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Characterization of a mouse model to study the relationship between apical periodontitis and atherosclerosis

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Abstract

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Aim First, to determine the feasibility of using the low-density lipoprotein receptor knockout (LDLR KO) mouse model to study apical periodontitis (AP). Secondly, to investigate the causal relationship between AP and atherosclerosis. It was hypothesized that it would be feasible to induce AP and atherosclerosis in LDLR KO mice and find a difference in atherosclerosis between AP and Sham groups.

Methodology Using a published methodology, AP was induced in LDLR KO mice by exposing the dental pulp of the four first molars (Tx). Shams received only anaesthesia. Mice were fed a high fat, Western-type diet (WTD), to induce atherosclerosis. At 16 weeks, mice were euthanized and aortas collected to measure atherosclerosis lesion burden (oil red O staining). Periapical lesions were validated using micro-CT and histology. Systemic inflammation was measured using a cytokine array.

Results Both groups developed a similar degree of atherosclerosis (mean lesion area 7.46 ± 0.44% in the Tx group compared with 7.65 ± 0.46%, in the Sham group, $P = 0.77$), and a similar degree of inflammation. Periapical lesions (PALs) in all four molars were only identified in a small subset of Tx mice.

Conclusions A novel mouse model, which combines AP and CVD, was created. This model allows investigation of the relationship between the two diseases, whilst avoiding other potential common confounders. Although no difference in the degree of atherosclerosis was found between the groups, more studies in which the number of periapical lesions, changes in systemic inflammation and the degree of atherosclerosis are correlated are necessary to ultimately determine the impact of AP on CVD.

Keywords: apical periodontitis, atherosclerosis, inflammation.

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Introduction

The relationship between oral and systemic health has been extensively researched and reviewed in the scientific literature. A subset of these addressed, specifically, the relationship between apical periodontitis (AP) and various factors related to cardiovascular disease (CVD) (reviewed in Berlin-Broner *et al.* 2017). These include associations between AP and hypertension (Segura-Egea *et al.* 2010), coronary heart disease (Hujoel *et al.* 2001, Caplan *et al.* 2006, Pasqualini *et al.* 2012, Costa *et al.* 2014, Liljestrand *et al.* 2016), acute CVD events/CVD mortality (Mattila *et al.* 1989, Jansson *et al.* 2001, Rutger Persson *et al.* 2003, Willershausen *et al.* 2014, Gomes *et al.* 2016) and atherosclerosis (Friedlander *et al.* 2010, Glodny *et al.* 2013, Petersen *et al.* 2014). Most of these studies were epidemiological, whilst a few correlated measures of inflammation with AP and

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CVD. To broadly summarize, most of the published studies listed did find a positive association between AP and CVD, although the quality of the existing evidence was moderate to low. New studies have emerged since publication of the systematic review (Berlin-Broner *et al.* 2017), also supporting an association between CVD and AP (An *et al.* 2016, Garrido *et al.* 2019, Messing *et al.* 2019).

Cardiovascular disease is the number one cause of death throughout the world [http://www.who.int], and an underlying cause is atherosclerosis, defined as a progressive inflammatory accumulation of lipid (plaque) involving large to medium-sized arteries (Hansson 2005). Atherosclerosis can lead to heart attack and stroke [http://www.nhlbi.nih.gov]. Metabolic risk factors, such as hypercholesterolaemia and hypertriglyceridemia, result in a pro-inflammatory endothelium, which triggers an immune response, and this initiates, propagates and activates atherosclerotic lesions in the arterial tree (Hansson 2005, Shapiro & Fazio 2016). Multiple risk factors contribute to atherosclerosis, including age, sex, sedentary lifestyle, high blood pressure and smoking (Hansson 2005).

Recently, increased CVD morbidity has been linked to other inflammatory diseases such as rheumatic diseases, obesity and diabetes; these may contribute to additive or synergistic systemic inflammation/oxidative stress (Castaneda *et al.* 2016). In the mouth, a degree of inflammation is almost always present in the form of the two most common oral diseases, which share bacterial aetiology, periodontal disease and apical periodontitis (AP).

The prevalence of AP, an 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). This is likely an underestimation due to the limited sensitivity of two-dimensional radiographs (Lennon *et al.* 2011, Petersson *et al.* 2012). Frequently, AP is detected only in later stages, when the size/location of the lesion is significant (Green *et al.* 1997, Petersson *et al.* 2012). Since AP is usually asymptomatic, patients may be unaware they are affected (Yu *et al.* 2012). Although the association between CVD and periodontal disease has been known for more than 20 years (Beck *et al.* 1996), oral inflammation, which includes AP, may be overlooked as a potential risk factor during clinical assessment of CVD, despite the fact that it is very common in the age group of patients prone to atherosclerosis (Dugas *et al.* 2003).

