared the relative plasma levels of 40 cytokines (refer to the detailed list of cytokines in section 2.14 below) in the Rx and the sham groups using a cytokine array (Proteome Profiler Mouse Cytokine Array Kit, Panel A, R&D Systems, Minneapolis, MN, USA).

The Proteome Profiler Mouse Cytokine Array Kit, Panel A is a membrane-based sandwich immunoassay. The array simultaneously detects cytokine differences between samples. The relative expression levels of 40 mouse cytokines can be determined (see full list of cytokines below).

Briefly, samples are mixed with a cocktail of biotinylated detection antibodies and then incubated with the array membrane which is spotted in duplicate with capture antibodies to

specific target proteins. Any cytokine/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, Streptavidin- horseradish peroxidase (HRP) and chemiluminescent detection reagents are added sequentially. Light is produced at each spot in proportion to the amount of cytokine bound.

The array was performed on the following four groups: Rx 8 weeks (n=4), sham 8 weeks (n=4), Rx 16 weeks (n=4) and sham 16 weeks (n=4). Briefly, 150µl of plasma from four mice was pooled to create each group, with a total of 600µL plasma per group.

Each of the four pooled samples (Rx/sham 8/16 weeks) was diluted, according to the manufacturer's instructions, mixed with a cocktail of biotinylated detection antibodies and then Incubated overnight at 4°C on a rocking platform shaker, with an array membrane (four membranes total), which is spotted in duplicate with capture antibodies to specific target proteins. Next, following a wash, the membranes were incubated with Streptavidin-HRP (HRP-Conjugated Streptavidin consists of streptavidin protein that is covalently conjugated to horseradish peroxidase (HRP) enzyme. Streptavidin binds to biotin and the conjugated HRP provides enzyme activity for detection using an appropriate substrate system.

Following incubation (30 minutes at room temperature on a rocking platform shaker) and wash, chemiluminescent detection reagent was added on each membrane.

Finally, the membranes were read in an imaging system (BioRad, ChemiDoc MP). The light which is produced at each spot is in proportion to the amount of cytokine bound. Images were taken after multiple exposure times in the range of 30-1000 sec and analyzed with image analysis software (Image Lab, BioRad). The software expresses the intensity of signal into pixels and the data can be exported into an Excel sheet. (Microsoft Excel).

The analysis was performed according to the manufacturer's instructions. Briefly, pixel densities of each pair of the spotted duplicates for each one of the 40 cytokines screened in the kit, were averaged. In order to compare the cytokine expression levels between the corresponding groups (each membrane represented one of the four groups), the pixel densities on the excel sheet were adjusted according to the control dots on each membrane.

The results are presented as the relative expression ('fold change') of a certain cytokine (Rx over sham) at a certain time point (8 or 16 weeks). 'Fold change', was calculated as follows: the average of signal intensity in sham group subtracted from the average signal intensity in the Rx group, divided by the signal intensity in the Sham group.

2.11 Peritoneal macrophage collection: Peritoneal macrophages were retrieved for cytokine levels analysis at the end-point (16 weeks) from Rx (n=4) and sham (n=4) mice. In the Rx group, one mouse had four PALs and three mice had three PALs. In addition to the high number of PALs, one of the mice had also four furcation lesions (FL), two of the mice had three FLs, and one mouse had two FLs. Therefore, the Rx mice that were pooled together for this test, were representatives of a high degree of potential dental inflammation.

Thioglycollate 4% solution, distilled water was prepared and autoclaved before use for elicitation of macrophages, mice were injected IP with 3ml sterile 4% thioglycollate four days prior to euthanasia. Following euthanasia with an overdose of IP pentobarbital injection (200mg/kg), macrophages were harvested by peritoneal lavage with sterile PBS.

Briefly, the abdomen of each mouse was soaked with 70% alcohol and then a small incision was made along the midline with sterile scissors. The abdominal skin was manually retracted to expose the intact peritoneal wall. Peritoneal cavity lavage was performed with 4mL of PBS and the fluid was aspirated and collected into a conical tube. This was repeated about three-four times until a clear liquid was retrieved; usually 12mL were collected in total.

The collected peritoneal fluid was centrifuged at 150G for five minutes at 21°C. The supernatant was discarded and the cell pellet was frozen (-20°C).

2.12 Protein content detection in the retrieved peritoneal macrophages: Since different amounts of macrophages were retrieved from each mouse, we measured the protein content in each sample, to normalize the protein amounts, before comparing the cytokines.

After thawing the samples at room temperature, we added the 100μL of following lysis buffer to the cells [lysis buffer: 10 mL of incomplete buffer:0.02M Tris, 0.15M NaCl, 1M EDTA, 1M Ethylene glycol tetraacetic acid (EGTA) was filter sterilized. Then one tablet of protease inhibitor cocktail 'complete mini EDTA free' (which is a mixture of several protease inhibitors, for the inhibition of serine proteases, cysteine proteases, but not metalloproteases) was added

together with 100µL of Triton 100)]. Of the lysed cells 2.5µL were diluted 1:10 with PBS for the protein assay to detect the amount of protein in each sample.

To quantify the amount of protein content in each sample (n=8), the Pierce™ Bicinchoninic Acid Kit (BCA) Protein Assay Kit was used, with different concentration of bovine serum albumin (BSA) as standards Briefly, in a multi-well plate, equal volumes (10µL) of samples and standards were loaded in duplicates.

The first step is the chelation of copper (Cu^{2+} to Cu^{1+}) with protein in an alkaline environment to form a light blue complex. In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one, resulting in an intense purple-colored reaction product. The reaction that leads to BCA color formation is strongly influenced by four amino acid residues, which are the amino acid sequence of the protein. The BCA/copper complex exhibits a strong linear absorbance at 562nm with increasing protein concentrations. The spectrophotometric microplate reader (Synergy H1, BioTek,) was used to read the plates, at wave length of 562 nm. After detection of the protein content in each sample, same amounts of protein were retrieved from each one of the eight samples and the four from the same group, Rx/sham.

2.13 Peritoneal macrophage cytokine profiling: For analysis and comparison of the cytokines content in the pooled samples of peritoneal macrophages, we used the two pooled samples that were prepared as described in section 2.12 'Protein content detection in the retrieved peritoneal macrophages' above. The procedure of cytokines detection was performed as was described in section 2.10 'Plasma cytokines profiling' above.

2.14 The 40 mouse cytokines that can be determined with the Proteome Profiler Mouse Cytokine Array Kit include (Proteome Profiler Mouse Cytokine Array Kit, Panel A, R&D Systems, Minneapolis, MN, USA)**:**

1. B lymphocyte chemoattractant (BLC) / C-X-C motif chemokine ligand /B cell-attracting chemokine 1 (BCA-1).
2. Complement Component C/C5a
3. Granulocyte-colony stimulating factor (G-CSF).
4. Granulocyte-macrophage colony-stimulating factor (GM-CSF).
5. I-309 (CCL1/TCA-3)

6. C-C motif chemokine ligand 11 (CCL11) / eosinophil chemotactic protein (Eotaxin).

7. Soluble intercellular adhesion molecule-1 (sICAM-1) / Cluster of differentiation 54 (CD54)

8. IFN-γ: Interferon gamma

9. IL-1α (IL-1F1): Interleukin1α

10. IL-1β (IL-1F2)

11. IL-1ra (IL-1F3): Interleukin1 receptor antagonist

12. IL-2

13. IL-3

14. IL-4

15. IL-5

16. IL-6

17. IL-7

18. IL-10

19. IL-13

20. IL-12 p70

21. IL-16

22. IL-17

23. IL-23

24. IL-27

25. C-X-C motif chemokine ligand 10 (CXCL10) / Interferon gamma-induced protein 10 (IP-10)

26. C-X-C motif chemokine ligand 11 / Interferon-inducible T-cell alpha chemoattractant (I-TAC)/Interferon-gamma-inducible protein 9 (IP-9).

27. C-X-C motif chemokine ligand 1(CXCL1). Keratinocyte chemoattractant (KC) / growth regulated oncogene 1 (GRO1) / GROα / neutrophil-activating protein 3 (NAP-3) / melanoma growth stimulating activity alpha (MSGA-α).

28. Macrophage colony-stimulating factor (M-CSF)

29., C-C motif chemokine ligand 2 (CCL2) / monocyte chemoattractant protein 1 (MCP-1) / small inducible cytokine A2 / JE

30. C-C motif chemokine ligand **12** (CCL12) / monocyte chemotactic protein 5 (MCP-5) / MCP-1-related chemokine.

31. Chemokine (C-X-C motif) ligand 9 / Monokine induced by gamma interferon (MIG).

32. Chemokine (C-C motif) ligand 3 (CCL3) / macrophage inflammatory protein 1-alpha (MIP-1- α).

33. Chemokine (C-C motif) ligand 4 (CCL4) / macrophage inflammatory protein 1-alpha (MIP-1- β).

34. Chemokine (C-X-C motif) ligand 2 (CXCL2)/ macrophage inflammatory protein 1-alpha (MIP-2).

35. Chemokine (C-C motif) ligand 5 (CCL5) / regulated on activation, normal T cell expressed and secreted (RANTES).

36. Chemokine (C-X-C motif) ligand 12 / stromal cell-derived factor 1 (SDF-1)

37. Chemokine (C-C motif) ligand 17 / thymus and activation regulated chemokine (TARC)

38. TIMP-1: metallopeptidase inhibitor 1

39. TNF-α: Tumor necrosis factor alpha

40. TREM-1: Triggering receptor expressed on myeloid cells 1

2.15 Euthanasia and fixation: At a terminal stage (four weeks or 16 weeks), mice were euthanized by IP injection of pentobarbital overdose (200mg/kg, IP). Following blood collection by cardiac puncture (previously described in section 2.8 'Blood collection') mice were fixed, in order to collect tissues for further investigation. Briefly, first mice were perfused twice with 10mL of PBS via cardiac puncture, to the left ventricle, with a 25G needle. This step is to wash out the blood from the vessels by applying positive pressure. Next, via the same technique, the mice were perfused with 10mL of 10% formaldehyde in order to fix the tissues to be collected for further study, as will be described in sections 2.10 'Aorta isolation and removal' and 2.21 'Heads removal and preservation' below.

2.16 Aorta isolation and removal: Following fixation with 10% formaldehyde, as described above (section 2.15 'Euthanasia and fixation'), the internal organs were removed from the abdominal area, including the intestine, liver, spleen, etc. leaving only the heart, aorta and both kidneys. At this stage, these remaining organs were extensively covered with fat, which was accumulated due to WTD. This fat was carefully removed, under a microscopic magnification, by using #4 and #5 forceps and scissors.

The aorta was identified and the fat was removed as much as possible of its outer surface. After removal of the fat, full exposure of the following was verified by visualization under microscopic magnification: heart, aortic arch with the three arteries that branch from the aortic arch (brachiocephalic trunk, left common carotid artery, and left subclavian artery), as shown in Figure 2.4, as well as the two renal arteries that branch from the aorta and lead to the kidneys and the aortic bifurcation into the two iliac arteries.

Next, the aorta was disconnected along its length and it was left stretched, connected only to the iliac arteries, heart, kidneys and the three branches leaving the arch. For successful retrieval of the aorta, it was first disconnected from the renal arteries, at the base (close to the aorta). Then, the three branches that leave the arch, were at their far ends, remaining connected to the aorta. Next the iliac arteries were cut. Lastly, the aorta was disconnected from the heart.

The entire aortic tree, from the heart, extending 3mm after bifurcation of the iliacs, including brachiocephalic trunk, left common carotid artery, and left subclavian artery, was dissected from the mouse, post-fixed in 10% formaldehyde, and stored at 4°C until the next steps, that will be described in details in the following sections aorta dissection and straining sections 2.17-20 below.

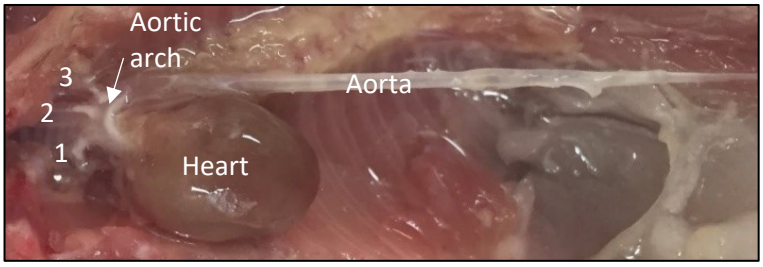


Figure 2.4: Aorta isolation. Aorta, isolated from mediastinal and abdominal fat, disconnected along its length, but still attached to the heart and to the brachiocephalic trunk (1), left common carotid (2) artery and left subclavian artery (3). The bifurcation of the iliacs is not shown in this image.

2.17 Aorta staining with oil red O: The fixed entire aortic (as was described in details in section 2.16 'Aorta isolation and removal' above), was stained with lipophilic dye (oil red O), in a well-established method (Febbraio M 2000).

Briefly, each fixed aorta was dipped into 1mL of oil red O solution and mixed manually occasionally over a period of 15 minutes. Next, the stained aorta was transferred into 100% methanol and rinsed by occasional manual mixing for 15 minutes to remove excess staining. Finally, each stained and washed aorta was stored in 1mL of 10% formaldehyde at 4°C. The whole staining process is shown in Figure 2.5 (A-D), bellow**.**

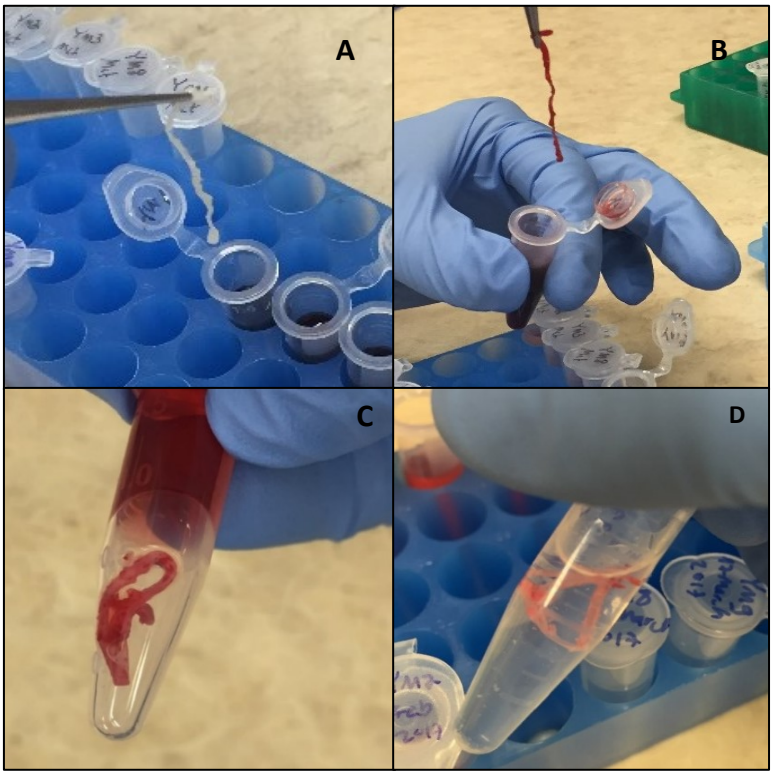


Figure 2.5 (A-D): Aorta staining with oil red O. **(A)** Aorta is dipped into 1ml of oil red O solution, mixed manually by shaking occasionally for 15 minutes. **(B)** The stained aorta is transferred into 100% methanol and rinsed by shaking manually occasionally for 15 minutes to remove excess staining. **(C)** Aorta in the process of de-staining in 100% methanol. (D) Stained and washed aorta is stored in 10% formaldehyde at 4°C.

2.18 Removal of outer fat and opening of the aortas: Each stained aorta was placed on a microscope slide, and a drop of PBS was added, to prevent over-drying. Then, each aorta was cleaned from the remaining of the outer fat, which appeared bright red due to the oil red O stain color, as shown in Figure 2.6 (A). The outer fat was removed by gently pulling it away from the aortic surface with fine tweezers and cutting it way and fine scissors, under microscopic magnification.

Next, aortas were longitudinally cut open by scissors. The incision started at the entrance of one of the renal arteries, and extended, along with the main trunk in two directions: towards and including each of the iliacs, and towards the aortic arch, including each one of the three arteries, branching from the aortic, as shown in Figure 2.6 (B). This allowed opening the aorta, exposing its entire inner surface area. In order to keep the aorta open on the microscope slide, access of moisture was gently absorbed with a ChemiWipe, preventing the reverse closure of the open aorta. The open aorta was fixed in place, by covering it with a coverslip. Then, a drop of PBS was added near the edge of the coverslip, to prevent dry out and sticking of the aorta to the slides (Figure 2.6 (C)).

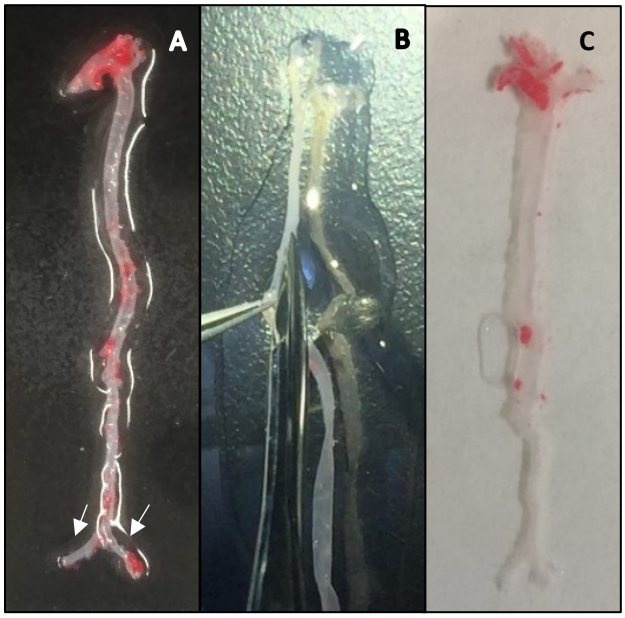
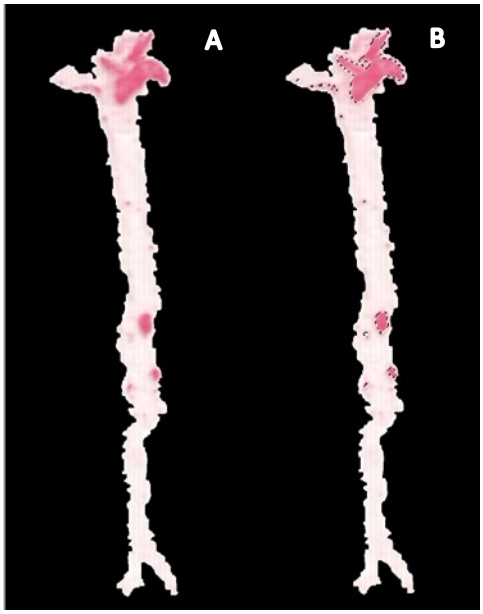


Figure 2.6 (A-C): Isolated and stained aorta, preparation for digital scanning. **(A)-**Entire aortic tree, including aortic arch on the top (with brachiocephalic trunk, left common carotid artery and left subclavian artery and iliacs on the bottom (white arrows), stained with oil red O stain. The bright red areas show fat stained with oil red O, attached to the outer surface of the aorta, just before it was removed**. (B)-** After removal of the fat from the outer surface of the aorta, the aorta was dissected longitudinally in order to be opened, exposing its entire inner surface. **(C)-**Image of a longitudinally open aorta, covered with a coverslip, before scanning. The red areas on the aorta, are the oil red O stained atherosclerotic lesions on the inner surface of the aorta.

2.19 Digital scanning of aortas: The open aorta, fixed between the microscope slide and coverslip, was placed in on the lower left corner of a scanner (CanoScan, Cannon) and digitally scanned with constant parameters. Each digitally scanned aorta was saved as a TIF file.

2.20 Atherosclerosis lesion percentage measurement: For atherosclerosis analysis, the aortas were renumbered continuously, to make the measurements blinded. The code was broken only after completion of all the measurements. Atherosclerosis was assessed by morphometry of the entire aortic tree after isolation and staining. Briefly, the TIF file of the scanned aorta was processed with Adobe Photoshop 5.5, Adobe Inc. software. First, the background around the aorta was blacked out as shown in Figure 2.7 (A). Next, the entire surface area of the aorta was selected and its surface was recorded in pixels as 'total aortic surface area'. Next, only the red color was selected on the surface area of each digitally scanned aorta, using the automatic 'select>color range' tool, Figure 2.7 (B). Finally, atherosclerosis lesion was expressed as a percent of lesion area (oil red O positive) of the total aortic area. This is a well-established protocol (Brown PM et al. 2015, Febbraio M et al. 2000) which was successfully performed in our lab in the past.

Figure 2.7 (A-B): Digitally scanned aorta**.** (A)- A screenshot of digitally scanned aorta, with a blacked background in Adobe Photoshop software. (B)- The red stained areas were selected by using the 'select color range' tool in Adobe Photoshop software. The percentage of atherosclerotic lesions area in this aorta was 9.49% of the total area of the aorta, as calculated by dividing the pixels of the red color selected area by the total area of the aorta.



2.21 Heads removal and preservation: Following euthanasia and fixation, as was previously described under section 2.15 'Euthanasia and fixation', the head was removed from each mouse, by a cut with a razor blade at the base of the skull. The skin was fully removed, and each head was then preserved in a 50mL conical tubes with formaldehyde 10% at 4ºC for 48 hours, on a rotating platform. Then, they were rinsed with PBS and kept at 4ºC until scanning with a micro–CT, followed by processing for histology, as described in details under sections 2.22 'micro-CT for analysis of PALs and 4.23 'histology for analysis of PALs ', below.

2.22 Micro-CT for analysis of PALs

2.22.1 Scanning of heads: The fixed heads were 3D micro-CT (Milabs U-CT, Utrecht, Netherlands) scanned at 25µm voxel size resolution using the following settings: 360 º, exposure 75MSec, voltage 50kV, 0.24mA current. The total procedure scanning time of one head was five minutes in total.

Each head was placed separately and scanned and saved as a 'nifti' file. Then the scanned image was reconstructed MiLabs Software, aligning the sagittal, coronal and axial planes (using the nasal septum and the occlusal plane of the first molars as reference planes. The reconstructed scan was saved as a 'parcel' file.

2.22.2 Detection of PALs on micro-CT scans: The reconstructed scans were analyzed using the Amira 5 software, (ThermoFisher Scientific). Each one of the four quadrants was aligned separately, according to the long axis of each of the roots (three roots in the maxillary first molars and two in the mandibular first molars).

Each root was examined simultaneously on three planes: sagittal, coronal and axial. In order to detect a PAL, a radiolucency should have been noticed in the periradicular area, wider than the size of the width of the PDL. If it was noticed in the PA area, it was recorded as a PAL, and if it was noticed in the furcation area, it was recorded as a furcation lesion (FL). Other data that were collected included the presence or absence of pulp exposure and pulp obliteration. Representative snapshots of all the teeth were taken and saved.

The data regarding presence or absence of PALs/FL was collected into a table, and the number of teeth with PALs or furcation radiolucent lesions (FL) was recorded (PALs 0, 1 ,2, 3, 4; Furcation 0, 1, 2, 3, 4). All the PALs that were recognized or not recognized on the micro-CT scans were compared to the recordings of the histological sections. If a PAL was questionable on micro-CT, it was recorded as PAL when confirmed on histology.

2.23 Histology for PALs analysis

Following the micro-CT scan, the heads were prepared for histology. To prepare the samples for histologic evaluation, they were first decalcified, then dehydrated, embedded, sectioned and finally stained, as described in the following sections.

2.23.1 0.5 M EDTA decalcification solution preparation protocol: Na_2 EDTA, disodium salt dehydrate powder (Fisher Scientific) was used to prepare 0.5M Na_2EDTA at pH 7.4. NaOH pellets were added gradually to keep the solution above pH 8.0 until the Na_2EDTA powder was fully dissolved. After the full dissolution of Na_2EDTA, the pH was adjusted to 7.4 with concentrated HCl. The prepared solution was filtered and sterilized by autoclaving.

2.23.2 Decalcification: For decalcification, each head was cut into two halves (right and left) in order to increase the surface area during the decalcification process, as shown in Figure 2.8. The skin was already removed, as described in 2.21 'Heads removal and preservation'. Soft tissue, such as the tongue and brain were removed from each one of the halves, in order to increase the

surface area of the hard tissue in contact with 0.5M Na_2EDTA during decalcification (Figure 2.8). Trimming of hard tissue at this stage would result in cracking and damaging the samples, therefore we did not trip the halves anymore at this stage. The two halves were transferred into sterile 0.5M Na_2EDTA, pH 7.4 for decalcification. Each pair was stored at room temperature in a single 15mL conical tube, filled with 0.5M Na_2EDTAsolution, gently agitated on a shaking platform and changed daily over 30 days.

