EXPLOITING DENTINE EXTRACELLULAR MATRIX COMPONENTS FOR TREATING APICAL PERIODONTITIS

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ABSTRACT

Conventional antimicrobial only approaches for treating apical periodontitis have consistently demonstrated limited successful treatment outcomes over the last five decades. This has resulted in growing interests for more biologically driven treatment strategies. One such approach is exploiting endogenous bioactive dentine extracellular matrix components (dECM) to upregulate the intrinsic regenerative capacity of recently discovered periapical lesion derived-mesenchymal stem cells (PL-MSCs). This mechanism is hypothesised to improve upon clinical outcomes when compared with a conventional approach. The overall aim of the present thesis was to therefore provide proof of concept for a biologically based regenerative clinical protocol for treating apical periodontitis that can be used in mature permanent teeth. Several *in vitro* and *in vivo* methodologies underpinned by systematic reviews and surveys were utilised, all of which culminated in a pilot triple blinded paralleled group randomised controlled clinical trial. Briefly, a series of *in vitro* investigations where sodium hypochlorite (NaOCl) was used at concentrations as low as 0.5 – 1.5% was found to inhibit release of dECMs, even after mechanically re-instrumenting the dentine. Treatment with 17% EDTA alone was thus considered the optimal irrigant regime for solubilising bioactive proteins from within the dentine matrix. This therapeutic protocol contrasted current trends in antimicrobial irrigant regimes, as evidenced in the national survey, and thus was tested *in vitro* against an *E.faecalis* biofilm and found to possess antimicrobial capabilities when combined with mechanical debridement. Furthermore, bioavailability was demonstrated *in vitro* for dECMs to interact with periradicular inflammatory tissues in mature permanent teeth with subsequent topical application to PL-MSC cultures revealing upregulation of

regenerative events at just picogram levels. This included increases in proliferation, migration, mineralisation and osteogenic differentiation without effecting cell viability. As some of these effects *in vivo* are likely to be undetectable using currently available clinical techniques, the process of sampling and analysing biomarkers from within periradicular tissue fluid was optimised. When clinically implemented, the proposed therapeutic irrigation protocol resulted in similar root canal treatment success rates to conventional regimes with no significant differences in pain scores or periradicular tissue-derived biomarker activity. The therapeutic potential for irrigant regimes that promote dECM release to treat periradicular disease has thus been demonstrated warranting the need for more extensive clinical investigation.

DEDICATION

This thesis is dedicated to my mother, Davinder Kaur Virdee; father, Jaspall Singh Virdee; and late uncle; Kuldip Singh Gahir.

It was through your unconditional support, unparalleled work ethic and selfless sacrifice that afforded me the privilege of completing this project.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Apical Periodontitis

1.1.1 The Microbial Attack

Apical periodontitis is a clinical term used to describe a localised inflammatory condition of the periodontium (Nair 2004). This condition, which affect approximately 50% of the global adult population, represents a dynamic equilibrium between putative endodontic micro-organisms and the interfacing host immune response (Tibúrcio-Machado et al. 2021). This disease process is initiated when constituents of the oral microbiome invade into the pulp space (Kakehashi et al. 1965). The most common portal of entry is following acid dissolution of hydroxyapatite through a carious process. Alternative routes however include iatrogenic injury; severe and acute non-carious tooth substance loss; complicated crown fractures; inconspicuous cracks; leaking restorations; and furcal, lateral and apical communications with the periodontal ligament. The bacterial front within dentinal tubules is preceeded by virulent by-products, which induce pulpal inflammation resulting in symptoms typical of mild to severe pulpitis (Bjørndal, 2008). Whilst pulp protective mechanisms such as tubular sclerosis and tertiary dentine deposition can delay this process, the pulp has a limited capacity to withstand a sustained microbial challenge and necroses in a coronal to apical direction in the absence of operative intervention (Bjørndal, 2008). At this stage, singular and free-floating planktonic micro-organisms infiltrate the exposed tissue, occupy vacant territory and begin forming surface-associated biofilms within the root canal. The exact mechanisms of how these endodontic biofilms form *in vivo* are currently not well understood however, their prevalence in teeth with apical periodontitis is well established (Nair 1987, Ricucci & Siqueira 2010). Next generation sequencing techniques characterise this established endodontic microbiome as being

one that is highly varied between individuals and dominated by gram-negative and some gram-positive anaerobic bacteria (Shin et al. 2018). As this highly resilient polymicrobial community colonises the apical portion of the canal, intrinsic and extrinsic bacterial by-products diffuse ahead of the microbial front, leak past the major apical foramen and initiate the immunological events within the periradicular tissues that leads to the development of apical periodontitis (Jönsson et al. 2011).

1.1.2 The Host Response

1.1.2.1 Initiation: The immune response is initiated when various virulent by-products, of which lipopolysaccharides (LPS) is the most notorious, activate membrane bound pathogen recognition receptors on host cells (Martinho et al. 2017, Machado et al. 2020). The first receptors to encounter such noxious stimuli are toll-like-receptors on the surface of fibroblastic periodontal ligament cells. The resulting receptor-ligand complexes initiate downstream intracellular signalling cascades that through nuclear factor kappa B (NF-κB) transcription, produces a myriad of pro-inflammatory cytokines (Jönsson et al. 2011). These auto- and paracrine signalling molecules attract cellular components of the innate response to the site of infection (Silva et al., 2007). Through similar pathways, LPS also stimulates local endothelial cells to upregulate expression of adhesion molecules, which activates the compliment system that stimulates further phagocytic chemotaxis, bacterial opsonisation and increases in local vascular permeability and vasodilation (Siqueira & Rôças 2007). The responses are limited to the interfacing periodontal ligament and neighbouring spongiosa with the resultant oedema compressing local nociceptors and giving rise to severe tenderness to percussion, hyperocclusion and radiographic widening of this anatomical structure

(Nair 1997). These elaborate networks of overlapping primary events serve to recognise noxious stimuli and activate the immune response.

1.1.2.2 Acute Phase: The principal feature of the acute phase is active periradicular bone resorption, which is mediated by the innate immune response. This begins when the immediate leukocyte infiltration at the periapex organises itself into an inner neutrophil layer, closest to the apical foramen, and an outer macrophage layer with small populations of eosinophil, dendritic and mast cells (Stashenko et al. 1992, Kawashima et al. 1996). The former layer acts as the first line of defence and phagocytoses accessible bacteria egressing from the root canal, but these cells rapidly undergo apoptosis (Kawashima et al. 1996, Nair 1997). Consequentially, proteolytic enzymes and reactive oxygen species are released into the surrounding periradicular tissues contributing to the on-going tissue destruction (Márton & Kiss 1993). Macrophages in the outer layer clear these disintegrated neutrophils and remaining micro-organisms and gradually become the dominant phagocyte (Kawashima et al. 1996). At the same time, LPS-induced macrophage activation initiates NF-κB transcription of pro-inflammatory cytokines (Márton & Kiss 1993 & 2000). Amongst these, interleukin [IL]-1α, IL-1β, IL-6 and tumour necrosis factor [TNF]-α have been implicated as potent stimulators of osteoclastic bone resorption (Nair 1997 & 2004, Márton & Kiss 2000). Moreover, arachidonic acid metabolites, such as leukotrienes and prostaglandins, are also released from cell membranes of phagocytes and act as permissive cofactors that work in synergy with pro-inflammatory cytokines to enhance their bone resorptive potential (Stashenko et al. 1987 &1995). Immunoregulatory cytokines such as IL-4, IL-10 and transforming growth factor [TGF]-β1 have also been detected in the acute phase with their concentrations negatively correlated with clinical symptoms (Walker et al. 2000, Kabashima et al. 2001, Colić et al. 2009, Gazivoda et

al. 2009). During active bone resorption, macrophages act as the interface between the innate and acquired immune response by presenting antigens to naïve T-helper/inducer lymphocytes (CD4+). These lymphocytes are more prevalent than their T-suppressor/cytotoxic (CD8+) counterparts (Stachenko et al. 1992, Lukić et al. 2006). Additionally, the pro-inflammatory CD4+ Type 1 (TH1) subgroup, which principally release TNF-α, Interferon [IFN]-ɣ, macrophage inflammatory protein [MIP]-1α and MIP-1β are more active than the anti-inflammatory CD4+ Type 2 (TH2) lymphocytes, which supresses TH1 cytokines via IL-4, IL-10 and IL-13 secretion (Stachenko et al. 1992). The pro-inflammatory actions of IFN-ɣ, MIP-1α and MIP-1β include attracting macrophages, stimulating IL-1β production and inhibiting TH2 subgroup differentiation (Jäger & Kuchroo 2010).

The net outcome of the above events culminates in the differentiation of mature osteoclast (Roodman 1993). This occurs indirectly by increased expression of transmembranous “Receptor Activator of Nuclear Factor Kappa B Ligand” (RANKL) in osteoblasts. The resultant osteoblastic RANKL and osteoclast RANK receptor complex formations stimulate differentiation of nearby osteoclast precursors into mature multinucleated resorptive cells with ruffled borders. In health, the physiological balance between the activity of these cell types is relatively balanced however, pro-inflammatory stimulation as in that of apical periodontitis, disturbs this ratio in favour of bony resorption (Márton & Kiss 2014). This has been observed to occur at a rapid rate between days one and 15 of pulp exposure, which is considered to be the active phase of lesion expansion (Wang & Stashenko 1991). Thereafter, there is a steady decline until bone resorption reaches near baseline levels at day 30 (Wang & Stashenko 1991). At this stage, the resultant bone loss may be visible on radiographic examination due to the disturbed integrity of the periradicular bone, cementum and

dentine. The lesion may progress into an abscess with sinus tract formation if the influx of neutrophils and bacterial byproducts exceeds the macrophage clearing capacity, such as when there are sudden increases in bacteria loads or when microbes evade host defences (Nair 2004). In most cases however, the lesion converts into a chronically stable but dynamic state (Nair et al., 1996).

1.1.2.3 Chronic Phase: Prolonged presence of microbial irritants induces chronic low-grade inflammation in periradicular tissues. It is here where the dynamic equilibrium between bacteria and host is such that the latter are unable to eliminate the pathogenic factors residing within the sanctuary of the root canal, and the former are restricted from spreading deeper into periradicular tissues (Márton & Kiss 2000). The resultant stability and space created through active lesion expansion relieves pressure on apical tissues and their respective nociceptors, resulting in alleviation of clinical symptoms and presence of a well demarcated radiographic lesion. Furthermore, bone resorbing activity has now declined to near baseline levels, which is the characteristic feature of apical lesions that have progressed into the more reparative chronic phase (Wang & Stashenko 1991).

Histologically, these lesions exist as granulomas, where 50% of the cellular content is occupied by inflammatory infiltrate (Stechenko et al., 1998). There are conflicting outcomes on whether T-lymphocytes (Márton & Kiss 1993, Yu & Stachenko 1987, Nilsen et al., 1984) or macrophages (Kawashima et al. 1999, Kopp & Schwarting 1989, Stern et al. 1981) dominate however, *in vivo* studies have found smaller lesions in athymic mice suggesting T lymphocytes are critical in facilitating progression of acute periradicular lesions into the chronic phase (Tani et al. 1995, Kawahara et al. 2004). This is supported by longitudinal immunohistochemical studies on induced rat periapical lesions that demonstrate a notable shift in the CD4+:CD8+ ratio in favour of

the former during active lesion expansion and the latter after 20 to 90 days pulp exposure (Stashenko & Yu 1989). The pro-inflammatory activity of the CD4+ may thus regulate bone resorption soon after immunological initiation, whilst CD8+ cells potentially serve as anti-inflammatory mediators that suppress osteoclast activity in the chronic phase. The exact mechanisms however are not yet fully understood. Interestingly, two recently discovered CD4+ subpopulations, T-helper type 17 (TH17) and regulatory T-lymphocytes (T-reg), have also been isolated from periapical lesions (Xiong et al. 2008, Colić et al. 2009). The T-reg cells, are recruited to the site of infection after antigen exposure and exert immunoregulatory activity via IL-10 and TGF-β1 secretion (Colić et al. 2009, Gazivoda et al. 2009). The anti-inflammatory effects of these cytokines allude to the protective nature of this subgroup. Conversely, TH17 cells are almost the exclusive source of IL-17, which attracts neutrophils, up-regulates cytokine expression and possesses the ability to reactivate acute inflammation (Colić et al. 2009, Yu & Gaffen 2008, Marton & Kiss 2014). This is evidenced by high concentrations detected in periradicular lesions with a sinus tract (Marçal et al. 2010). The pleotropic TGF-β1 molecule fundamentally regulates the differentiation of these subtypes, suggesting a potential critical but at present poorly understood role in determining the outcome of periapical disease in terms of lesion expansion or healing (Marçal et al. 2010).

The remaining composition of periapical granulomas consists of an outer fibrous connective tissue capsule; highly vascular networks of endothelium and the resulting inflammatory exudate, formally termed “periradicular tissue fluid” (PTF); fibroblasts, osteoclasts and osteoblasts; and proliferating stratified squamous epithelial rest cells (Márton & Kiss 2000, Nair 2004). This structure ultimately serves to generate space

for the body's defensive components and establish an equilibrium with bacteria housed within the root canal.

1.2 Periapical lesion-derived mesenchymal stem cells

Stem cells are essential to any repair or regenerative process as they possess high proliferation rates, self-renewal capabilities, and potential for multi-lineage differentiation. Embryonic stem cells are pluripotent as they develop into stromal cells from any of the three germinal layers whereas multipotent postnatal stem cells are restricted to organ-specific lineages (Huang *et al.* 2009). The latter are more amenable to clinical translation due to their autologous nature and presence within almost all adult tissues. The mesoderm subdivision, "mesenchymal stem cells" (MSC), has attracted particular interest within regenerative dentistry as dense reservoirs have been isolated within many odontogenic hard and soft tissues (Huang *et al.* 2009, Kim *et al.* 2018). Based on their tissue of origin, established dental MSC niches include "dental pulp stem cells" (DPSC), "stem cells from human exfoliated deciduous teeth" (SHED), "periodontal ligament stem cells" (PDLSC), "dental follicle precursor cells" (DFPC), "stem cells of the apical papilla" (SCAP), "gingival MSCs," "alveolar bone MSCs," and "tooth germ progenitor cells.

In recent years, *in vitro* investigations of human periapical granulomas have identified a distinct MSC niche, which mostly reside in the peripheral capsule layer (Liao et al. 2011. Estrela *et al.* 2019). More specifically, these periapical lesion-derived MSCs (PL-MSCs) were found to possess the minimum criteria necessary to be defied as MSCs in that they demonstrated plastic adherent, highly proliferative and multipotent properties (Liao et al. 2011, Đokić et al. 2012, Marrelli et al. 2013). Moreover, their

immunophenotype expressed mesenchymal surface markers, namely Cluster of Differentiation [CD]-13, -29, -44, -73, -90, -105 and -166, but did not express hematopoietic markers such as CD-14, -19, -34 and -45 (Liao et al. 2011, Đokić et al. 2012, Marrelli et al. 2013, Paduano et al. 2016). When investigated for their therapeutic potential, PL-MSCs demonstrated tremendous immunosuppressive and regenerative potential. For example, co-cultures with leukocytes revealed reductions in leukocyte proliferation; less production in pro-inflammatory cytokine; and increases in expression of immunoregulatory molecules such as TGF-β1 (Đokić et al. 2012). Moreover, their demonstrable ability to differentiate into osteoblasts, cementoblasts, adipocytes, astrocytes and chondrocytes are relevant for regenerating the various periradicular tissues that were lost to the immune response (Đokić et al. 2012, Marrelli et al. 2013 & 2015). The mineralisation needed for these cells to be considered functional has also been confirmed *in vitro* and in *in vivo* mouse models (Đokić et al. 2012, Marrelli et al. 2013). Collectively, these findings implicate PL-MSCs as being contributory factors in arresting periapical disease progression and key determinants of the wound healing processes that ultimately leads to the regeneration of periradicular tissues. Their discovery offers novel opportunities to develop more biologically driven therapies for treating apical periodontitis that actively engage with the endogenous mechanisms of periradicular wound healing and regeneration. Preliminary methodological experiments revealed demonstrated multipotent potential of plastic adherent cells isolated from human periapical granulomas from extracted teeth diagnosed with apical periodontitis (Figure 1).