In spite of the numerous epidemiological studies suggesting a link between AP and CVD, causality has yet to be demonstrated (Berlin-Broner *et al.* 2017). Performing a longitudinal study in humans to demonstrate causality is challenging due to the complexity of the systemic conditions influencing inflammatory status and the difficulty in controlling all potential confounders along with AP. The absence of animal studies 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 knowledge regarding the causality of the relationship between AP and atherosclerosis, and the mechanism(s) by which they may be linked, and an animal model is essential to study the role of AP as a separate risk factor.

Like AP, epidemiological and experimental research support an association between periodontal disease and CVD, but the mechanism(s) underlying this association are not fully understood (Schillinger 2006, Bahekar *et al.* 2007, Lockhart *et al.* 2012, Dietrich *et al.* 2013, Schenkein & Loos 2013). Several studies, using genetically engineered mouse models of atherosclerosis, have given support to a causal contribution of periodontal disease to the development of atherosclerosis (Li *et al.* 2002, Lalla *et al.* 2003, Brown *et al.* 2015, Velsko *et al.* 2015, Yang *et al.* 2017). Thus, the aim was to investigate the potential causative role of AP in atherosclerosis by using the classic technique of Kakehashi *et al.* (1965) to generate periapical lesions in a mouse model of atherosclerosis. Since every mouse is of the same genetic background and will be treated exactly in the same manner, except in terms of AP induction, there are few confounders that would exist in a human study setting, including periodontal disease, medications and other systemic conditions that will be avoided. No studies to date have addressed the relationship between AP and atherosclerosis in a single animal model. Thus, the combined use of a technique to generate AP and a genetically engineered atherosclerosis mouse model represents a novel paradigm to study a potential causal role for AP in atherosclerosis.

The overall goal of this study was twofold: first, to determine the feasibility of using the low-density lipoprotein receptor knockout (LDLR KO) mouse, a classic and recognized model in the field for reliably mirroring aspects of human atherosclerotic disease (Zadelaar *et al.* 2007), to study AP. Secondly, to investigate the causal relationship between AP and atherosclerosis. It was hypothesized that it would be

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feasible to utilize the LDLR KO mouse model to study the relationship between AP and atherosclerosis and that mice with experimentally induced AP would have an increased atherosclerosis lesion burden, with increased levels of systemic inflammation as a contributing factor, compared with Sham controls.

Materials and methods

Mice, surgery and diet

The Animal Care and Use Committee of the University of Alberta approved all animal procedures prior to study initiation (Protocol #00001782). Male LDLR KO mice (B6.129S7-Ldlr$^{tm1Her/J}$, strain #002207, Jackson Laboratory, Bar Harbor, ME, USA), age 12–14 weeks were used. Mice were housed in a specific pathogen-free environment with *ad libitum* access to food and water. The number of mice in each group was based on a power calculation from a previous periodontal disease study. In that study, to achieve a P value < 0.05 with 90% power, the sample size was 11 mice per group. Based on the length of the study and the degree of dermatitis experienced in the current facility, the number of mice was increased; 17 in the treatment group (Tx) and 22 in the Sham group completed the 16-week regimen.

At time 0, all mice in the treatment (Tx) and Sham groups were anaesthetized by intraperitoneal (IP) injection of ketamine (80 mg kg^{-1}) and xylazine (12 mg kg^{-1}). The four first molars were accessed to expose the pulp in the Tx group, as described in Wang & Stashenko (1991). No teeth were prepared in the Sham group. Pain was minimized by subcutaneous Buprenorphine-HCl (0.03 mg kg^{-1}) injection in the Tx group; Sham mice also received this injection. Immediately after the surgery/sham procedure, all mice had their normal chow diet replaced with a Western-type diet (WTD), Teklad Diet #88317 (Envigo, Indianapolis, IN, USA), for 16 weeks to induce atherosclerosis. Weight was recorded regularly during the study and was used as a surrogate sign of distress, as all the mice in the Tx group were expected to eat, gain weight and behave in a similar manner to mice in the Sham group.

Blood and macrophage collection

Blood (up to 100 μL) was collected at study midpoint (8 weeks) by tail bleeding into tubes containing dipotassium ethylenediaminetetraacetic acid (K_2EDTA, 4 mM L^{-1} final concentration). Four days prior to euthanization, mice were injected with 2 mL of 4% thioglycolate (IP). Mice were euthanized (euthanyl, 200 mg kg^{-1} IP, Bimeda-MTC, Cambridge, ON, Canada) at 16 weeks. Macrophages were collected by peritoneal lavage into sterile phosphate-buffered saline (PBS) and blood (up to 1 mL) collected by cardiac puncture, again into EDTA containing tubes. Collected blood (8 and 16 weeks) and macrophages were immediately centrifuged (5 min, RT at 1100 ***g***). Macrophage pellets and plasma were stored at −20 °C for further analyses.