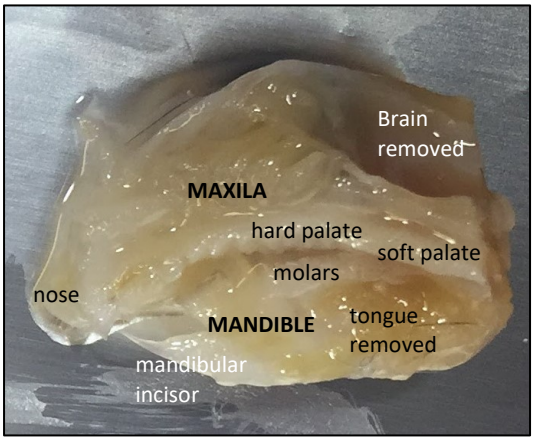


Figure 2.8: Head preparation for decalcification. An unskinned head, cut in the middle, with a razor blade, dividing it into two symmetrical halves. The right half is shown on this picture, internal part of the half facing upward. The brain and tongue were removed in order to increase the surface of hard tissue in contact with the 0.5M Na_2EDTAdecalcification solution. The mandibular molars can be seen in this image. However, only the palate is seen in the maxilla, as the molars are located deeper. The titles on the image were added for orientation.

After 30 days, when the hard tissue became softer, each half of the head (R and L sides) was further cut into two halves, dividing the upper and lower jaws to an upper (MAXILLA) and lower (MANDIBLE) quadrant (Q), as shown in Figure 2.9. This resulted in four samples (four quadrants) from each mouse: Q1 (right upper/maxilla), Q2 (left upper/maxilla), Q3 (left lower/mandible), Q4 (right/ lower/mandible). Each quadrant was trimmed so that all hard and soft tissues were removed, leaving about 1.5 mm of tissue on the buccal, lingual/palatal, mesial to the first molar and distal to the third molar. The incisor was kept only in the mandibles, for orientation purposes (Figure 2.9). Each pair (upper and lower quadrants of the same side) was further stored 5mL tubes with EDTA solution for farther decalcification. The endpoint for decalcification was determined with a physical test, by bending and probing (27G needle) the mandibular incisor, which also became transparent at that point (due to the loss of mineral).

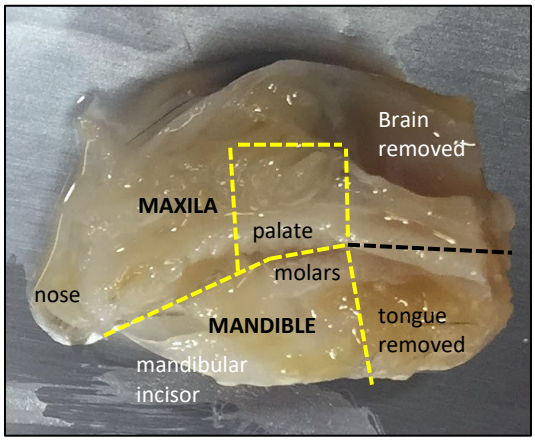


Figure 2.9: Jaws trimming for decalcification. An unskinned head, cut in the middle, with a razor blade, dividing it into two symmetrical halves. The right half is shown on this picture, the internal part of the half facing upward. The brain and tongue were removed in order to increase the surface of the hard tissue in contact with the 0.5M Na_2EDTA decalcification solution. Black interrupted line shows the cutting line to split the maxilla from the mandible. Yellow interrupted line represents the cutting line to trim each one of the jaws for embedding.

2.23.3 Dehydration and processing: After decalcification was verified, as described in section 2.23.2 'Decalcification' samples were transferred into 50% ethanol for an at least 2 hours. Then samples were placed in an automatic tissue processor for overnight processing (Leica TP1020, Leica Biosystem) using the following settings: 70% ethanol for 1hour, 90% ethanol for 1hour, 100% ethanol for 2 hours, xylene for 3hr and finally to paraffin. The samples stayed in paraffin until they were transferred into the embedding machine, as described in section 2.23.4 'Embedding', below.

2.23.4 Embedding: Embedding was performed using the Embedding Center (Leica EG1160, Leica Biosystems) through a surgical microscope magnification (Carl Zeiss F-125, Germany). Each of the four molars with its surrounding tissues (trimmed as was described in section 2.23.2 'Decalcification) was embedded separately in embedding wax (Histoplst, Fisher Scientific, Cheshire, UK).

A consistent orientation of the samples was kept during the embedding process, as follows: The buccal side of each specimen was placed on the bottom and the lingual/palatal side facing up, as shown in Figure 2.10. The occlusal surface of the molars (mandibular/maxillary) was always facing the edge of the plastic cassette that had the label. In order to assure that the specimens will be sectioned as parallel to the long axis of the roots as possible, they were embedded with the occlusal table of the first molar in 90 ^{o} to the floor. The incisor was kept on all the mandibular samples for easy orientation (Figure 2.10). Each mouse had four samples,

which were labeled with the number of the mouse and one of the following (R-right and L-left): R-maxilla, R-mandible, L- maxilla and, L- mandible. The consistent orientation, allowed us a more predictable collection of slices of interest, as will be described in 2.23.5 'sectioning and hematoxylin and eosin (H&E) staining', below.

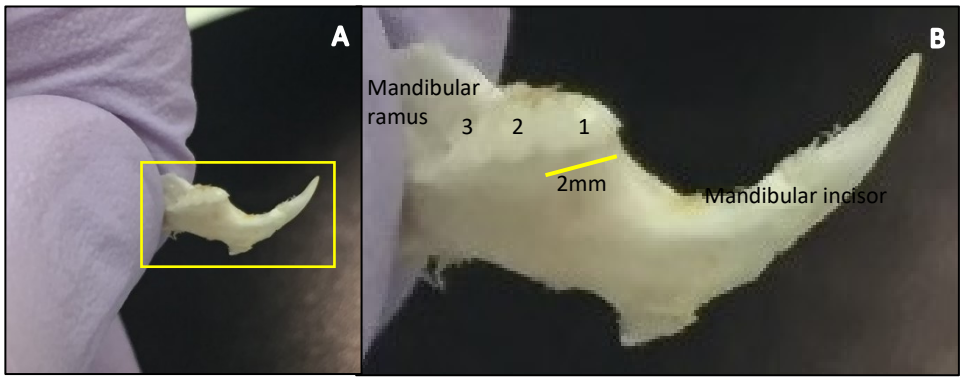


Figure 2.10 (A-B): Left trimmed mandible before embedding, demonstrating the small dimensions next to the holding gloved fingers. In image **(B)** the enlarged image of the yellow rectangle from **(A)** showing the three molars (first, second and third). The incisor was kept on all the mandibular specimens for embedding orientation. *In this sample, the soft tissue was almost fully removed to show the anatomy, although it was not fully removed in our experimental samples.

2.23.5 Sectioning and hematoxylin and eosin (H&E) staining: The services of the 'HistoCore', Alberta Diabetes Institute at the University of Alberta for sectioning and H&E staining processes were used. We worked closely with the facility operator to ensure a good understanding of our needs. Our area of interest included the sections that contained dental tissue of the roots and the crown of the first molars. Therefore, the sections started being collected on the microscope slides (Superfrost Plus, 25X75X1.0mm, Fisher Scientific), from the moment that roots or crowns appeared (points of orientation). The slices were collected continuously until the roots and the coronal pulp disappeared. This ensured that we did not miss the significant areas of interest. Since our trimming of the specimens and embedding orientation were consistent, overall, it took 10 slides, with 5-6 sections of 10µm thickness on each of them, to include the whole area of interest.

For each tooth, the 10 slides (40 slides per mouse) were examined under X50-200 microscopic magnification (Leica DMRE, Leica Microsystems, Concord, Canada), in order to identify the best slides for H&E staining. The slides that contained sections going through the

whole canal system (including the pulp chamber, roots, and apical foramina) were chosen. In cases when there were no orthoradial slides that include all these anatomical regions, the next best slides, which include one of the listed above areas were chosen, to have at least one image of each of these areas.

2.23.6 Histological examination and images: Although the examiner was blinded to the group at the time of evaluation, it was inevitable to see the condition of the crown (drilled or not). The teeth and peri-radicular areas were visualized using a histology microscope (Leica DMRE, Leica Microsystems, Concord, Canada). For each tooth, at least one representative image of a stained section was taken with a camera for histology images (Leica MC170 HD, Concord, Canada) with the available magnification range (X16-X1,000).

The following data was collected from the histology slides and gathered into a table: the presence of pulp exposure, the presence of inflammatory infiltrate in the furcation or in the periapical area. Other findings that were also recorded: the presence of a dentinal bridge in the area of the drill, obliteration of the pulp chamber or canals.

Histological examination was based on the H&E staining and morphological features. When inflammatory infiltrate was seen in the periapical area, or increase in the PDL space was noticed, it was recorded as a PAL. The presence of PALs on histology would be compared with the data obtained from the micro-CT analysis and recorded into a chart.

2.24 Statistical analysis: Results are presented as means ± S.E. Results were subjected to Kolmogorov-Smirnov normality and equality of variances tests. If the results were normally distributed and had equal variances, they were assessed by a non-paired, two-tailed t-test or the One-Way ANOVA test. If normality had not been met, then a non-parametric, Mann-Witney or Kruskal-Wallis test would have been used. Statistical significance will be set at $p<0.05$. GraphPad Prism 5 software was used for statistical analysis.

CHAPTER 3: RESULTS

3.1 Experimental Design Feasibility Analysis

A pilot study was first carried out in order to determine the best surgical methodology. By practicing on cadaver mice (n=10), we determined that only the first molars, 4 out of 12 in each mouse, would be used, due to the small size of the oral cavity and the inability to access the second and third molars. At this stage, micro-surgical skills were gained, including performing precise drilling (0.5mm diameter in a 1.5-2mm occlusal surface size) using a surgical microscope. Also at this stage, we learned how to effectively retract the surrounding soft tissues (tongue and cheeks) and how to avoid perforation of the furcation as much as possible. At this stage, the entire surgical procedure of drilling 4 molars took about 40 minutes.

The standard anesthesia regimen in the lab used for mice was ketamine/xylazine. Because this provided ~20 minutes of anesthesia, we investigated other anesthetic cocktails, in consultation with and recommended by veterinary staff at the University of Alberta that would provide a 40 minutes surgical plane.

First, we used dexdormitor (0.15mg/kg), midazolam (4mg/kg), butorphanol 5mg/kg – in subcutaneous (SC) injection. Atipamezole (1.5mg/kg) SC injection was given for recovery. However, we noticed that with this combination, mice did not enter surgical plane predictably, and did not lose their intra-oral reflexes. We then tried adding acepromazine 1mg/kg to ketamine 80mg/kg and xylazine 6mg/kg, however, with this combination mice had a long recovery time, (1.5-hour post injection) and there was high mortality (n=4). It should be noted that veterinary staff were present or informed immediately of all issues during this pilot.

As a result of the additional surgical practice, we were able to improve our skills and to shorten the total procedure time from 40 minutes to 15-20 minutes per mouse (the time from start to finish of drilling four molars in each). Therefore, we returned to ketamine (80mg/kg) and xylazine 12 (mg/kg) and determined that it provided the optimal anesthesia for the animals with least amount of morbidity/mortality.

3.2 Study Design and Results

Based on previous experience in the lab with this mouse strain (LDLR KO) and diet, we planned to have 17 mice per group (Rx and sham) for end-point (16 weeks) analysis (n=34) and 6 mice per group for the 4 weeks analysis of PALs (n=12). We also had a group of mice (n=5) with no intervention to serve as baseline comparisons. However, surprisingly, the mice in both groups fed the WTD started experiencing dermatitis, some with very early onset (beginning at 4-14 weeks). Since the purpose of the 4 weeks group was to give information as to our surgical success, and according to the literature, the lesions are stable after formation, we reasoned that mice greater than 4 weeks could be placed in this category. Therefore, mice that had to be terminated before the endpoint due to severe dermatitis were placed in this category, to conform with the animal ethics dictate of using the minimal number of animals to meet study goals. Unfortunately, the rate of dermatitis (as will be discussed in section 3.3 'dermatitis') exceeded the planned numbers to be included in the 4-week time point, thus the number of mice in this group is greater than in our original study design. Additional mice were enrolled to be included in the end-point (16 weeks) groups.

Eventually, 65 mice were fed a WTD in total: 34 in the Rx group and 31 in the sham group and were terminated either for micro-CT or end-point analysis. For the latter, we had 17 in the Rx group and 22 in the sham group. The final study design is summarized in Figure 3.1.

3.3 Dermatitis

Amongst all the mice that reached the experimental end-point, 13.6% (3/22) in the sham group and 29.4% (5/17) in the Rx group had dermatitis. The total incidence of dermatitis in the sham group was 12.3% (8/65) and in the Rx group, 29.2% (19/65). This was far greater than any other previous study in our lab at the University of Alberta or at the lab's previous institution, the Cleveland Clinic, encompassing nearly 15 years of work with this strain of mouse and this diet.

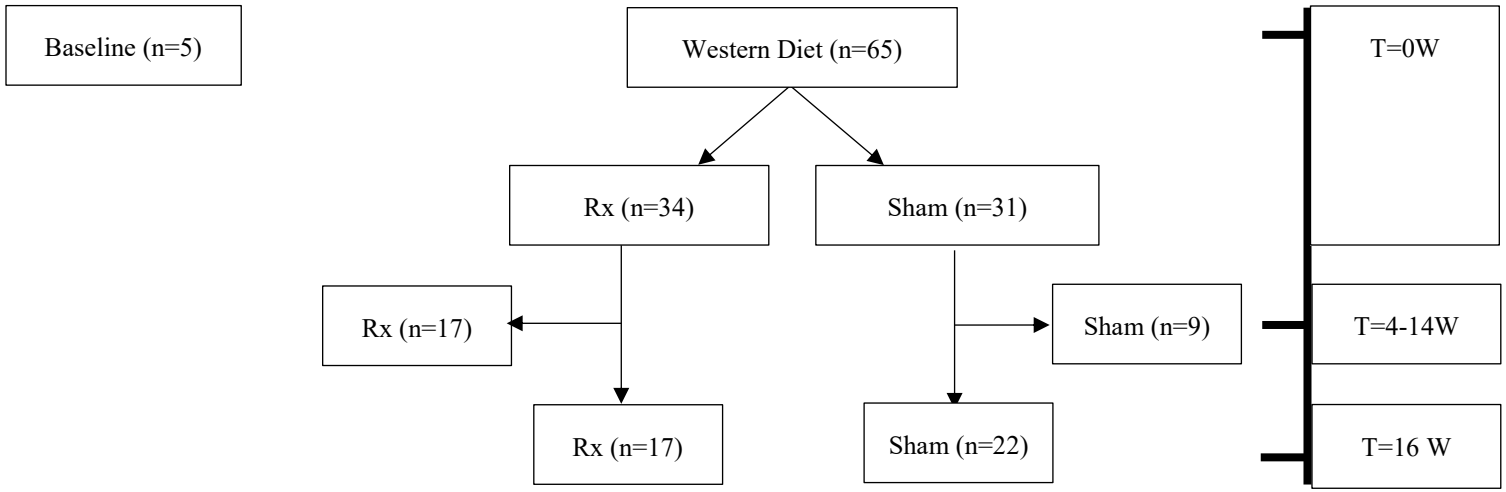


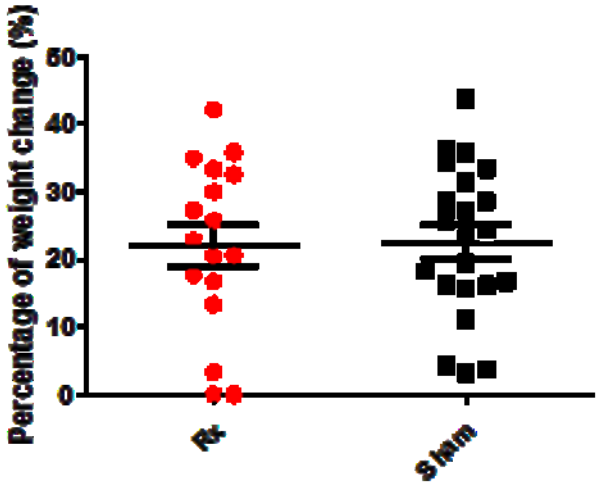
Figure 3.1: Final study design. At time 0 (T=0 weeks) 65 mice in total started WTD, right after surgery/sham anesthesia, Rx group (n=34), sham (n=34). At various time points (T=4-14 weeks), mice were euthanized, Rx (n=17) and sham (n=9) mainly due to dermatitis and for early verification of PALs. After 16 weeks (T=16 weeks), the remaining, Rx (n=17) and sham (n=22) euthanized. Five mice were used as baseline. They were not placed on WTD and were euthanized at baseline (T=0 weeks).

3.4 Body weight: The mean percentage of body weight gained between weeks 0 and 16 was 22.16± 3.06% in the Rx group (n=17) and 22.58± 2.39% in the sham group (n=22). Since the percentage of body weight gained between 0 and 16 weeks shows a normal distribution in both groups, as demonstrated by the Kolmogorov-Smirnov test ($p > 0.10$), and the variances are equal, as demonstrated by the F test for equality of variances (p=0.59), we used a Student's unpaired two -tailed t-test to compare between the two groups. The mean percentage of body weight gained between weeks 0 and 16 in Rx and sham groups was not significantly different (p=0.9139, Table 3.1 and Figure 3.2).

Table 3.1: Body weight (percentage) gained between weeks 0 and 16 in Rx (n=17) and sham (n=22) groups, showing no significant difference, p=0.9139.

Group	**Rx (n=17)**	**Sham (n=22)**
Minimum	0.0	3.230
Median	22.86	23.83
Maximum	42.22	43.75
Mean	22.16	22.58
Std. Deviation	12.64	11.22
Std. Error	3.067	2.392
Lower 95% CI of mean	15.66	17.61
Upper 95% CI of mean	28.67	27.56
Kolmogorov–Smirnov normality	p > 0.10	p > 0.10
F test to compare variances	p=0.5988	
Mean ± S.E	22.16±3.06	22.58±2.39
Difference between means	-0.4167±3.829	
95% confidence interval	-8.179 to 7.346	
Unpaired two-tailed t test	p=0.9139	

Figure 3.2: Percentage body weight gained between weeks 0 and 16 in Rx (n=17) and sham (n=22) groups.

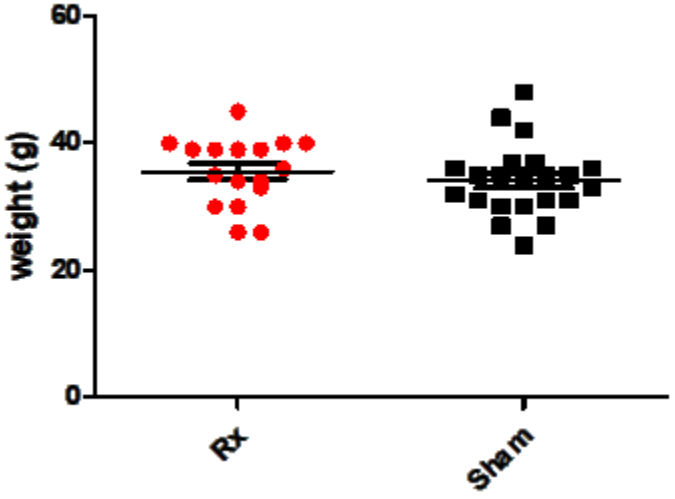


At 16 weeks, the mean weight of mice in the Rx group was 35.59±1.2g and in the sham group, 34.14±1.15g. The variances were equal, as demonstrated by the F test for equality of variances (p=0.86), but since the body weight at 16 weeks showed a non-normal distribution in the Rx group, as demonstrated by the Kolmogorov-Smirnov test (p=0.045), we used the non-parametric Mann-Whitney test to compare between the two groups. There was no significant difference in median weight at 16 weeks between Rx and sham groups (two-tailed Mann Whitney test, p = 0.3269, Table 3.2 and Figure 3.3).

Table 3.2: Body weight (g) at 16 weeks in Rx (n=17) and sham (n=22) groups, showing no significant difference between the groups, p=0.3269.

Group	**Rx (n=17)**	**Sham (n=22)**
Minimum	26.00	24.00
Median	36.00	35.00
Maximum	45.00	48.00
Mean	35.59	34.14
Std. Deviation	5.316	5.575
Std. Error	1.289	1.189
Lower 95% CI of mean	32.86	31.66
Upper 95% CI of mean	38.32	36.61
Kolmogorov-Smirnov normality	p=0.0446	p > 0.10
F test to compare variances	p=0.8587	
Mean ± SEM	35.59±1.28	33.91±1.15
Difference between means	1.67±1.744	
95% confidence interval	-1.85 to 5.20	
Two-tailed Mann Whitney test	p=0.3269	

Figure 3.3: Body weight (g) at 16 weeks in Rx (n=17) and sham (n=22) groups.

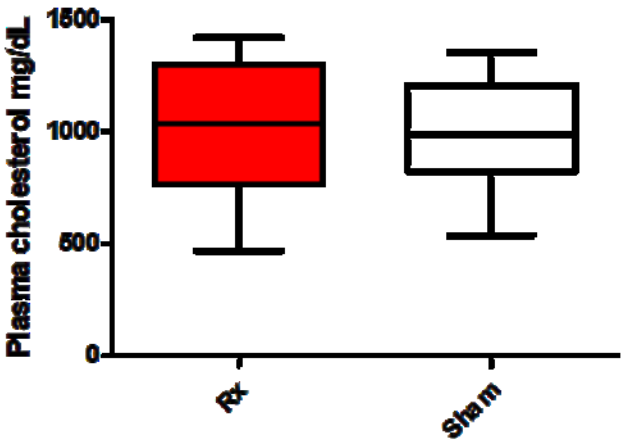


3.5 Plasma total cholesterol levels The mean plasma total cholesterol 16 weeks (end-point) was 1007±74.57 mg/dL in the Rx group and 996.9±46.17 mg/dL in the sham group. Since the total cholesterol levels were normally distributed in both groups, as demonstrated by the Kolmogorov-Smirnov test ($p > 0.10$), and variances were equal, as demonstrated by the F test for equality of variances ($p=0.13$), we used an unpaired two-tailed Student's t-test to compare between the two groups. There was no significant difference in the mean plasma total cholesterol levels at 8 weeks ($p=0.9014$, Table 3.3 and Figure 3.4).

Table 3.3: Plasma total cholesterol levels at 16 weeks (mg/dL) in Rx (n=17) and sham (n=22) groups, showing no significant difference between the groups ($p=0.9014$).

Group	**Rx (n=17)**	**Sham (n=22)**
Number of values	17	22
Minimum	469.9	539.5
Median	1034	989.3
Maximum	1419	1351
Mean	1007	996.9
Std. Deviation	307.5	216.5
Std. Error	74.57	46.17
Lower 95% CI of mean	849.2	900.9
Upper 95% CI of mean	1165	1093
Kolmogorov-Smirnov normality	$p > 0.10$	$p > 0.10$
F test to compare variances	$p=0.1327$	
Difference between means	10.47±83.89	
95% confidence interval	-159.6 to 180.5	
Unpaired t test two-tailed P value	$p=0.9014$	

Figure 3.4: Distribution of plasma total cholesterol levels at 16 weeks (mg/dL) in Rx (n=17) and sham (n=22) groups.



3.7 Success rate of PAL induction

Originally, we planned to have two groups of mice Rx (n=6) and sham (n=6) at four weeks post-surgery, in order to evaluate the success rate of PALs induction by micro-CT. Based on the findings, we planned to add more mice to have enough mice with four PALs. However, by the time we had an active micro-CT n our facility, all our 16 weeks mice reached their end-point and were terminated. Therefore, we ended up with analyzing the success rates of PALs induction in the Rx 16 weeks' group.

When analyzing the endpoint Rx group, we found that out of the 68 molars drilled (four molars per mouse, 17 mice), the pulp was successfully exposed in 92% (62/68), as verified by micro-CT scans and matching histological sections (Table 3.4). However, only 71% (44/62) of the teeth with exposed pulps showed a PAL, as verified by micro-CT scans and matching histological sections. Additionally, 76% (47/62) of teeth developed furcation lesions, which were verified by micro-CT scans and matching histological sections (Table 3.4).

Table 3.4: Number of teeth with pulp exposure, PALs and furcation lesions in each one of the 17 mice Rx group at 16 weeks.

YM#	Pulp exposures	PALs	Furcation lesions
1	3	3	3
2	3	2	1
3	4	4	3
8	4	1	4
11	3	3	2
22	3	1	3
23	4	1	2
24	4	1	2
25	4	2	2
29	4	1	1
31	4	4	4
35	4	4	4
36	4	4	4
46	4	4	3
48	4	3	4
65	3	3	2
66	3	3	3
N=17 (68 teeth)	92% (62/68)	71% (44/62)	76% (47/62)

Out of the mice in the Rx group, 27.7% (5/17) presented with four PALs, 27.7% (5/17) had three PALs, 11.8% (2/17) had two PALs, and 27.7% (5/17) had only one PAL, as verified by micro-CT and histology (Table 3.5). Additionally, furcation lesions were also recorded and their prevalence appears in Table 3.5.

Table 3.5: Distribution of number of PALs and furcation lesions in Rx group mice (n=17) at 16 weeks.

Number	**4 of 4**	**3 of 4**	**2 of 4**	**1 of 4**
PALs	27.7% (5/17)	27.7% (5/17)	11.8% (2/17)	27.7% (5/17)
Furcation	27.7% (5/17)	27.7% (5/17)	27.7% (5/17)	11.8% (2/17)

Table 3.6 presents, below, is a summary of all the PALs and furcation lesions in the Rx group, using micro-CT and histology. Figures 3.5-8, below, show representative histological sections of molars without and with pulp exposures.