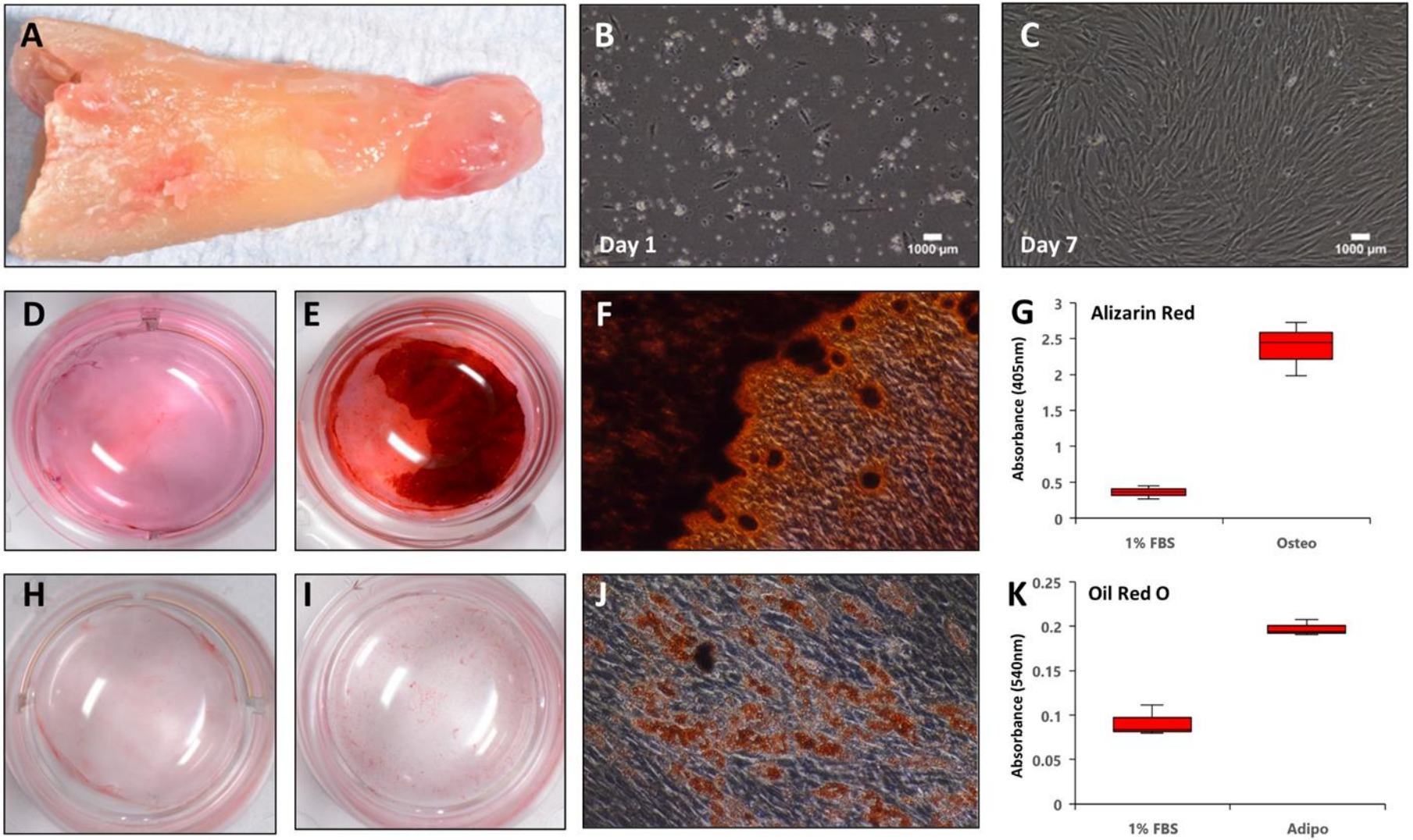
A
B
Day 1
1000 µm
C
Day 7
1000 µm
D
E
F
G
Alizarin Red
Absorbance (405nm)
3
2.5
2
1.5
1
0.5
0
1% FBS
Osteo
H
I
J
K
Oil Red O
Absorbance (540nm)
0.25
0.2
0.15
0.1
0.05
0
1% FBS
Adipo

Figure 1

Figure 1: Pre-liminary investigation into the multipotent potential of primary PL-MSCs. (A) PL-MSCs were isolated from the apical granuloma of extracted teeth via a collagenase type 1 enzyme digestion technique (*n* = 3). Cells were cultured in a T25 flask with 20% foetal bovine serum supplemented α-MEM media, which was changed every two days. (B & C) Phase contrast microscopy at 10 x magnification of PL-MSC cultures at day 1 (B) and day 7 (C). D, E, F & G: Osteogenic differentiation following 21 days culture with control or osteogenic induction media (α-MEM, 20% FBS, 1% penicillin/streptomycin, 2mM glutamine, 0.2mM ascorbic acid, 100nm dexamethasone, 10 mM β-glycerophosphate). Staining with Alizarin Red S confirmed absence in control wells (D) and presence in test wells of mineral deposits (E & F). Staining was solubilised with 10% acetic acid and subsequent intensity quantified using a microplate reader with an excitation wavelength set at 405 nm (G). (H, I, J & K) Adipogenic differentiation following 21 days culture with control or adipogenic induction media (α-MEM, 20% FBS, 1% penicillin/streptomycin, 2mM glutamine, 0.5 mM IBMX, 200 µM indomethacin, 10 µM insulin, 1 µM dexamethasone).Staining with Oil Red O confirmed absence in control wells (H) and presence in test wells of lipid droplets (I & J). Staining was solubilised with isopropanol and subsequent intensity quantified using a microplate reader with an excitation wavelength set at 540 nm (K). All experiments were conducted up to passage 2 using three biological replicates. Scale bars represent 1000 µm.

1.3 Periapical Healing

Periapical healing is fundamentally a regenerative process that involves a highly co-ordinated sequence of overlapping cellular events that are regulated by numerous cell-cell and cell-matrix signalling interactions (Lin & Rosenberg 2011). These are in turn mediated by a range of growth factors, cytokines, chemokines and neuropeptides (Robson 1997, Barrientos et al. 2008). These endogenously secreted proteins function by binding to transmembranous receptors, via auto- or para-crine mechanisms, and activating downstream signalling cascades that ultimately bring about changes in cellular growth, metabolism, motility and polarity. Noteworthy examples include TGF-

β1, Bone Morphogenetic Proteins (BMP), Fibroblast Growth Factors (FGF) and Vascular Endothelial Growth Factors (VEGF), amongst many others (Robson 1997, Barrientos et al. 2008). Whilst the exact mechanisms are poorly understood, these processes likely recapitulate those exhibited in the wound healing of other bodily connective tissues. It could be argued however, that regeneration of the periapical tissues is a more complex feat owing to the various hard and soft tissue types that comprise the periodontium; namely cementum, periodontal ligament and alveolar bone. Nevertheless, the generic stages of wound healing include inflammation, cell proliferation and tissue remodelling and are initiated after adequate cessation of the microbial stimulus (Gurtner et al. 2008, Velner et al. 2009).

1.3.1 Inflammation: The inflammatory phase described above serves to prevent infection, achieve haemostasis and eliminate devitalised tissues. It is here where the lack of an inflammatory stimulus starves pro-inflammatory cells of the vital mediators they need to continue proliferating and causing local tissue destruction. Many of the redundant cellular components consequentially apoptose into smaller membrane bound vesicles, which get phagocytosed by local macrophages (Greenhalgh 1998). These cells also secrete a myriad of growth factors, namely members of the FGF, epidermal growth factor (EGF), TGF-α and TGF-β families, which activate local MSCs and endothelial cells to initiate tissue repair (Gurtner et al. 2008, Velnar et al. 2009). At the same time, the extracellular matrix is being remodelled by various host-derived metalloproteinases (MMP) whilst a fibrin matrix is formed in its place via platelet aggregation (Gurtner et al. 2008). This acts as an initial scaffold for infiltrating cells. Collectively, these events cause the lesion to begin regressing in size which in turn provides room for the original tissues to be reinstated.

1.3.2 Proliferation: The proliferative phase of periapical healing marks the transition to a reparative stage. Initially, VEGF-A and FGF within the extracellular matrix stimulate local endothelial cell proliferation (Takeshita et al. 1994, Baum & Arpey 2005). These new capillary networks, formed via angiogenesis, restore the oxygen and nutrient supply to the lesion and deliver progenitor cells to enhance the regenerative potential. At the same time, undifferentiated progenitor/MSCs from various sources including the adjacent periodontal ligament, endosteum, granulation tissue and bone marrow are recruited to and proliferate over the denuded root surface (Grzesik & Narayanan 2002, Seo 2004). Here, FGFs, insulin growth factors [IGF], platelet-derived growth factors [PDGF] and TGF-βs released from stromal cells, osteoblasts, platelets and bone matrix following MSC stimulation, mediate stem cell migration, attachment, proliferation and differentiation into cementoblasts, osteoblasts and fibroblasts (Linkhart et al. 1996). These events begin to respectively regenerate the cementum, alveolar bone and periodontal ligament whilst the wound continues to contract.

1.3.3 Maturation: The final maturation phase is characterised by continued hard and soft tissue deposition and organisation; apoptosis of all redundant cellular components; and MMP mediated remodelling of a now relatively acellular collagen type I extracellular matrix (Gurtner et al. 2008). These events, which can take up to four years, ultimately leads to the regeneration of a periodontal ligament such that one group of Sharpey's collagen fibres insert into newly formed cementum, and their counterparts into newly formed alveolar bone (Orstavik 1996, Lin & Rosenberg 2009, Ng et al. 2011). This signifies the completion of periapical wound healing.

1.3.4 Repair and regeneration: The aforementioned processes aim to mimic embryonic tissue development where under the correct temporal and spatial

conditions, injured tissues are regenerated with the same cell type for complete restoration of biological function and architecture (Kim et al. 2018). From a clinical perspective, it is remarkable how the periodontal ligament, cementum and alveolar bone are all restored in such an orderly fashion after conventional root canal therapy, which in some part could be attributed to the enclosed bony compartment that naturally excludes epithelial cell proliferation (Melcher 1976). When interactions between these components are dysregulated, such as when buccal and lingual cortical plates are perforated, the same mechanisms lead to repair where damaged tissues are replaced with fibrous scar tissue leading to a loss in biological function (Lin & Rosenberg 2009). With respect to apical periodontitis, complete regeneration without some reparative fibrous tissue, albeit subclinical, is unlikely to occur due to the post-natal nature of this process. Periapical lesions will thus always heal by a combination of regeneration and some degree of repair (Lin & Rosenberg 2009 & 2011).

1.4 Treatment strategies

1.4.1 Conventional endodontic therapy

The ultimate aim of endodontic therapy in teeth with apical periodontitis is to reinstate the original architecture and biological functions of the injured periradicular tissues with as minimal scar tissue as possible (Lin & Rosenberg 2011). To date, treatment strategies strive to achieve this by focusing exclusively on the microbial component through a process of chemomechanical debridement. Briefly, infected root canals are sequentially enlarged with mechanical instruments and irrigated with antimicrobial solutions to reduce intraradicular bacterial load (Schilder 1974). Over the last five decades, considerable advancements have been made in disinfection

armamentarium, knowledge and technique to assist clinicians achieve this goal. This however has not proportionally translated into improved clinical outcomes. More specifically, using strict success criteria approximately 20% of all teeth that are root treated to a guideline standard consistently fail to exhibit periapical healing after appropriate review periods (Ng et al. 2008). These cases ultimately go onto require more complex resource intensive remedial therapy.

The principal issue associated with this antimicrobial only approach is that whilst it produces the endodontic micro-environment necessary to initiate subsequent periapical healing processes, there is no further stimulus to the endogenous mechanisms thereafter. The disproportionate focus on the microbial component of this dynamic equilibrium is such that subsequent host derived mechanisms, which are the driving force behind periapical healing, are neglected. This is to the extent where key cellular and signalling components essential for periapical regeneration after root canal treatment are even sacrificed, albeit unintentionally, to facilitate more powerful disinfection protocols that do not yield greater clinical outcomes (Paredes-Vieyra et al. 2012, Liang et al 2013, Verma et al. 2019). Persistent periapical lesions may therefore not only represent the presence of an intra- or extra-radicular residual biofilm, but also an inadequate capacity to stimulate physiological regeneration within injured periapical tissues. This could be one explanation for why success rates decrease in larger lesions (Ng et al. 2011, Ricucci et al. 2011). The continued efforts into increasingly aggressive disinfection protocols could be derived from the fact that the critical threshold for an endodontic micro-environment to be considered conducive for healing is currently unknown (Siqueira & Rôças 2008). Nevertheless, it can be surmised that the effects of antimicrobial only approaches on periradicular healing may

have now saturated and to improve success rates further, it will be necessary to adopt more biologically driven therapeutic approaches.

1.4.2 Regenerative endodontic therapy

Regenerative endodontic therapies are biologically based procedures designed to replace damaged tooth structures (Murray et al. 2007). These treatment modalities have typically been reserved for reinstating the dentine-pulp complex in teeth with immature apices (Galler et al. 2016). Desirable outcomes are achieved by bringing together the triad of components for tissue engineering, namely stem cells, signalling molecules and scaffolds (Kim et al. 2018). Broadly, this can be through a cell-based manner, where MSCs are transplanted into injured tissues, or the more clinically amenable cell-free homing technique, where autogenous MSCs are recruited to the site of injury and stimulated *in situ* by supplying the damaged compartment with signalling molecules (Kim *et al.* 2018). The recent discovery of a clinically accessible PL-MSC niche makes it conceivable to extend the latter treatment modality to mature teeth diagnosed with apical periodontitis (Liao et al. 2011). By developing biologically driven protocols that produce a healthy endodontic micro-environment, preserve the biological integrity of the root canal system and supply injured periradicular tissues with the very signalling molecules responsible for co-ordinating healing events, more predictable periapical healing could potentially be achieved. This would translate into greater clinical outcomes than those currently observed for antimicrobial only approaches, which have remained stagnant for five decades (Ng et al. 2007). Fortunately, abundant autologous reservoirs of bioactive molecules are known to exist in a sequestered state within the dentine's extracellular matrix (Smith et al. 2012).

1.5 Dentine extracellular matrix components

Bioactive molecules within the dentine's extracellular matrix, termed dentine extracellular matrix components (dECM), are deposited by odontoblasts during dentinogenesis and become sequestered by subsequent mineralisation processes (Smith et al. 2012). Thereafter, their biological activity remains highly preserved through the formation of proteoglycan bonds, as well as lack of dentine turnover, but can be immediately reinstated on command upon their release (Schönherr & Hausser 2000, Baker et al. 2009). This has clinically been achieved through the use of various demineralising irrigant solutions (Galler et al. 2015, Zeng et al. 2016, Chae et al. 2018), pulp capping agents (Graham et al. 2006, Tomson et al. 2017), epigenetic modifiers (Duncan et al. 2017) and dental adhesives (Ferracane et al. 2013).