Plasma and macrophage lysate assays

Plasma cholesterol levels were measured in 16-week plasmas using the Total Cholesterol Assay Kit (colorimetric, Cell Biolabs, Inc., San Diego, CA, USA). Relative levels of 40 cytokines were measured by using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems, Minneapolis, MN, USA), in 8- and 16-week plasmas and in 16-week peritoneal macrophage lysates (four pooled samples from each Tx/Sham group at each time-point). For normalization of protein content in the macrophage lysates, protein concentration was determined using the Pierce^{TM} Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Aorta dissection and atherosclerosis lesion morphometry

Following blood collection, mice were perfused with PBS followed by 10% formaldehyde. The entire aorta was dissected free of fat and stained with oil red O. Aortas were laid open and digitally scanned (CanoScan LiDE 210; Canon, Melville, NY, USA) as described by Febbraio *et al.* (2000) and Brown *et al.* (2015). Atherosclerosis lesion burden was determined in a blinded manner using Adobe Photoshop software (Adobe Inc., San Jose, CA, USA). Atherosclerosis lesion (oil red O positive) was expressed as a percentage of the total aorta.

Micro-Computerized tomography (micro-CT) analysis

Three-dimensional (3D) micro-CT scans of mouse heads were taken at 25 μm, 360°, 75 MSec, 50 kV and 0.24 mA (Milabs U-CT, Utrecht, Netherlands). Scans were reconstructed in MiLabs Software and

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analysed with Amira software (ThermoFisher Scientific, Ottawa, ON, Canada). Any periradicular radiolucency wider than the size of the width of the periodontal ligament (PDL) was recorded.

Histological validation of AP

Following micro-CT scan, maxilla and mandible were decalcified (0.5 mol L^{-1} Na_2EDTA, pH 7.4) and processed for histology. Tissues were dehydrated (Leica TP1020, Leica Biosystems, Concord, ON, Canada) and embedded in Histoplast paraffin (Fisher Scientific, Loughborough, UK) using an embedding centre (Leica EG1160). Microscopic examination ensured optimal and consistent orientation of the specimens (Carl Zeiss F-125, Oberkochen, Germany). Samples were sectioned at 10 μm thickness and stained with haematoxylin and eosin (H&E) using the HistoCore of the Alberta Diabetes Institute (Edmonton, AB, Canada).

Histological examination was performed using a Leica DMRE microscope (16–1000× magnification) (Leica Microsystems). Any inflammatory infiltrate in the periradicular area and increase in the PDL space were recorded as a periradicular lesion: periapical lesion (PAL) or furcation lesion (FL), depending on location.

Statistical analysis

Tx and Sham groups were compared. Tx mice were further sub-grouped by the number of teeth with PALs per mouse, by the number of teeth with FLs per mouse and by the combination of the number of PALs and FLs per mouse (PALs + FLs = 'lesion load' score). 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 nonpaired, two-tailed *t*-test or ANOVA. If normality was not met, then a nonparametric, Mann–Whitney or Kruskal–Wallis test was employed. Statistical significance was set at $P < 0.05$. GraphPad Prism software (San Diego, CA, USA) was used for statistical analysis. Results are presented as means ± SE.

Results

Several criteria were used to determine whether the LDLR KO model was acceptable for studying AP and atherosclerosis. First, by verifying that PALs could be successfully induced (using micro-CT and histology, as detailed below) and secondly, by monitoring the animals to determine if they could reach the relatively long-term end-point of the study (16 weeks) in order to compare atherosclerosis.

Mice in both groups gained weight similarly during the experimental period (mean weight gain percentage in Tx: 22.16 ± 3.06%, Sham: 22.58 ± 2.39%, $P = 0.9139$). The final absolute weights were also similar (Tx: 35.59 ± 1.2 g, Sham: 34.14 ± 1.15 g, $P = 0.3269$). There was no difference in plasma total cholesterol levels (Tx: 1007 ± 74.57 mg dL^{-1}, Sham: 996.9 ± 46.17 mg dL^{-1}, $P = 0.9014$).

Pulps were exposed in 92% of molars, as verified by micro-CT scans and matching histological sections. Of those, PALs were identified in 71% and FLs in 76%. A representative micro-CT image and matching histological section are shown in Fig. 1. The variability in number of PALs and FLs amongst mice is summarized in Table 1.

Atherosclerotic lesion burden was not different between Tx and Sham groups (Tx: 7.46 ± 0.44%, Sham: 7.65 ± 0.46%, $P = 0.7766$; Fig. 2a,b). Tx mice were sub-grouped by the number of teeth with PALs per mouse, by the number of teeth with FLs per mouse and by 'lesion load' score, which is the combination of the number of PALs and FLs per mouse. No significant differences in atherosclerotic lesion burden were found neither between any of the Tx sub-groups nor between any of the sub-groups and Sham group (Fig. 2c–e).