Table 3.6: Summary of all the PALs and furcation lesions in the Rx group. Each mouse was coded as 'YM#'. For each mouse the histology/micro-CT findings on each of the four drilled teeth is presented. In total there were n=17 mice in the Rx group. The number of teeth with pulp exposure (expo), periapical lesions (PALs) and furcation lesions (F). Q-quadrant, R-right, L-left, MAX-maxilla, MAN-mandible, LL-lesion load (F+PAL) is summarized. 'X' - histological sections were not available for the sample, for example due to lack for appropriate section that did not include the apical area of the roots in a longitudinal orientation.

YM#	evaluation method	Q1 R-MAX	Q2 L-MAX	Q3 L-MAN	Q4 R-MAN	Pulp expo	PALs	F	LL: F+PALs
YM1	Histology	No expo	X	X	PAL, F	3	3	3	6
	Micro-CT	No expo	PAL, F	PAL, F	PAL, F				
YM2	Histology	X	PAL	PAL, F	X	3	1	1	2
	Micro-CT	pin point expo	PAL	F	no expo				
YM3	Histology	PAL, F	PAL, F	X	F	4	4	3	7
	Micro-CT	PAL, F	PAL, F	PAL	PAL, F				
YM8	Histology	necrosis, F	F	F	F, F perfo	4	1	4	5
	Micro-CT	F	PAL, F	F	F				
YM11	Histology	no expo	PAL, F	PAL	PAL, F	3	3	2	5
	Micro-CT	no expo	PAL, F	inconclusive	PAL, F				
YM22	Histology	no expo	X	X	PAL, F	3	1	3	4
	Micro-CT	no expo	F	F	F				
YM23	Histology	PAL, F, F perfo	X	X	no expo	4	1	2	3
	Micro-CT	F	pin point drill	F	no expo				
YM24	Histology	tertiary dentin	sections inconclusive	X	X	4	1	2	3
	Micro-CT	small expo	pin point expo, PAL	F	F				

YM25	Histology	PAL	necrosis	F, F perfo, necrosis	necrosis	4	2	2	4
	Micro-CT	PAL	PAL, F	F	inconclusive				
YM29	Histology	PAL, F	X	No necrosis in canals	transvers sections, necrosis is seen	4	1	1	2
	Micro-CT	PAL	expo	No/pin expo, canal obliteration	No/pin expo, canal obliteration				
YM31	Histology	PAL, F, F perfo	PAL, F	PAL	F	4	4	4	8
	Micro-CT	PAL, F, F perfo	PAL	F	PAL, F, F perfo				
YM35	Histology	PAL, F	X	PAL, F	X	4	4	4	8
	Micro-CT	PAL, F	PAL, F, F perfo	PAL, F,	PAL, F				
YM36	Histology	PAL, F	PAL, F,F perfo	PAL, F, F perfo	PAL, F,F perfo	4	4	4	8
	Micro-CT	PAL	PAL, F	F	F				
YM46	Histology	PAL, F	partial necrosis, dentinal bridge in crown	X	PAL, F	4	4	3	7
	Micro-CT	F	PAL, F	PAL	PAL				
YM48	Histology	PAL F, F perfo	X	necrosis (limited sections)	X	4	3	4	7
	Micro-CT	PAL, F	PAL, F, F perfo	F	PAL, F				
YM65	Histology	PAL	X	X	no expo	3	3	2	5
	Micro-CT	F	PAL	PAL, F	no expo				
YM66	Histology	PAL	no expo	F,F perforation	transverse sections	3	3	3	6
	Micro-CT	F, PAL	no expo	F, PAL	F, PAL				

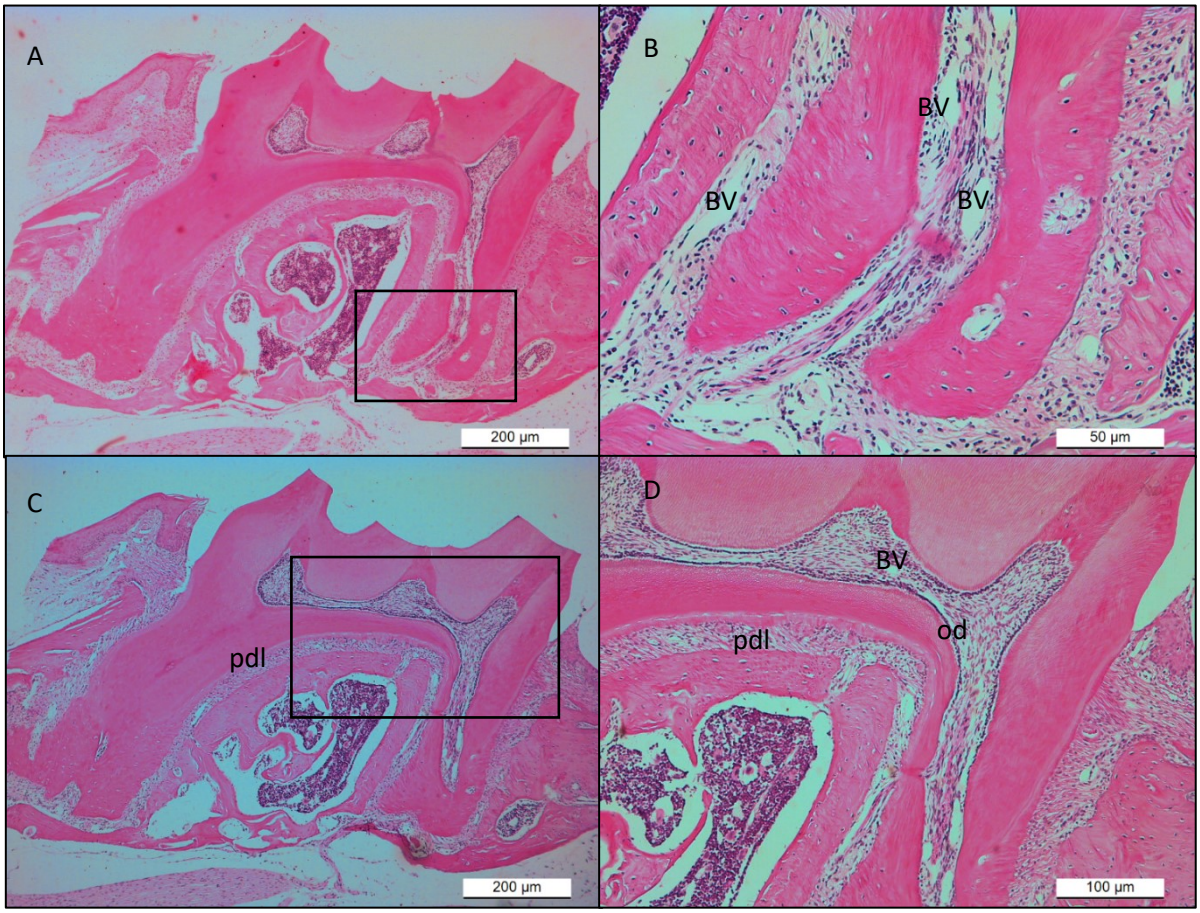


Figure 3.5 (A-D): Sagittal section through a right mandibular first molar, showing the normal pulp in the coronal and canal areas, no pulp exposure. Images **(A)** and **(C)** taken in magnification X50; images image **(D)** taken in magnification X100 and image **(C)** in magnification X200. Blood vessels (BV) are seen in the pulp area **(D)** as well as in the periradicular area **(B).** A circumferential layer of odontoblasts (od) is seen in the periphery of the pulp **(D).** The width of the periodontal ligament (pdl) is uniform around the roots and is about 30µM **(C)** and **(D).**

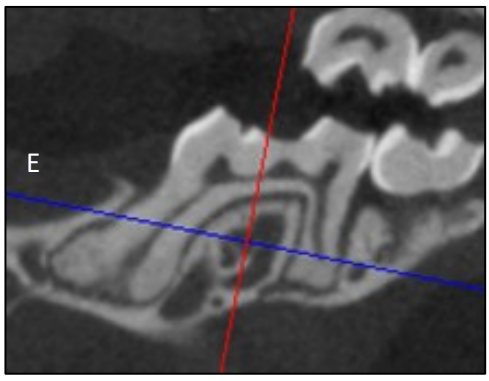


Figure 3.5 (E): Corresponding micro-CT scan, sagittal section through a right mandibular first molar with no pulp exposure. Uniform pdl width is seen in the periradicular and furcation area.

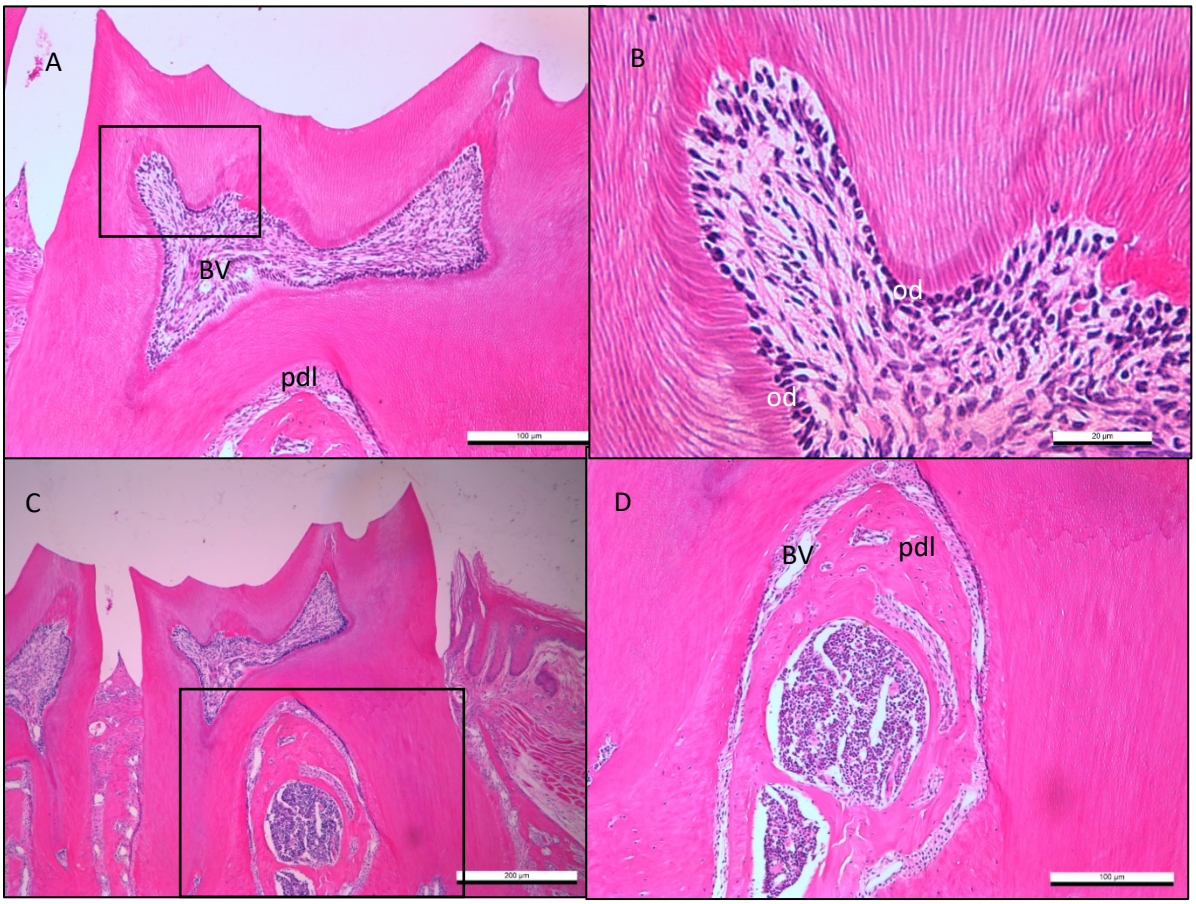


Figure 3.6 (A-D): Sagittal section through a right mandibular first molar, showing the normal pulp in the coronal and furcation areas, no pulp exposure. Images **(A)** and **(D)** taken in magnification X100, image **(C)** X50, image **(B)** X400. A circumferential layer of odontoblasts is seen in the periphery of the pulp. The width of the pdl is uniform around the roots and is about 30μM.

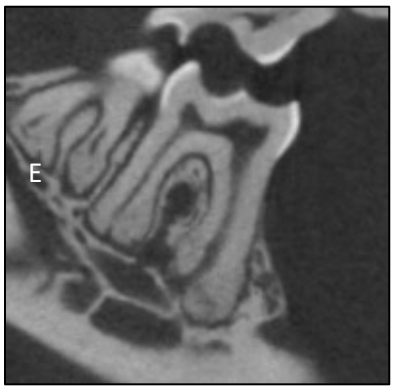


Figure 3.6 (E): Corresponding micro-CT scan, sagittal section through a right mandibular first molar, with no pulp exposure. Uniform pdl width is seen in the periradicular and furcation areas.

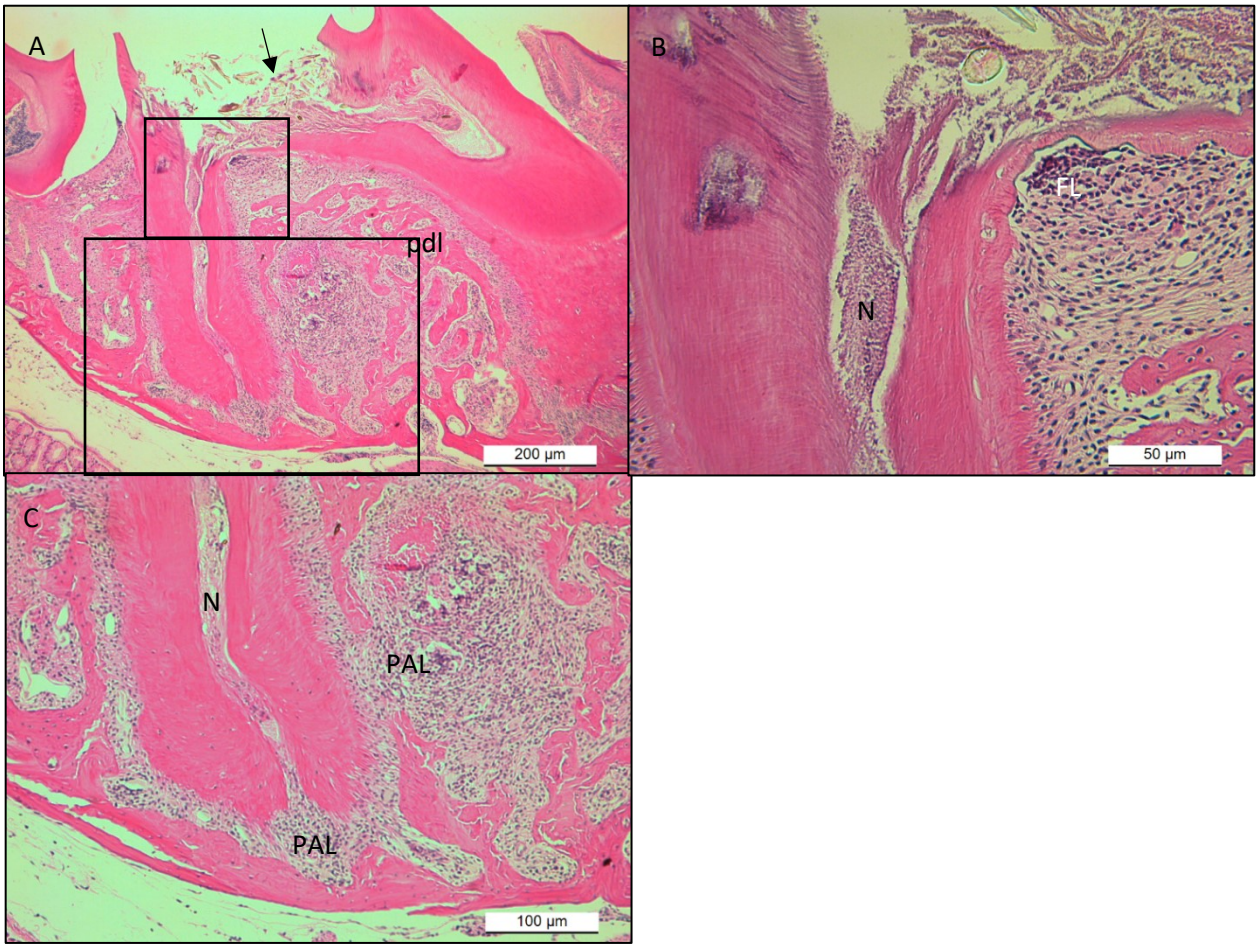
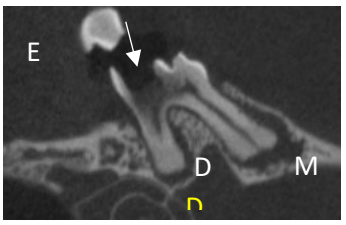


Figure 3.7 (A-D): Sagittal section through a drilled left maxillary first molar. Image **(A)** taken in magnification X50, image **(C)** X100, image **(B)** X200. Arrow is pointing towards the pulp exposure/drill site on **(A)**. Necrosis is seen in the canal (N). Furcation lesion (FL) is seen on image **(B).** Image **(A)** showing the periapical area of the distal root, and PALs are seen. The width of the pdl is not uniform around the roots and is more than 30µM in the furcation and periapical area.



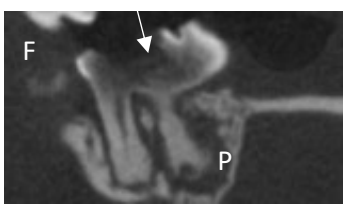


Figure 3.7 (E-F): Corresponding micro-CT scan, sagittal section through a right mandibular first molar, with pulp exposure (arrow). PALs are seen associated with the palatal-P, mesial M and distal-D roots.

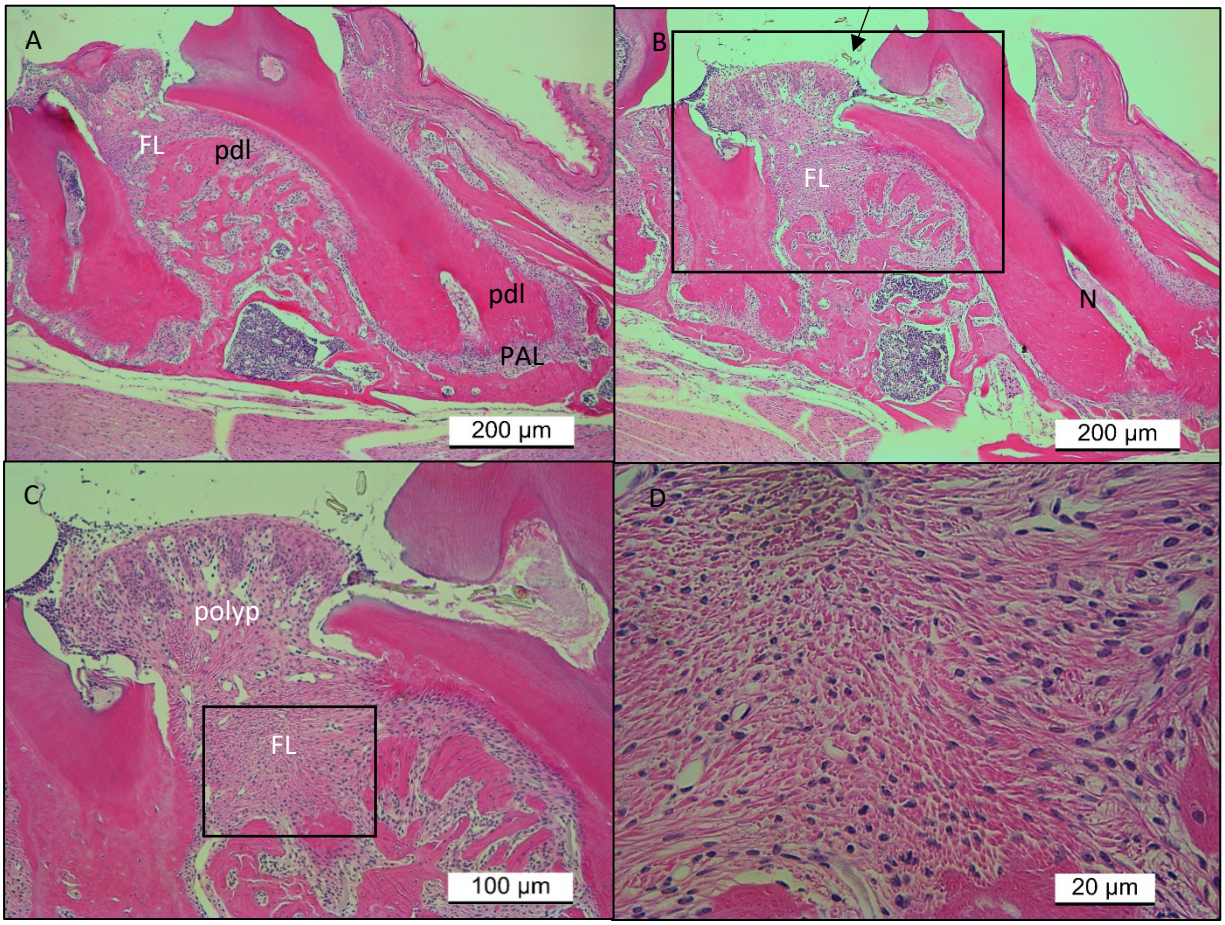


Figure 3.8 (A-D): Sagittal section through a drilled left maxillary first molar. Images **(A)** and **(B)** are taken in magnification X50, image **(C)** X100 and image **(D)** at X400. Arrow is pointing towards the pulp exposure/drill site on (B). Furcation lesion (FL) is seen on images **(A), (B)** and **(C),** just below the furcation perforation. Image (A) showing the periapical area of the distal root, and PALs are seen. (D) Inflammatory infiltrate in the furcation area. The width of the pdl is not uniform around the roots and is more than 30µM in the furcation and periapical area. Necrosis is seen in the canal (N).

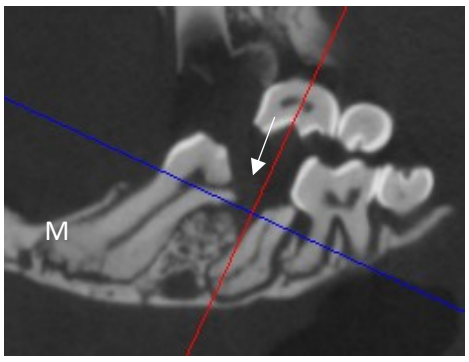


Figure 3.8 (E): Corresponding micro-CT scan, **s**agittal section through a right mandibular first molar, with pulp exposure and furcation perforation (arrow). The surface of the mesial root-M is irregular.

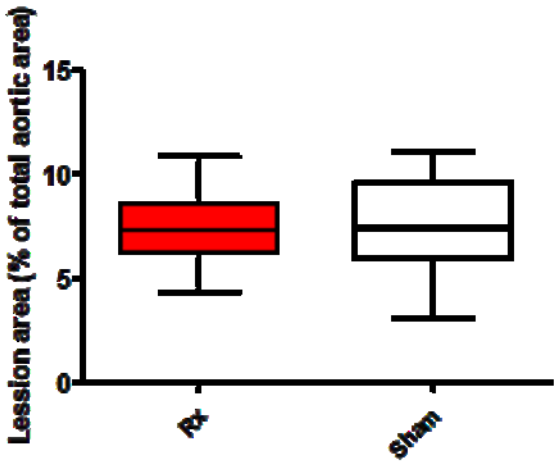
3.8 Atherosclerosis

Atherosclerotic lesions covered an area of 7.46±0.44% of the total aortic surface in the Rx group and 7.65±0.46% in the sham group. Since lesion burden was normally distributed in both groups, as demonstrated by the Kolmogorov-Smirnov test ($p > 0.10$), and the variances were equal, as demonstrated by the F test for equality of variances ($p=0.46$), we used an unpaired two-tailed t-test to compare between the two groups. There was no significant difference in mean atherosclerotic lesion area ($p = 0.7766$, Table 3.7 and Figure 3.9).

Table 3.7: Atherosclerotic lesion area (%) in Rx (n=17) and sham (n=22) groups, showing no difference (p=0.7766).

Group	**Rx (n=17)**	**Sham (n=22)**
Minimum	4.32	3.12
Median	7.32	7.39
Maximum	10.84	11.10
Mean	7.46	7.65
Std. Deviation	1.82	2.19
Std. Error	0.44	0.46
Lower 95% CI of mean	6.52	6.60
Upper 95% CI of mean	8.40	8.62
Kolmogorov-Smirnov normality	p > 0.10	p > 0.10
F test to compare variances	p = 0.4567	
Difference between means±S.E	-0.1888 ± 0.6604	
95% confidence interval	-1.528 to 1.150	
Unpaired two-tailed t test	p = 0.7766	

Figure 3.9: Atherosclerotic lesion area (%) among mice in Rx (n=17) and sham (n=22) groups.