1.5.1 Composition

Approximately 280 bioactive molecules have been identified within demineralised dentine extracts (Jágr et al. 2012). The majority of these are non-collagenous extracellular matrix proteins that are crucial for dentinogenesis (Smith et al. 2012). Growth factors constitute large proportions of this cohort are implicated in regulating dentine-pulp reparative and regenerative responses. Members of the TGF-β, BMP, VEGF, FGF, IGF, PDGF, hepatocyte growth factor (HGF), placental-derived growth factor (PlGF) and epidermal growth factor families are frequently detected, with TGF-β1 often found in the greatest abundance in solubilised extracts (Duncan et al. 2017, Tomson et al. 2017). VEGFs, FGFs, PDGFs and PlGFs are also known mediators of angiogenesis, which as described above is a critical to the proliferative phase of periapical wound healing (Zhang et al. 2011). Closely associated with these are neurotrophic factors that are responsible for developing intricate innervations within

the dentine-pulp complex. Key examples include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 and 4 (NT3/NT4) and glial cell–line derived neurotrophic factor (Austah et al. 2019). Furthermore, a broad range of pro- and anti-inflammatory cytokines, namely IL-1α, -1β, -4, -6, -8, -10, -12 and granulocyte-macrophage colony-stimulating factor, have also been detected within solubilised dECMs (Graham et al. 2006). These NF-κB signalling molecules likely contribute to immunoregulatory pulp mechanisms as indicated by their capacity to induce a wide array of inflammatory events (Lara et al. 2003).

Other non-collagenous protein families released from the dentine matrix are those associated with regulating mineralisation and maturation processes of human calcified tissues (Orsini et al. 2012). Briefly, these include small integrin-binding ligand n-linked glycoproteins (dentine matrix acid phosphoprotein [DMP]-1, BSP, osteopontin [OPN], dentine phosphoprotein [DPP], dentine sialoprotein, dentine glycoprotein, matrix-extracellular-phosphoglycoprotein [MEPE]); vitamin K-dependant glycoproteins (osteocalcin [OCN]); small leucine-rich proteoglycans (decorin, biglycan, fibromodulin, lumican, osteoadherin); secretory calcium-binding phosphoproteins (ON) and large aggregating proteoglycans (versican). Many of these require enzymatic activation and therefore it is not unexpected the dentine substrate also contains MMP-2, -3, -8, -9, -20 and tissue inhibitors of MMPs ([TIMP]-1,-2; Niu et al. 2011, Mazzoni et al. 2011), which also regulate extracellular matrix remodelling in later phases of healing.

Given the above, dentine can no longer be considered an inert structural tissue but instead, a reservoir of auto- and paracrine cell-signalling molecules that could be exploited for novel therapies. Their endogenous nature overcomes many ethical issues associated with clinically using exogenous substitutes and the synergistic activity within solubilised dECMs; exhibit a greater potency than single recombinant

molecules at just picogram levels (Lee et al. 2015, Widbiller et al. 2018). For these reasons, dECM extracts have been extensively studied for their ability to initiate regenerative events within multiple previously established endodontic MSC niches. Of these, the most studied include DPSCs, SCAPs, PDLSCs and SHEDs.

1.5.2 Biological Effects

Dentinal morphogens induce their bioactive effects when binding to complimentary receptors on the surfaces of target cells. These auto- and paracrine interactions initiate downstream signalling cascades to the nucleus that ultimately bring about changes in genetic expression, phenotype commitment and cellular activity (Massagué 2000). In the context of odontogenic MSCs, those biological behaviours observed to be influenced by dEMC application that are relevant to tissue regeneration and repair include:

1.5.2.1 Migration: dECMs have repeatedly confirmed their chemotactic potential in vitro by way of transwall migration, matrigel invasion and scratch wound assay techniques (Smith et al. 2012, Tomson et al. 2017, Okamoto et al. 2018). When solubilised, these extracts exhibit a high degree of potency, with DPSC recruitment starting at just picogram levels and then increasing thereafter in a dose-dependent manner (Widbiller et al. 2018). Moreover, even root segments pre-conditioned with demineralising agents induce similar migratory effects in DPSCs and SHEDs, which contrasts the relatively inert properties of their deproteinised counterparts (Galler et al. 2016b, Gonçalves et al. 2016, Zeng et al. 2016). Other laboratory studies using single recombinant growth factors suggest that these capabilities can be largely attributed to the presence of known chemoattractants, such as TGF-β, HGF and to a lesser extent,

FGF molecules (Melin et al. 2000, Howard et al. 2010, Mathieu et al. 2013, Tomson et al. 2013, Zeng et al. 2016).

1.5.2.2 Proliferation: Solubilised dEMCs induce time- and dose-dependent proliferation within MSCs. These properties however are observed only up to a critical threshold, after which anti-mitogenic events become more apparent. For example, dEMC applications of less than 100 µg ml-1 and 7 days have generally been found to enhance DPSC proliferation in vitro (Lee et al. 2015, Tabatabaei & Torshabi 2016, Tomson et al. 2017, Okomato et al. 2018), whereas greater concentrations and contact-times prevent further growth (Sadaghiani et al. 2016, Widbiller et al. 2018). This pattern of stimulation, which is also witnessed in endothelial cell cultures and angiogenic tube formation assays (Zhang et al. 2011), could be explained as being the net outcome induced by the bioactive cocktail of polypeptides that is found within dEMCs. Some of these constituents, namely TGF-β (Massagué 2000, He et al. 2008), inhibit proliferation in several cell types but may also act to attenuate the effects of other known stimulatory growth factors, such as MEPE, PDGF, VEGF, IGF and FGF (Nakashima 1992, Denholm et al. 1998, Onishi et al. 1999, Nakao et al. 2004, d'Alimonte et al. 2011, Wei et al. 2012, Mathieu et al. 2013). Moreover, the ability of dEMCs to promote terminal differentiation could further contribute to reducing cell numbers over time (Sadaghiani et al. 2016).

1.5.2.3 Apoptosis: Despite the tumour supressing capabilities of TGF-β1 (Schuster & Krieglstein 2002, He et al. 2008), solubilised extracts induce little to no apoptotic effects in odontogenic MSCs (Smith et al. 2012, Lee et al. 2015, Galler et al. 2016b, Widbiller et al. 2018). In fact, higher concentrations of dEMCs have even been found to aid DPSC viability, as indicated by reduced caspase-3 activity and increased serine threonine kinase (Akt1) gene expression (Lee et al. 2015). This phenomenon could be

credited to those dentine morphogens that possess anti-apoptotic potential such as DPPs and PDGF, which activate downstream signalling cascades for cell survival (i.e. Akt1 and Akt2; Romashkova & Makarov 1999, Fujisawa et al. 2008).

1.5.2.4 Differentiation: Numerous in vitro studies using DPSCs, SCAPs and SHEDs indicate that dEMC extracts are powerful inducers of osteo- and odontoblastic differentiation. For instance, topical applications rapidly stimulate organisation and formation of elongated cellular processes that can extend into the tubules of pre-conditioned dentine disks (Liu et al. 2005, Chun et al. 2011, Pang et al. 2014, Galler et al. 2016b). This is accompanied by significant increases in mRNA expression for genes that are characteristic of odonto- and osteogenic commitment. These have to date included DSPP, DMP-1, OPN, OCN, BSP, RunX2, MEPE, type 1 collagen, alkaline phosphatase, distal-less homeobox 5 and msh homeobox 2 (Casagrande et al. 2010, Pang et al. 2014, Lee et al. 2015, Sadaghiani et al. 2016, Okamoto et al. 2018, Widbiller et al. 2018). More remarkably, when DPSCs and SHEDs are implanted subcutaneously alongside dEMCs, many of the aforementioned events have been found to still transpire (Smith et al. 1990, Tziafas et al. 1995 & 1998, Sakai et al. 2004, Galler et al. 2011). Understandably, no single molecule can be wholly responsible for initiating such a multifaceted process; however, dentine-derived BMP-2 unequivocally plays an essential role. This was demonstrated when blockage of BMP-2 signals, which are otherwise transduced down osteogenic canonical smad-1/5/8 and non-canonical p38 mitogen-activated-protein-kinase pathways, overtly inhibited expression of odontoblastic genes in SHED cultures (Casagrande et al. 2010). Furthermore, many studies using commercial growth factors continue to display its potent differentiating capabilities (Nakashima et al. 1994, Iohara et al. 2004, Nakao et al. 2004, Saito et al. 2004). Nevertheless, other dentine morphogens that may act

concomitantly with BMP-2 include TGF-β1, although this molecule has exhibited suppressive effects via smad-3 dependant mechanisms in SCAPs (Bégue-Kirn et al. 1992, He et al. 2014); PDGF; FGF; BMP-4; IGF; HGF; VEGF; NGF; BDNF; NT3; NT4; MEPE and TNF-α (Nakashima et al. 1992 & 1994, Onishi et al. 1999, Nakao et al. 2004, Mizuno et al. 2007, He et al. 2008, Paula-Silva et al. 2009, d'Alimonte et al. 2011, Li et al. 2011, Wei et al. 2012, Tomson et al. 2013).

1.5.2.5 Mineralisation: Based on colorimetric methods for calcium quantification, dEMCs have been found to significantly accelerate mineralised matrix production within MSCs (Liu et al. 2005, Lee et al. 2015, Sadaghiani et al. 2016, Okamoto et al. 2018, Widbiller et al. 2018). From a visual perspective, the calcified nodules indicative of functioning osteo- and odontoblasts can be observed as early as five days post exposure and become more prominent thereafter (Lee et al. 2015). When tested in vivo, using subcutaneous implantation models, this deposition has ultimately lead to the formation of osseous, dentinal and collagenous like tissues (Smith et al. 1990, Tziafas et al. 1995, Sakai et al. 2004, Li et al. 2011, Widbiller 2018). These potent effects can be ascribed to the ability of dentinal morphogens to upregulate genes, both of which are mentioned above, that code for the production of extracellular matrix proteins in teeth and bone.

1.5.3 Existing therapies

To date, the aforementioned findings have been clinically translated into several well recognised pulp preservation and regeneration cell-free homing protocols (Galler et al. 2016a, ESE 2019). For example, the release of endogenous dECMs by calcium silicate pulp capping materials in vital pulp therapy enhance chemotaxis (Tomson et al 2017) , angiogenesis and the differentiation of progenitor cells into dentinogenic cells (Liu et al. 2005, Zhang et al. 2011). This could in part explain why histologically, their

use in these procedures leads to a less porous higher quality tertiary dentine bridge formation in comparison to those induced by traditional calcium hydroxide liners and in turn, greater success rates (Nair et al. 2008, Mente et al. 2014). Revitalisation protocols in immature permanent teeth also recommend the use of less aggressive irrigation protocols so to preserve the integrity of natural biological components of the dentine and apical papilla (Galler et al. 2016a). The former is then intended to provide endogenous signalling to local autogenous SCAPs for regeneration of the pulp.

Overall, these protocols provide proof of concept for the cell-free therapeutic potentials of endogenous dECMs and their bioactive properties that if applied to PL-MSCs, would be of clinical utility for periradicular tissue regeneration.

1.6 Potential clinical protocol

There are several conceivable clinical strategies to exploit endogenous dEMC for the purpose of enhancing periradicular tissue regeneration in mature permanent teeth diagnosed with apical periodontitis. The present body of work however focuses on only a single cell-free protocol (Figure 2). Briefly, the root canal would need to be adequately disinfected soon after it is accessed in order to create the pre-requisite endodontic micro-environment that would permit regenerative procedures. Whether this could be achieved without the use of sodium hypochlorite (NaOCl), which has shown to negatively impact biological components at even low concentrations, is currently unknown (Galler et al. 2015, Gonçalves et al. 2016). Thereafter, viable dECMs would need to be solubilised into the lumen of the root canal via irrigation protocols that maintain their integrity and produce a high yield. This has previously been achieved with common chelating agents, namely 17%

ethylenediaminetetraacetic acid (EDTA), irrigant activation with passive ultrasonic techniques and intracanal medicaments such as calcium hydroxide (Graham et al. 2006, Widbiller et al. 2017, Tavares et al. 2021).

Upon release, these solubilised morphogens will then need to be encouraged to egress into periapical tissues, where they can interact and induce their biological effects on resident PL-MSC populations. This could be achieved by way of manual dynamic activation or patency filing. This interaction will also likely require some prior enlargement of the apical foramen after accurately determining its position to the increase surface area of the interface (Laureys et al. 2013). With this approach, multi-visit treatment with calcium hydroxide intracanal medication may serve several functions. Firstly, it could prolong dECM solubilization given the ability of this medicament to also liberate molecules from within dentine (Graham et al. 2006, Galler et al. 2015). Secondly, it could provide additional disinfection and in doing so, potentially compensate for the lack of NaOCl. Thirdly, it could help confirm absence of active disease prior to obturation. Thereafter, appropriate clinical and radiographic examination would be required to monitor periapical healing. It must be stressed however this protocol is at present only speculative and there are several key challenges that require further investigation prior to clinical translation.

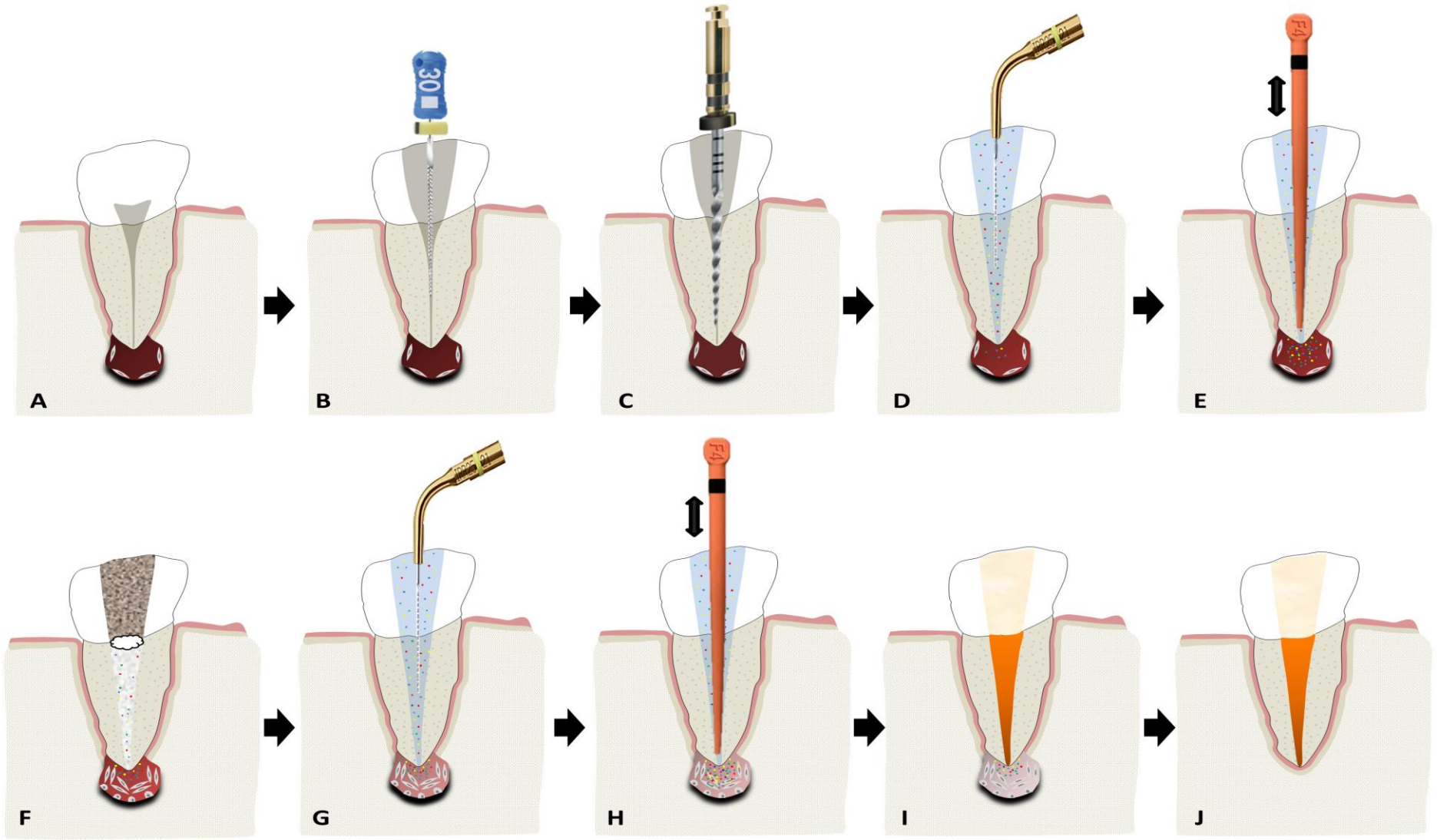

Figure 2

Figure 2: Schematic illustration of the proposed protocol for enhancing periradicular tissue regeneration in mature permanent teeth using endogenous dentine extracellular matrix components (dECM). (A) Single rooted mature permanent tooth diagnosed with apical periodontitis; (B) accessing pulp chamber and conservative pre-enlargement of apical foramen; (C) chemomechanical root canal preparation using chelating agent; (D) passive ultrasonic activation to stimulate dECM release; (E) encourage periapical bioavailability of dECMs; (F) interappointment calcium hydroxide medicament; (G) irrigation and passive ultrasonic activation to release dECMs; (H) manual dynamic activation to encourage periapical bioavailability of dECMs; (I) obturation; (J) annual clinical and radiographic review.