The fold changes of plasma cytokines after 8 and 16 weeks, and in peritoneal macrophage lysates after 16 weeks, between the Tx and Sham groups are summarized in Figs 3 and 4.

Discussion

In this study, a novel mouse model was created that combines oral inflammation and CVD. This model allows investigation of the relationship between periradicular inflammation, as would occur in AP, and atherosclerosis, whilst avoiding potential common confounders that would exist in a human population, including periodontal disease, medications and other systemic conditions.

Whilst atherosclerosis takes decades to develop in humans, WTD-fed LDLR KO mice develop significant atherosclerosis throughout the aortic tree at 16 weeks (Ishibashi *et al.* 1993). This mouse model is extensively used in the CVD field, as it mirrors certain aspects of human atherosclerosis development,

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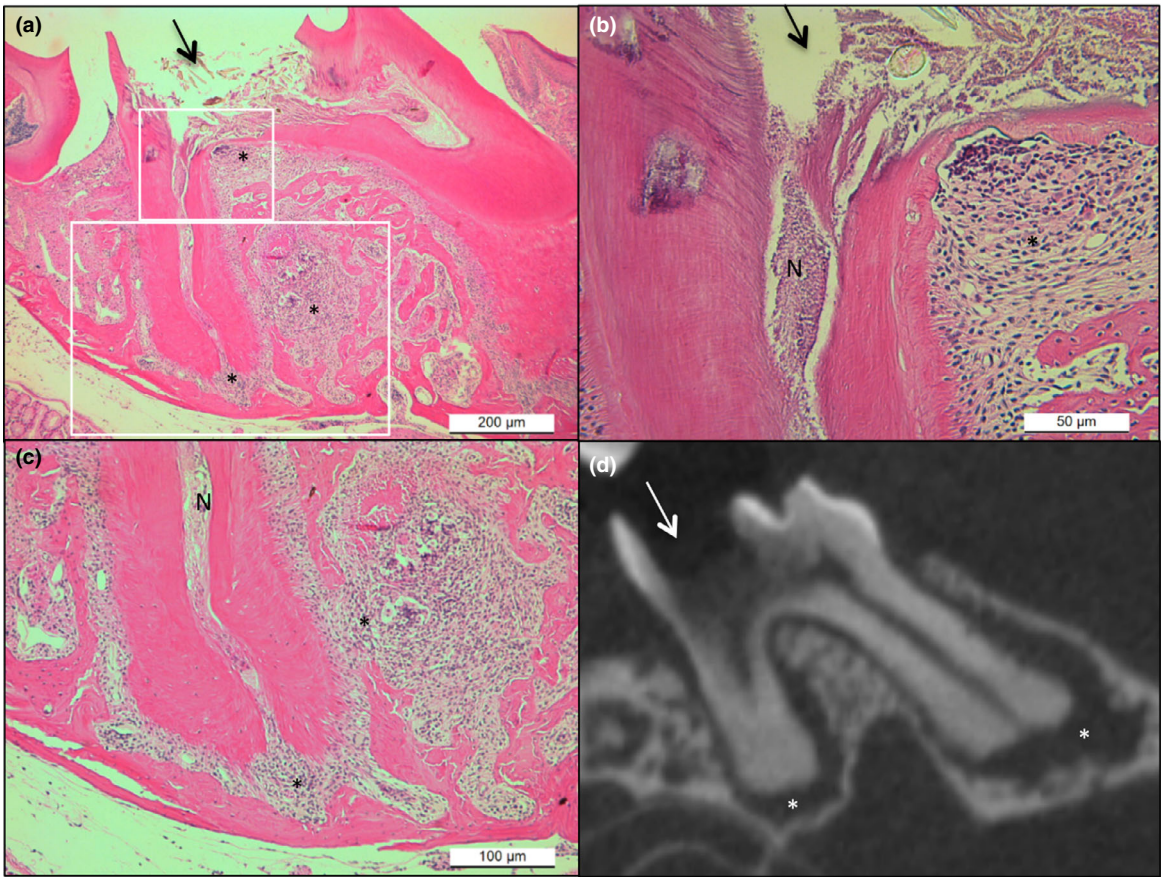

Figure 1 (a-d) Sagittal section through a left maxillary first molar, Tx group. Haematoxylin–eosin stain (a,b) and micro-CT section (c). Pulp exposure/drill site with debris (arrow); necrotic pulp tissue (N) and debris are seen in the pulp chamber and canal. Inflammatory responses in the periradicular (apical and furcation) are evident (*) (c). The width of the PDL is not uniform around the roots and enlarged demonstrating radiolucent periradicular lesions (d).