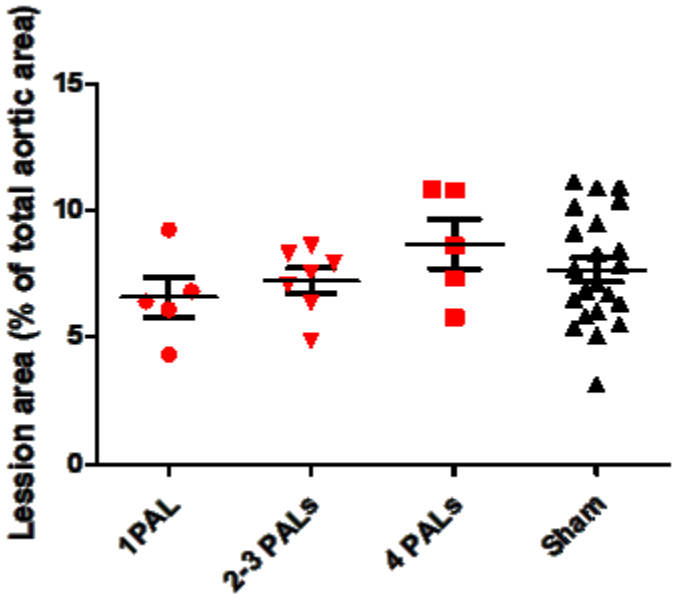
Due to the variability in number of PALs per mouse, we performed additional statistical analysis and grouped the Rx mice into three sub-groups by the number of PALs per mouse: one, two-three, and four. Since there were only two mice with two PALs, they were joined with the three PALs group.

Mice with one PAL had a mean atherosclerosis lesion area of 6.58±0.79%, mice with two-three PALs had 7.24±0.48% and mice with four PALs had 8.67±0.98%; sham mice had a mean atherosclerosis lesion area of 7.65±0.46%. Due to variability in group sizes, we used the Kruskal-Wallis overall test to compare the medians of the four groups, which showed that there was not enough evidence for a difference between the groups (p=0.5129 Table 3.8 and Figure 3.10). Although insignificant, a trend can be noticed: the higher the number of PALs, the greater atherosclerosis lesion burden (Table 3.8 and Figure 3.10).

Group	**1 PAL (n=5)**	**2-3 PALs (n=7)**	**4 PALs (n=5)**	**Sham (n=22)**
Minimum	4.320	4.860	5.790	3.12
Median	6.410	7.530	8.610	7.390
Maximum	9.240	8.610	10.84	11.10
Mean	6.578	7.237	8.673	7.654
Std. Deviation	1.768	1.289	2.200	2.197
Std. Error	0.7906	0.4873	0.9840	0.4683
Lower 95% CI of mean	4.383	6.045	5.949	6.680
Upper 95% CI of mean	8.773	8.429	11.40	8.628
Kolmogorov-Smirnov normality	p > 0.10	p > 0.10	p > 0.10	p > 0.10
Bartlett's test for equal variances	p = 0.5375			
Kruskal-Wallis test	p = 0.5129			

Table 3.8: Atherosclerotic lesion area (%) among mice in the Rx and sham group. The Rx mice were also grouped into three sub-groups by the number of PALs per mouse: one (n=5), two-three (n=7) and four (n=5).

Figure 3.10: Atherosclerotic lesion area (%) among mice in Rx sub-groups (by the number of PALs per mouse) and sham group (p=0.5129, Kruskal-Wallis test).



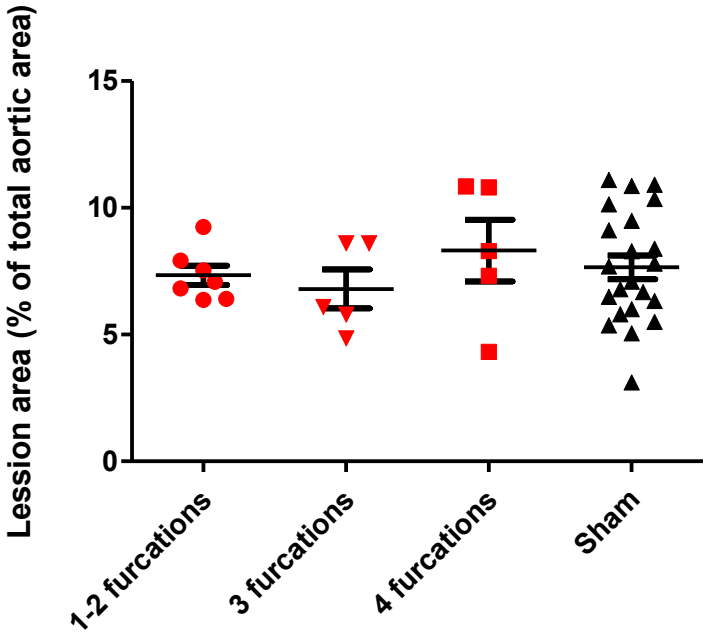
Due to the variability in the number of furcation lesions per mouse, we performed additional statistical analysis and grouped the Rx mice into three sub-groups by the number of furcation lesions: one-two, three, and four (since there were only two mice with one furcation, they were joined with the two furcation group).

Mice with one to two furcation lesions had a mean atherosclerosis lesion area of 7.34%±0.38%, mice with three furcation lesions had 6.79±0.76% and mice with four furcation lesions had 8.31±1.21%; sham mice had a mean atherosclerosis lesion area of 7.65±0.46%. Due to the variability in group sizes, we used the Kruskal-Wallis overall test to compare the medians of the four groups and found that there was not enough evidence for a difference between the groups ($p = 0.7094$, Table 3.9 and Figure 3.11).

Groups	**1-2 FL (n=7)**	**3 FL (n=5)**	**4 FL (n=5)**	**Sham (n=22)**
Minimum	6.370	4.860	4.320	3.12
Median	7.080	6.100	8.290	7.390
Maximum	9.240	8.610	10.84	11.10
Mean	7.339	6.794	8.314	7.654
Std. Deviation	1.010	1.719	2.716	2.197
Std. Error	0.3819	0.7690	1.215	0.4683
Lower 95% CI	6.404	4.659	4.942	6.680
Upper 95% CI	8.273	8.929	11.69	8.628
Kolmogorov–Smirnov normality test	$p > 0.10$	$p > 0.10$	$p > 0.10$	$p > 0.10$
Bartlett's test for equal variances	$p= 0.1885$			
Kruskal-Wallis test	$p = 0.7094$			

Table 3.9: Atherosclerotic lesion area (%) among Rx and sham mice. Rx mice are sub-grouped by the number of furcation lesions: one-two (n=7), three (n=5) and four (n=5).

Figure 3.11: Atherosclerotic lesion area (%) in the Rx sub-groups, grouped by the number of furcation lesions, and sham group. The difference between the groups is not significant, p=0.7094, Kruskal-Wallis test.



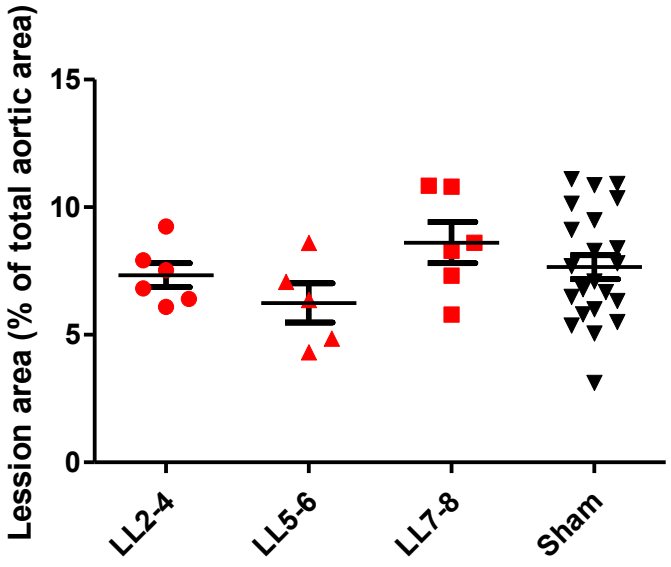
Finally, we combined the number of PALs and furcation lesions in each mouse into a 'lesion load' score (LL), which is the sum of number of PALs and furcation lesions. The maximum possible score is 8 (four PALs and four furcation lesions, one in each drilled tooth). Due to variability in the LL score per mouse Rx mice were grouped into three sub-groups by the lesion load score: LL two-four (n=6), five-six (n=5) and seven-eight (n=6).

Mice with a LL score of two-four had a mean atherosclerosis lesion area of 7.33±0.47%, mice with a LL score of five-six had 6.25±0.77% and mice with the highest LL score of seven-eight had 8.06±0.80%; sham mice had a mean atherosclerosis lesion area of 7.65±0.46%. Due to variability in group sizes, we used the Kruskal-Wallis overall test to compare the medians of the four groups and found that there was not enough evidence for a difference between the groups ($p = 0.3328$, Table 3.10 and Figure 3.12).

Groups	**LL 2-4 (n=6)**	**LL 5-6 (n=5)**	**LL 7-8 (n=6)**	**Sham (n=22)**
Minimum	6.100	4.320	5.790	3.12
Median	7.175	6.370	8.450	7.390
Maximum	9.240	8.610	10.84	11.10
Mean	7.337	6.248	8.608	7.654
Std. Deviation	1.154	1.727	1.974	2.197
Std. Error	0.4710	0.7723	0.8060	0.4683
Lower 95% CI	6.126	4.104	6.537	6.680
Upper 95% CI	8.547	8.392	10.68	8.628
Kolmogorov–Smirnov normality	$p > 0.10$	$p > 0.10$	$p > 0.10$	$p > 0.10$
Bartlett's test for equal variances	$p=0.4701$			
Kruskal-Wallis test	$p=0.3328$			

Table 3.10: Atherosclerotic lesion area (%) among Rx and sham mice. Rx mice, are grouped by the LL score.

Figure 3.12: Atherosclerotic lesion area (%) among mice in the Rx sub-groups, grouped by the LL score, and sham group. The difference between the groups was not significant, p=0.3328, Kruskal-Wallis test.



3.9 Plasma cytokine profiling

We analyzed plasma from sham and Rx mice at 8 weeks and found the following cytokines to have increased expression in the Rx group compared with the sham group, using a cytokine array: M-CSF, IL1-ra, TIMP-1, SDF-1/CXCL12, C5/C5a and BLC/CXCL13. sICAM-1/CD54 was increased in the sham group. KC/CXCL1 could be detected only in the sham group (Figure 13).

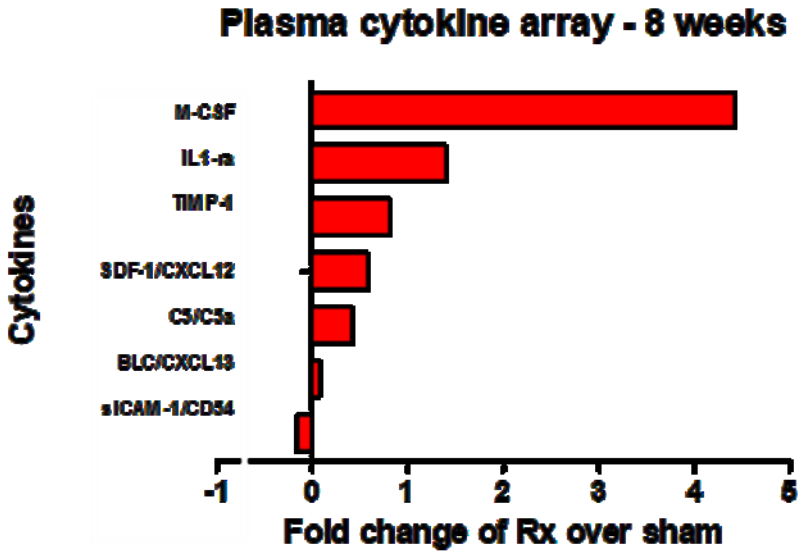


Figure 3.13: Plasma cytokine array results expressed as 'fold change': (Rx-Sham)/Sham, at 8 weeks.

We also analyzed plasma isolated at the endpoint and found the following cytokines were increased in expression in the Rx group compared with the sham group: M-CSF, IL1-ra, TIMP-1, SDF-1/CXCL12, and KC/CXCL1. sICAM-1/CD54, BLC/CXCL13 and C5/C5a were increased in the sham group.
(Figure 3.14).

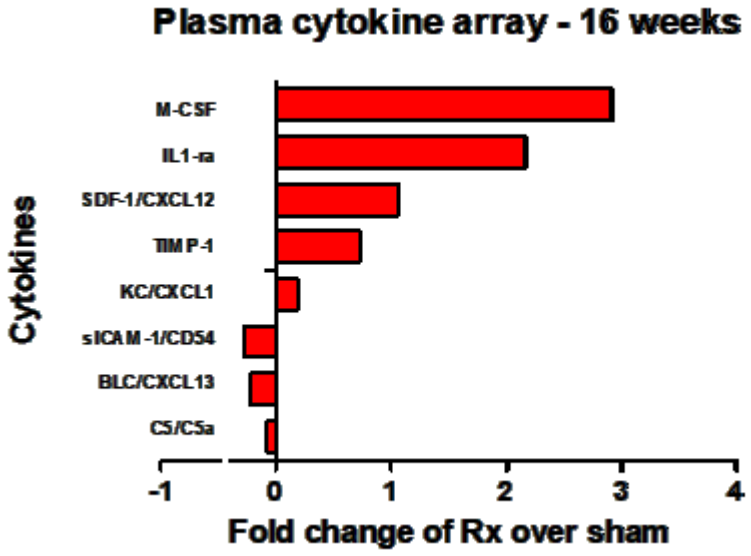


Figure 3.14: Plasma cytokine array results expressed as 'fold change': (Rx-Sham)/Sham, at 16 weeks.

3.10 Cytokine profiling of peritoneal macrophage lysates

We analyzed cytokine expression in lysates of peritoneal macrophages isolated at endpoint. The following cytokines were increased in the Rx group compared with the sham group: TIMP-1, SDF-1/CXCL12, M-CSF and BLC/CXCL13. TNF-alpha and TREM-1 were detected only in the Rx group. JE/CCL2/MCP-1, IL1-ra, MIP-1-alpha/CCL3, IL-16. IL1-alpha, sICAM-1/CD54 and C5/C5a showed higher levels in the sham group. (Figure 3.14).

Figure 3.15: Macrophage cytokines levels expressed as a 'fold change: (Rx-Sham)/Sham, at 16 weeks.

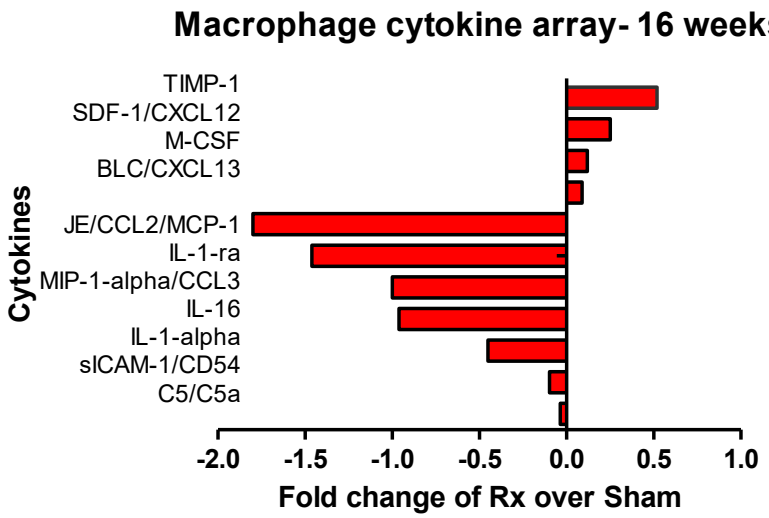


Table 3.11: Preliminary data on the variability of histologic appearance of PALs. Pulp exposure (expo), periapical lesions (PALs) and furcation lesions (F). Q-quadrant, R-right, L-left, MAX-maxilla, MAN-mandible, pulp expo-pulp exposure, PAL-periapical lesion, F-furcation lesion. LL-lesion load (F+PAL) is summarized. The percentage of atherosclerotic lesion burden is presented on the right. . 'X' - histological sections were not available for the sample, for example due to lack for appropriate section that did not include the apical area of the roots in a longitudinal orientation.

YM#	**Q1**	**Q2**	**Q3**	**Q4**	**Atherosclerosis percentage**
YM1	no expo	X	X	necrosis, PAL, F	8.61%
YM2	X	Necrosis, PAL (large)	necrosis, F, PAL, apical resorption, severe crown damage	X	7.92%
YM3	PAL (large), F	PAL (large), F (large)	X	necrosis, F (large)	8.61%
YM8	necrosis, F	F (large), no sections through canal	F, no sections through canal	F (large), F perforation, apical resorption, no sections through canal	4.32%
YM11	no expo	PAL, F (large)	PAL, no sections through canal, apical resorption	PAL, F, crown damage	6.37%
YM22	no expo	X	X	PAL, F, apical resorption	6.10%
YM23	PAL, F, polip, apical resorption	X	X	no expo, partial necrosis in D canal	6.41%
YM24	tertiary dentin	necrosis in M canal, sections inconclusive	X	X	9.24%
YM25	PA, apical resorption	partial necrosis in P, severe crown damage	F (large), F perforation, apical resorption, partial necrosis	necrosis , apical resorption	7.53%
YM29	PAL, F, apical resorption	X	Apical resorption	transvers sections, necrosis is seen	6.82%
YM31	PAL, F (perfo), apical resorption, F polip	PAL (large), F , apical resorption	PAL (large) , apical resorption, i	F, apical resorption	10.84%

YM35	PAL, F	X	PAL, F	X	10.80%
YM36	necrosis, big bone marrow! PAL, F	necrosis, PAL?, F (perfo, polip), apical resorption	necrosis, large bone marrow. PAL, F (perfo, polip)	necrosis, large bone marrow. PAL, F (perfo, polip), apical resorption	7.32%
YM46	necrosis, apical resorption, PAL, F	partial necrosis, dentinal bridge in crown, normal pulp in D canal, PDL loss direction	X	M necrosis, D PAL, F (wide PDL), M+D apical root resorption	5.79%
YM48	severe inflammation, pulp polip, large PAL, F	X	necrosis (limited sections)	X	8.29%
YM65	Large F, PAL, Pulp necrosis and	X	X	No expo, Normal structures, large invagination in D root, directional PDL	7.08%
YM66	Classical section through the whole canal necrosis, PAL classic	No expo	F lesion, canal not seen in sections, F perforation ***2 mandibular nerves, PA status not conclusive.	transverse sections	4.86%

CHAPTER 4: DISCUSSION

4.1 Rational

The goal of our study was to test whether AP had a causative contributive role in the development of atherosclerosis. The importance as to why to explore the connection between these two diseases was extensively discussed in our recently published systematic (Berlin-Broner Y et al. 2017[a]) and narrative (Berlin-Broner Y et al. 2017[b]) reviews. The systematic review identified a positive association between AP and cardiovascular disease, however, no studies were found that examined causality (Berlin-Broner Y et al. 2017[a]). Therefore, to our best knowledge, this was the first study to use an animal model to investigate the connection between AP and atherosclerosis.

4.2 Innovation

The use of a mouse model allowed us to study the influence of an isolated factor (AP) on a certain disease (atherosclerosis), overcoming a common limitation of human studies, various confounders (Berlin-Broner Y et al. 2017 [a]), which may impact both the influence factor (in this case, AP), as well as the response factor (atherosclerosis). These confounders include systemic diseases, medications, genetic background etc. By using a mouse model we addressed this limitation by providing a uniform genetic background and identical external conditions (environment). Since atherosclerosis takes decades to develop in humans, using mice also allowed us to evaluate atherosclerosis after a short time, 16 weeks, which is the time it takes for LDLR KO mice to develop significant atherosclerosis in the aortic tree (Ishibashi S et al. 1993).

Weight gain in both groups was similar (22.16 ± 3.06% in the Rx group and 22.58 ± 2.39% in the sham group) over the experimental period, demonstrating that the drilled four molars in the Rx group mice did not compromise the ability of the mice to eat, nor lead to significant pain or distress that impacted their eating behavior. Since, to our knowledge, this is the first study that induced PALs in LDLR KO mice, the similar weight gain in both groups demonstrates the feasibility of using this model for future AP studies.

In both groups, there were three mice that gained less than 5% of their starting weight. The lack of weight gain in these mice was not associated with lack of atherosclerosis or dermatitis. If these were excluded from the analysis, it did not change the results.

After 16 weeks WTD feeding, mice in both groups had similarly increased levels of total plasma cholesterol (1007 ± 74.57 mg/dL in the Rx group and 996.9 ± 46.17 mg/dL in the sham group), similar to the range previously reported for LDLR KO mice fed the same diet (Brown PM et al. 2015). The normal cholesterol levels in these strain of mice, without the LDLR KO mutation, is only 146 mg/dl without WTD, and on WTD 149 mg/dL (Ishibashi S et al. 1993).

4.3 Induction of PALs

Since the classical study of Kakehashi S and colleagues who induced PALs in rats (Kakehashi S et al. 1965), others adopted their protocol and used in mice (Wang CY & Stashenko P 1991, Balto K et al. 2000, von Stechow D et al. 2003, Shah A et al 2018). This method, of exposing the pulp to the oral flora by drilling, requires a single intervention (general anesthesia to drill the cavities) and does not require extra step as needed in other techniques that can be found in the literature, such as adding an intra-pulpal infection with a mixture of anaerobic pathogens common in endodontic infections post pulpal exposure (Hou L et al. 2000, Rider D et al. 2016).

Due to the difficulty of the surgical technique used to induce PALs, we first practiced on cadavers. Our initial study design was to induce PALs in all 12 molars. We considered this would induce the maximum inflammation and would have the greatest chance to influence atherosclerosis lesion development. However, due to technical constraints, we eventually redesigned our experiment to include the first molars only (four per mouse), for the reasons listed below.

In our study, we drilled four molars. While practicing in cadavers, it took us about 40 minutes to do this. With practice, the time was reduced to 20 minutes. This was important because it reduced the time that mice need to be anesthetized, therefore reduced morbidity and mortality. It also allowed us to use the common anesthesia combination of ketamine/xylazine, as used in other similar studies (Balto K et al. 2000,

Balto K et al. 2002, von Stechow D et al. 2003), rather adding other anesthetics, which provide longer anesthesia but increase mortality, such as acepromazine (Arras M et al.2001)

The time it takes to drill and expose the pulp in molars in mice is not mentioned in the literature and is dependent on the skill and experience of the investigator. Previous studies that induced PALs in mice drilled a maximum of two molars per mouse (Balto K et al. 2000, Balto K et al. 2002, von Stechow D et al. 2003, da Silva RA et al. 2012) or only one molar per mouse (Shah A et al 2018).

Another reason why we revised our experimental plan was that we quickly realized that there was limited access to the second and third molars and they are smaller in size compared to the first molars (smaller then 1.5mm). These factors increased the potential for mortality, as to access these teeth would impede the airway of the mice, and increase the surgical time.

Finally, by exposing only the first molar teeth, without involving the other two in each quadrant, mice can keep their eating function without a severe compromise.

4.4 Success rate of PALs induction

Based on the literature, we thought it would be straightforward to induce apical periodontitis via pulp exposure in every tooth since studies usually do not address the 'success' rates of induction of PALs upon pulp exposure, and the assumption was that probably all of them developed PALs (Wang CY & Stashenko P 1991, Balto K et al. 2000, von Stechow D et al. 2003, Shah A et al 2018).

In fact, we observed a variability in the number of PALs: 27.7% had only 1 PAL and an equal percentage had 4, as verified by histology and micro-CT. In our study, out of 68 teeth drilled in 17 mice (four molars per mouse), we successfully exposed the pulp in 92% (62/68).

Overall, we identified PALs in 72% (44/62) of the teeth with exposed pulps, as verified by micro-CT and histology. One group reported similarly that PALs were induced in 70% (7/10) of drilled teeth using micro-CT and histology for evaluation (Balto K et al. 2000, von Stechow D et al. 2003). These authors reported that upon closer

examination of the corresponding histological sections of the teeth that showed no PALs, revealed that the pulp exposures in these teeth were very small, resulting in delayed pulpal infection and necrosis and slowed periradicular bone destruction (Balto K et al. 2000, von Stechow D et al. 2003).

Although the success rate of PAL induction was reported in the two papers above, both were from the same research group, and it may be that the same mice were analyzed. Most of the other published studies by this group and others did not mention success rate and only described the successful PALs (Balto K et al. 2002, da Silva RA at al. 2012). We speculate that perhaps in those with a small group size (5 mice) and only the 2 lower molars drilled per mouse, PALs were induced in all the drilled teeth, and therefore the success rate was not specifically mentioned (Balto K et al. 2000, Balto K et al. 2002), but we cannot be certain. In a larger study of 27-28 mice, with 2 lower molars drilled per mouse (da Silva RA et al. 2012), we assumed, because it was not discussed, 100% success rate; in hindsight, this was probably incorrect.

Inducing PALs in four molars per mouse turned out to be a challenging task. Other studies in mice usually reported that they drilled up to two molars (Balto K et al. 2000, Balto K et al. 2002, von Stechow D et al. 2003, da Silva RA et al. 2012) or one molar per mouse in total (Shah A et al 2018), and usually the drillings were confined to the lower molars. In hindsight, we speculate that due to the technical difficulty of the procedure, the skill required of the operator and anesthesia time constraints, this was the reason most studies exposed a maximum of two molars per mouse.

When conducting a study that involves induction of PALs by drilling cavities, it was important to take into consideration that PALs would not occur in 100% of cases. We originally planned to assess PALs at an intermediate time point at 4 weeks, to evaluate the rate of PALs development due to our pulp exposure. Four weeks after exposure, is the early time point that allows detection of PALs after pulp exposure (Wang CY & Stashenko P 1991). In parallel, the early onset of dermatitis started showing upland and we decided to use the mice that had to be terminated early due to severe dermatitis, towards the group of early evaluation of PALs.

However, due to technical limitations as the micro-CT machine arrived and became available in our facility only later. Therefore, we could not use micro-CT at the early evaluation time point, and therefore, we proceeded with the later time points of 16 weeks.