1.7 Challenges to clinical implementation

1.7.1 Irrigation protocol: At present, there is currently no consensus on the ideal irrigant protocol that could achieve adequate disinfection whilst preserving integrity of dECMs and local stem cell populations. The details that require further clarification include the solution types, combinations and strengths; exposure time within the root canal; use of irrigant activation techniques and intracanal medicaments. Interestingly, whilst NaOCl is considered the ideal solution for antimicrobial only approaches, its use is paradoxical to the aims of regenerative endodontic procedures (Galler et al. 2015, Gonçalves et al. 2016). For example, aggressive applications into the root canal certainly fulfils the disinfection criteria but at the cost of the losing integrity of local biological components. This can be attributed to its proteinaceous properties and whilst revitalisation guidelines advocate limited exposure to weaker concentrations, this recommendation is based on the viability of MSCs only (Trevino et al. 2011, Martin et al. 2014) and not preserving the potential potent positive contribution that dentine could provide. Consequentially, it is currently not known how these regimes influence release of bioactive dEMCs. It is therefore apparent that if operators were to administer

NaOCl even in a limited capacity, consideration would need to be taken to mitigate its deleterious effects. Theoretically, this could include enhancing the tubular penetration of demineralising agents to access deeper portions of dentine unaffected by NaOCl or even mechanically removing the affected dentinal substrate itself. These strategies would require a prerequisite understanding of NaOCl's penetrative capabilities. However, should these methods lead to no avail; NaOCl will need to be entirely substituted for alternative antimicrobial strategies. For instance, the thicker and less fragile root canal walls in mature permanent teeth allow larger emphasis to be placed on conventional instrumentation and intracanal medicaments, which have *in vivo* shown a greater contribution to endodontic disinfection than lower concentrations of NaOCl (Windley et al. 2005, Rodríguez-Benítez et al. 2014). Moreover, in recent years, EDTA, which is currently considered a weak antifungal agent (Mohammadi et al. 2013), has been found to destabilise the outer cell membranes of gram-negative bacteria and deteriorate the macrostructures of established biofilms (Finnegan & Percival 2015, de Almeida et al. 2016). Whilst these effects alone may not always induce cell death, they could potentially be enhanced enough to do so when combined with mechanical instrumentation and irrigant agitation techniques in a closed system. The reductions in microbial load achieved through these mechanisms may equate to that of NaOCl and exceed the threshold necessary to control infection whilst preserving the biological components within dentine (Siqueira & Rôças 2008). Further investigations are required to test these theories using a clinically applicable model and develop the appropriate irrigant protocol.

1.7.2 Periradicular bioavailability: The therapeutic use of dECMs for periradicular tissue engineering via the proposed protocol would require these signalling molecules to mobilise to the vicinity of the injured tissues. In mature permanent teeth, the

interface between solubilised growth factors released within the canal space and the target tissues is likely to be restricted by the diameter of the minor apical foramen, which physiological ranges between 0.1 and 0.2 mm (Chapman 1968). It is therefore currently not known if dECMs solubilised within root canals are capable of interacting with the periradicular compartment in these circumstances. Whilst there are recommendations to pre-enlarge this anatomical structure to between 0.5 and 1.0 mm, this could unnecessarily compromise the structural integrity of the mature root and increase risk of chemical and bacterial extrusions reducing overall tooth survival (Kim et al. 2018). Furthermore, these parameters were based on the need for the intraradicular influx of sufficient blood and cellular components for subsequent pulp regeneration, which would require a larger interface than that for extraradicular efflux of dECMs molecules (Fang et al. 2018, Kim et al. 2018). Therefore, further investigations are needed to determine if only minimally pre-enlarging the apical foramen facilitates periradicular bioavailability of dECMs solubilised into the root canal.

1.7.3 Regenerative effects on PL-MSCs: As one of the most recently discovered endodontic niches, PL-MSCs have not been subjected to as much interventional study as other more established dental MSCs. It is therefore currently unknown if they will exhibit similar regenerative responses following topical applications of dECMs as DPSCs, PDLSCs and SHEDs. This is particularly in light of the fact that these cells are isolated from inflamed tissues and have already demonstrated some differences in their stem-like characteristics when compared to their healthy counterparts (Đokić *et al.* 2012). Therefore, further cell culture investigations are required to deduce this information. This preliminary data would provide proof of concept for the aforementioned therapeutic irrigant protocol that promotes release of dECMs during

root canal treatment and be necessary to justify more resource-intensive appropriately powered randomised controlled clinical interventional studies.

1.7.4 Detecting biological changes: The paradigm shift towards more biologically driven endodontic treatment strategies calls for more sophisticated methods to better monitor the periradicular disease status. Traditional methods such as visual assessments for swellings and sinus', tenderness to percussion and palpation or plain film radiographic assessment lack sensitivity, specificity, poorly correlate to the histological status and are too rudimentary to detect subtle changes in the healing processes (Dummer et al. 1980, Klausen et al. 1985). More detailed radiographic examination improve upon this however, multiple exposures are required for temporal assessment that could last for up to four years, at which the patient could be lost to follow up (Lofthag-Hansen et al. 2007). The true effects of novel biologically driven treatment strategies for apical periodontitis may thus go unnoticed. A growing number of clinical studies however have begun sampling and analysing the myriad of analytes within PTF to gain a greater appreciation of various interventions (Shimauchi et al. 1996, Martinho et al. 2016). Whilst this shows proof of concept for local biomarkers within PTF to be harvested, and their diagnostic, prognostic and predictive potential, very little is known about these techniques and there still remains an absence of universal protocols for sampling, analysis and biomarker selection. This could be attributed to the fact that PTF remains one of the only oral exudates that has not been characterized using more contemporary high-throughput methods of proteomic analysis, an approach that better reflects the complex multifaceted nature of periradicular pathophysiology. Such analyses could provide highly sensitive data on the periradicular changes that could occur as a result of the aforementioned protocol (Zehnder & Belibasakis 2022).

1.8 Hypothesis

The overarching hypothesis was that dentine extracellular matrix components can be solubilised via conducive irrigant regimes and made biologically available to local mesenchymal stem cells found within diseased periradicular tissues. These bioactive molecules will initiate regenerative healing mechanisms at a molecular level to improve clinical outcomes of root canal treatment in mature permanent teeth diagnosed with apical periodontitis (Figure 3).

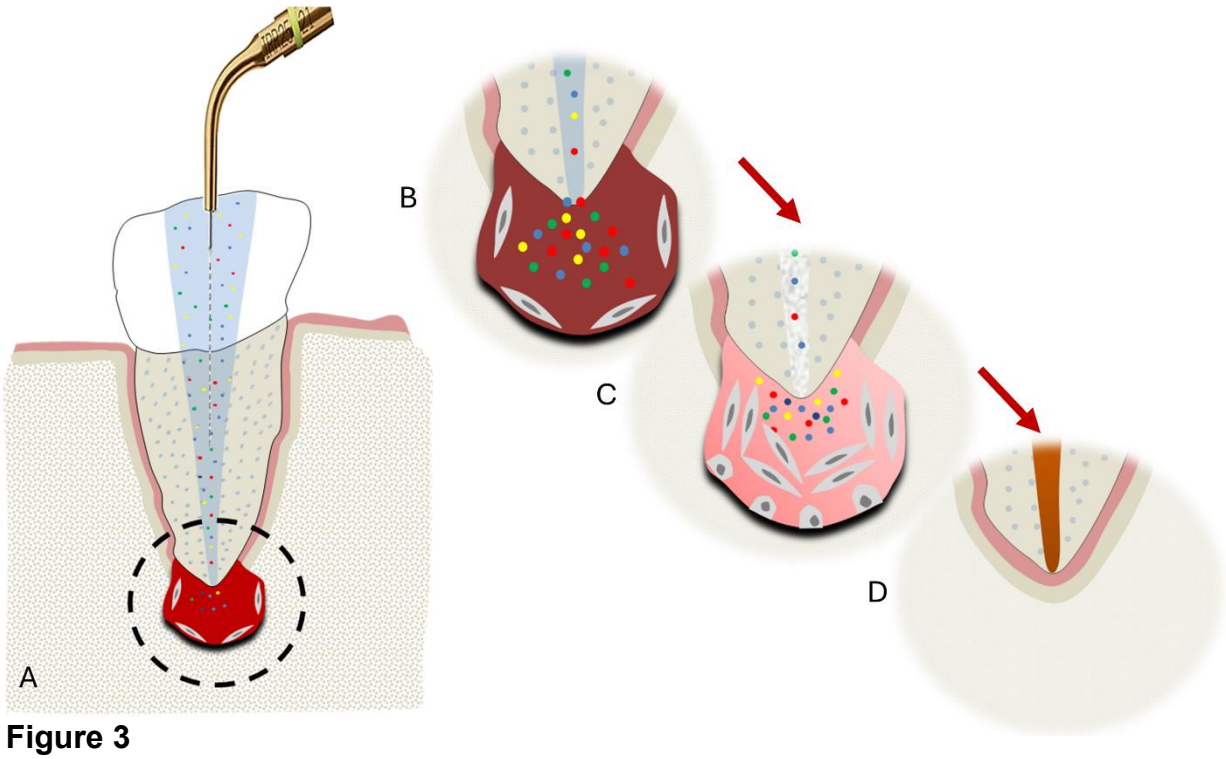

Figure 3

Figure 3: Schematic diagram illustrating the proposed hypothesis. (A) Single rooted mature permanent tooth diagnosed with apical periodontitis. The therapeutic irrigant regime encourages release of dECMs into the canal. (B) dECMs interact with the periradicular tissues via a pre-enlarged apical foramen; (C) dECMs stimulate regenerative events within local populations of PL-MSCs; (D) Leads to improved clinical outcomes.

The individual hypotheses investigated were as follows:

1. Clinically recommended protocols in regenerative endodontics facilitate solubilisation of a range of viable dECMs (Chapter 3)
2. dECMs solubilised into the root canals of mature permanent teeth interact with the periradicular tissues via the apical foramen (Chapter 3)
3. dECMs solubilised with a clinically translatable irrigant regime promote regenerative events in PL-MSCs (Chapter 3)
4. EDTA irrigant regimes have antimicrobial properties when combined with mechanical debridement (Chapter 4)
5. PTF contains a source of biomarkers that could be utilised for monitoring periradicular disease status during root canal treatment? (Chapter 5)
6. The success rates of root canal treatment when conducted with therapeutic irrigant regimes that promote dECM solubilisation are equivalent to conventional irrigant regimes (Chapter 6)
7. Patients undergoing root canal treatment with therapeutic irrigant regimes promoting dECM solubilisation experience less pain than those patients treated with conventional irrigant regimes (Chapter 6)

1.9 Research Questions

The overall research question was that can dECMs be exploited via therapeutic irrigant regimes to induce periradicular healing events. The specific questions that were investigated were as follows:

1. Do clinically recommended protocols in regenerative endodontics facilitate solubilisation of viable dECMs? (Chapter 3)

2. Is there periradicular bioavailability of dECMs when these molecules are solubilised into the root canals of mature permanent teeth? (Chapter 3)
3. What range of dECMs can be detected with a multiplex array on dECMs solubilised with the clinically translatable irrigant regime (Chapter 3)
4. Do dECMs solubilised with a clinically translatable irrigant regime promote regenerative events in PL-MSCs? (Chapter 3)
5. What are the current trends in endodontic irrigation? (Chapter 4)
6. What depth of dentinal tubular penetration is achieved by NaOCl solutions? (Chapter 4)
7. Do EDTA irrigant regimes have any antimicrobial properties when combined with mechanical debridement? (Chapter 4)
8. What are the optimum methods of sampling and analysing PTF-derived biomarkers? (Chapter 5)
9. What are the most reliable PTF-derived biomarkers for monitoring periradicular disease status during root canal treatment? (Chapter 5)
10. What are the success rates of root canal treatment when conducted with therapeutic irrigant regimes that promote dECM solubilisation? (Chapter 6)
11. Do patients undergoing root canal treatment with therapeutic irrigant regimes promoting dECM solubilisation, experience more pain than those patients treated with conventional irrigant regimes? (Chapter 6)

1.9 Research Aims & Objectives

The overarching research aim was to investigate the clinical efficacy of dECMs in inducing periradicular healing events. The specific objectives to be examined included:

1. Determine an optimal and clinically translatable irrigant regime that can be used to solubilise dECMs during root canal treatment (Chapter 3 & 4)
2. Determine the periradicular bioavailability of dECMs in mature permanent teeth to potentially enhance healing of periradicular tissues (Chapter 3)
3. Determine the regenerative effects of dECMs solubilised with optimised irrigant regimes on PL-MSCs (Chapter 3)
4. Determine the optimum method of analysing PTF-derived biomarkers during root canal treatment in mature permanent teeth diagnosed with apical periodontitis (Chapter 5)
5. Compare clinical and patient reported outcomes of root canal treatment when conducted with therapeutic irrigant regimes that promote dECM solubilisation in mature teeth diagnosed with apical periodontitis (Chapter 6)

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CHAPTER 2

PRESENTING THE HYPOTHESIS

Publication 1: Virdee, S. S., Bashir, N., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2022). Exploiting Dentine Matrix Proteins in Cell-Free Approaches for Periradicular Tissue Engineering. *Tissue Engineering. Part B, Reviews*, 28(4), 707–732.

The narrative literature review (Publication 1) presented in this chapter sets out an entirely new concept and approach to treating periradicular disease and the hypothesis of which is investigated in the subsequent thesis. It draws upon findings from a broad volume of literature across the medical and dental disciplines to highlight current pitfalls of antimicrobial only treatment strategies for apical periodontitis; the discovery of a novel niche of endodontic MSCs isolated from apical granulomas; dECMs and their regenerative potential; and how these latter two components could be clinically exploited to upregulate periradicular regenerative events during root canal treatment of mature permanent teeth diagnosed with apical periodontitis. This information is utilised to present a novel clinical protocol that relies on alternative endodontic irrigation regimes to implement such principles. The challenges preventing clinical translation are also discussed, with areas of future research outlined.