Table 1 Variability in number of periradicular lesions

Lesions	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)
FLs	27.7% (5/17)	27.7% (5/17)	27.7% (5/17)	11.8% (2/17)

Data presented as percentages of mice with periapical lesions (PALs) and furcation lesions (FLs): amongst Tx cohort (n = 17) as verified by micro-CT scans and matching histological sections 16 weeks after pulp exposure.

including diet-induced hyperlipidemia, hypercholesterolaemia, dyslipidemia, lesion location and morphology (Zadelaar *et al.* 2007).

Animal models used in the investigation of atherosclerosis have included rabbits, pigs and non-human primates, each of which has its advantages and disadvantages (Getz & Reardon 2015). Mice, which are relatively inexpensive to maintain, are generally resistant to atherosclerosis, but genetically engineered models were created in the 1990s to take advantage of the relative ease to experimentally study and manipulate. The two most common models used in atherosclerosis studies are the apolipoprotein E knockout (apoE KO) and LDLR KO (Getz & Reardon 2016). Both of these models are characterized by hypercholesterolaemia, which is a prerequisite for the development of atherosclerosis in murine models, but there are some differences. The apoE KO will develop plaque when fed either a standard low-fat chow diet or a high fat/cholesterol diet, whilst the LDLR KO

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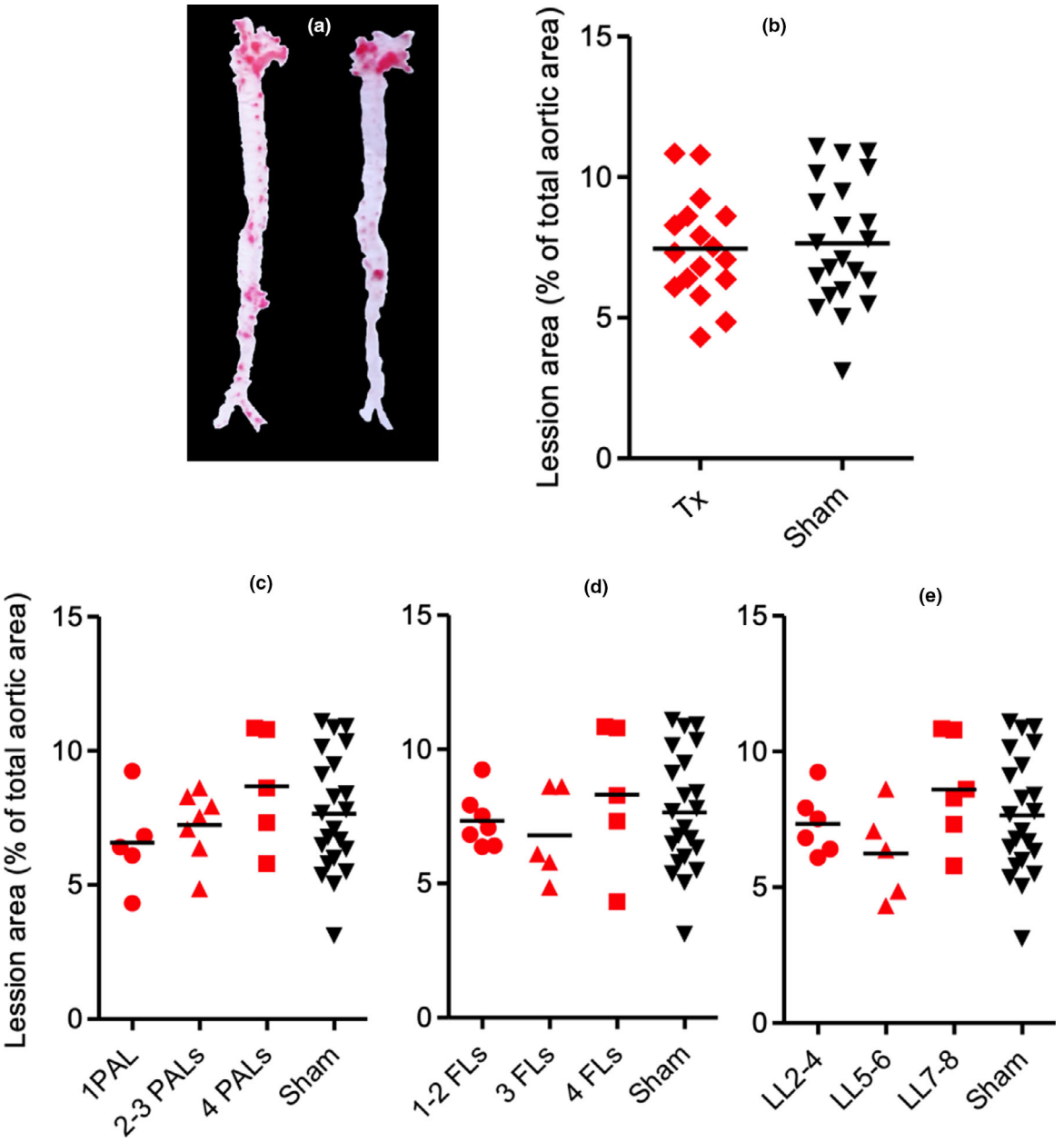