Unfortunately, instead of having a group of at least 11 mice with four PALs in the Rx group (based on sample size calculation that can be found in section 4.3 'Sample size'), we ended up with only five mice with four PALs. Interestingly, four out of these five mice were in the latter 50% of mice which were drilled (Table 3.4). We noticed that the last seven mice in which PALs were induced (Rx group), all had 3-4 PALs, as opposed to the first ten mice in the Rx group, which had only 3 mice with 3-4 PALs (Table 3.4). Therefore, improved surgical skills would help to create a more predictable pulp exposure.

4.5 Furcation lesions

In our study, 76% (47/62) of teeth developed furcation lesions, which were verified by micro-CT and matching histological sections. Since the etiology of a furcation lesion, is triggered by microbial infection and similarly to PALs, the presence of furcation lesion might have an influence on the results of our study. Therefore we performed statistical analysis, which took their presence into account. However, the statistical test did not show any difference in atherosclerosis between mice with/without furcation lesions, nor when they were added to the PALs, to a score of 'lesion load'.

Interestingly, other studies which used a mouse model to induce AP with methods similar to ours did not report the rate of furcation lesions. In some cases, the authors mentioned that they were present in some specimens (Akamine A et al. 1994, Balto K et al. 2000), but in other cases, there was no mention, even though they could be observed in the published images (von Stechow D et al. 2003). Due to the absence of reported incidence of furcation lesions in other studies, we cannot compare the rate we observed. Since we have not noticed the presence of furcation canals in micro-CT or histological sections, we speculate that the high prevalence of furcation lesions in our study may be attributed to the thin dentin in the furcal floor, which may not be enough of a barrier against microbial penetration. As a result, as soon as the pulp chamber became infected, the microbial infection easily traveled to the furcation area. Furcation lesions can be

reduced if furcation perforations are avoided during drilling of pulp exposure (depends on the skills of the operator), however, they will still appear in some instances even without perforation.

Based on the above, when evaluating the influence of PALs on the development of atherosclerosis in our study, considering the Rx group as a single and homogenous variable may result in bias, with underestimation of the potential influence of AP in atherosclerosis development. Dividing the Rx group into subgroups, based on the number of PALs/mouse, may lead to more accurate results, however, in our study, it resulted in 0-5 mice per group, weakening the strength of statistical tests. Even if we were able to induce four PALs per mouse in all the mice in the Rx group, this may still not be enough of local inflammation to cause a potential increase in atherosclerosis. As such, this study is suggested to be viewed as a pilot and can be used as a launch pad for future studies.

4.6 Micro-CT for detection of PALs

Although AP, which manifests as PALs is an inflammatory process, it can be detected using x-rays due to its location, surrounded by hard tissue. There are several methods to detect PALs, including two dimensional (2D) radiographs, three dimensional (3D) radiographs and the 'gold standard', histological sections. In animal models of PALs all these methods can be used for research (Kalatzis-Sousa NG et al. 2017).

In spite of micro-CT being a common tool in research, the authors of a recent systematic review concluded that there was no defined protocol for acquiring and analyzing micro-CT images of PALs in rats and mice Kalatzis-Sousa NG et al, 2017). Furthermore, acquisition, reconstruction, and analysis parameters were not adequately explained, which may compromise the reproducibility and the scientific impact of the studies (Kalatzis-Sousa NG et al. 2017). We used a voxel size resolution of 25μM, which is in the acceptable range for our purposes (Kalatzis-Sousa NG et al. 2017). Other parameters of the micro-CT scan can be found in section 2.22.1 'Micro-CT Scanning of heads).

In examining micro-CT scans, we noticed that some anatomical considerations made it challenging to recognize PALs. When the area of the PAL was large, it could be

identified immediately and the micro-CT was very useful. However in cases when, for example, the PAL was small, or located in proximity to the mandibular nerve canal, the determination whether it actually a PAL was more difficult due to the fusion of the radiolucency of the mandibular canal and the periapical area. Therefore, when we were judging about presence vs absence of a PAL, we examined the area, on the three planes (coronal, sagittal, axial) simultaneously.

The last issue was not addressed in the systematic review and we are interested to look further into the challenges of detection of questionable PALs micro-CT. In this study, the questionable PALs were further examined by histological sections as described in section 2.23 'Histology for PALs analyses below'.

4.7 Histological evaluation of PALs

For histological evaluation, we used H&E staining of 10μM micron sagittal (going through the mesial and distal roots simultaneously). The landmarks we used were the coronal pulp chamber and the periapical area of the roots. An 'ideal' section would go through the pulp chamber, canal and will include the apical foramina. Morphological evaluation of PALs in such sections has been used for decades and the different infiltrating cells (macrophages, lymphocytes, PMNs, and osteoclasts) can be differentiated (Metzger Z 2000),

For analysis of the results, we recorded the periradicular status of each tooth as 'PAL' (when PDL widening/inflammatory infiltrate was noticed in the periapical/periradicular area), 'F' (when PDL widening was noticed in the furcation PDL area). Since the sections include the coronal portion of the tooth, where pulp exposure can be seen, the histological evaluation was not blinded. We categorized the mice based on the number of PALs, F for the sum of PALs and F (LL-lesion load) and compared atherosclerosis lesion burden between.

While examining the histological sections, we also noticed a variability in the appearance of the PALs and furcation lesions, ranging from some inflammatory infiltrate, small lesions and up to large/severe lesions with dense inflammatory infiltrate. Other features that were noticed included apical resorption areas (irregularities on the outer surface of the root), size of pulp exposure, furcation perforation and degree of crown

destruction. Sections in which there was some ambiguity, were shown to an oral pathologist to confirm the findings. The features above were recorded and consequently, as a continuation project we plan to categorize the mice level of histologic inflammation. Preliminary records of these data can be found in Table 3.11.

Since we hypothesized that the inflammatory nature of PALs can play a contributive role in the development of atherosclerosis, the degree of tooth associated inflammation might be significant. Consequently, we are currently working on a categorization of the various histological appearances, as a continuation project, to test whether the severity of the inflammation, for example, size of the PAL, is associated with higher atherosclerotic lesion burden.

4.8 Atherosclerosis lesion burden

There are some advantages and limitations to the use of mouse models of atherosclerosis. A major advantage is that atherosclerosis in a mouse model develops over a period of months, rather than decades as in humans, and this time frame makes it feasible to study the disease in the time of an MSc project. Second, it is possible to perform genetic manipulation to allow for investigation of certain proteins or cells involved in the atherogenic process. Third, mice have a short gestation time, and finally, lower amounts of drugs are needed due to their small size (Getz GS & Reardon CA et al. 2015).

Despite the advantages listed above, mouse models also have limitations. There are some known differences between mice and humans in terms of the atherogenic process. Mice do not develop unstable plaques, which are characteristic of human lesions, nor coronary artery atherosclerosis. However, they do develop significant atherosclerosis in the aortic sinus, which is not a common site of lesion formation in humans (Getz GS & Reardon CA et al. 2015). In this project, we used LDLR KO mice, which is a classic model and recognized in the field as reliably mirroring certain aspects of human disease (Zadelaar S et al. 2007).

Despite our hypothesis that mice in the Rx group would show an increase in atherosclerosis lesion burden as compared to the sham group, we found they, in fact, developed a similar degree of atherosclerosis (lesion area in the Rx group = 7.65 ±

0.47%, compared with 7.46 ± 0.44% in the sham group, p=0.77). Our hypothesis was based in part on studies using a periodontal disease model, which showed significantly increased atherosclerosis lesion area in mice infected with Pg by oral lavage compared to control mice (Brown PM et al. 2015).

The reason why the periodontal disease study showed increased atherosclerosis in the treated mice while our study did not, can be potentially explained by two factors. First, the variability in the number of PALs among the Rx group, and second, the bacterial challenge (even four PALs might be not enough), which might be below the threshold to influence atherosclerosis, as will be discussed below.

The variability in the number of PALs among mice in the Rx group resulted in few mice with four PALs (n=5). Based on our power calculation, we needed 11 mice/group to determine if there was a significant difference in atherosclerosis lesion. Although the group with the highest number of PALs (four) had the highest average percent of atherosclerosis lesion area (8.67 ± 0.98%), as compared to the average in the sham group (7.65±0.46%) as well as the groups with one PAL (6.58±0.79%) or two-three PALs (7.24±0.48%), the power of statistical tests is low due to the small size of the groups and the variability in atherosclerosis area in the groups.

Based on the means and standard deviations in the sham and Rx sub-group with four PALs in our study, a sample size of 43 mice per group is needed for a power of 0.8 or 58 mice for a power of 0.9 [https://www.stat.ubc.ca]. Based on the fact that not 100% of mice in Rx group had four PALs, but 27% (5/17), even higher numbers area need. However, when taking into account that improved surgical skills would help to create a more predictable pulp exposure (based on the success rate of the last seven mice in Rx group, with 57% (4/7) mice with four PALs as opposed to only 27.7% (5/17) with four PALs in total, fewer mice will be needed.

Second, the difference between our results and the results of periodontal disease study may be explained by the fact that in our model, there was no exogenous introduction of a pathogenic bacteria. In our study, pulps of molars were exposed to the oral cavity and the pulps were infected with the endogenous oral flora. Mice in the periodontal disease study (Brown PM et al. 2015) were challenged with a perio-

pathogens that were applied by oral lavage. Although theoretically, perio-pathogens are part of the natural oral flora of mice, they might not be present in enough quantity "to push" the system towards significant inflammation that leads to changes in atherosclerosis.

4.9 Plasma and peritoneal macrophages cytokines

We used a cytokine array to measure cytokines in the plasma and peritoneal macrophages. This array can be used to determine the relative levels of specific mouse cytokines. The advantage of this method is that it allows detection and relative comparison of multiple cytokines at the same time.

Out of the 40 cytokines that can be compared, we identified eight that were variable (increase/decrease) between Rx over sham group in the plasma or peritoneal macrophages, at eight/16 weeks after PALs induction or sham anesthesia, including M-CSF, IL-1ra, TIMP-1, SDF-1/CXCL12, C5/C5a, BLC/CXCL13, sICAM-1/CD54. KC/CXCL1 (see list of cytokines' abbreviations, in section 2.14) and TNF-alpha and TREM-1, JE/CCL2/MCP-1, MIP-1-alpha/CCL3, IL-16. IL1-alpha were variable in the peritoneal macrophages. The highest fold changes were noted for M-CSF (4.24-2.92 at eight and 16 weeks) and IL-1ra (1.42 - 2.16 at eight and 16 weeks) in the plasma.

As opposed to our findings, data from the CVD study that used a periodontal disease model driven by Pg in LDLR KO mice, showed that sham-treated mice had a mean lesion area of 6% and Pg treated mice had a mean lesion area of 12%. (SE+/- 3%), and a larger variety of cytokines showed a change via the array. Also, pro-atherogenic cytokines were increased in that study. The lower variety of cytokines in our study and the lack of some key atherogenic cytokines are aligned with the fact that there was no difference in the atherosclerotic lesion burden between Rx and sham mice. For the cytokines that did show some change, and are known to be involved in atherogenesis (such as M-CSF), maybe the change was too small to induce a change in atherosclerosis.

M-CSF is a hematopoietic growth factor which is involved in the differentiation, proliferation and survival monocytes, macrophages and stem cell progenitors, and it stimulates increased phagocytic and chemotactic activity of macrophages (Stanley ER et al. 1997). It can be locally produced by endothelial cells and smooth muscle cells in

atherosclerotic plaques and therefore contributes to the development of atherosclerosis (Rajavashisth T. et al. 1998). Disruption of the M-CSF gene resulted in mice that were deficient in circulating monocytes, tissue macrophages, and osteoclasts and they were highly protected from atherosclerosis in the setting of hypercholesterolemia, demonstrating the importance of the role of macrophages in the pathogenesis of atherosclerosis (Ait-Oufella H et al. 2011).

Expression of Interleukin 1 family members and their receptors has been demonstrated in atherosclerotic plaques. Consistently, IL-1ra, a natural antagonist of IL-1, exhibits anti-inflammatory properties, mainly through the inhibition of IL-1 signaling. Overexpression of IL-1ra in LDLR KO mice markedly decreased the size of atherosclerotic lesions (Ait-Oufella H et al. 2011).

TIMPs are key regulators of the metalloproteinases that degrade the extracellular matrix and shed cell surface molecules. Since TIMPs can be measured in the plasma, they can allow monitoring of matrix turnover. TIMP-1 is synthesized by most connective tissue cell types, including mesangial cells and macrophages and is indicted in cardiovascular disease development and prognosis. Its circulating levels have been related to cardiovascular disease risk factors in a large community-based sample (Hansson J. et al. 2011). In a human study, 1 concluded that increased expression of TIMP might determine the evolution of advanced atherosclerotic plaques and contribute to its vulnerability since proteolytic imbalance might determine arterial remodeling and plaque destabilization in atherosclerotic vessels (Orbe J et al. 2003). Although TIMP-1 is an inhibitor of matrix degradation, increased levels of it in the plasma could possibly show that there is an increased turn-over of the matrix (increasing vulnerability of atherosclerotic plaques), which triggered an increase in the inhibitor.

SDF-1/CXCL12 is highly expressed in smooth muscle cells, endothelial cells and macrophages in human atherosclerotic plaques but not in normal vessels. It controls the homing of endothelial progenitor cells (EPCs) from bone-marrow to areas of vascular injury for angiogenesis and repair (Brunner S et al. 2009). It can also induce platelet activation and this suggests a role for SDF-1 in the pathogenesis of atherosclerosis and

thrombo-occlusive diseases (Abi-Younes S et al. 2012). Soluble intercellular adhesion molecule (sICAM-1/CD54) is related to the severity of atherosclerosis.

In a systematic review of human studies, it was concluded that AP is associated with increased levels of C-reactive protein (CRP), IL-1, IL-2, IL-6 in the plasma (Gomes MS et al. 2013). However, in our study, we did not find increased levels for IL1, 2 nor 6 and we did and we did not measure levels of CRP. Possibly, the difference may be due to the fact that in humans, other systemic condition had influence on the systemic cytokines and that in our study the level of the local inflammation due to the PALs or furcation lesions (of all the variable appearances), was not enough to have an influence in these cytokines in the plasma.

We compared the cytokines at the time point of eight and 16 weeks post-pulp exposure. It is known that the period of acute growth for PALs takes place within 3 weeks post exposure (Wang CY & Stashenko P 1991), and a slower bone destruction activity is taking place in the following time. Maybe if we would have performed the cytokine array earlier, three weeks post-pulp exposure, we would find a bigger variety of cytokines expressed and differ between the Rx and sham groups.

4.10 Morbidity/Dermatitis:

Although 16 weeks is a short time to develop atherosclerosis in humans, proportionally, it is a long period in a life of a mouse. Due to the longevity of these experiments, complications may arise. One of the known morbidities of WTD feeding in atherosclerosis-prone mice, which we witnessed in our study, was dermatitis. Unfortunately, we experienced unusually high rates of dermatitis in our study (sham group 12.3% (8/65), Rx group 29.2% (19/65)). The higher rate of dermatitis in the Rx as compared to the sham group may be attributed to a potential discomfort that the Rx mice were experiencing and therefore were scratching themselves more. Due to increased rates of dermatitis at early onset (ranging between 4-14 weeks), to make sure that we have an appropriate group size at the endpoint of 16 weeks, we were forced to enroll higher numbers of mice.

The dermatitis rate was far greater than any other previous study in our lab at the University of Alberta or at the lab's previous institution, the Cleveland Clinic,

encompassing nearly 15 years of work with this strain of mouse and this diet. The rate was more comparable to that observed in static cages (a cage with a micro-isolator roof, without airflow). At the same time as our experiment, there was another ongoing study in our lab, using the same LDLR KO mice and diet, but instead of PALs, periodontal disease was induced by oral lavage. Mice in both studies were housed in the same facility but in different rooms. Dermatitis rates were much lower compared to our study (2/40). Due to the unusually high rates of dermatitis, we carried out an extensive investigation, together with the Health Sciences Laboratory Animal Services (HSLAS), University of Alberta and concluded that it had to do with the airflow to the cages that apparently cannot be recorded or monitored. The facility had no methods to measure the airflow in the cage so we could not do anything about it.

Idiopathic, ulcerative dermatitis (UD) is a spontaneous, chronic disease generally affecting C57BL/6 mice. Prevalence rates of 4.1% to 26% have been reported (Hampton AL et al. 2015). Mice on WTD demonstrated increased UD lesions and impaired healing of induced wounds (Hampton AL et al. 2015, Zabalawi M et al. 2007).

Perhaps decreased air flow to the cages, together with the general anesthesia and drilling, added stress to the mice and increased their scratching, causing wounds on their skin. The LDLR KO mice fed a WTD have 10 times higher cholesterol content, which makes dermatitis even worse (Zabalawi M et al. 2007). Perhaps using airflow monitoring system in the cages should be a condition to perform this study in the future. Another way to reduce dermatitis rate could be cutting nails, to prevent scratching.

Despite the high rates of dermatitis, it does not correlate nor with the atherosclerotic lesion burden in this study, and this has previously been noted by the Febbraio lab, neither with the number of PALs/furcation lesions.

4.11 Limitations and future directions

Since our aim was to study the potential causal contribution of AP to atherosclerosis, and we hypothesized that the connection might be inflammatory, one limitation of our study is that we performed only a limited quantification of the amount of inflammation related to PALs or furcation lesions. The variability among mice in the Rx group (in terms of number of PALs/ furcation lesions, their size, and their inflammatory

nature (composition of inflammatory cells), might have masked on a potential influence that they have systemically, in case the local system would have pushed more to its edge, challenging higher local inflammation. The variability resulted only in a small group of mice with severe inflammation, which is too small to have statistical power. To overcome this limitation, since we noticed that the experience of the operator plays a role in the successful induction of PALs, the success rate PALs induction per mouse is expected to be improved in our future studies. Additionally, now that we have the micro-CT device available, we can evaluate our success rate of PALs induction as early as three weeks post drilling and adjust our technique as needed. Live imaging is also possible now, allowing us to evaluate the dynamics of PALs without the need to euthanize the mice.

Regarding the variability in the characteristics of the PALs, in the future, the PALs can be categorized by the inflammatory infiltrate/presence of apical resorption. Monoclonal antibodies against subsets of T-lymphocytes, B-lymphocytes, macrophages, dendritic cells, as well as plasma cells and PMNs, resulted in a major breakthrough in the understanding of the immune-biology of periapical host response, in both naturally occurring human PALs and those experimentally induced in the rat (Metzger Z 2000). Immune-histochemical staining will allow us to differentiate and quantify different cell populations in the periapical area. For example, specific staining for the various immune cell can be done in order to sub-group the Rx groups and evaluate their association with atherosclerosis

In order to increase the bacterial challenge, a combination of periodontal disease with AP can be considered, mimicking the common situation in humans, having periodontal disease it the background of AP. Maybe the fact that humans have a periodontal disease in addition to apical periodontitis, promotes atherosclerosis formation and explains the positive association findings in epidemiologic human studies (Berlin-Broner Y et al. 2017 [a]).

The relatively high rate of dermatitis in our study should be addressed in future studies to reduce the number of early euthanized mice. One way to reduce the rate of

dermatitis could be cutting the nails, to reduce self-trauma of the skin by scratching. Also, the airflow in the cages should be monitored to allow proper ventilation.

Since we found no difference in the atherosclerosis lesion area between Rx and sham mice, we did not compare the oxidative stress between the groups, although it was originally planned in our study. If we would have found a difference in the atherosclerosis lesion area, we would have checked to see if oxidative stress is involved could explain the difference.

CHAPTER 5: CONCLUSIONS

Our study aimed to compare mice with AP to mice without AP to explore a possible causative contribution of AP to atherosclerosis. We hypothesized that mice with AP, as compared to mice without AP, would show increased atherosclerosis lesion burden, with increased systemic inflammation. Although we found no difference in atherosclerosis lesion area between Rx and sham mice, the lack of difference could be possibly attributed to several issues that came up in the study. Most importantly, the group size of mice with four PALs was less than half the size (n=5) of what was required, based on power calculation (n=11). Therefore, the statistical test comparing the Rx and sham groups was not sufficiently strong enough to draw conclusions.

Overall, a question raised by our study is whether the LDLR KO mouse is a good model for studying the connection between AP and atherosclerosis, considering the challenge of inducing predictable numbers of PALs, and the high dermatitis rate. The latter issue may have been due to environmental controls and thus perhaps cannot be fully attributed to the genetic model.

Based on our results, in future studies, we recommend that the operator who is performing the drilling to induce PALs to be highly skilled. Measures to reduce dermatitis should be taken immediately. Finally, challenging the system with a periodontal disease pathogen along with AP, in order to increase the inflammatory load, may further help in exploring the contributive role of AP to atherosclerosis. Due to the issues above, we recommend that this study to be viewed as a pilot study and a launch pad for future studies.

To our best knowledge, this is the first study using the LDLR KO mouse model for atherosclerosis to study the influence of AP. Although no difference was found between the groups in terms of atherosclerotic lesion burden, we recommend that this study is viewed as a launch pad for future studies, implementing the suggestions to overcome the challenges which were discussed in the manuscript. Since AP might still have the potential to have a contributive role in the development of atherosclerosis, this area should be further explored.

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APPENDIX 1: SYSTEMATIC LITERATURE REVIEW

INTERNATIONAL ENDODONTIC JOURNAL

doi:10.1111/iej.12710

REVIEW

Association between apical periodontitis and cardiovascular diseases: a systematic review of the literature

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Abstract

Berlin-Broner Y, Febbraio M, Levin L. Association between apical periodontitis and cardiovascular diseases: a systematic review of the literature. *International Endodontic Journal*, **50**, 847–859, 2017.

A systematic review was conducted to assess the association between apical periodontitis (AP) and cardiovascular disease (CVD). Studies published from the earliest date available until September 2015 were retrieved from the Medline, PubMed and Embase databases. The included studies reported the results from observational studies and assessed the link between AP and CVD as confirmed by one of the following criteria: diagnosed coronary artery disease, angina pectoris, acute myocardial infarction, stroke or mortality caused by cardiac pathology. The study characteristics were abstracted by independent researchers following the PRISMA standard protocol. NOS criteria were used to rate the quality of the studies, and the GRADE was used for level of evidence evaluation. Nineteen epidemiological studies fulfilled the predetermined inclusion criteria: 10 case–control studies, five cross-sectional studies and four cohort studies. There was considerable heterogeneity amongst the included studies in terms of their study design, population, outcomes of interest and AP evaluation methods. Considering the limited availability and the heterogeneity amongst the studies, meta-analysis was not attempted. Thirteen of the 19 included studies found a significant positive association between apical periodontitis and cardiovascular disease, although in two of them, the significance was present only in univariate analysis. Five studies failed to reveal positive significance, and one study reported a negative association. In conclusion, although most of the published studies found a positive association between apical periodontitis and cardiovascular disease, the quality of the existing evidence is moderate–low and a causal relationship cannot be established.

Keywords: alveolar bone, atherosclerosis, inflammation, plaque, root canal treatment.

Received 6 July 2016; accepted 18 October 2016

Introduction

Rationale

The prevalence of apical periodontitis (AP), inflammation of the periapical tissue as a result of bacterial infection of the dental pulp, is as high as 34–61%, and it increases with age (Dugas *et al.* 2003, Jimenez-Pinzon *et al.* 2004, Lopez-Lopez *et al.* 2012). Five per cent of all teeth without root canal treatment (RCT)

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and 25% with a RCT have a periapical radiolucent lesion, which is the radiographic characteristic of AP (Pak *et al.* 2012). Overall, AP is likely underestimated, due to the limited sensitivity of radiographs.

Cardiovascular disease (CVD) remains the number one cause of death throughout the world, accounting for three in every 10 deaths in 2012, despite the prevalence and success of lipid lowering drugs [http://www.who.int]. Cerebrovascular disease (stroke, transient ischaemic attack) results from some of the same processes as CVD. Although CVD is recognized as a chronic inflammatory condition, with contributions from known risk factors such as age, gender, tobacco smoking, diabetes and high cholesterol levels, less focus has been directed towards the potential contribution of inflammatory processes that may further contribute to CVD (Jousilahti *et al.* 1999). In this category is AP.

The potential association between CVD and chronic inflammatory processes of endodontic origin or chronic AP has been thoroughly reviewed (Cotti & Mercuro 2015, Segura-Egea *et al.* 2015). Although endodontic infections cause a local tissue response, there is compelling evidence that like periodontitis, chronic AP may not be exclusively a local phenomenon, and may contribute to systemic inflammation (Jousilahti *et al.* 1999, Gomes *et al.* 2013, Brown *et al.* 2015, Cotti & Mercuro 2015, Segura-Egea *et al.* 2015). To date, no systematic review with a quality assessment has been published to investigate studies on the association between CVD and chronic AP.

Objective

The objective was to conduct a systematic review of the literature for evidence of an association between AP and CVD or cerebrovascular disease in humans. Participants, Interventions, Comparisons, Outcomes, Study Designs (PICOS) questions were predetermined in order to specifically address and achieve the aforementioned aims and objectives (Moher *et al.* 2009, 2015, Shamseer *et al.* 2015) (Table 1).