CHAPTER 2

PUBLICATION 1

Exploiting Dentine Matrix Proteins in Cell-Free Approaches for Periradicular Tissue Engineering

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Exploiting Dentine Matrix Proteins in Cell-Free Approaches for Periradicular Tissue Engineering

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The recent discovery of mesenchymal stem cells within periapical lesions (PL-MSC) has presented novel opportunities for managing periradicular diseases in adult teeth by way of enhancing tissue regeneration. This discovery coincides with the current paradigm shift toward biologically driven treatment strategies in endodontics, which have typically been reserved for non-vital immature permanent teeth. One such approach that shows promise is utilizing local endogenous non-collagenous dentine extracellular matrix components (dECM) to recruit and upregulate the intrinsic regenerative capacity of PL-MSCs *in situ*. At picogram levels, these morphogens have demonstrated tremendous ability to enhance the cellular activities in *in vitro* and *in vivo* animal studies that would otherwise be necessary for periradicular regeneration. Briefly, these include proliferation, viability, migration, differentiation, and mineralization. Therefore, topical application of dECMs during ortho- or retrograde root canal treatment could potentially enhance and sustain the regenerative mechanisms within diseased periapical tissues that are responsible for attaining favorable clinical and radiographic outcomes. This would provide many advantages when compared with conventional antimicrobial-only therapies for apical periodontitis (AP), which do not directly stimulate healing and have had stagnant success rates over the past five decades despite significant advances in operative techniques. The aim of this narrative review was to present the novel concept of exploiting endogenous dECMs as clinical tools for treating AP in mature permanent teeth. A large scope of literature was summarized to discuss the issues associated with conventional treatment modalities; current knowledge surrounding PL-MSCs; composition of the dECM; inductive potentials of dECM morphogens in other odontogenic stem cell niches; how treatment protocols can be adapted to take advantage of dECMs and PL-MSCs; and finally, the challenges currently impeding successful clinical translation alongside directions for future research.

Keywords: dentine extracellular matrix components, endodontics, regenerative medicine, stem cells, tissue regeneration, wound healing

Impact Statement

Apical periodontitis (AP) is an inflammatory condition that is associated with a great degree of morbidity and ultimately leads to tooth loss. The purpose of this review was to summarize the current evidence pertaining to stem cell therapy in endodontics and present a novel clinical methodology through which they may be utilized to address AP. A comprehensive overview of the basic science, clinical translation, and potential challenges are presented in this review.

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Introduction

Apical periodontitis (AP) is an inflammatory condition of the periodontium that exists when there is a dynamic equilibrium between putative endodontic microorganisms and host defense mechanisms.[1] The ideal objective for treating this disease is to restore architecture and functions of the periradicular tissues that were lost to the immune response. Conventional therapies achieve these outcomes indirectly by reducing the microbial load within infected root canals to create a pro-healing environment.[2] Although this approach may be enough to initiate periapical wound healing, which involves a highly co-ordinated sequence of hemostasis, inflammation, proliferation, and remodeling,[3] it offers no additional stimulus for biological regeneration thereafter.[4]

Unaided, these endogenous processes are often insufficient to achieve complete tissue regeneration and will instead be compensated by reparative scar tissue.[4] Persistent periapical radiolucencies may, therefore represent not only failure to eradicate intraradicular infection but also inadequate physiological regenerative processes, which could explain why larger lesions demonstrate higher treatment failure rates.[5,6] It also suggests that to attain more predictable outcomes, it would be necessary to employ alternative strategies that simultaneously manage the microbial load and directly enhance intrinsic regenerative events within damaged periradicular tissues.

Stem cells are essential to wound-healing processes, as they possess high proliferation rates, self-renewal capabilities, and potential for multi-lineage differentiation.[7,8] Embryonic stem cells are pluripotent, as they can develop into stromal cells from any of the three germinal layers whereas multipotent postnatal stem cells are more restricted to organ-specific lineages.[9] The latter are more amenable to clinical translation due to their autologous nature and presence within almost all adult tissues.[10] A subset of multipotent progenitors derived from the mesoderm germ layer, called ''mesenchymal stem cells'' (MSC), has attracted particular interest within regenerative endodontics as they can give rise to several mineral producing mesoderm lineages, including bone (Fig. 1).[11]

Moreover; although they are known to be harvested from bone marrow, other reservoirs have been isolated from within the pulp and associated periodontal tissues of permanent and deciduous teeth.[9,12,13] Named according to their tissue of origin, these ''dental MSC'' niches include ''dental pulp stem cells'' (DPSC), ''stem cells from human exfoliated deciduous teeth'' (SHED), ''periodontal ligament stem cells'' (PDLSC), ''dental follicle precursor cells'' (DFPC), ''stem cells of the apical papilla'' (SCAP), ''gingival MSCs,'' ''alveolar bone MSCs,'' and ''tooth germ progenitor cells.''[9,12] When transplanted into *in vivo* human and animal models, these dental MSCs have demonstrated a potent capacity to regenerate pulp-like tissue in empty root canals,[14–16] dentine-like tissues in endodontic perforation defects,[17] and periodontal tissues in surgically created periodontal defects.[18–20]

Further, the positive outcomes revealed from their applications to other non dento-alveolar tissues, including the treatment of autoimmune, cardiovascular, endocrine, hepatic, musculoskeletal, neurodegenerative, ophthalmic, dermatological, and respiratory diseases, confirm their potential to be utilized as powerful therapeutic tools (Supplementary Table S1). Recent studies, however, have identified another clinically accessible dental MSC population directly within the inflamed periradicular tissues of infected mature permanent teeth.[21,22] These periapical lesion-derived MSCs (PL-MSC) possess tremendous immunosuppressive and regenerative potential and could, therefore, provide exciting opportunities to develop therapies for AP that actively engage with the endogenous mechanisms of periradicular tissue regeneration.

The cellular events required for periradicular regeneration are co-ordinated by various growth factors, cytokines, chemokines, and angiogenic and neurotrophic signaling molecules.[23] Noteworthy examples include members of the transforming growth factor-beta (TGF-β), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and insulin growth factor (IGF) families, among many others.[24] Although these polypeptides are endogenously secreted by host cells at the site of disease, they rapidly deplete due to their relatively short half-life within the extracellular environment.[23] Fortunately, abundant reservoirs of these molecules are locally sequestered within the dentine's extracellular matrix.[25] They are deposited by secreting odontoblasts during dentinogenesis and become fossilized during subsequent mineralization.

Thereafter, their bioactivity remains highly preserved through the formation of proteoglycan bonds but these can be immediately reinstated on release.[26,27] This has previously been achieved on command through demineralizing irrigants,[28–30] pulp capping agents,[31–33] epigenetic modifiers,[34] and dental adhesives.[35] The resulting extracts, formally termed ''dentine extracellular matrix components'' (dECM), have demonstrated a potent capacity to upregulate regenerative events within various odontogenic MSC niches.[33,36,37] It is, therefore, plausible to expose PL-MSCs *in situ* to this cocktail of bioactive molecules to enhance local tissue healing. This approach could overcome current limitations associated with conventional treatments for AP and provide clinicians with unique capabilities to actively apply a biologically driven therapy to the diseased periradicular tissues.

The aim of this narrative review was to explore the novel concept of exploiting endogenous dECMs to upregulate local MSC-mediated periradicular tissue regeneration in mature permanent teeth diagnosed with AP. All abbreviations used in this article are provided in Table 1.

Current Issues Associated with Conventional Root Canal Therapy

Kakehashi *et al.* confirmed a direct causal relationship between putative endodontic microorganisms and periapical disease.[38] Consequentially, therapeutic strategies for AP have focused exclusively on disinfecting necrotic root canals with the aim of relieving clinical signs and symptoms of inflammatory disease, preventing systemic bacterial spread, and ultimately retaining natural and functioning teeth.[2] These outcomes are typically achieved through the use of antimicrobial solutions, primarily sodium hypochlorite (NaOCl), which possesses potent bactericidal and proteonacious properties, in conjunction with canal enlarging instruments.[39]

Significant advances in the chemo-mechanical debriding armamentarium have been made over the past 50 years, with some of the most revolutionary developments including highly

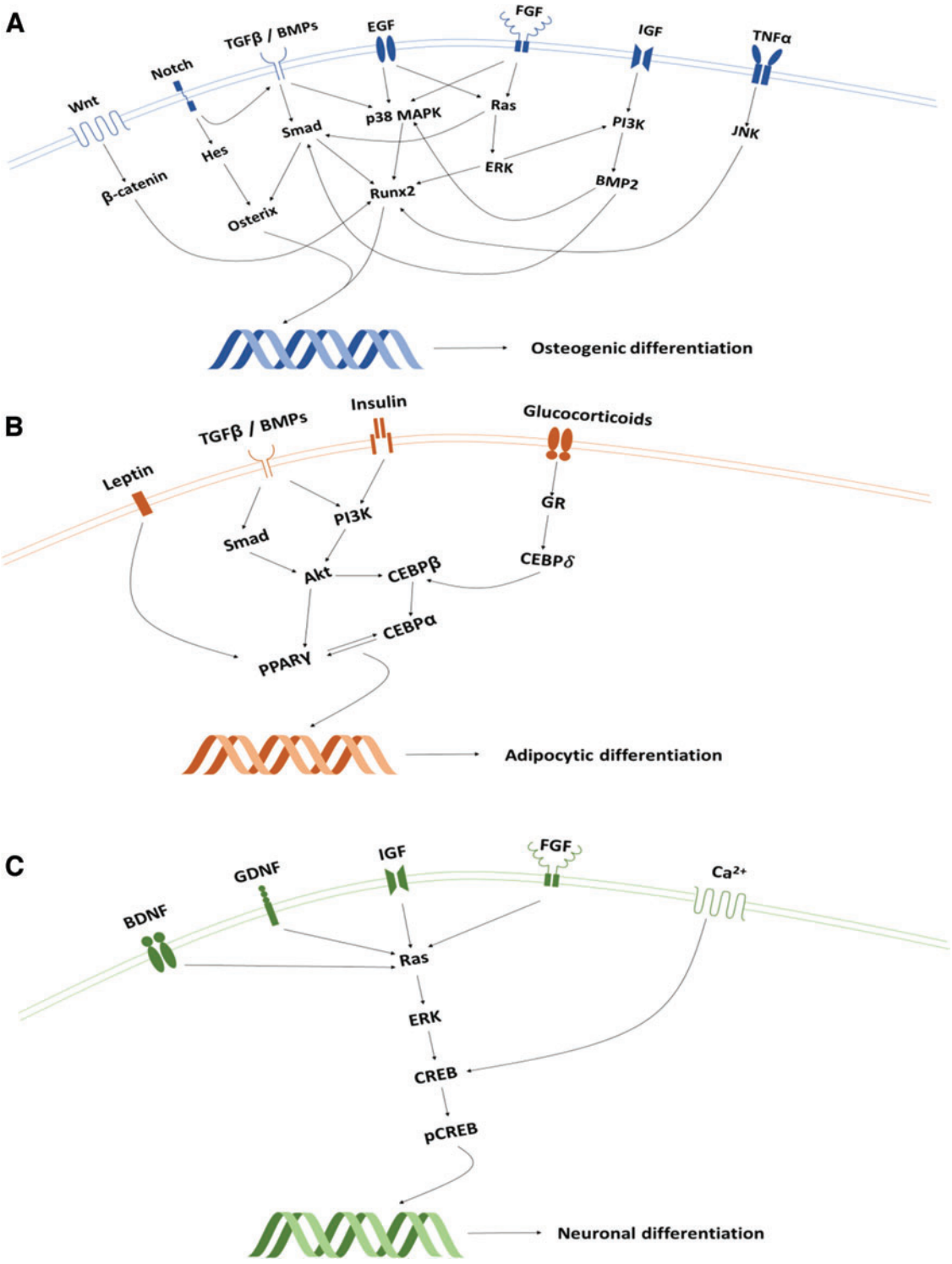

FIG. 1. (**A–C**) A schematic illustration of osteogenic, adipocytic, and neuronal differentiation pathways in mesenchymal stem cells. Akt, protein kinase B; BDNF, brain-derived neurotrophic factor; BMP, bone matrix protein; Ca^{2+}, calcium ions; CEBP, enhancer binding protein; CREB, cAMP response element-binding protein; EGF, epithelial growth factor; ERK, extracellular signal-regulated kinases; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; GR, glucocorticoid receptor; HES, hairy and enhancer of split-1; IGF, insulin-like growth factor; Jnk, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; Runx2, runt-related transcription factor 2; TGFβ, transforming growth factor beta; TNF-α, tumour necrosis factor-alpha; Wnt, wingless/integrated. Color images are available online.

Table 1. Definitions of Abbreviations Found in Text

Abbreviation	*Definition*
AP	Apical periodontitis
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic proteins
BSP	Bone sialoprotein
CD	Cluster of differentiation
CXCR4	Chemokine receptor type 4
dECM	Dentine extracellular matrix components
DFPC	Dental follicle precursor cells
DMP-1	Dentine matrix protein 1
DPP	Dentine phosphoprotein
DPSC	Dental pulp stem cells
DSPP	Dentin sialophosphoprotein
EDTA	Ethylenediaminetetraacetic acid
ESE	European Society of Endodontology
FGF	Fibroblast growth factors
HGF	Hepatocyte growth factor
IGF	Insulin growth factor
IL	Interleukin
MEPE	Matrix extracellular phosphoglycoprotein
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem cells
NaOCl	Sodium hypochlorite
NGF	Nerve growth factor
NT3	Neurotrophin 3
NT4	Neurotrophin 4
OCN	Osteocalcin
ON	Osteonectin
OPN	Osteopontin
PDGF	Platelet-derived growth factor
PDLSC	Periodontal ligament stem cell
PlGF	Placental-derived growth factor
PL-MSC	Periapical lesion-derived mesenchymal stem cell
RUNX2/ CBFA1	Runt-related transcription factor 2
SCAP	Stem cells of the apical papilla
SDF-1	Stromal-derived factor 1
SHED	Stem cells from human exfoliated deciduous teeth
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitors of matrix metalloproteinases
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factors

flexible rotary/reciprocating file systems and machine-assisted irrigant agitation techniques. When compared with more conventional approaches, these now widely used practices facilitate deeper irrigant penetration into root dentine,[40] greater intracanal debris and smear layer removal,[41] and reductions in endodontic bacterial load and viability.[42,43] It is, therefore, apparent that the operator's ability to disinfect root canals has significantly improved since the fundamental principles of endodontic therapy were first established.

Unfortunately, the aforementioned progress has not translated into improved clinical outcomes as success rates for root canal treatment have remained static for five decades. For instance, a systematic review by Ng *et al.* (2007) revealed that pooled success rates of all prior observational studies at 1 year follow-up ranged between 68% and 85% according to strict plain-film radiographic criteria.[44] Thereafter, several prospective cohort studies reported comparable results of 83.0% (2–4 year follow-up) and 82.7% (5 year follow-up),[5,6] with those reviewing patients over longer periods revealing even less favorable outcomes of 65.3% (20 year follow-up).[45] Therefore, one out of five teeth with primary AP will in the short to medium term fail to heal after root canal treatment and eventually require more complicated and invasive remedial therapy.

Moreover, these figures likely underestimate the true incidence of treatment failure, as plain-film radiographs lack sensitivity for detecting periapical pathosis when compared with three-dimensional imaging techniques.[46,47]

Another issue is that microorganisms, and their by-products, cannot be completely eradicated from root canal systems due to complicated anatomy.[48] Even root-filled teeth exhibiting no clinical or radiographic signs of AP harbor vital bacteria.[49,50] Several inferences can be drawn from this finding. First, the ultimate objective of conventional approaches may be too idealistic, as residual bacteria are postoperatively unavoidable. Second, below a certain microbial load host mechanisms are capable of initiating, but not necessarily sustaining, regenerative events. Third, after surpassing this critical threshold, further disinfection provides no additional stimulus for endogenous periapical healing. These concepts are supported by several robust clinical investigations by Paredes-Vieyra *et al.*, Liang *et al.*, and Verma *et al.* who, respectively, demonstrated that intracanal medicaments, irrigant agitation techniques, and concentrated NaOCl solutions do not increase treatment success when compared with less aggressive disinfection protocols.[51–53]

It can be surmised that the effects of antimicrobial-only approaches on periradicular healing are finite and alternative methods, designed to initiate and sustain tissue healing, may yield more predictable outcomes. It must be stressed, however, that adequate endodontic disinfection still remains a fundamental prerequisite to provide an adequate microenvironment for any tissue repair strategy.