Figure 2 (a–e) Atherosclerotic lesion burden. Representatives of digitally scanned aortas stained with oil red O for lipid (atherosclerotic lesion areas: left-Rx; right-S) (a). Distribution of atherosclerotic lesion area percentage out of total aortic area in Tx and Sham group mice (b); distribution of atherosclerotic lesion area percentage out of total aortic area in Sham group and Tx grouped by: number of periapical lesions (PALs) (c), number of furcation lesions (FLs) (d) and by lesion load (LL) which is the sum of PALs + FLs per mouse (e).

requires a high fat/cholesterol diet (Getz & Reardon 2016). One reason that the LDLR KO model was used in the current study 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 WTD. As a result, older, larger mice could be used without worrying that the process of atherosclerosis had already begun. Older mice were used because weight at the time of anaesthesia proved to be important for survival. Another reason to use

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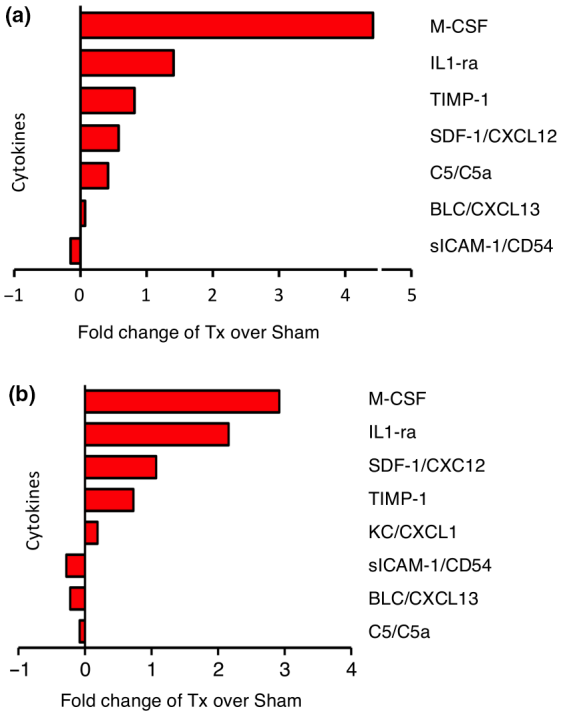

Figure 3 (a,b) Cytokine levels in 8-week (a) and 16-week (b) plasmas. Results are expressed as 'fold change' (Tx-Sham)/Sham. Keratinocyte chemoattractant/CXC chemokine ligand-1 (KC/CXCL1) could be detected only in the Sham group. Abbreviations: BLC/CXCL13, B lymphocyte chemoattractant; C5/C5a, complement component 5/5a; IL1-ra, interleukin-1 receptor antagonist stromal cell-derived factor 1; M-CSF, macrophage colony-stimulating factor; SDF-1/CXCL12, stromal cell-derived factor-1; sICAM-1/CD54, soluble intercellular adhesion molecule-1/Cluster of differentiation-54; TIMP-1, tissue metallopeptidase inhibitor 1.

the LDLR KO is because loss of the LDL receptor results in accumulation of LDL in plasma, which is similar to what occurs in human disease.

The similar weight gain observed in both groups confirmed that the overall condition of the mice in the Tx group was not compromised due to the procedure to induce AP (Wang & Stashenko 1991) and that they most likely did not experience pain/distress. Thus, weight was not a confounder in this study. Given the length of this study compared with others that use the AP model, the monitoring data suggest the LDLR KO can be feasibly used to study AP and atherosclerosis. A similar increase in cholesterol levels was also observed in a range that was previously reported for LDLR KO mice fed this diet (Brown *et al.* 2015).

Since the classical study by Kakehashi *et al.* (1965), others have adopted their protocol for AP induction by pulp exposure (Wang & Stashenko 1991, Balto *et al.* 2000, von Stechow *et al.* 2003, Shah *et al.* 2018). In this study, the pulps of the four first molars per mouse were exposed. This number was based on the desire to induce increased inflammation (more involved teeth = more inflammation) balanced against morbidity/mortality, technical difficulty and anaesthesia constraints. The latter factors may provide a reason why most studies exposed only one or two molars per mouse (Balto *et al.* 2000, 2002, von Stechow *et al.* 2003, da Silva *et al.* 2012, Shah *et al.* 2018).