Review

Eligibility criteria

They are presented in Table 1. At first, a search was undertaken to identify all types of studies that researched the relationship between AP and CVD or cerebrovascular disease with no language limit applied. The search was conducted to identify all the available literature, from the earliest available date until September, 2015. Next, for the quality assessment part of this review, the language of the included studies was restricted to English and studies were systematically selected according to strict criteria, using the PICOS inquiries, as detailed in Table 1.

Information sources

The Medline, PubMed and Embase databases were electronically searched from the earliest available date until September, 2015. The electronic search was further supplemented with an additional hand-search citation mining process.

Search strategy

The search strategy was developed and conducted with an experienced reference librarian. The search was assembled from synonyms for AP and CVD.

Table 1 PICOS – Participants, interventions, comparisons, outcomes, study designs

Participants	Dentate human subjects (primary and permanent dentition), without age or gender restriction.
(Interventions) Exposures	Subjects with number of radiolucent periapical lesions >= 1 Apical periodontitis included radiographically diagnosed (periapical radiographs, panoramic radiographs or computerized tomography (CT)). Studies assessing the presence of apical periodontitis based on patients' self-report or a clinical examination without a radiographic assessment were excluded.
Comparator (control)	Subjects with number of radiolucent periapical lesions = 0
Outcomes	Cardiovascular disease: Established diagnosis of angina pectoris, myocardial infarction, stroke, coronary disease, atherosclerosis measured by angiography or other methods, measurements of cardiovascular factors (initial endothelial impairment (reduced endothelial flow rate), increased oxidative stress, hypertension or mortality caused by cardiac pathologies or self-report of a cardiovascular disease event.
Study designs	Clinical trials, case–control studies, cross-sectional and cohorts

Medical Subject Headings (MeSH) were included in the PubMed strategy, as presented below:

Database: Ovid MEDLINE(R)

1. periapical diseases/or periapical periodontitis/or periapical abscess/or periapical granuloma/or pulpitis/or 'Root Canal Therapy'/or Endodontics/(18637)
2. ((apical or periapical or periradicular or radicular) adj2 periodontitis).ti,ab. (1353)
3. ((periapical or periradicular or radicular or tooth or dent*) adj2 granuloma).ti,ab. (170)
4. ((periapical or periradicular or radicular) adj2 (abscess or disease* or infection* or lesion* or patho* or Inflammat* or condition* or process*)).ti,ab. (3185)
5. (apical adj2 (granuloma or abscess or disease* or condition* or process* or infection* or lesion* or patho* or inflammat*)).ti,ab. (1326)
6. (tooth or teeth or dent* or oral).mp. (1012929)
7. 5 and 6 (530)
8. (Pulpiti* or Endodontics).ti,ab. (3776)
9. (pulp* adj2 (inflammat* or infect*)).ti,ab. (808)
10. (endodont* adj2 (therap* or treatment* or inflammat* or origin or variables or patho* or failure* or lesion*)).ti,ab. (5385)
11. ('root canal' adj1 (therap* or treatment*)).ti,ab. (2942)
12. ((dent* or odontogenic) adj2 infect*).ti,ab. (2658)
13. 1 or 2 or 3 or 4 or 7 or 8 or 9 or 10 or 11 or 12 (26840)
14. exp Cardiovascular Diseases/(1988975)
15. exp Cerebrovascular Disorders/(299299)
16. (Cardio* or cardiac or coronary or heart or myocardi* or angiocardio* or hypertens* or atherosclero* or vascular or vasculo* or cerebr* or stroke or angiopath* or ischemi*).ti,ab. (2522419)
17. 14 or 15 or 16 (3284867)
18. 13 and 17 (840)

Data management

Mendeley reference manager (Mendeley, Inc., New York, NY, USA) was used for record and data management throughout the review. Duplicates were identified and discarded.

Selection process

Titles and abstracts of the studies selected in the preliminary analysis were scanned, and potentially qualifying studies were identified by two independent examiners (YB, LL). These studies received full-text assessment with respect to inclusion criteria (Table 1). The authors adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standard for reporting systematic reviews (Moher *et al.* 2009). Any disagreement between the examiners was resolved by discussion until agreement was reached.

Data collection process

The relevant studies were subjected to a comprehensive text evaluation, including data extraction and methodological quality analysis. Any disagreement between the examiners was resolved by discussion until agreement was reached.

Data items

The following information was collected from each included study: names of the authors, year of publication, study design, total number of subjects at baseline, population characteristics (age, gender, country), inclusion years, follow-up period, exposure, outcome or end-point, evaluation methods, adjusting factors, odds ratio (OR), relative risk (RR), main results and study limitations.

Outcomes

To evaluate the association between AP and CVD, CVD was defined as the outcome, as detailed in Table 1.

Quality and risk for bias in individual studies assessment

The quality of each study was rated independently, based on established criteria according to the Newcastle-Ottawa Scale (NOS) for 'assessing the quality of nonrandomized studies in meta-analysis' (Wells *et al.* 2014), and a consensus was reached (Tables S2 and S3). Although the NOS was originally formulated for use for case–control and cohort studies, it was adapted for assessing cross-sectional studies in this review, as previously described (Gomes *et al.* 2013).

Data synthesis

Due to heterogeneity amongst the studies, quantitative synthesis was not appropriate. If a statistically

significant association between AP and CVD was reported by an individual study, it was coded by the authors as present, 'Yes'; if absent, it was coded by the authors as 'No', as shown in Tables S4 and S5.

Meta-biases

Study limitations were assessed and presented under 'limitations' in Tables S4 and S5.

Confidence in cumulative evidence

The strength of the body of evidence was assessed according to the GRADE approach (Balshem *et al.* 2011).

Search results

Figure 1 is a PRISMA flow chart of the search results, adopted from Moher *et al.* 2009, 2015 and Shamseer *et al.* 2015. A total of 2101 records were identified using the three databases. After duplicate removal and title and abstract review, 47 studies were identified for full-text assessment. References from these 47 studies were inspected, and an additional 11 studies were identified for full-text assessment. Therefore, a total of 58 studies underwent full-text assessment, after which 39 studies were excluded (Table S1). The remaining 19 studies were finally included for quality assessment and systematic review (Table 2).

Availability of literature

There were 19 studies which fulfilled the predetermined inclusion criteria (Table 2). Of these, 10 were case–control studies (Grau *et al.* 1997, Rutger Persson *et al.* 2003, Oikarinen *et al.* 2009, Willershausen *et al.* 2009, 2014, Friedlander *et al.* 2010, Segura-Egea *et al.* 2010, Cotti *et al.* 2011, Pasqualini *et al.* 2012, Cotti & Mercuro 2015). Five were cross-sectional studies (Aleksejuniene *et al.* 2000, Frisk *et al.* 2003, Glodny *et al.* 2013, Costa *et al.* 2014, Petersen *et al.* 2014). Four were cohort studies (Jansson *et al.* 2001, Caplan *et al.* 2006, Inchingolo *et al.* 2014, Gomes *et al.* 2016).

Studies that presented only general dental scores, where AP was a part of other dental conditions without specific assessment for AP, and where no separate data regarding AP status were presented, were excluded from analysis in this review (Mattila *et al.* 1989, 1995, 2000, Syrjanen *et al.* 1989, DeStefano *et al.* 1993, Hujoel *et al.* 2001, Meurman *et al.* 2003, Janket *et al.* 2004, Haikola *et al.* 2013, Budin *et al.* 2014) (Table S1).

There was considerable heterogeneity amongst the included studies in terms of their study design, study population, outcomes of interest and AP evaluation methods (Tables S4 and S5). As a result, meta-analysis was not attempted.

Causal versus noncausal associations

According to the findings of the present review, although some epidemiological evidence supports an association between chronic AP and CVD, a causal influence of AP on CVD remains unclear and should be further investigated. The Bradford Hill criteria are a group of minimal conditions necessary to provide adequate evidence of a causal relationship between an incidence and a possible consequence (McMichael 2005). To date, the Bradford Hill criteria have not been fulfilled and causality cannot be established for AP and CVD. Below, each criterion is presented, defined according to McMichael (2005) and addressed in relationship to the results of this study.

Strength of association

A strong association is more likely to have a causal component than is a modest association. Amongst the 13 studies which found a positive significant association between AP and CVD, only four reported the OR, which ranged from 1.54 to 4.45 (Pasqualini *et al.* 2012, Willershausen *et al.* 2014, Costa *et al.* 2014, Tables S4 and S5). The wide variation in OR values can be explained by the variability in methods and in definitions of exposure and outcomes, etc. (as further discussed under the title 'Heterogeneity' below). The significant ORs reported by these four studies may have clinical significance, as an example a systematic review published in 2009, investigating the association between periodontal disease and CVD (Blaizot *et al.* 2009) can be used. In that review, a meta-analysis was performed and the pooled odds ratio calculated from the 22 case–control and cross-sectional studies was 2.35 (95% CI [1.87; 2.96], $P < 0.0001$) (Blaizot *et al.* 2009). The risk of developing cardiovascular disease was found to be significantly (34%) higher in subjects with periodontal disease compared to those without periodontal disease (pooled relative risk from the seven cohort studies was 1.34 (95% CI [1.27; 1.42], $P < 0.0001$) (Blaizot *et al.* 2009). In this study, due to heterogeneity, a meta-analysis was not

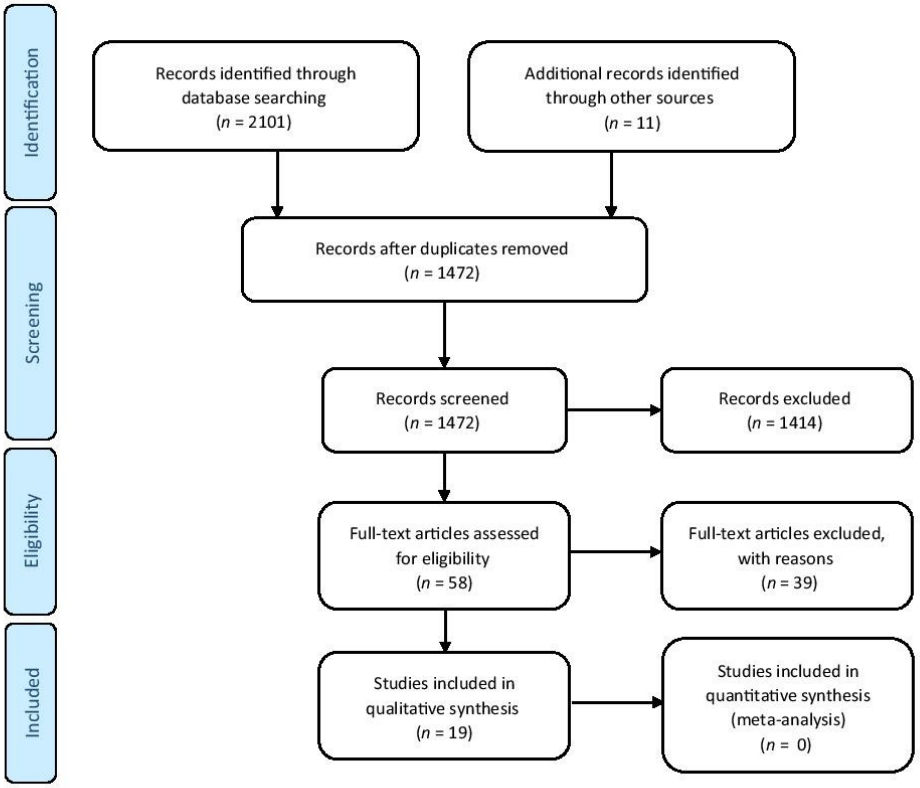

Figure 1 PRISMA flow chart, adopted from Moher *et al.* 2009.

attempted and a pooled odds ratio was not calculated. Although the total contribution to CVD from oral disease may be small, it may a have critical influence, if combined with other risk factors, especially if it is considered that CVD has a multifactorial aetiology.

Consistency

A relationship is observed repeatedly. Thirteen of the 19 included studies found a significant positive association between AP and CVD (Grau *et al.* 1997, Jansson *et al.* 2001, Caplan *et al.* 2006, Oikarinen *et al.* 2009, Willershausen *et al.* 2009, 2014, Cotti *et al.* 2011, 2015, Pasqualini *et al.* 2012, Glodny *et al.* 2013, Costa *et al.* 2014, Inchingolo *et al.* 2014, Petersen *et al.* 2014), although two studies did not demonstrate significance following multivariable analysis (Grau *et al.* 1997, Glodny *et al.* 2013). Five studies failed to reveal significance (Frisk *et al.* 2003, Rutger Persson *et al.* 2003, Friedlander *et al.* 2010, Segura-Egea *et al.* 2010, Gomes *et al.* 2016) and one study found a negative association (Aleksejuniene *et al.* 2000).

Specificity

A factor influences specifically a particular outcome or population. According to the Bradford Hill criteria, the value of this rule lies in its combination with the strength of an association. For example, whilst it may be observed that the population with periapical lesions (PALs) is at higher risk for CVD, it still cannot be concluded that PALs influence specifically the cardiovascular system, due to lack of studies. As a result of the multifactorial aetiology of CVD, and the presence of other influencing factors that can serve as

Table 2 Included studies

Authors, year	Title
Grau *et al.* 1997	Association between acute cerebrovascular ischemia and chronic and recurrent infection
Aleksejuniene *et al.* 2000[a]	Apical periodontitis and related factors in an adult Lithuanian population
Jansson *et al.* 2001	Relationship between oral health and mortality in cardiovascular diseases.
Rutger Persson *et al.* 2003[a]	Chronic periodontitis, a significant relationship with acute myocardial infarction
Frisk *et al.* 2003	Endodontic variables and coronary heart disease
Caplan *et al.* 2006	Lesions of endodontic origin and risk of coronary heart disease
Friedlander *et al.* 2010	Radiographic quantification of chronic dental infection and its relationship to the atherosclerotic process in the carotid arteries
Oikarinen *et al.* 2009	Infectious dental diseases in patients with coronary artery disease: an orthopantomographic case–control study
Willershausen *et al.* 2009	Association between chronic dental infection and acute myocardial infarction
Segura-Egea *et al.* 2010	Hypertension and dental periapical condition.
Cotti *et al.* 2011	Association of endodontic infection with detection of an initial lesion to the cardiovascular system.
Pasqualini *et al.* 2012	Association among oral health, apical periodontitis, CD14 polymorphisms, and coronary heart disease in middle-aged adults
Petersen *et al.* 2014	The association of chronic apical periodontitis and endodontic therapy with atherosclerosis
Glodny *et al.* 2013	The occurrence of dental caries is associated with atherosclerosis
Inchingolo *et al.* 2014	Influence of endodontic treatment on systemic oxidative stress.
Willershausen *et al.* 2014	Association between chronic periodontal and apical inflammation and acute myocardial infarction
Costa *et al.* 2014	Association between chronic apical periodontitis and coronary artery disease
Gomes *et al.* 2016	Apical periodontitis and incident cardiovascular events in the Baltimore Longitudinal Study of Ageing
Cotti *et al.* 2015	Endodontic infection and endothelial dysfunction are associated with different mechanisms in men and women.

[a]Studies added from manual search of references.

confounders, such as medications and systemic health, demonstrating a specific influence of PALs on CVD becomes a complex mission.

Temporality

The factor must precede the outcome it is assumed to affect. This criterion has not been fulfilled for AP and CVD, as most of the studies are cross-sectional. To date, only four cohort studies have been published (Jansson *et al.* 2001, Caplan *et al.* 2006, Inchingolo *et al.* 2014, Gomes *et al.* 2016) and three of them found a positive association between AP and CVD (Jansson *et al.* 2001, Caplan *et al.* 2006, Inchingolo *et al.* 2014). Longitudinal experiments in humans, intending to study the temporality between the two conditions, are logistically difficult. Atherosclerosis takes decades to develop in humans, and during that experimental time frame, a number of other factors, unrelated to endodontic disease, may accrue. Those factors can influence the cardiovascular system and add to the risk of developing cardiovascular disease.

Biological gradient

The outcome increases monotonically with increasing dose of exposure or according to a function predicted by a substantive theory. The Bradford Hill criteria favour linear relationships between exposure level and outcome; for instance, in the case of this study, increasing number of PALs should lead to a linear increase in the risk for CVD. Only one study reported results that follow that rule (Glodny *et al.* 2013).

Plausibility

The observed association can be plausibly explained by substantive matter (e.g. biological) explanations. As per Bradford Hill criteria, the presence of a biological explanation supports a causal conclusion. In one study, endothelial flow rate (EFR), which is widely considered a measure of vascular health, was evaluated in male AP patients (Cotti *et al.* 2011). EFR is a reproducible index of endothelial-dependent vasodilation, indicative of endothelial function. EFR is dependent on levels of plasma asymmetrical dimethylarginine (ADMA). ADMA inhibits the enzyme nitric oxide synthase (NOS), which regulates vascular tone. Elevated ADMA levels are suggestive of endothelial dysfunction. Cotti *et al.* (2011) designed a prospective observational cross-sectional trial to investigate whether the state of chronic inflammation that accompanies AP may significantly alter endothelial

function, and serve as a predictor of possible future cardiovascular problems (Cotti *et al.* 2011). The results demonstrated that serum levels of IL-1, IL-2, IL-6 and ADMA were increased significantly, whilst EFR was significantly reduced in male patients with AP when compared with healthy controls. The data suggest an early endothelial dysfunction in young male adults with AP, in the absence of CVD (Cotti *et al.* 2011).

Another systematic review concluded that chronic AP may significantly increase systemic inflammation (Gomes *et al.* 2013). This biological state may contribute to the development of CVD, which is known to have an inflammatory component (Hansson 2005). Thus, at least two studies suggest a plausible biological explanation for the association between AP and CVD, supporting causality.

Coherence

A causal conclusion should not fundamentally contradict present substantive knowledge. The potential causal association between AP and CVD does not contradict present knowledge.

Experiment

Causation is more likely if evidence is based on randomized experiments. This criterion is not fulfilled, as there is a lack of randomized experiments in the field.

Quality and risk for bias assessment of included studies

Quality assessment according to the NOS criteria

The detailed assessment of quality for all included studies, based on the NOS criteria (Wells *et al.* 2014), is presented in Tables S2 and S3. Each case–control or cross-sectional study could be awarded a maximum of eight stars, whilst a maximum of nine stars could be awarded for cohort studies. All of the four included cohort studies were awarded six or more stars (Jansson *et al.* 2001, Caplan *et al.* 2006, Inchingolo *et al.* 2014, Gomes *et al.* 2016). Only four of the 10 case–control studies were awarded six or more stars (Rutger Persson *et al.* 2003, Cotti *et al.* 2011, 2015, Pasqualini *et al.* 2012), and one received only three stars (Segura-Egea *et al.* 2010). Two of the five included cross-sectional studies were awarded six or more stars (Glodny *et al.* 2013, Costa *et al.* 2014) and one received only two stars (Aleksejuniene *et al.* 2000). Analysis of the results of the 10 studies which received six or more stars (four cohort, four case–controls, two cross-sectional; Jansson *et al.* 2001, Rutger Persson *et al.* 2003, Caplan *et al.* 2006, Cotti *et al.* 2011, Pasqualini *et al.* 2012, Glodny *et al.* 2013, Inchingolo *et al.* 2014, Costa *et al.* 2014, Cotti *et al.* 2015, Gomes *et al.* 2016) revealed that all but two (Rutger Persson *et al.* 2003, Gomes *et al.* 2016) found a significant positive association between AP and CVD. One of them, however, did not show significance following multivariable analysis (Glodny *et al.* 2013). The quality assessment showed a lack of high-quality longitudinal studies investigating the association between AP and CVD. To date, only four cohort studies have been published (Jansson *et al.* 2001, Caplan *et al.* 2006, Inchingolo *et al.* 2014, Gomes *et al.* 2016) and three of them found a positive association between AP and CVD (Jansson *et al.* 2001, Caplan *et al.* 2006, Inchingolo *et al.* 2014, Gomes *et al.* 2016). An existing validated tool was used for quality analysis, the NOS, to give a quantitative value to the quality of evidence. The number of stars is a tool for evaluation and a cut-off point (six stars) was selected by the authors. The quantitative analysis of the quality of the studies does not stand alone, but is considered together with the level of evidence. The level of evidence in this study was based on the GRADE (Grading of Recommendations Assessment, Development and Evaluation) approach (Balshem *et al.* 2011, see under 'Level of Evidence' below). Please note that although the type of radiograph used in the diagnosis of AP is an important factor in the quality of each study, it was not included in Tables S2 and S3, because the criteria here are based solely on the NOS (Wells *et al.* 2014). The influence of radiograph type on the quality of the study is addressed separately in Tables S4 and S5 and is further discussed below.

Quality and type of radiographs

The method used for radiographic evaluation of radiolucent AP may have a critical influence on the study results. The specificity and sensitivity vary amongst panoramic radiographs, periapical radiographs and cone beam computerized tomography (CT) images to detect a periapical lesion. The sensitivity of these three modalities is 0.28, 0.55 and 0.91–0.98, respectively, and the specificity is 1, 0.98 and 0.73, respectively (Lennon *et al.* 2011, Petersson *et al.* 2012). These differences influence the ability of a clinician to detect an AP lesion. Most of the included studies (10 of 19) used panoramic radiographs as the only

method for periapical lesion screening (Grau *et al.* 1997, Frisk *et al.* 2003, Oikarinen *et al.* 2009, Friedlander *et al.* 2010, Cotti *et al.* 2011, 2015, Gomes *et al.* 2016), or as a primary method followed by additional radiographic methods, only when a radiolucent lesion was detected on the panoramic radiographs (Aleksejuniene *et al.* 2000, Willershausen *et al.* 2009, 2014) (Tables S4 and S5). Some studies used full-mouth intra-oral periapical radiographs (Jansson *et al.* 2001, Caplan *et al.* 2006, Segura-Egea *et al.* 2010, Pasqualini *et al.* 2012, Costa *et al.* 2014), or only whole-body CT (Glodny *et al.* 2013, Petersen *et al.* 2014). In two studies, the radiographic method was not specified (Rutger Persson *et al.* 2003, Inchingolo *et al.* 2014). Use of panoramic radiographs may have caused an underestimation in the prevalence of AP lesions, as this method only detects 28% of lesions (Petersson *et al.* 2012). This may partially explain the results of one of the cohort studies, which found a nonsignificant positive association between AP with CVD (Gomes *et al.* 2016). When root filled teeth were added to the analysis, the association became significant and independent between (AP + RCT) >= 3 and CVD (RR = 1.77, CI = 1.04–3.02) (Gomes *et al.* 2016). Thus, addition of treated teeth may have compensated for the underestimation caused by the use of panoramic radiographs for PA lesion detection in this study.

Reliability of radiographic interpretation

The radiographic interpretation may have a critical influence on the study results. Radiolucent lesions are not always easy to detect by two-dimensional radiograph, especially when they are small. When specific criteria are applied, such as a cut-off point to define an AP lesion, this may contribute to variability. When six experienced examiners were asked to determine whether an area of rarefaction was or was not present on one radiograph, the agreement was <50% (Goldman *et al.* 1972). In a more recent study, similar results were obtained, even with the addition of cone beam CT for AP evaluation (van der Borden *et al.* 2013).

Delayed radiographic evidence of the inflammatory process

As an inflammatory periapical lesion does not immediately appear radiographically, this may result in an underestimation. Only 70–74% of histological inflammatory processes are detected on the radiograph, meaning that in 26–30% of cases, inflammation was histologically present but no AP could be detected radiographically (Green *et al.* 1997, Barthel *et al.* 2004). The appearance of a radiolucent lesion on the radiograph is often but not always dependent on its relationship to the cortical bone size and mineral loss percentage (Bender 1982, Lee & Messer 1986, Marmary *et al.* 1999, Bender & Seltzer 2003a,b).

Multivariable analysis and confounders

Bivariate analysis investigates two variables to test an association, for example AP (exposure) and CVD (outcome). However, by itself, it cannot determine causality and does not take into consideration the potential influence of other variables that may affect the association. Multivariable analysis takes into account the simultaneous effects of other variables and calculates the strength of the relationship between an exposure and an outcome more accurately. In two studies, the positive association that was found between AP and CVD lost significance after multivariable analysis (Grau *et al.* 1997, Glodny *et al.* 2013). Periodontitis is a known risk factor for CVD and is a potential confounder that may be responsible for the positive association between AP and CVD in some studies, as it was not controlled (Oikarinen *et al.* 2009, Willershausen *et al.* 2009, Inchingolo *et al.* 2014). The positive association between endodontic burden (AP + RCT) >= 3 and CVD was found to remain significant only in patients with moderate/severe periodontitis, but lost its significance in patients with no/slight periodontitis (Gomes *et al.* 2016).

Selection bias

Study population varied and included only males (Caplan *et al.* 2006, Inchingolo *et al.* 2014), mostly males (Oikarinen *et al.* 2009, Willershausen *et al.* 2009, 2014, Friedlander *et al.* 2010, Pasqualini *et al.* 2012) or only females (Frisk *et al.* 2003). In one study, the gender was not specified (Aleksejuniene *et al.* 2000). The remainder of the studies included both genders. Male gender is a known risk factor for CVD and may explain the lack of an association between AP and CVD in some of the studies, especially in the large cross-sectional study which included only women (Frisk *et al.* 2003). This may suggest that AP lesions are a contributory risk factor in an already susceptible population. Another selection bias may be present in those studies which used whole-body CT scans of patients who had other medical conditions, including cancer, arthritic degeneration or trauma (Glodny *et al.* 2013, Petersen *et al.* 2014). These diseases may lead to distortion of

the real relationship between inflammatory AP and CVD by increasing systemic inflammation or by modifying the immune response due to various therapies directed at the primary disease, and this could result in wrong data interpretation. Future studies should control for the background health status of the study population.