Periapical Lesion-Derived MSCs

In 2004, Maeda *et al.* successfully isolated ''fibroblastic cells'' from within the inflamed periradicular granulation tissues of mature infected teeth.[54] Thereafter, Liao *et al.*, Đokić *et al.*, and Marrelli *et al. in vitro* all confirmed their highly proliferative, multipotent, and clonogenic properties.[21,55,56] Further, mesenchymal surface markers, Cluster of Differentiation [CD]-13, -29, -44, -73, -90, -105, and -166 were highly expressed; whereas hematopoietic markers, namely CD-14, -19, -34, -45, and human leukocyte antigen-DR isotype, were not.[21,55–61] These characteristics fulfilled the minimum criteria necessary for this population to be recognized as a distinct MSC niche. Although many terms have been used to refer to this group, ''PL-MSCs'' is considered most accurate in the absence of explicit histological diagnoses and thus is the preferred designation (Table 2).

Figure 2 outlines preliminary data on the multipotent potential of primary PL-MSCs cultured from the apical granulomas of extracted teeth diagnosed with AP.

Clinical observations of periradicular regeneration after endodontic therapy indicate that PL-MSCs primarily contribute to local intrinsic periapical wound-healing processes. This is supported by *in vitro* investigations confirming that

Table 2. Key Characteristics of Periapical Lesion-Derived Mesenchymal Stem Cells

	Immunophenotype		*Proliferation*		*Differentiation markers*				
Aliases	*Positive*	*Negative*	*Niche*	*Rate*	*Cell type*	*Genetic*	*Staining*	*Mineralisation*	*Immunoregulatory effects*
Granulation tissue-derived stem cells Human fibroblastic cells Human periapical cyst-derived mesenchymal stem cells Inflamed periapical progenitor cells Periapical lesion-derived stem cells	CD-13, CD-29, CD-44, CD-46, CD-73, CD-90, CD-105, CD-146, CD-166, Stro-1	CD-14, CD-19, CD-34, CD-45, HLA-DR	DFPC DPSC PDLSC PL-MSC SCAP SHED	++ ++ +++ + +++ ++	Odontoblast Osteoblast Cementoblast Chondrocyte Adipocyte Astrocyte	*DMP-1, DSSP* *ALP, BSP, MEPE, ON, OPN, RunX2/ Cbfa1* *BSP, OCN, OPN* — *ADIPOQ, GLUT-4, LPL, PPARγ* *DAT, En1, Foxa2, GFAP, MAP2, MSX1, NF-H, NF-M, Nurr1, Pitx3, TH, β-III tubulin*	Alizarin Red S Alizarin Red S Alizarin Red S Alcian Blue Oil Red O —	Calcific Tissues Fibrous Tissues	Increases leukocytic production of TGF-β Inhibits differentiation of dendritic cells Reduces leukocytic production of IL-1β, -2, -5, -6, TNF-α, and IFN-γ Reduces leukocytic proliferation

ADIPOQ, adiponectin; ALP, alkaline phosphatase; BSP, bone sialoprotein; CD, cluster of differentiation; DAT, dopamine transporter; DFPC, dental follicle precursor cells; DMP-1, dentine matrix protein 1; DPSC, dental pulp stem cell; DSSP, dentin sialophosphoprotein; En1, engrailed-1; Foxa2, forkhead box protein A2; GFAP, glial fibrillary acidic protein; GLUT-4, glucose transporter type 4; HLA-DR, human leukocyte antigen-DR isotype; IFN-γ, interferon gamma; IL, interleukin; LPL, lipoprotein lipase; MAP2, microtubule-associated protein 2; MEPE, matrix extracellular phosphoglycoprotein; MSX1, msh homeobox 1; NF-H, neurofilaments heavy; NF-M, neurofilaments medium; Nurr1, nuclear receptor related 1 protein; OCN, osteocalcin; ON, osteonectin; OPN, osteopontin; PDLSC, periodontal ligament stem cell; Pitx3, paired-like homeodomain transcription factor 3; PL-MSC, periapical lesion-derived stem cell; PPARγ, peroxisome proliferator-activated receptor gamma; RunX2/Cbfa1, runt-related transcription factor 2; SCAP, stem cells of the apical papilla; SHED, stem cells from human exfoliated deciduous teeth; TGF-β, transforming growth factor-beta; TH, tyrosine hydroxylase; TNF-α, tumour necrosis factor alpha; +, low; ++, medium; +++, high.

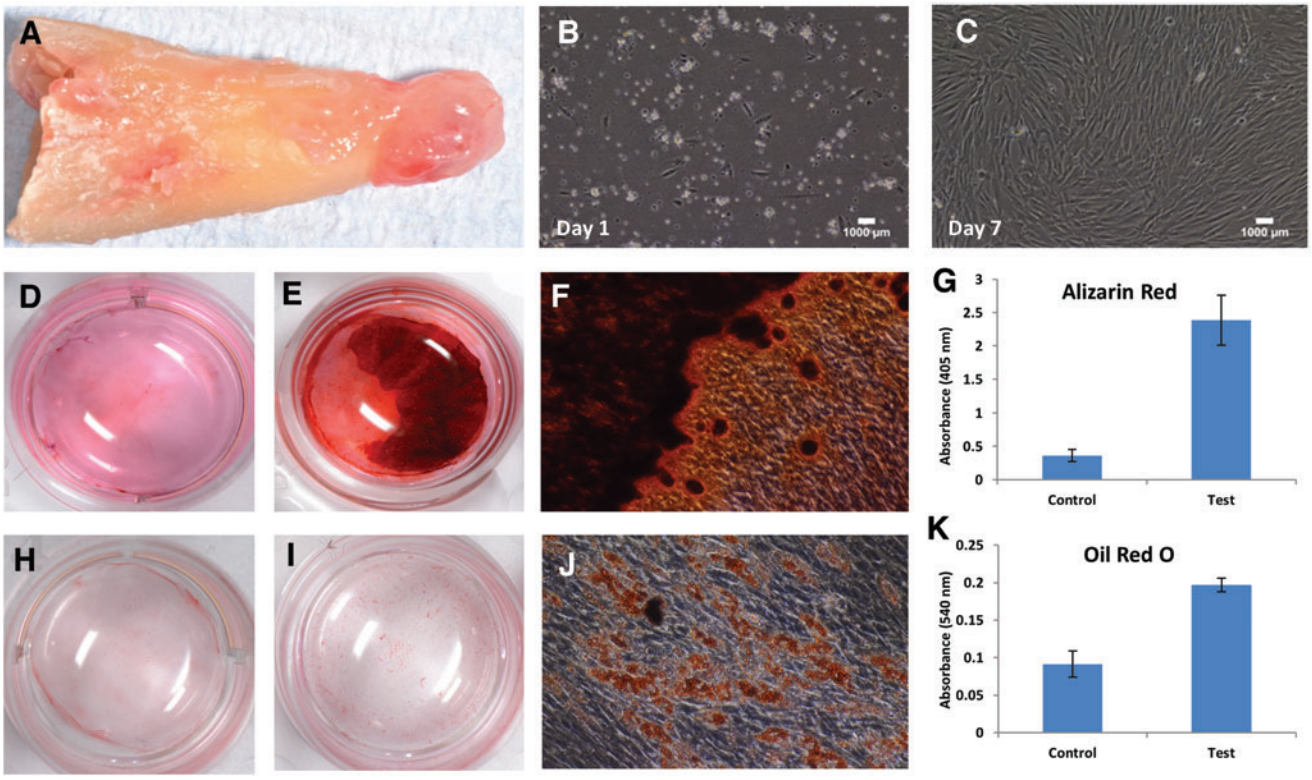

FIG. 2. Multipotent potential of primary PL-MSCs. **(A)** PL-MSCs were isolated from the apical granuloma of extracted teeth via a collagenase type 1 enzyme digestion technique. Cells were cultured in a T25 flask with 20% fetal bovine serum supplemented α-MEM media, which was changed every 2 days. **(B, C)** Phase-contrast microscopy at 10× magnification of PL-MSC cultures at day 1 **(B)** and day 7 **(C)**. **(D–G)** Osteogenic differentiation after 21 days of culture with control or osteogenic induction media (α-MEM, 20% FBS, 1% penicillin/streptomycin, 2 mM glutamine, 0.2 mM ascorbic acid, 100 nm dexamethasone, 10 mM β-glycerophosphate). Staining with Alizarin Red S confirmed absence in control wells **(D)** and presence in test wells of mineral deposits **(E, F)**. Staining was solubilised with 10% acetic acid, and subsequent intensity was quantified by using a microplate reader with an excitation wavelength set at 405 nm **(G)**. **(H–K)** Adipogenic differentiation after 21 days of culture with control or adipogenic induction media (α-MEM, 20% FBS, 1% penicillin/streptomycin, 2 mM glutamine, 0.5 mM IBMX, 200 µM indomethacin, 10 µM insulin, 1 µM dexamethasone). Staining with Oil Red O confirmed absence in control wells **(H)** and presence in test wells of lipid droplets **(I, J)**. Staining was solubilized with isopropanol, and subsequent intensity was quantified by using a microplate reader with an excitation wavelength set at 540 nm **(K)**. All experiments were conducted up to passage 2 by using three biological replicates. Scale bars represent 1000 µm. Color images are available online.

these cells possess the necessary capabilities to restore such tissues. For instance, with appropriate cues PL-MSCs differentiate into osteoblasts, cementoblasts, adipocytes, astrocytes, and chondrocytes, all of which are relevant for regenerating the periodontium.[54–56,58]

In addition, when compared with other odontogenic MSCs, these multipotent properties are more directed toward osteogenesis.[55,62] This was demonstrated through gene expression analyses where upon osteogenic induction, PL-MSCs exhibited transcriptional profiles more indicative of osteogenic differentiation than DPSCs (osteonectin [*ON*], bone sialoprotein [*BSP*], runt-related-transcription-factor 2 [*RUNX2/CBFA1*]), which instead greatly expressed odontogenic markers (dentin sialophosphoprotein [*DSPP*], dentine matrix protein [*DMP*]-1).[62] The mineralization needed for these cells to be considered functional has also been confirmed through several *in vitro* differentiation assays,[55,56,60] as well as *in vivo* subcutaneous implantation mouse models.[21]

Such stem-like characteristics, however, do vary with CD146-positive PL-MSC subpopulations exhibiting lower proliferative, clonogenic, and osteogenic potential than CD146-negative subpopulations.[59] These properties may also be dampened by the inflammatory microenvironment, as indicated by weaker proliferation rates when compared with healthy DPSCs and PDLSCs.[21,55,63]

Stem cells from periradicular lesions also possess immunomodulatory properties. For instance, Đokić *et al.* initially demonstrated that PL-MSC co-cultures significantly reduced leukocytic proliferation, differentiation, and pro-osteoclastic cytokine production (Interleukin [IL]-1β, -2, -5, -6, tumor necrosis factor [TNF]-α, Interferon-γ), while simultaneously increasing anti-inflammatory growth factor secretion (TGF-β).[55,57] These results were corroborated by Araujo-Pires *et al.*, who *in vivo* detected a converse immunological profile in Chemokine Receptor Type [CXCR]4 knockout mice and higher expression of transcriptional markers for MSC mobilization (CD-29, -44, -73, CXCR4), differentiation (NANOG, Stro-1), and transmigration (CD-106, -166) within chronic, as opposed to acute, human periapical granulomas.[22]

More recently, Estrela *et al.* also observed a higher presence of MSCs within stable periradicular lesions. Collectively, these findings suggest that the immunosuppressive properties of PL-MSCs actively contribute to arresting progression of periapical diseases.[64]

Overall, the study investigations described earlier highlight the tremendous regenerative and immunomodulatory capabilities of PL-MSCs. They lay a strong foundation for preclinical *in vivo* studies, which should be performed, that explore their therapeutic potentials. Dentoalveolar, neurodegenerative, and skeletal diseases may particularly benefit from advances in this area due to the enhanced neurogenic and osteogenic commitment of this niche.[58,61,62,65] Moreover, the immunomodulatory and mineralized regenerative properties demonstrated by PL-MSCs *in vitro* and *in vivo*, respectively, indicate that these cells are modulators of the periapical lesion healing process and thus making them ideal targets in novel tissue regeneration strategies for AP.[21] One such approach would involve enhancing their regenerative capacity *in situ* by liberating endogenous signaling molecules from within the dentine's extracellular matrix.

dECM Components

More than 280 bioactive molecules have been identified within demineralized dentine.[66,67] A vast majority of these are non-collagenous extracellular matrix proteins,[66] which comprise $\sim$10% of the dentine's organic phase and are considered crucial for dentinogenesis.[68] Growth factors constitute large proportions of this cohort and have been implicated in regulating dentine-pulp reparative and regenerative responses. Members of the TGF-β, BMP, VEGF, FGF, IGF, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), placental-derived growth factor (PlGF), epidermal growth factor, and adrenomedullin families are frequently detected, with TGF-β1 often found in the greatest abundance.[28,29,33,34,69–71]

Several of these, namely VEGFs, FGFs, PDGFs, and PlGFs, are also known mediators of angiogenesis, which is a critical wound-healing process involving the formation of new blood vessels.[72,73] Closely associated with these are neurotrophic factors that are responsible for developing intricate innervations within the dentin-pulp complex.[74] Isolated examples include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 and 4 (NT3/NT4), and glial cell–line derived neurotrophic factor.[75] Further, a broad range of pro- and anti-inflammatory cytokines, namely IL-1α, -1β, -4, -6, -8, -10, -12, and granulocyte-macrophage colony-stimulating factor, have also been detected within solubilized dECMs.[32,76] These NF-κB signaling molecules likely contribute to immunoregulatory pulp mechanisms, as indicated by their capacity to induce a wide array of inflammatory events.[77]

Other non-collagenous protein families released from the dentine matrix are those associated with regulating mineralization and maturation processes of human calcified tissues.[78] Briefly, these include small integrin-binding ligand n-linked glycoproteins (*DMP-1*, *BSP*, osteopontin [*OPN*], dentine phosphoprotein [*DPP*], dentine sialoprotein, dentine glycoprotein, matrix-extracellular-phosphoglycoprotein [*MEPE*]); vitamin K-dependent glycoproteins (osteocalcin [*OCN*]); small leukine-rich proteoglycans (decorin, biglycan, fibromodulin, lumican, osteoadherin); secretory calcium-binding phosphoproteins (*ON*); and large aggregating proteoglycans (versican).

Many of these require enzymatic activation and therefore it is not unexpected that the dentine substrate also contains matrix metalloproteinases ([MMP]-2, -3, -8, -9, -20) and tissue inhibitors of MMPs ([TIMP]-1,-2),[79–82] which also regulate extracellular matrix remodeling. Although serum proteins (albumin, Immunoglobulin-A, -M, Transferin, Fetuin-A) are also present, currently their functions are unknown.[83,84]

Given what has been cited earlier, dentine can no longer be considered an inert structural tissue but instead, a reservoir of potentially exploitable therapeutic auto- and paracrine cell-signaling molecules that resembles other connective tissues such as bone.[85] Figure 3 represents the results of a broad human anti-body array conducted by our own research group on lyophilized dECM components extracted from dentine powder using ethylenediaminetetraacetic acid (EDTA). The endogenous nature of these morphogens overcomes many ethical issues associated with clinically using exogenous substitutes and the synergistic activity within solubilized dECMs; it exhibits a greater potency than single recombinant molecules.[37,86,87] For these reasons, dECM extracts have been extensively studied for their ability to initiate regenerative events within various oral and dental MSC niches.

Effects of dECM Components on Dental Stem Cells

The effects of dECM application on dental MSC niches are summarized in Table 3.