Micro-CT is a useful tool to study AP, but a defined protocol for acquiring/analysing micro-CT images of PALs in rodents is lacking (Kalatzis-Sousa *et al.* 2017). Parameters found in similar previously published work (Kalatzis-Sousa *et al.* 2017) were used. All radiolucent areas, including those that were questionable, were further examined and confirmed in corresponding H&E stained histological sections (Metzger 2000). Although success was achieved in exposing the pulp of the surgical teeth in >90% of cases, development of periradicular lesions was less common. Overall, PALs were verified in 72% of teeth with exposed pulps; these results are similar to those reported by others (Balto *et al.* 2000, von Stechow *et al.* 2003).

Due to variability in number/size of periradicular lesions and their clinical parameters, ranging from some inflammatory infiltrate to dense inflammatory infiltrate with extensive alveolar bone destruction, and variability in size of pulp exposure and crown destruction, analysing the Tx group as a single homogenous variable might have led to bias. Therefore, the Tx mice were grouped according to number and types of lesions. However, this then resulted in a small sample size (n = 5–7), weakening the strength of statistical tests and no differences were found between the groups.

Despite the hypothesis that mice in the Tx group would show an increase in atherosclerosis lesion burden as compared to the Sham group, they developed a similar degree of atherosclerosis. This hypothesis was based, in part, on studies using a periodontal disease model, which revealed significantly increased atherosclerosis lesion area in aortas of LDLR KO mice orally infected with *Porphyromonas gingivalis* (*P. gingivalis*) compared with control mice (12 ± 3% vs. 6 ± 3%, Brown *et al.* 2015). Mechanistically, the

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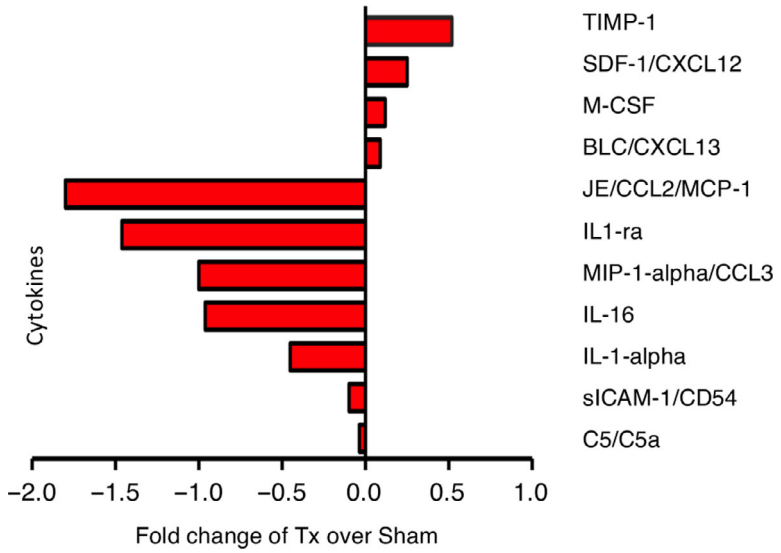

Figure 4 Cytokine levels in 16-week peritoneal macrophage lysates. Results are expressed as a 'fold change': (Tx-Sham)/Sham. Tumour necrosis factor alpha (TNF-alpha) and triggering receptor expressed on myeloid cells-1 (TREM-1) were detected only in Tx group. Abbreviations: BLC/CXCL13, B lymphocyte chemoattractant; C5/C5a, complement component 5/5a; ICAM-1/CD54, soluble intercellular adhesion molecule 1/Cluster of differentiation 54; IL-16, interleukin 16; IL1-alpha, interleukin-1-alpha; IL1-ra, interleukin-1 receptor antagonist stromal cell-derived factor 1; JE/CCL2/MCP-1, monocyte secretory protein JE/CC chemokine ligand 2/monocyte chemoattractant protein 1; M-CSF, macrophage colony-stimulating factor; MIP-1-alpha/CCL3, macrophage inflammatory protein 1-alpha; SDF-1/CXCL12, stromal cell-derived factor 1/CXC chemokine ligand 12; TIMP-1, tissue metallopeptidase inhibitor 1.

authors reported increased systemic inflammation by cytokine array that may have resulted from oral inflammatory processes augmenting WTD feeding in this model.

The present study has limitations that preclude the unequivocal conclusion that there is no causality. The variability in the number, size and clinical aspects of periradicular lesions (PALs and FL) amongst the Tx group may have contributed to the outcome. Although the four 1st molars were included, it may be that even four PALs are not sufficient to increase systemic inflammation above the threshold to influence atherosclerosis. As opposed to the periodontal study, in the current study, there was no introduction of exogenous pathogenic bacteria (exposed pulps were naturally infected by endogenous oral flora). Patients with AP often present with chronic periodontal disease, which is prevalent in 46% of the population (Eke *et al.* 2015). Since both are common oral diseases in the adult population, it may be that CVD correlates more strongly when both conditions are present, and this accounts for the positive association findings in epidemiologic human studies (Berlin-Broner *et al.* 2017). Although 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. It would be interesting in future studies to introduce a periodontal disease pathogen at the time of pulp exposure, mimicking the common situation in humans.