Publication Bias

Publication bias is another important issue which needs to be taken into account in coming to conclusions. This limitation exists because results from every study performed are not necessarily published. Results may not be published because they are negative, contrary to the expectations of the authors, or because they may not be of great interest. There may also be bias as a result of the interests of the sponsor of the study.

Heterogeneity

As already mentioned, due to the high degree of heterogeneity amongst the included studies, in terms of their study design, study population, outcomes of interest and AP evaluation methods (Tables S4 and S5), a meta-analysis was not attempted. The degree of diversity between studies is due to lack of published guidelines and recommendations in the field. Different attempts were made to study various aspects of CVD, rather than concentrating on one aspect (see 'Outcome definitions' below). Regarding the case/exposure (PAL) definition, the high degree of heterogeneity here was due to the various definitions of PALs (based on size) and diversity of accepted or less accepted methods for evaluation of AP (two-dimensional images, such as periapical radiographs and panoramic radiographs versus three-dimensional radiographs, including cone beam CT and CT) (see 'Case/Exposure definitions of AP across studies', 'Quality and type of radiographs', 'Reliability of radiographic interpretation'). Based on the heterogeneity that was uncovered in the present systematic review, professional associations should develop a set of core methods and outcomes to recommend for future studies.

Outcome definitions of CVD across studies

The outcome of interest was heterogenic amongst the studies (Tables S4 and S5). CVD was represented by many conditions, including angina pectoris, myocardial infarction, cerebrovascular insult, acute myocardial infarction, chronic ischaemic heart disease, coronary calcified atheroma, hypertension, initial endothelial impairment/reduced endothelial flow rate, aortic atherosclerosis, oxidative balance and mortality related to CVD. Different evaluation methods were used, including self-reporting of CVD/hypertension (Aleksejuniene *et al.* 2000, Frisk *et al.* 2003, Segura-Egea *et al.* 2010), measuring the aortic atherosclerotic burden by a calcium scoring method (Glodny *et al.* 2013, Petersen *et al.* 2014), Doppler ultrasound (Friedlander *et al.* 2010) and measurement of plasma markers (Cotti *et al.* 2011, 2015). The heterogeneity in the outcome definitions between studies makes it difficult or ill-advised to combine the results from different studies. Clinicians, researchers and professional associations are called upon to publish definitive guideline and recommendations for the best existing and reliable method(s) to evaluate the state of the cardiovascular system (such as blood inflammatory markers, chronic versus acute signs and symptoms). There is an imperative need for a uniform protocol to allow indisputable comparison between different studies.

Case/Exposure definitions of AP across studies

Heterogeneity also existed in the size of the PALs to be included in the study and in scoring methods. These different definitions of AP may serve as a potential source of discrepancy across studies (Tables S4 and S5). The question as to whether the size of the PAL has influence on the association remains to be answered. Studies that used self-reporting questionnaires to evaluate the presence of endodontic status (e.g. 'Have you ever had an endodontic therapy?'), without radiologic confirmation, were excluded from the quality assessment part of this systematic review, due to the unreliability of the self-report method (Caplan *et al.* 2006, Joshipura *et al.* 2006, Arroll *et al.* 2010).

Level of evidence

The overall level of evidence of the included studies is moderate–low, according to GRADE (Balshem *et al.* 2011). The reader should be aware that even though some of the studies sum up to more than six of eight or nine stars based on the NOS, the level of evidence is still moderate–low (GRADE). Clinicians and researchers are called upon to perform more high-quality research in the field to fill the gaps of knowledge.

Limitations of the study

The major limitation of this review was the clinical heterogeneity amongst the included studies with regard to outcome (CVD) and exposure (AP) definitions and diagnostic methods. As a result, a meta-analysis was not attempted. The entire range of CVD and cerebrovascular disease outcomes included hypertension, morbidity and mortality in cohort studies and coronary heart disease and acute myocardial infarctions in cross-sectional and case–control studies. Methods of AP assessment also widely varied amongst studies and included panoramic radiographs, full-mouth radiographs and CT. Moreover, different definitions of AP lesion size and scoring systems were used.

Comparison of findings with other systematic reviews

This is the first systematic review of the evidence on the association between AP and CVD. There were no identified systematic reviews specifically investigating the association between AP and CVD. However, one recently published systematic review investigated the association between AP and systemic inflammatory markers (Gomes *et al.* 2013). Although the available evidence was limited, the authors found that AP was associated with increased levels of C reactive protein (CRP), IL-1, IL-2, IL-6, ADMA, IgA, IgG and IgM in humans. These findings suggest that AP contributes to a systemic immune response not confined to the localized lesion, potentially leading to increased systemic inflammation (Gomes *et al.* 2013), and would be consistent with the present findings related to CVD. The association between periodontitis and CVD has been more widely investigated, and a positive association has been established in a systematic review of observational studies (Blaizot *et al.* 2009). A meta-analysis concluded that periodontal disease appears to be associated with a 19% increase in risk of future coronary heart disease and the increase in RR was more prominent (44%) in persons aged 65 or greater (Blaizot *et al.* 2009).

Implications for clinical practice and research

Within the limitations of the available literature, clinicians should bear in mind that PALs may not only have local manifestations but also systemic ramifications, as has been already shown for periodontitis (Beck *et al.* 1996, Madianos *et al.* 2002). Due to the lack of long-term longitudinal studies, strong evidence for the association between AP and CVD could not be obtained. A causative relationship, if established, may have a critical impact in both the dental and medical fields. Mechanisms linking endodontic disease to CVD risk might be similar to those hypothesized for associations between periodontal disease and CVD, in which a localized inflammatory response to a bacterial infection leads to release of cytokines into the systemic circulation and subsequent deleterious vascular effects (Lockhart *et al.* 2012). A recently published study has revealed more extensive mechanistic detail (Brown *et al.* 2015). The prevailing theory is that localized oral infections, by increasing systematic inflammation/oxidative stress, exacerbate CVD, contribute to future events or decrease the threshold for CVD events (Lockhart *et al.* 2012, Gomes *et al.* 2013, Brown *et al.* 2015). The presence of AP can be considered an additive risk factor for CVD, along with active periodontal disease.

Although the contributing absolute risk to CVD from oral infections may be relatively small, risk factors for CVD are additive. Because PALs are often silent, they may be overlooked during clinical assessment of CVD, despite the fact that they are very common in the age group of patients prone to atherosclerosis.

If AP truly caused CVD, treatment of oral conditions such as PALs may reduce atherosclerosis development, and sequel such as heart attack and stroke, especially in high-risk populations. Recognition of PALs as a contributory risk factor for systemic inflammatory conditions such as atherosclerosis will provide incentive for prevention and early intervention. Educating dental health professionals, medical professionals, patients and policymakers about the impact of oral inflammation stemming from chronic AP on systemic health will increase awareness of the need to include oral health assessment, treatment and preventive strategies as a means to improve health outcomes in the population worldwide.

Future directions

Future research should be directed towards studies with long-term follow-up and better control of confounders and clinical trials that test underlining mechanisms linking inflammatory AP and CVD. Animal experimentation is also key to a better understanding of the pathophysiology of AP and CVD. There is significant research potential in this area, as oral inflammatory status may contribute to the severity of other systemic chronic inflammatory conditions as well.

Conclusions

In conclusion, although most of the published studies found a positive association between AP and CVD, the quality of the existing evidence is moderate–low and a causal relationship cannot be established.

Acknowledgements

The authors would like to thank Ms. Ruti Souhami – head of search section, Gitter-Smolarz Library of Life Sciences and Medicine Library, Tel-Aviv University, Israel, for her contribution in developing the search strategy.

Conflict of interest

The authors have stated explicitly that there is no conflict of interest in connection with this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Studies considered but excluded and reasons of exclusion.

Table S2. Methodological quality assessment of cohort studies according to NOS criteria 10 ($N = 4$).

Table S3. Methodological quality assessment of case-control and cross-sectional studies according to NOS criteria ($N = 15$).

Table S4. Characteristics, main results, and limitations of the cohort studies included in the systematic review.

Table S5. Characteristics, main results, and limitations of the cross-sectional and case-control studies included in the systematic review.

Table S1: Studies considered but excluded and reasons of exclusion

Authors, year	Reason for exclusion
Robbins 1980	Case reports
Mattila *et al.* 1989*	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Syrjänen *et al.* 1989	No separate data regarding PAL, only the sum of PALs and other dental conditions.
DeStefano F *et al.* 1993*	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Mattila *et al.* 1993	Review
Mattila *et al.* 1995	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Lorber *et al.* 1998	Letter to the editor
Ziegler *et al.* 1998	Study published in German
2012 *et al.* 1999	Review
Beck *et al.* 2000*	Review
Murray *et al.* 2000	Review
Li X, Kolltveit *et al.* 2000	Review
Mattila *et al.* 2000	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Hujoel *et al.* 2001	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Jansson *et al.* 2002	Cardiovascular diseases were excluded.
Meurman *et al.* 2003	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Marton *et al.* 2004*	Review
Caplan *et al.* 2004 *	Review
Janket *et a*l. 2004*	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Joshipura *et al.* 2006	No clinical or radiographic examinations were performed, endodontic treatment diagnosis based on self-report.
Frisk *et al.* 2007	The same cohort subjects as Frisk 2003, same study population as Frisk 2003,but different statistical analysis
Marton *et al.* 2007*	Review (Hungarian)
Hama *et al.* 2008	Analysis of human PA granulomas, association between AP and CVD not investigated.
Maloney *et al.* 2009	Letter to the editor
Caplan *et al.* 2009	No clinical or radiographic examinations were performed, endodontic treatment diagnosis based on self-report.
Arroll et al. 2010	No clinical or radiographic examinations were performed, endodontic treatment diagnosis based on self-report.
Cotti *et al.* 2011	Review
Somma *et al.* 2011	Review
Segura-Egea *et al.* 2011	Association between smoking and PALs tested, not between AP and CVD not investigated
Brilhante *et al.* 2012	Animal study
Haikola *et al.* 2013	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Pyysalo *et al.* 2013	Microbial genome assessment in ruptured intracranial aneurysms.
Gomes *et al.* 2013	Review
Slutzky-Goldberg *et al.* 2013	Analysis of human PA granulomas, association between AP and CVD not investigated.
Budin *et al.* 2014	No separate data regarding PAL, only the sum of PALs and other dental conditions.
Al QM, J.H. M. 2014	A letter to the editor
Cotti *et al* 2015 a	Review
Segura-Egea *et al.* 2015	Review

*Studies added from manual search of reference

Table S2 Methodological Quality Assessment of Cohort Studies According to NOS Criteria 10 (N=4)

NOS criteria	Jansson *et al.* 2001	Caplan *et al.* 2006	Inchingolo *et al.* 2013	Gomes *et al.* 2015
Selection				
1. Representativeness of the exposed cohort	*	*	*	*
2. Selection of the non-exposed cohort	*	*	*	*
3. Ascertainment of exposure	*	*	---	*
4. Demonstration that outcome of interest was not present at start of study	---	*	*	---
Comparability				
1. Comparability of cohorts on the basis of the design or analysis	**	**	---	**
Outcome				
1. Assessment of outcome	*	*	*	*
2. Was follow-up long enough for outcomes to occur	*	*	*	*
3. Adequacy of follow-up of cohorts (<20%)	*	*	*	*
Total awarded stars	8	9	6	8

- A study can be awarded a maximum of 1 star for each numbered item within the selection and exposure categories.
- A maximum of 2 stars can be given for comparability. The two factors that that were the most important for comparability were selected to be age and gender (as they are known as strong risk factors to CVD). Adjustment or matching for each of them awards with one star.
- Adjustment or matching for other important risk factors (such as social status, smoking, periodontitis etc. are presented in tables 6S, 7S).
- Total number of awarded stars could be 9.

Table S3 Methodological Quality Assessment of Case-control and Cross-sectional Studies According to NOS Criteria (N = 15)

NOS criteria	Grau 1997 CC	Rutger 2003 CC	Friedlander 2009 CC	Oikarinen 2009 CC	Willershausen 2009 CC	Segura-Egea JJ 2010 CC	Cotti 2011 CC	Pasqualini 2012 CC	Willershausen 2014 CC	Cotti. 2015b CC	Aleksejunien 2000 CS	Costa 2014 CS	Frisk. 2003 CS	Glodny 2013 CS	Petersen 2013 CS
Selection															
1 Is the case definition adequate	*	*	*	*	*	---	*	*	*	*	---	*	---	*	*
2 representativeness of the cases	*	---	---	---	*	---	*	*	*	*	---	*	*	*	---
3 Selection of controls	---	*	---	---	---	---	---	*	---	---	---	----	---	---	---
4 Definition of controls	*	*	*	*	*	*	*	*	*	*	---	----	----	----	---
Comparability															
1 Comparability of cases and controls on the basis of the design or analysis	**	*	**	**	**	---	**	**	**	**	---	**	*	**	**
Exposure															
1 Ascertainment of exposure	*	--	*	*	*	*	**	*	*	*	*	*	*	*	*
2 Same method of ascertainment for cases and controls	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3 Nonresponse rate	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Total awarded stars	5	6	5	5	5	3	7	6	5	7	2	6	4	6	5

- Abbreviations: CC – case control study, CS – cross sectional study, NA - does not apply.
- A study can be awarded a maximum of 1 star for each numbered item within the selection and exposure categories.
- A maximum of 2 stars can be given for comparability. The two factors that that were the most important for comparability were selected to be age and gender (as they are known as strong risk factors to CVD). Adjustment or matching for each of them awards with one star.
- Adjustment or matching for other important risk factors (such as social status, smoking, periodontitis etc. are presented in tables 6S, 7S)
- Total number of awarded stars could be 8.

Table S4 Characteristics, Main Results, and Limitations of the Cohort Studies Included in the Systematic Review

-Study -Study design	-mean age -Age range -gender N°	- Subjects N° -cases /controls	Population characteristics	-Exposure -Evaluation Method -AP definition	-Outcome - Evaluation method	Inclusion Period follow up (y)	Adjusting factors	OR or RR [CI 95%]	Main results	Association between AP and CVD (P value)	Observations and limitations
Caplan et al. 2006	mean age at baseline: 47.4 years 31-65 5 intervals defined by cut points at 40, 45, 50, and 55 yrs M 708	708	Participants (who were not VA patients) were men enrolled in the VA Dental Longitudinal Study and the concurrent Normative Aging Study, ongoing epidemiologic studies of male adults in the Boston area.	- Incident PAL " lesion years" estimated the cumulative burden of chronic endodontic Inflammation experienced by each subject. Full-mouth dental radiographic series. PAL: PDL space that was >= 2 mm wide, with absence of intact lamina dura.	Time to First Diagnosis of CHD: AMI, CIHD or ANP	-1960s-2001 FU every 3 yrs. -mean 24 -max 32	Age Education Income BMI Smoking Diabetes HT TG TC N° of teeth N° of teeth with PAL N° of RF teeth Mean ABL "number of teeth with PALs at baseline" as a covariate in multivariable regression analyses	---	Significant association between incident PALs and subsequent CHD among younger (<40 yr), but not older, men.	Yes significant p < 0.05	---
Jansson et al. 2001	38.7 18-65 M W	1393 Of them 88% had radio-graph =1225	County of Stockholm. At a follow-up in 1997, the mortality rate of the sample during the years 1970–1996 was registered as	PAL 18 intraoral radiograph in fully dentate persons.	Death from CVD	Dental - 1970 - 1997 FU mortality rate and causes of death registration:	Age Gender N° of teeth marginal bone loss smoking caries plaque CVD in 1970	---	The independent variable: number of PALs, was significantly correlated to death due to CVD when adjusted	Yes p<0.05	Only mortality of CVD was taken into account, but not CVD

				criteria (De Moor et al. 2000).		from dental examination and through 2011	CAD Stroke PD No of teeth <> 20	AP ≥ 3 RCT ≥3 29% CVE vs 19% non CVE subjects: had AP>=1 not significant association of AP with incident of CVE	No, P = 0.081 No, P = 0.055 Not significant Not significant		available in the BLSA.

Abbreviations: ASB - Atherosclerotic burden , BMI – body mass index, CAD – coronary artery disease, CAP – chronic apical periodontitis, CC – Case control study, CCA – calcified carotid artery CHD – coronary heart disease, CIHF – chronic ischemic heart disease, CS – Cross sectional study, CT - computed tomography, CVD – cardiovascular disease CVE – cardiovascular event, CVI - cerebrovascular ischemia, DBP - diastolic blood pressure, DM – diabetes mellitus, EFR - endothelial flow reserve, ET – endodontic treatment, HDL, high-density lipoprotein, HL – hyperlipidemia, HT – hyper tension, LDL, low-density lipoprotein, , M- men, MPI - Mattila pantomography index, N° - number, NS – not specified RAL - radiolucent periapical lesions, RF – root filled teeth, SBP-systolic blood pressure; TC- total cholesterol, TG - triglycerides, PAI –Periapical index score , PAN – panoramic radiograph, PD - periodontal disease , TIA - transient ischemic attack, , RCT – root canal treatment, SD – standard deviation, W – women, WBC- white blood cells.

			in the 3 months before recruitment, No smoking No obesity, no systemic inflammatory or metabolic diseases, No poor oral hygiene.						found to have progressively returned to normal in the cases group.		
Gomes et al. 2015	55.0 +/- 16.8 base line M 143 W 135	278	participants in the Baltimore Longitudinal Study of Aging (BLSA).	AP or RCT = 0 AP or RCT >=1 EB=AP+RCT PAN according to pre-established	Incident CVE: -ANP -MI -CV death: stroke, CAD, etc. (N=62)	Follow up: 17.4 -/+ 11.1 1-44yrs Dental data: 1962-1995 Outcome measured	Age Sex Education yrs Smoking Dyslipidemia BMI HT DM Cancer	EB RR = 1.77 (1.04–3.02) RR = 1.46 (0.91–2.33)	EB (AP+RCT) ≥3 Independently associated with incident CVE (only in moderate/severe PD, not in non/slight PD).	Yes P=0.03	No. of AP was recorded only at base line so this no. might have changed over time. No dental longitudinal data were

											periodontitis and CHD.
Glodny et al. 2013	54.1+/-17.3 NS W137 M 155	292	-Departments of Internal Medicine, Surgery, and Traumatology. Austria. -None of the patients were referred from the School of Dentistry. -Indications for CT examination: tumor staging, staging of multiple myeloma, morphological evaluation of presumed, arthritic degeneration or trauma.	-PAL -CT	-Aortic Atherosclerotic burden -CT-a calcium scoring method	2008 - 2010	Age Caries Gender Bone height N°of teeth Indication for CT scan Incisal point to bone crest	---	Univariate analyses: atherosclerotic burden is higher in patients with chronic AP compared to patients without chronic AP: chronic AP >=1 (0.24+/-0.6 mL) CAP=0 (0.51+/-1.13 mL) The greater the number of teeth affected by chronic AP, the greater the ASB. Logistical regression analyses of the target variable "**presence of** aortic atherosclerosis: chronic AP was not an independent risk factor.	Yes P<0.00 Not significant	-No adjustment for CAD conventional risk factors (smoking, BMI, DM, etc). -It is not anticipated that the diagnosis of a tumor disease or an accident, which applied to most of the patients in the study, could have an effect on the status of the parameters studied.

Petersen et al.2013	50 ±15.7 8–89 259 W 272 M	531 11,191 teeth	-Indications for CT examination: arthritis, identify and staging of tumors, evaluating trauma. -Innsbruck Medical University	-chronic AP -whole-body CT .	-Aortic atherosclerotic burden -calcium scoring method -Whole-body CT.	NS	Age Gender Caries per tooth N° of teeth Distance from the crown to the bone	---	Positive correlation, independent of the effect of marginal periodontitis between chronic AP and aortic atherosclerotic burden. ASB increased with: -age -N° of chronic AP without RCT but not with N° of CAP with RCT	Yes p <0.05 each	-It is not anticipated that the diagnosis of a tumor disease or an accident, which applied to most of the patients in the study, could have an effect on the status of the parameters studied No adjustment to smoking
Costa et al. 2014	- 61.9 -NS M 52 W51	103	Patients who underwent coronary angiography in the University Hospital Presidente Dutra, Federal University of Maranhoao, Brazil	-CAP Periapical Index score (PAI >2) -full-mouth set of PA radiographs.	CAD: coronary artery obstruction >50% in at least 1 coronary artery angiography (n=67)	2009 - 2010	Age ><60 Gender Race Education Marital status HT DM CAD Stroke Smoking Alcoholism Sedentarism BMI SBP DBP Waist and Hip circum-ference Fasting -glycemia	RR= 2.79 (1.1-7.3)	Chronic AP was independently associated with CAD.	Yes P=.012	-No adjustment for periodontitis

			examination at the Central Hospital Kristainstad by recognized specialists in Cardiology. -Matched for: age, gender smoking status, socio-economic factors -None of the subjects had received dental or periodontal treatment within the preceding 2 months to the AMI event								
Friedlander et al. 2009	65±10 >=40 M 35 W 1	72 36/36	-Cases: calcified atheroma. Patients from Veterans Administration (VA) dental clinic. Controls: no atheroma, attending the UCLA Medical Center's reduced-fee dental clinic. -California -No type1 DM No history of TIA/stroke. -Matched by: age, gender, N° of missing teeth & atherogenic risk factors (HT, medications to type II DM, Hyperlipidemia, BMI, economic status & smoking history)	PAL PAN (Mattila pantomography index (MPI)	CCA/ calcified atheroma PAN confirmed by by Doppler US	NS	NS	---	N° of teeth with PAL was non significantly higher among cases than controls: 1.5 +/-2.4 vs 0.8+/- 1.5	not-significant P=0.12	-Small group size. - Sample consists predominantly of males. .

			mia & no other severe diseases -Matched for: gender, age, ethnicity and smoking habits. -At least 5 residual teeth present.						Controls: greater number of PALs on teeth with ET (26.4% without versus 74.6% with ET.		
Segura-Egea JJ et al. 2010	56.8 ±11.1 Cases: 59.5±9.7 41-73 Controls: 56.4 ±9.9 40–74 M 43 W 48	40/51 91 40 – HT 51-control	Cases: - well-controlled HT - receiving treatment for HT. Controls: - No history of HT - did not receive treatment for HT . No difference in age or gender between study and control groups. Patients looking for routine dental care, University of Seville, Faculty of Dentistry	PAL ((PAI >2) full-mouth radiographic survey - of 14 PA radiographs	HT Self-report	NS	Age Gender N° of teeth N° of RCT	OR=1.94; (0.78–4.81)	AP>= 1 75% of HT patients 61% of controls % teeth with AP of all teeth: HT : 5.2% Control: 4.2% % teeth with AP of ET teeth: HT: 65% Control: 43%	No P = .15 No P > .05 No P > .05	-Self-report of HT No adjustment for periodontitis . And smoking
Cotti et al. 2011	20-40 Case: 35 ± 5 22–40 Controls: 27 ± 3 21–33 M 40	40 20/20	- cases and controls were age mached and: - No periodontitis - No arterial hypertension - No dyslipidemia - No DM - No obesity - No smoking history - Presence of at least 25 teeth	AP PAN radiograph followed by selected PA radiographs	Endothelial dysfunction by plasma markes: 1. ADMA 2. EFR	2009	N° of teeth N° of RCT	---	significant increase in ADMA in patients with AP compared to the controls (0.73 +/- 0.14 mmol/L vs 0.65 +/- 0.09 mmol/L) EFR values in the lower limit	Yes ADMA P<0.05 Yes EFR	EFR was used to represent initial CVD.

			-Cardiology Department of San Giovanni, Battista University Hospital, Turin, Italy							
Willershausen et al. 2014	62.3 51–83 cases: 62.3 ±10.1 Controls: 63.5 ± 10.5 Cases: M 201 F 47 Control: M 179 F 70	248/249 497	-Cases: recent AMI, followed by a diagnostic catheterization and reperfusion therapy such as percutaneous coronary intervention, bypass surgery or thrombolytic therapy at the Department of Cardiology and Angiology of the University Medical Center. Oral Assessment in the dental school, Mainz, Germany. -Controls: healthy patients from the Department of Operative Dentistry of the University Medical Center, Mainz, Germany. Good general health, no clinical signs of CVD, no hypercholesterolemia and no any other serious diseases. -Matched for: age, gender, number of residual teeth and smoking habits.	PALs (check within 5M after AMI) PDL space more than 2 mm wide + absence of an intact lamina dura -PAN -PA radiographs -in special cases CT PALs recorded before and after ET	AMI ECG and serum enzymes	2007-2011	Age Gender Periodontitis DMFT N° of teeth	OR= 1.54 (1.10–2.16)	PALs were found significantly more often in AMI patients vs controls Logistic regression showed that the probability of PALs >=1 was significantly higher in the AMI patient group. AMI group: 95/248 patients showed a total N° of 150 PALs. Control group: 59/249 patients had a total N° of 76 PALs.	Yes significant p = 0.001 Yes significant p = 0.012

			-At least 5 residual teeth present.							
Cotti et al. 2015b	31 ±5.71 20–40 Cases: M 20 W 21 Controls : M 20 W 20	81 41/40	- Similar demographic data (race, sex, and age distribution) - No periodontitis - No arterial HT - No dyslipidemia - No DM - No obesity - No smoking history - Presence of at least 25 teeth - No non-endodontic lesions in the maxillary bones - No previous and/or current CV or CVD - No chronic inflammatory conditions in other districts involving systemic health - For women, the use of anti-conceptive hormones. - University of Cagliari, Italy	AP PAN followed by selected PA radiographs	Initial injury to the CV system: Endothelial dysfunction Plasma levels of: 1. ADMA 2. EFR	NS	Gender N° of teeth N° of RCT	---	Patients with AP of both sexes showed a significant reduction in EFR ADMA levels were unchanged in women with AP, but they were significantly increased in men. No difference in the EFR values between male and female	Yes significant EFR P<0.05 Yes significant ADMA in men P<0.05	EFR was used to represent initial CVD.