Migration

Dentine matrix components have demonstrated chemotactic properties *in vitro* via transwell migration, matrigel invasion, and scratch wound assays.[33,90,92] When solubilized, these extracts exhibit considerable potency with DPSC recruitment occurring at just picogram levels.[37] Moreover, root segments pre-conditioned with demineralizing agents induce similar migratory effects in DPSCs and SHEDs, which contrasts the relatively inert properties of their deproteinized counterparts.[29,98,99] Other *in vitro* studies using single recombinant growth factors indicate that these properties can be attributed to the presence of known chemoattractants such as TGF-β1, HGF, and FGF.[29,71,106,112,116]

Proliferation

Solubilized dECMs induce time- and dose-dependent MSC proliferation. These properties, however, are observed only up to a critical threshold, after which anti-mitogenic events become apparent. For example, dECM applications less than $100\,\mu g mL^{-1}$ enhance DPSC proliferation *in vitro*,[33,87,91,92] whereas greater concentrations inhibit further growth.[36,37] This observation, which is also witnessed in endothelial cell cultures and angiogenic tube formation assays,[73] could be explained as being the net outcome induced by various molecules within dECM extracts. Some constituents, namely TGF-β1,[117,119] inhibit proliferation in several cell types but may also attenuate effects of other stimulatory growth factors such as

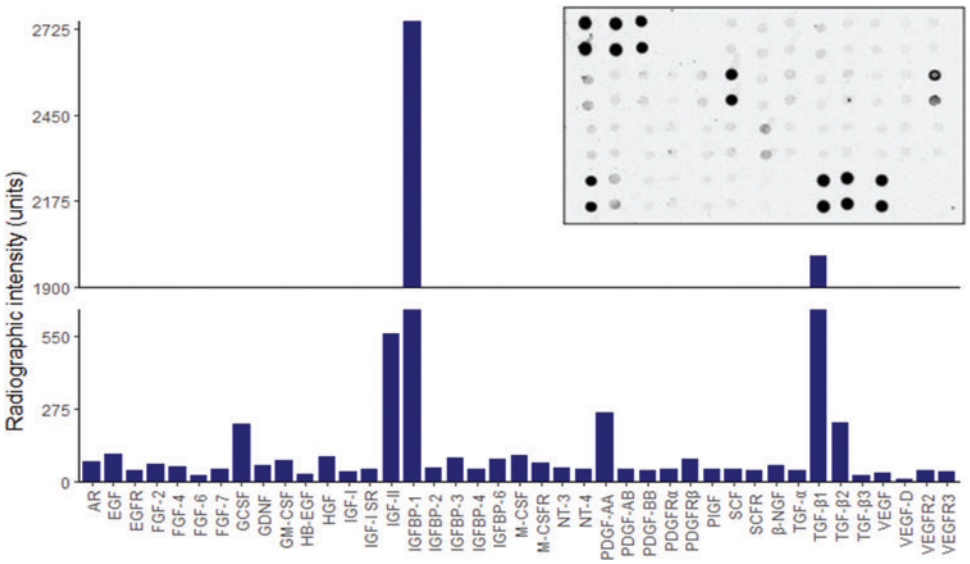

FIG. 3. Human growth factor anti-body array of lyophilized dECM components extracted from dentine powder using 10% EDTA. A semi-quantitative autoradiographic image analysis technique was used to determine the relative radiographic intensity for a total of 41 different cytokines. A representative autoradiographic image is displayed in the top left corner. AR, amphiregulin; EGFR, epidermal growth factor receptor; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB-EGF, heparin-binding epidermal growth factor; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor binding protein; M-CSF, macrophage colony-stimulating factor; M-CSFR, macrophage colony-stimulating factor receptor; NGF, nerve growth factor; NT, neurotrophin; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PlGF, placental growth factor; SCF, stem cell factor; SCFR, stem cell factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor. Color images are available online.

MEPE, PDGF, VEGF, IGF, and FGF.[102,104,105,107,113,115,116] Moreover, dECM-induced terminal differentiation may further contribute to reducing cell numbers over time.[36]

Apoptosis

Solubilized extracts induce limited apoptotic effects in MSCs.[37,87,90,98] Higher dECM concentrations have even been found to aid DPSC viability, as indicated by reduced caspase-3 activity and increased serine threonine kinase gene expression.[87] This could be accredited to dentinal morphogens that possess anti-apoptotic potential such as DPPs and PDGF, which activate downstream signaling cascades for cell survival.[120,121]

Differentiation

Numerous *in vitro* studies using DPSCs, SCAPs, and SHEDs indicate that dECM extracts are powerful inducers of osteo- and odontoblastic differentiation. For instance, topical applications stimulate organization and formation of elongated cellular processes that extend into tubules of preconditioned dentine disks.[88,89,97,98] This is accompanied by significant increases in mRNA expression for genes characteristic of odonto- and osteogenic commitment. These include *DSPP*, *DMP-1*, *OPN*, *OCN*, *BSP*, *RUNX2/CBFA1*, *MEPE*, type 1 collagen, alkaline phosphatase, distal-less homeobox 5, and msh homeobox 2.[36,37,87,92,96,97]

In addition, when DPSCs and SHEDs are implanted subcutaneously alongside dECMs, differentiation events still transpire.[93–95,100,101,118] Dentine-derived BMP-2, in particular, is essential in this process, as is demonstrated when blockade of BMP-2 signals, which are otherwise transduced down osteogenic smad-1/5/8 and *p*38 mitogen-activated-protein-kinase pathways, inhibited odontoblastic gene expression in SHEDs.[96] Further, many studies using recombinant growth factors continue to display the potent differentiating activity of BMP-2.[103,107–109] Nevertheless, other dentine morphogens that may act concomitantly include TGF-β1, although it exhibits suppressive effects via smad-3 dependent mechanisms in SCAPs; PDGF; FGF; BMP-4; IGF; HGF; VEGF; NGF; BDNF; NT3; NT4; MEPE; and TNF-α.[71,102,103,105,107,110,111,113–115,122]

Mineralization

Colorimetric methods for calcium quantification demonstrate that dECMs significantly accelerate mineralised matrix production within MSCs.[37,84,87,88,92] Calcified nodules indicating functioning osteo- and odontoblasts can be visually observed as early as 5 days post-exposure and become more prominent thereafter.[87] When tested *in vivo,* using subcutaneous implantation models, this deposition leads to osseous, dentinal, and collagenous-like tissue formation.[93–95,100,114] This feature can be ascribed to the ability of dentinal morphogens to upregulate genes that code for extracellular matrix protein production in teeth and bone.

Overall, dECMs possess bioactive properties that, if applied to PL-MSCs, could be of clinical utility for periradicular tissue regeneration.

Potential Therapeutic Approach

The principles underlying cell-free homing techniques, where MSCs are recruited and stimulated *in situ* by supplying damaged tissues with signaling molecules,[13] could be utilized to exploit endogenous dECMs for the treatment

Table 3. Regenerative Effects of Dentine Extracellular Matrix Component on Odontogenic Stem Cell Niches

Study		*dECMs*			*MSC*							
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Bégue-Kirn *et al.*[86]	*In vitro*	Solubilized	Human	—	Mouse	SCAP				+		Solubilized dECMs increased differentiation in SCAPs
Liu *et al.*[88]	*In vitro*	Solubilized	Porcine	—	Human	DPSC				+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs
Chun *et al.* [89]	*In vitro*	Solubilized	Human	—	Human	DPSC				+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs
Smith *et al.*[90]	*In vitro*	Solubilized	Bovine	—	Rodent	DPSC		/	+			Solubilized dECMs increased migration in DPSCs but did not affect viability
Lee *et al.*[87]	*In vitro*	Solubilized	Human	—	Human	DPSC	+	+	/	+	+	Solubilized dECMs increased proliferation (dose-dependent), viability, differentiation, and mineralization in DPSCs but did not affect migration
Sadaghiani *et al.*[36]	*In vitro*	Solubilized	Human	—	Human	DPSC	-			+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs but decreased proliferation
Tabatabaei & Torshabi[91]	*In vitro*	Solubilized	Human	—	Human	DPSC	+					Solubilized dECMs increased proliferation (dose-dependent) in DPSCs

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*								
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Tomson *et al.*[33]	*In vitro*	Solubilized	Human	—	Rodent	DPSC	+		+			Solubilized dECMs increased proliferation (dose-dependent) and migration in DPSCs
Okamoto *et al.*[92]	*In vitro*	Solubilized	Human	—	Rodent	DPSC	+		+	+	+	Solubilized dECMs increased proliferation, migration, differentiation, and mineralization in DPSCs
Widbiller *et al.*[95]	*In vitro*	Solubilized	Human	—	Human	DPSC	-	/	+	+	+	Solubilized dECMs increased migration, differentiation, and mineralization in DPSCs, did not affect viability, and decreased proliferation (dose-dependent)
Smith *et al.*[93]	*In vivo*	Solubilized	Rabbit	—	Ferret	DPSC				+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs
Tziafas *et al.*[94]	*In vivo*	Solubilized	Rabbit	—	Dog	DPSC				+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs
Chun *et al.*[89]	*In vivo*	Solubilized	Human	—	Human	DPSC				+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs
Widbiller *et al.*[37]	*In vivo*	Solubilized	Human	—	Human	DPSC				+	+	Solubilized dECMs increased differentiation and mineralization in DPSCs

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*							
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Casagrande *et al.*[96]	*In vitro*	Disk/ Slice	Human	—	Human	SHED	/			+		Dentine disks/ slices preconditioned with 10% EDTA increased differentiation in DPSCs but did not affect proliferation
Pang *et al.*[97]	*In vitro*	Disk/Slice	Human	—	Human	DPSC				+	+	Dentine disks/slices preconditioned with 17% EDTA increased differentiation and mineralization in DPSCs
Galler *et al.*[98]	*In vitro*	Disk/Slice	Human	—	Human	DPSC		/	+	+		Dentine disks/slices preconditioned with 10% EDTA increased differentiation and mineralization in DPSCs but did not affect viability
Gonçalves *et al.*[99]	*In vitro*	Disk/Slice	Human	—	Human	SHED			+			Dentine disks/slices preconditioned with 10% EDTA, 2.5% NaOCl and PBS increased migration in SHEDs
Sadaghiani *et al.*[36]	*In vitro*	Disk/Slice	Human	—	Human	DPSC				+		Dentine disks/slices preconditioned with 10% EDTA and 10% citric acid increased differentiation in DPSCs
Zeng *et al.*[29]	*In vitro*	Disk/Slice	Human	—	Human	DPSC			+			Dentine disks/slices preconditioned with 1.5% NaOCl followed by 17% EDTA increased migration in DPSCs

(*continued*)

Table 3. (Continued)

Study		dECMs			MSC								
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Chae *et al.*[30]	*In vitro*	Disk/Slice	Human	—	Human	SCAP		/				Dentine disks/slices preconditioned with saline, 17% EDTA, 10% citric acid, and 37% phosphoric acid did not affect viability in DPSC
Sakai *et al.*[100]	*In vivo*	Disk/Slice	Human	—	Human	SHED				+	+	Subcutaneously implanted dentine disks/slices induced differentiation and mineralization in SHEDs
Casagrande *et al.*[96]	*In vivo*	Disk/Slice	Human	—	Human	SHED				+		Subcutaneously implanted dentine disks/slices preconditioned with 10% EDTA induced differentiation in SHEDs
Galler *et al.*[101]	*In vivo*	Disk/Slice	Human	—	Human	DPSC				+		Subcutaneously implanted dentine disks/slices preconditioned with 17% EDTA induced differentiation in DPSCs
Bégue-Kirn *et al.*[86]	*In vitro*	Recomb.	—	BMP-2	Mouse	SCAP				/	+	Recombinant TGF-β1 increased mineralization but did not affect differentiation in SCAPs
				TGF-β1	Mouse	SCAP				/	+	Recombinant BMP-2 increased mineralization but did not affect differentiation in SCAPs

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*								
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Nakashima[102]	*In vitro*	Recomb.	—	EGF	Bovine	DPSC	/			/	/	Recombinant EGF did not affect proliferation, differentiation, and mineralization in DPSCs
				FGF-1	Bovine	DPSC	/			+	+	Recombinant FGF-1 increased differentiation and mineralization in DPSCs but did not affect proliferation
				FGF-2	Bovine	DPSC	/			+	/	Recombinant FGF-2 increased differentiation in DPSCs but did not affect proliferation and mineralization
				IGF-1	Bovine	DPSC	+			/	+	Recombinant IGF-1 increased proliferation (dose-dependent) and mineralization in DPSCs but did not affect differentiation
				IGF-2	Bovine	DPSC	/			/	+	Recombinant IGF-2 increased mineralization in DPSCs but did not affect proliferation and differentiation
				PDGF	Bovine	DPSC	+			+	/	Recombinant PDGF increased proliferation (dose-dependent) and differentiation in DPSCs but did not affect mineralization

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*							
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
				TGF-β	Bovine	DPSC	/			+	/	Recombinant TGF-β increased differentiation in DPSCs but did not affect proliferation and mineralization
Nakashima *et al.*[103]	*In vitro*	Recomb.	—	BMP-2	Bovine	DPSC				+		Recombinant BMP-4 increased differentiation in DPSCs
				BMP-4	Bovine	DPSC				+		Recombinant BMP-4 increased differentiation in DPSCs
				TGF-β	Bovine	DPSC				-		Recombinant TGF-β decreased differentiation in DPSCs
Denholm *et al.*[104]	*In vitro*	Recomb.	—	IGF-1	Human	DPSC	+					Recombinant IGF-1 increased proliferation in DPSCs
				PDGF	Human	DPSC	+					Recombinant PDGF increased proliferation in DPSCs
Onishi *et al.*[105]	*In vitro*	Recomb.	—	IGF-1	Dog	DPSC	+			+		Recombinant IGF-1 increased proliferation and differentiation in DPSCs
				IGF-2	Dog	DPSC	+			+		Recombinant IGF-2 increased proliferation and differentiation in DPSCs
Melin *et al.*[106]	*In vitro*	Recomb.	—	TGF-β1	Human	DPSC	+		+			Recombinant TGF-β1 increased proliferation and migration in DPSCs

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*								
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Nakao *et al.*[107]	*In vitro*	Recomb.	—	BMP-2	Rodent	DPSC	/			/	/	Recombinant BMP-2 did not affect proliferation, differentiation, and mineralization in DPSCs
				BMP-4	Rodent	DPSC	/			/	/	Recombinant BMP-4 did not affect proliferation, differentiation, and mineralization in DPSCs
				FGF-2	Rodent	DPSC	+			+	+	Recombinant FGF-2 increased proliferation, differentiation, and mineralization in DPSCs
				FGF-8	Rodent	DPSC	/			/	/	Recombinant FGF-8 did not affect proliferation, differentiation, and mineralization in DPSCs
				NGF	Rodent	DPSC	/			/	/	Recombinant NGF did not affect proliferation, differentiation, and mineralization in DPSCs
Iohara *et al.*[108]	*In vitro*	Recomb.	—	BMP-2	Porcine	DPSC	/			+	+	Recombinant BMP-2 increased differentiation and mineralization in DPSCs but did not affect proliferation
Saito *et al.*[109]	*In vitro*	Recomb.	—	BMP-2	Human	DPSC	/			+	+	Recombinant BMP-2 increased differentiation and mineralization in DPSCs but did not affect proliferation

(*continued*)

TABLE 3. (CONTINUED)

Study		*dECMs*			*MSC*								
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Mizuno *et al.*[110]	*In vitro*	Recomb.	—	BDNF	Human	DPSC	/			+	+	Recombinant BDNF increased differentiation and mineralization in DPSCs but did not affect proliferation
				NGF	Human	DPSC	+			+	+	Recombinant NGF increased proliferation, differentiation, and mineralization in DPSCs
				NT3	Human	DPSC	/			+	+	Recombinant NT3 increased differentiation and mineralization in DPSCs but did not affect proliferation
				NT4	Human	DPSC	/			+	+	Recombinant NT4 increased differentiation and mineralization in DPSCs but did not affect proliferation
He *et al.*[111]	*In vitro*	Recomb.	—	FGF-2	Human	DPSC	/			/	/	Recombinant FGF-2 did not affect proliferation, differentiation, and mineralization in DPSCs
				TGF-β1	Human	DPSC	+			+	+	Recombinant TGF-β1 increased proliferation, differentiation, and mineralization in DPSCs
Casagrande *et al.*[96]	*In vitro*	Recomb.	—	BMP-2	Human	SHED				+		Recombinant BMP-2 increased differentiation in SHEDs