It is known that the period of acute PAL growth takes place within the first three weeks post-pulp exposure (Wang & Stashenko 1991) and a slow rate of bone destruction follows. It may be that if the cytokine array was performed earlier, during the acute development phase, a larger number of differentially expressed cytokines would have been observed. The low number of cytokines differentially related to atherosclerosis expressed at 8 and 16 weeks in plasma, and at 16 weeks in macrophage lysates, suggests an absence of sufficient increased oral inflammation to contribute to overall systemic inflammation. This lack of increase in some key atherogenic cytokines, such as interferon-gamma (INF-gamma) and IL-6 (Brown *et al.* 2015), aligns with the atherosclerotic lesion burden data showing no difference between Tx and Sham mice.

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Of the 40 cytokines that could be compared using the cytokine array, several were identified that were variable (increased/decreased) between Tx and Sham groups in the plasma (Fig. 3) or peritoneal macrophages (Fig. 4), at 8/16 weeks after AP induction or sham anaesthesia. The highest fold changes were noted for M-CSF (4.24–2.92 at 8 and 16 weeks) and of interleukin-1 receptor antagonist (IL-1ra, 1.42–2.16 at 8 and 16 weeks) in the plasma. M-CSF is a haematopoietic growth factor, which is involved in the differentiation, proliferation and survival of monocytes, macrophages and stem cell progenitors and stimulates increased phagocytic and chemotactic activity of macrophages (Stanley *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 *et al.* 1998). Expression of IL-1family 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 signalling. Overexpression of IL-1ra in LDLR KO mice markedly decreased the size of atherosclerotic lesions (Ait-Oufella *et al.* 2011).

TIMPs are key regulators of metalloproteinases that degrade the extracellular matrix and shed cell surface molecules. Since TIMPs can be measured in the plasma, they can be monitored as a marker of matrix turnover. TIMP-1 is associated with CVD development and prognosis: circulating levels of TIMP-1 have been related to CVD risk factors (Hansson *et al.* 2011). 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 *et al.* 2009). It can also induce platelet activation, and this suggests a role for SDF-1 in thrombo-occlusive diseases (Abi-Younes *et al.* 2000). Soluble intercellular adhesion molecule (sICAM-1/CD54) is related to the severity of atherosclerosis.

A systematic review concluded that AP is associated with increased levels of C-reactive protein (CRP), IL-1, IL-2 and IL-6 in human plasma (Gomes *et al.* 2013). No increased levels were found for IL1, 2 nor 6 (CRP was not measured). Possibly, the difference may be due to the fact that in humans, other systemic conditions, in combination with AP, influenced systemic cytokine expression. Alternatively, it might be that in the present study, the level of local inflammation due to PALs or FLs was not enough to influence the expression of these cytokines. Finally, it may be related to the difference between the models, where in the current study infection was induced by pulp exposure, whilst other periodontal disease models had introduced oral infection with *P. gingivalis,* which might have systemic effects (Lalla *et al.* 2003, Brown *et al.* 2015).

One of the known morbidities of WTD feeding in atherosclerosis-prone mice, which was witnessed in this study, is dermatitis. Idiopathic ulcerative dermatitis (UD) is a spontaneous, chronic disease generally affecting C57BL/6 mice with prevalence rates of 4.1%–26% (Hampton *et al.* 2015). Moreover, mice fed a WTD demonstrate increased UD lesions and impaired healing of induced wounds (Zabalawi *et al.* 2007, Hampton *et al.* 2015). LDLR KO mice fed a WTD have 10 times higher plasma cholesterol levels, which leads to xanthomas that worsen dermatitis (Zabalawi *et al.* 2007). A higher rate of dermatitis was observed in the Tx as compared to the Sham group in the current study (13.6% (3/22) in the Sham group and 29.4% (5/17) in the Tx group had dermatitis); however, as observed previously, dermatitis did not impact systemic inflammation, as demonstrated by results of the cytokine array.

Conclusions

This is the first study using the LDLR KO mouse model to study the influence of AP on atherogenesis. This model allows investigation of the relationship between the two diseases, whilst avoiding other potential common confounders. In the current study, no differences were found between the Tx and the Sham groups in terms of atherosclerotic lesion burden and inflammation, but there were substantial limitations due to study challenges. This study can be viewed as a launch pad for future studies, implementing changes in study design to overcome the challenges encountered, as discussed. 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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Conflict of interest

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

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