Abberivation: ABL - alveolar bone loss, ADMA - asymmetrical dimethylarginine, AMI - acute myocardial infarction, ANP - angina pectoris, AP - apical periodontitis, ASB - Atherosclerotic burden , BMI - body mass index, CAD - coronary artery disease, CAP - chronic apical periodontitis, CC - Case control study, CCA - calcified carotid artery CHD - coronary heart disease, CIHF - chronic ischemic heart disease, CS - Cross sectional study, CT - computed tomography, CVD - cardiovascular disease CVE - cardiovascular event, CVI - cerebrovascular ischemia, DBP - diastolic blood pressure, DM diabetes mellitus, EFR - endothelial flow reserve, ET - endodontic treatment, HDL - high-density lipoprotein, HL - hyperlipidemia, HT - hyper tension, LDL - low-density lipoprotein, MPI - Mattila Pantomography Index, N° - number, NS - not specified, M- men, PAI –Periapical index score, RAL - radiolucent periapical lesions, RF - root filled teeth, SBP-systolic blood pressure; TC- total cholesterol, TG - triglycerides, PAN - panoramic radiograph, PD - periodontal disease , TIA - transient ischemic attack, RCT - root canal treatment, SD - standard deviation, W - women, WBC- white blood cells.

APPENDIX 2: LITERATURE REVIEW

QUINTESSENCE INTERNATIONAL
PERIODONTOLOGY

Yuli Berlin-Broner

Apical periodontitis and atherosclerosis: Is there a link? Review of the literature and potential mechanism of linkage

Yuli Berlin-Broner, DMD[1]/Maria Febbraio, PhD[2]*/Liran Levin, DMD[3]*

Atherosclerosis is a progressive narrowing of arteries that may lead to occlusion as a consequence of lipid deposition. It underlies coronary heart disease, as well as myocardial and cerebral infarctions. Recent attention has been directed towards the potential contribution of chronic inflammatory processes that may amplify vascular inflammation in atherosclerosis, as it is recognized as a chronic inflammatory disease. In this category are two of the most prevalent oral diseases: periodontal disease and apical periodontitis (AP). There is increasing epidemiologic evidence for a positive association between periodontal disease and cardiovascular disease (CVD) as well as between AP and CVD. A review of the literature, as well as a potential mechanism for the linkage between AP and atherosclerosis, are presented in this article. *(Quintessence Int 2017;48:527–534; doi: 10.3290/j.qi.a38162)*

Key words: blood pressure, bone loss, heart disease, plaque

Atherosclerosis is a progressive narrowing of arteries that may lead to occlusion as a consequence of lipid deposition. Atherosclerosis has a multifactorial etiology, and a large majority of cases can be traced to known risk factors. Increased awareness and knowledge of risk factors has played a large part in prevention and reduction of atherosclerosis prevalence and complications.

Recent attention has been directed towards the potential contribution of chronic inflammatory processes that may amplify vascular inflammation in atherosclerosis, as it is recognized as a chronic inflammatory disease. In this category are two of the most prevalent oral diseases: periodontal disease and apical periodontitis (AP). There is increasing epidemiologic evidence for a positive association between periodontal disease and cardiovascular disease (CVD) as well as between AP and CVD. Although a causal effect was demonstrated between periodontal disease and atherosclerosis in mice, no studies evaluating the potential causal relationship between AP and atherosclerosis have been reported. Given the similarity between AP and periodontal disease and the increasing amount of evidence that AP might not be locally limited, research in this area is essential to our understanding of atherosclerosis disease risk. In this article, periodontal disease and AP, and the relationship between each of them and CVD, is discussed, and potential mechanism for the linkage between AP and atherosclerosis is presented.

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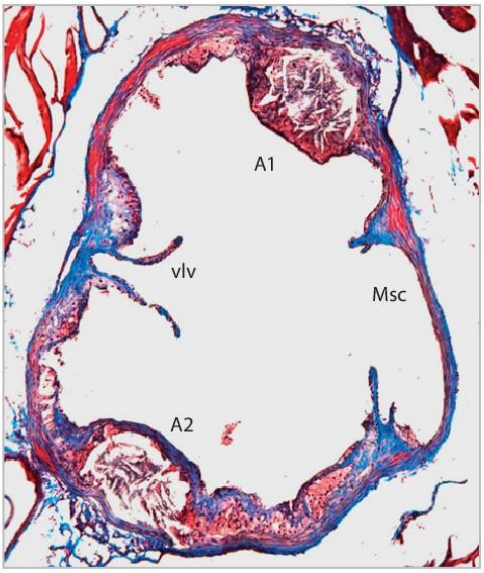


Fig 1 Atherosclerotic lesions in a mouse aortic sinus. Mouse aortic sinus section cut at the level of the valve leaflets (vlv), stained with Trichrome stain: red, cytoplasm; blue, collagen; purple, nuclei. Msc, normal muscle wall. A1 and A2, atherosclerotic lesions. The atherosclerotic lesions cause narrowing of the arterial space.

ATHEROSCLEROSIS

Cardiovascular disease (CVD) and cerebrovascular disease (stroke, transient ischemic attack) share common mechanisms and often result from atherosclerosis.[1] Atherosclerosis can lead to coronary heart disease, as well as myocardial and cerebral infarctions.[2] Atherosclerosis is a progressive narrowing of arteries that can lead to occlusion due to lipid deposition. According to the World Health Organization, in 2012, CVD accounted for 3 in every 10 deaths despite the prevalence and success of lipid-lowering drugs.[3,4]

Atherosclerotic lesions, or atheromata, are asymmetric focal thickenings of the innermost layer of the artery, which is known as the intima (Fig 1).[5] The intima is in contact with blood and is lined by endothelial cells. Atheromata begin when lipids from high fat diets activate endothelial cells. Increased blood lipids leads to changes in sheer stress, and this is perceived by mechano-sensors on endothelial cells as a danger signal.[6] These blood lipids also accumulate in the space beneath the endothelial cells, known as the sub-intima. Endothelial cells respond to these abnormalities by expressing chemoattractants for monocytes, which are the body's immune surveillance cells.[7] The endothelial cell surface also changes, and expresses receptors, such as a vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), to guide monocyte migration and extravasation to the site of lipid accumulation in the sub-intima.[7] As a result of these signals, monocytes mature into macrophages, which can take up and degrade the lipid. However, this process can be overwhelmed by chronic high fat, leading to entrapment of macrophages within the sub-intima.

The initial lesion of atherosclerosis is known as the fatty streak and consists almost entirely of lipid-engorged macrophages. As the disease progresses, these lesions grow, attract other immune cells and smooth muscle cells, which encapsulate the lesion. Cells die as a result of lipotoxicity, and lipid, cholesterol, and cellular debris accumulate, along with connective tissue elements, as the body attempts to heal this wound.[8] Atherosclerosis plaques may be characterized as stable or unstable, depending upon the strength and thickness of the smooth muscle cell cap.[9] Unstable plaque can rupture, spilling contents that promote blood clotting and occlusion of the vessel.[9] This may result in myocardial infarction or stroke and potential fatal consequences. Atherosclerosis is recognized as a chronic inflammatory disease in that metabolic risk factors, such as hypercholesterolemia and hypertriglyceridemia, lead to an activated endothelium which triggers an immune response, and this initiates, propagates, and activates lesions in the arterial tree.[4,5]

Atherosclerosis has a multifactorial etiology (Fig 2). Major risk factors include unhealthy blood cholesterol and/or triglyceride levels, high blood pressure, smoking, insulin resistance, diabetes, overweight or obesity, lack of physical activity, unhealthy diet, age, gender (male), and family history of early heart disease.[10] High plasma levels of the acute phase response protein,

C-reactive protein (CRP), is a sign of chronic vascular inflammation and correlates with atherosclerosis disease risk.[10] Emerging risk factors include heavy alcohol consumption and sleep apnea.[10-12] Some risk factors are fixed, including family history, age, and gender, but others can be modified by medication (such as controlling blood pressure or cholesterol levels) or by behavioral changes (such as diet and physical activity). Increased knowledge of potential risk factors allows for future prevention and reduction of atherosclerosis.

It is important to note that a substantial proportion of atherosclerotic CVD events occur in individuals without hyperlipidemia.[4] This has turned the attention to other pathophysiologic drivers of atherosclerosis. Because inflammation is involved in all stages of atherosclerosis, interventions modulating systemic or local inflammatory responses have become attractive means to alter CVD risk.[4] Recently, more attention has been directed towards the identification of potential contributors to chronic inflammatory processes that may amplify vascular inflammation in atherosclerosis. In this category are two of the most prevalent oral diseases: periodontal disease (periodontitis) and AP.

CHRONIC ORAL INFLAMMATORY DISEASES

Periodontal disease and AP share a common bacterial etiology. Bacteria that are normally found in the oral cavity create a pathologic micro-environment in the periodontal pocket or root canal that leads to a continuous host inflammatory reaction. The host tries to eliminate the bacteria, but without success, due to continued bacterial flow (from the oral cavity) and the inability of the immune system to access these two niches. The two diseases are mostly painless and create minimal discomfort to patients, and as a result they may persist unnoticed for years.[13] Periodontal disease and AP can be eliminated by appropriate therapy, which involves removal and prevention of resettlement of the microbial factor.

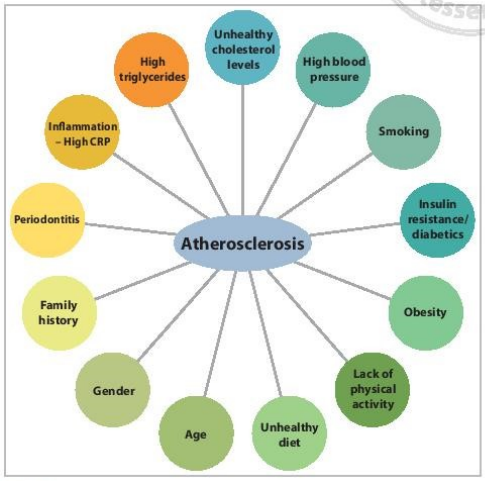


Fig 2 Atherosclerosis risk factors. Atherosclerosis is a chronic inflammatory condition with a multifactorial etiology.

Periodontal disease

Periodontal disease is inflammation of the tissues surrounding the tooth, including the periodontal ligament, cementum, and alveolar bone. It develops as a response to (mostly) gram-negative, anaerobic bacteria, which originate in the oral cavity and accumulate on the tooth surface as dental plaque.[2,14] The host mounts an inflammatory response to the bacteria in plaque. Whether acute or chronic, the inflammation can eventually lead to loss of bone supporting the tooth structures. Severe periodontal disease is found in 15% to 20% of middle-aged (35 to 44 years) adults. More than 70% of adults 65 and older have moderate to severe periodontal disease.[15]

The association between periodontal disease and CVD has been widely investigated and a positive association has been reported in a systematic review and meta-analysis of observational studies.[16] The meta-analysis concluded that periodontal disease is associated with a 19% increase in the risk of future coronary heart disease.[16] Furthermore, the increase in relative risk was more prominent (44%) in persons aged 65 years and

older.[16] A cross-sectional human study found that levels of triglycerides were higher in periodontitis patients compared to subjects without periodontitis (178 mg/dL vs 165 mg/dL; $P < .05$), and levels of high-density lipoproteins were lower (44 mg/dL vs 50 mg/dL; $P < .05$).[17] This lipid profile is consistent with greater potential for CVD. Several basic science studies have shown a causative association between atherosclerosis and periodontal disease.[18,19] A recent study, using a genetically engineered atherosclerosis mouse model, identified CD36 as essential to host inflammatory signaling in response to the gram-negative bacteria, *Porphyromonas gingivalis*, which is a known perio-pathogen (and also found in infected root canals in AP) and suggested CD36-dependent interleukin 1 beta (IL-1β) generation as a link between periodontal disease and CVD.[19]

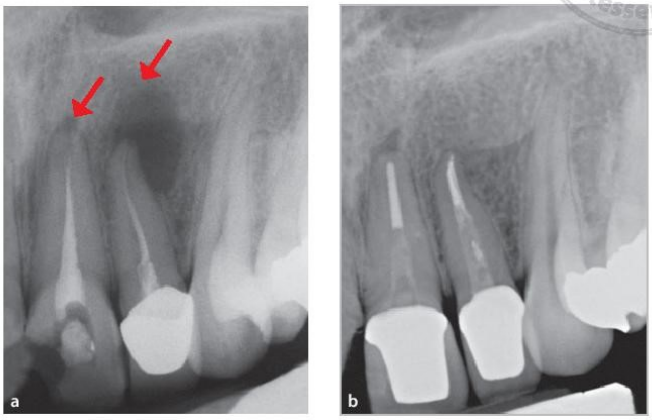
Apical periodontits (AP)

The term "apical periodontitis" describes inflammation in the alveolar bone, adjacent to the apical opening of an infected root canal, in reaction to infection harbored in the root canal system. This infection, similar to periodontal disease, consists primarily of gram-negative anaerobic bacteria. AP may be detected radiographically as a radiolucent lesion in the surrounding alveolar bone adjacent to the apical part of the infected tooth, and is then referred to as a periapical lesion (PAL) (Fig 3). PALs are highly prevalent, occurring in 34% to 61% of patients.[20-22] Five percent of all teeth without root canal treatment (RCT) and 25% with RCT present with PALs.[23] These numbers are likely an underestimation, due to the limited sensitivity of radiographs. Because PALs are usually not painful, they may go undetected and remain chronic and untreated for years.[24] In a recent publication, the long-term (at least 4 years) dynamics of PALs were evaluated and described.[25] In this study, 51% of the PALs worsened, 28.5% of PALs remained unchanged, and 20% improved over time. The authors concluded that further endodontic treatment was indicated in non-healed cases, those with poor root fillings or poor coronal restorations, because of the likelihood for the lesions to worsen.[25] The treatment of choice, tooth retention or extraction, should be evidence-based, taking into account the various factors and related treatment outcomes, as well as some perceptions based on clinical experience.[26]

Most radiographically detected PALs (97.2%) represented lesions of endodontic origin and included periapical granulomas (60%), radicular cysts (36.7%), periapical fibrous scars (0.27%) and periapical abscesses (0.23%).[13] High-quality endodontic treatment or retreatment led to PAL resolution in 80% of cases.[27] A small subset (2.8%) of PALs represent a group of lesions of non-endodontic origin.[13] These lesions require a different and more radical treatment, including surgical intervention, due to their aggressive behavior, tendency to recur, and risk of malignancy; they should not be left untreated. Therefore, in cases when there is no resolution/healing despite intervention, a biopsy is required for further diagnosis and reevaluation. The potential histopathologic diagnoses of these lesions include odontogenic keratosis, benign fibro-osseous lesions, ameloblastoma, Langerhans cell disease, and central giant cell granuloma or malignancy.[13] In addition to the fact that long-standing, unhealed PALs raise suspicion for one of these different diagnoses, the chronic inflammatory process may, like periodontal disease, have systemic influence.

EPIDEMIOLOGIC EVIDENCE FOR LINKAGE BETWEEN AP AND CVD

New studies continue to emerge regarding the epidemiologic evidence for the association between AP and CVD, and this area of research draws interest within the dental research community.[28-31] A recent systematic review identified 19 epidemiologic studies in humans (4 cohort, 5 cross-sectional, and 10 case-control) that examined the association between radiographically detected AP and CVD.[28] Of these, 13 showed a significant positive association between AP and CVD, although two lost significance after multivariable analysis. It was concluded that evidence exists for a positive association between AP and CVD; however, the level was moderate to low, and no causal relationship could be established.[28] Since that analysis, new epidemiologic evi-



Figs 3a and 3b Radiolucent periapical lesions (PALs)/apical periodontitis (AP). *(a)* Radiolucent PALs (red arrows) associated maxillary left incisors, poor endodontic treatments, and poor restorations. *(b)* One year following endodontic retreatment and new restorations in the maxillary left incisors; maxillary left lateral incisor with a healed PAL; maxillary left central incisor with an unchanged PAL.

dence supporting the positive association between AP and CVD has emerged, including a study, with 364 participants, that found that the likelihood of subjects with radiographically detected AP to also have CVD was 5.3-fold higher compared with subjects without AP.[29]

Evaluation of the influence of AP as a separate risk factor for CVD is challenging for several reasons. First, many factors contribute to the risk of atherosclerosis, as it is known to have a multifactorial etiology (Fig 2).[10] These factors can be addressed during study design and analysis as potential confounders, but based on previous publications, these factors are not always easily controlled.[28] Another challenge is the method used in the studies to measure outcome (CVD) and level of exposure (AP). For example, different radiographic methods are used to evaluate for the presence of AP, including periapical radiographs, panoramic radiographs, cone beam computed tomography (CBCT), and whole body CT.[28] The variation in method is significant because the type of radiograph influences the ability to detect a PAL. The specificity and sensitivity for detection of a PAL varies among panoramic radiographs, periapical radiographs, and CBCT images: sensitivity 0.28, 0.55, 0.91 to 0.98, respectively; and specificity 1, 0.98, and 0.73, respectively.[32,33] This means that when a panoramic radiograph is used for screening only 28% of PALs would be detected. Some studies have used patients' self-report of having a RCT in the past as an indicator of AP, without a complementary radiographic evaluation.[34-36] This method would be considered the least reliable. For these reasons, studying the direct association between CVD and AP is challenging in an experimental setting that involves humans, and a causal relationship between the two has not yet been established.

Possible underlying mechanism of the association between AP and CVD

Although periapical infections cause a number of local tissue responses that limit the spread of infectious elements, AP may not be an exclusively local phenomenon.[37] Results from a meta-analysis by Gomes et al[38] show that the presence of AP is associated with a systemic increase in inflammatory markers including C-reactive protein (CRP), IL-1, IL-2, IL-6, asymmetric dimethylarginine (ADMA), and immunoglobulins (IgA, IgG, and IgM) in humans.[38] Overall those findings suggest that AP contributes to a systemic immune response that may mediate accelerated atherosclerosis.[38] The potential role of these inflammatory mediators is as follows:

- CRP, which is increased in patients with AP,[38] is a marker associated with atherosclerotic cardiovascular disease, independent of traditional risk factors for CVD.[4,39]
- It is known that endothelial cells can undergo a dramatic modulation in their functional phenotype in response to certain bacterial products, such as gram-negative endotoxins, and other pathogen-associated molecular patterns (PAMPs) or cytokines, such as IL-1, tumor necrosis factor (TNF), and interferon-gamma (INF-γ).[40,41]
- ADMA, an endogenous inhibitor of the endothelial enzyme nitric oxide (NO) synthase, is a cardiovascular risk marker.[42] Low levels of NO are associated with endothelial dysfunction.
- Endotoxin or lipopolysaccharide (LPS) is an important gram-negative bacteria virulence factor. After its release from bacteria, LPS initially binds to a plasma protein called LPS-binding protein and then is delivered to the cell receptor for LPS, CD14, which is expressed primarily on the surface of macrophages.[43] In most situations, CD14 facilitates signaling by the innate immune receptor, Toll-like receptor 4 (TLR4), leading to the host response, which includes activation of the transcription factor nuclear factor kappa B (NF-κB) and consequent expression of pro-inflammatory cytokines and chemokines.[43]

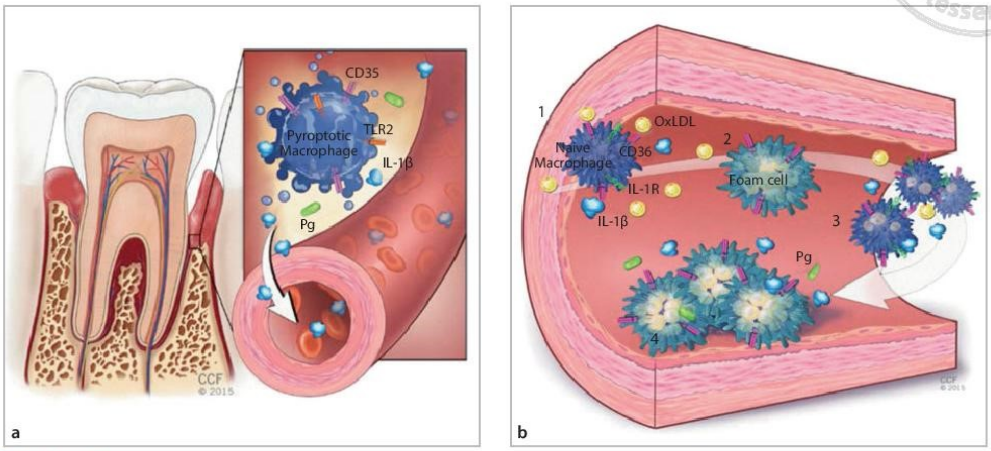
Interestingly, LPS from a major causative pathogen in periodontal disease and AP, the anaerobic gram-negative bacteria *P gingivalis*, has been found to activate macrophages through an alternative TLR, TLR2.[44] Bacterial activation of this inflammatory cascade may underlie the association between periodontitis (and potentially AP) with CVD; locally released cytokines may gain access to the systemic circulation, inducing or perpetuating an elevated chronic systemic inflammatory status.[45] Indeed, a recent study, using a mouse model of *P gingivalis*-induced periodontal disease found evidence for a systemic pro-inflammatory effect, through increasing overall oxidative stress and levels of IL-1β.[19] This study implicated the macrophage receptor CD36, as essential to *P gingivalis*-induced TLR2 signaling and cytokine production, which provides a new target for therapeutic strategies (Fig 4).[19]

Since the etiology and the pathophysiology of periodontal disease and AP are similar, there may also be similar underlying mechanisms for systemic effects from these chronic oral infections. This area remains to be further investigated. A summary of the suggested mechanism of association between AP and atherosclerosis, based on the studies uncovered, is presented in Fig 5. Briefly, it is known that AP is a local inflammatory reaction, but systemic influence has been demonstrated.[38] Chronic inflammatory mediators may be carried via the circulation to various areas in the body. In those areas with a pre-existing atherosclerotic lesion, this could enhance the immune reaction at that site. This may potentially result in acceleration of the pre-existing atherosclerosis process.

CONCLUSION

Although there is epidemiologic evidence for an association between AP and CVD, a recent systematic review concluded that the level of evidence was moderate to low.[28] Atherosclerosis has a multifactorial etiology and AP may potentially contribute to atherosclerotic lesion development in certain subjects, similar to periodontal disease. Since the etiology and the pathophysiology of periodontal disease and AP are similar, it is reasonable to hypothesize that similar underlying mechanisms could be responsible for a link between AP and CVD, but this remains an area that needs further study. The question of causality also remains unanswered.

In addition to these questions, there are also other important issues that need to be considered: is there a critical periapical lesion number/size that determines systemic influence? Is the length of time with an AP important? Can AP lead to CVD in the absence of hyperlipidemia or other more traditional risk factors of CVD? Given the number of patients with AP and the potential impact, further longitudinal study is warranted, with better control of confounders, and investigation of mechanisms of linkage. Mechanistic understanding of how a chronic long-term inflammatory



Figs 4a and 4b Inflammation: From the tooth to the arteries. (a) Activation of macrophages by periodontal disease bacteria (*Porphyromonas gingivalis*, Pg) in the oral cavity is mediated by CD36 and TLR2 and leads to systemic release of pro-atherosclerotic IL1β. *(b)* Systemic IL1β activates naive (to Pg) vascular macrophages to secrete IL1β (1), and promotes CD36-mediated uptake of lipid (2) and enhanced atherosclerosis (3). Although it is controversial as to whether Pg/PgLPS is found in the vasculature, the presence of lipid would promote the development of greater atherosclerotic plaque (4). Reproduced with permission from Brown et al.[19]

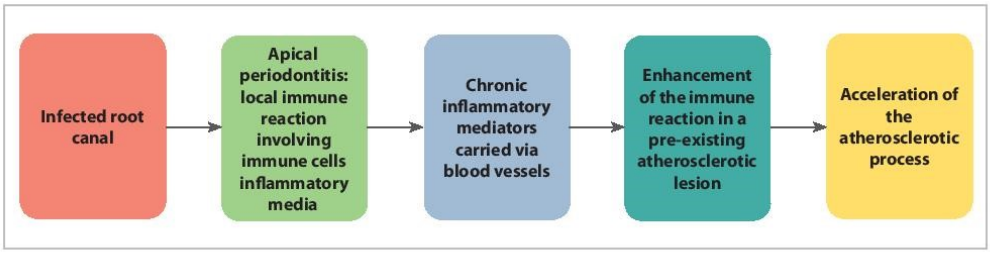


Fig 5 Suggested potential biologic mechanism for linkage between AP and atherosclerosis.

condition, such as AP, increases risk of atherosclerosis may be applicable to other conditions, including rheumatoid arthritis, inflammatory bowel disease, chronic kidney disease, etc. Overall, there is a need for more research to fill the gaps in our knowledge.

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