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*								
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
				BMP-7	Human	SHED				/		Recombinant BMP-7 did not affect differentiation in SHEDs
Howard *et al.*[112]	*In vitro*	Recomb.	—	EGF	Human	SHED			/			Recombinant EGF did not affect migration in SHEDs
				FGF	Human	SHED			+			Recombinant FGF increased migration in SHEDs
				TGF-β	Human	SHED			+			Recombinant TGF-β increased migration in SHEDs
d' Alimonte *et al.*[113]	*In vitro*	Recomb.	—	VEGF	Human	DPSC	+			+		Recombinant VEGF increased proliferation (dose-dependent) and differentiation in DPSCs
Li *et al.*[114]	*In vitro*	Recomb.	—	TGF-β1	Rodent	DPSC	/			+	+	Recombinant TGF-β1 increased differentiation and mineralization in DPSCs but did not affect proliferation
Wei *et al.*[115]	*In vitro*	Recomb.	—	MEPE	Human	DPSC	+			+	+	Recombinant TGF-β increased proliferation (dose-dependent), differentiation, and mineralization in DPSCs
Mathieu *et al.*[116]	*In vitro*	Recomb.	—	FGF-2	Human	DPSC	+	/	/			Recombinant FGF-2 increased proliferation (dose-dependent) in DPSC but did not affect viability and migration

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*							
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
				TGF-β1	Human	DPSC	/	/	+			Recombinant TGF-β1 increased migration in DPSC but did not affect proliferation and viability
Tomson *et al.*[71]	*In vitro*	Recomb.	—	HGF	Rodent	DPSC	/		+	+	+	Recombinant HGF increased migration, differentiation, and mineralization in DPSCs
He *et al.*[117]	*In vitro*	Recomb.	—	TGF-β1	Human	SCAP	-			-	-	Recombinant TGF-β1 reduced proliferation (dose-dependent), differentiation, and mineralization in SCAPs
Gonçalves *et al.*[99]	*In vitro*	Recomb.	—	TGF-β1	Human	SHED			+			Recombinant TGF-β1 increased migration in SHEDs
Tabatabaei & Torshabi[91]	*In vitro*	Recomb.	—	TGF-β1	Human	DPSC	+					Recombinant TGF-β1 increased proliferation in DPSCs
Zeng *et al.*[29]	*In vitro*	Recomb.	—	FGF-2	Human	DPSC			/			Recombinant FGF-2 did not affect migration in DPSCs
				TGF-β1	Human	DPSC			+			Recombinant TGF-β1 increased migration in DPSCs
Widbiller *et al.*[95]	*In vitro*	Recomb.	—	TGF-β1	Human	DPSC	/	/	+			Recombinant TGF-β1 increased migration in DPSCs but did not affect proliferation and viability

(*continued*)

Table 3. (Continued)

Study		*dECMs*			*MSC*							
Reference	*Design*	*Model*	*Source*	*Molecule*	*Source*	*Niche*	*Proliferation*	*Viability*	*Migration*	*Differentiation*	*Mineralisation*	*Key findings*
Tziafas *et al.*[118]	*In vivo*	Recomb.	—	FGF-2	Dog	DPSC				/	+	Recombinant FGF-2 increased mineralization in DPSCs but did not affect differentiation
				IGF	Dog	DPSC				/	+	Recombinant IGF increased mineralization in DPSCs but did not affect differentiation
				TGF-β1	Dog	DPSC				+	+	Recombinant TGF-β1 increased differentiation and mineralization in DPSCs
Li *et al.* (2011)[114]	*In vivo*	Recomb.	—	TGF-β1	Rodent	DPSC				+	+	Recombinant TGF-β1 increased differentiation and mineralization in DPSCs

Studies have been arranged primarily on the dECM model used, followed by study design and then date.

BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; dECM, dentin extracellular matrix component; DPSC, dental pulp stem cell; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin growth factor; MEPE, matrix extracellular phosphoglycoprotein; MSC, mesenchymal stem cell; NGF, nerve growth factor; NT3, neurotrophin 3; NT4, neurotrophin 4; PDGF, platelet-derived growth factor; Recomb, recombinant; SCAP, stem cells of the apical papilla; SHED, stem cells from human exfoliated deciduous teeth; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; +, increased; -,decreased; /, nil. Color images are available online.

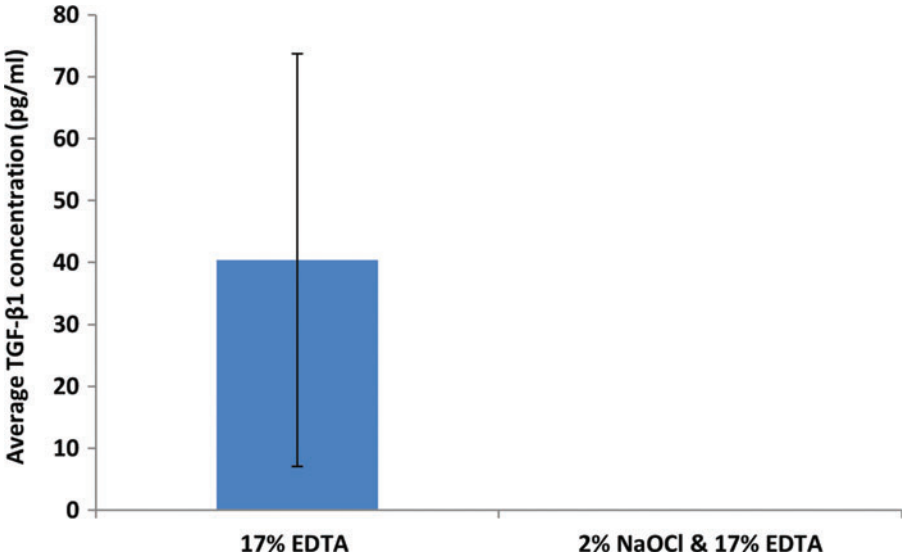

FIG. 4. Data obtained from our research group demonstrating the deleterious effects of sodium hypochlorite on the solubilization of dentine extracellular matrix components when delivered into prepared root canals of extracted mature permanent human teeth ($n = 10$) via conventional needle irrigation. A sandwich ELISA technique was used to detect TGF-β1 concentration (pg/mL) from 100 μL of EDTA after irrigation with or without 2% sodium hypochlorite. Error bars represent standard deviation. Color images are available online.

of AP in mature permanent teeth. Conceptually speaking, common chelating agents, namely, 17% EDTA, can be used as the primary irrigant throughout chemo-mechanical debridement to preserve and maximize the release of dentine matrix proteins into root canals. These would otherwise be negatively impacted by the proteonacious properties exhibited by even low concentrations of NaOCl.[28,99] This observation is consistent with pilot data obtained by our own research group (Fig. 4).

Ultrasonic agitation has been found to significantly assist dECM release and, thus, is an essential irrigant adjunct after instrumentation.[123] These solubilized morphogens could then be encouraged to egress into periapical tissues, by way of manual dynamic activation and patency filing. Subsequently, they would act as chemoattractants to local tissue PL-MSCs present within the peripheral capsular region of granulomas, and subsequently enhance their regenerative potential.[124] This interaction will likely require precise pre-enlargement of the apical foramen after accurately determining its position.[13]

Moreover; antimicrobial inter-appointment medicaments, namely calcium hydroxide, may further prolong dECM exposure due to their ability to liberate bioactive dentine molecules.[31,33] This two-stage approach provides additional disinfection, potentially compensating for the absence of NaOCl, and helps confirm resolution of active disease before obturation. Thereafter, routine clinical and radiographic examination would be required to monitor periapical healing (Fig. 5).

The theoretical basis of the proposed approach is derived from preclinical animal studies that have utilized recombinant components of the dentine matrix to regenerate dentoalveolar tissues. For instance; Kim *et al.* reported that BMP-7 and stromal-derived factor-1 (SDF-1), delivered subcutaneously into rats via 200 μm micro-channels in bioprinted human molar scaffolds, increased both recruitment of endogenous MSCs and angiogenesis and ultimately led to regeneration of an anatomically shaped tooth like-structure.[125]

Remarkably, in this model a *de novo* periodontal ligament and alveolar bone was also observed as integrating with the native bone at the scaffold interface after 9 weeks. When the same molecules, plus FGF, were used to coat the roots of intentionally avulsed mandibular premolars in beagle dogs, they were found to contribute to the re-establishment of highly organized periodontal ligament tissues after delayed reimplantation.[126,127] These neo-fibers inserted deeply into the adjacent cementum and alveolar bone and prevented the onset of external replacement or inflammatory root resorption.

Kim *et al.* were also able to demonstrate that without the use of stem cell transplantation, re-cellularized and revascularized dental pulp-like tissue was regenerated across the entire length of endodontically treated human-sized root canals after 3 weeks of exposure to FGF, VEGF, PDGF, NGF, and BMP7 in a subcutaneous implantation mouse model.[128] Similar observations were reported by Suzuki *et al.*[129] These studies, in particular, provide the strongest support for the proposed protocol as they demonstrate *in vivo* regeneration of the very tissues necessary for a *de novo* periodontium using a cell-free approach.

Further support for the homing potential of dECMs, however, comes from the applications of other prevalent dentine matrix proteins, such as DPP and DMP-1, in rat models. For example, exposure to DPP induced odontoblastic differentiation and subsequent reparative dentine bridge formation in inflamed pulp tissue and DMP-1 impregnated scaffolds exhibited marked extracellular matrix deposition and neovascularization in endodontic perforation defects.[17,130]

In other areas of medicine, stem cell homing techniques utilizing TGF-β3 molecules have successfully contributed to regenerating entire humeral condyles in rabbits after radical resection.[131] Collectively, these findings have to date been clinically translated into novel pulp preservation and regeneration protocols and provide proof of concept for the therapeutic potentials of dECMs when used in stem cell homing techniques as described earlier.[132–134] Nevertheless, although the proposed approach circumvents many ethical issues related to cell-based transplantation strategies, it is at present only speculative. Numerous hurdles are still required to be overcome before successful clinical translation.

Challenges to Successful Clinical Translation and Directions for Future Research

The greatest challenge associated with implementing the protocol cited earlier is developing chemo-mechanical

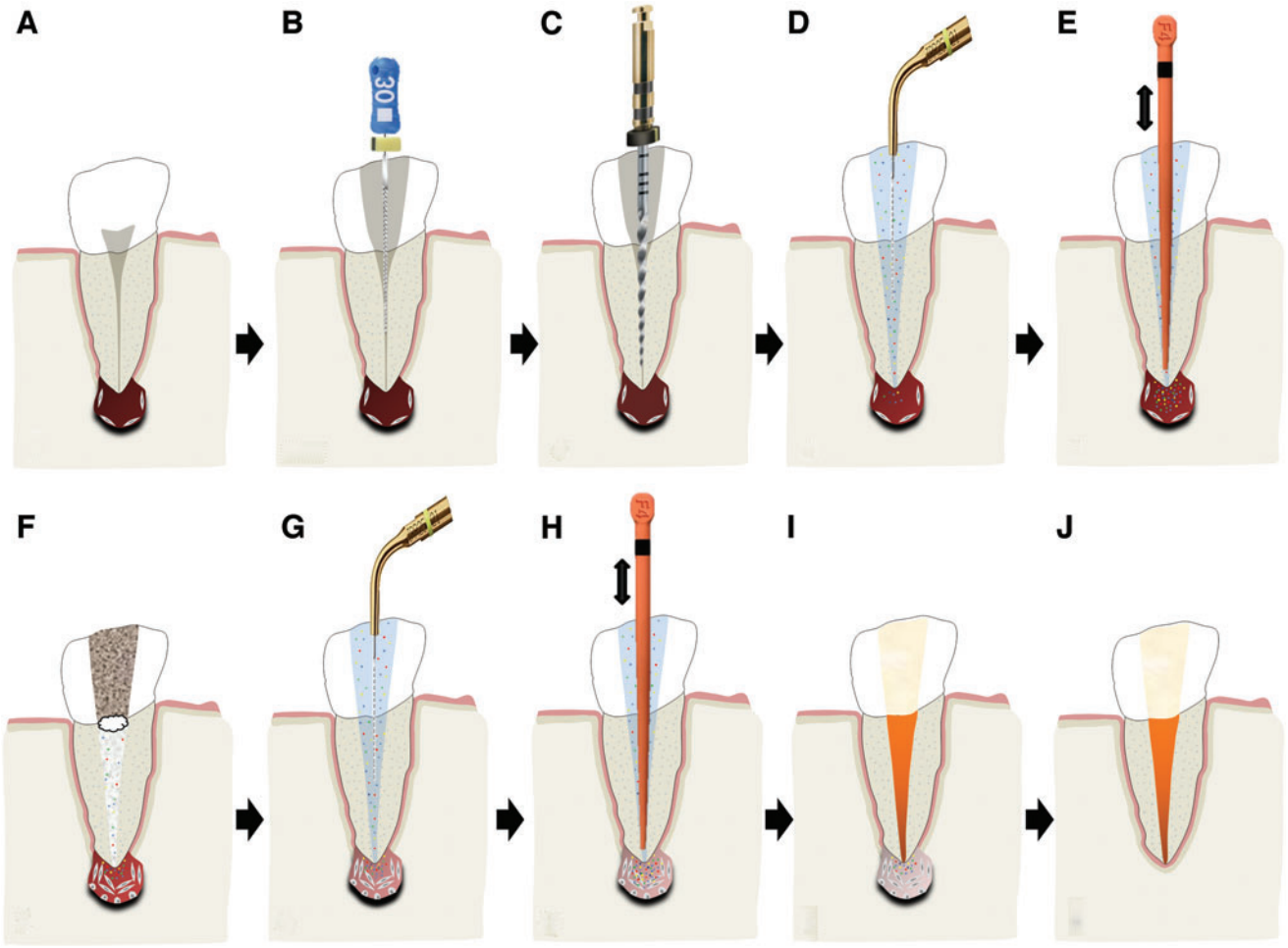

FIG. 5. A schematic illustration of the proposed protocol for enhancing periradicular tissue regeneration in mature permanent teeth by using endogenous dECM components. **(A)** Single-rooted mature permanent tooth diagnosed with apical periodontitis; **(B)** accessing pulp chamber and conservative pre-enlargement of apical foramen; **(C)** chemomechanical preparation of root canal using a chelating agent; **(D)** passive ultrasonic activation of irrigant to stimulate release of dECMs into the root canal; **(E)** manual dynamic activation to encourage periapical bioavailability of dECMs; **(F)** interappointment calcium hydroxide medicament; **(G)** irrigation and passive ultrasonic activation to release dECMs; **(H)** manual dynamic activation to encourage periapical bioavailability of dECMs; **(I)** obturation; **(J)** annual clinical and radiographic review. Color images are available online.

debridement regimes that sufficiently disinfect root canals while preserving dECMs and PL-MSCs. This would particularly affect NaOCl use, which has proven detrimental to stem cell viability,[135,136] dentine matrix growth factor bioavailability,[28,99] and induction of key tissue regeneration events.[96,101] Although lower concentrations and contact times of 1.5% and 5 min, respectively, have been advocated for regenerative endodontic treatments, these parameters are derived from studies only investigating MSC viability.[98,135,136] Therefore, it is currently unknown how they influence dECM release. Further, the ideal strength for NaOCl's antimicrobial efficacy is reported as 2.5%,[137] which is otherwise cytotoxic to MSCs and significantly reduces the bioavailability of dECMs.[99,136]

What has been cited earlier suggests that if NaOCl were to be administered even in a limited capacity, its deleterious effects on dECMs would need mitigating, which is supported by pilot data (Fig. 4). This could perhaps be achieved by enhancing the activity of demineralizing agents or mechanically removing the affected dentinal substrate, the latter of which requires a prerequisite understanding of NaOCl's penetrative capabilities. However, should these methods lead to no avail, NaOCl will need to be substituted for alternative antimicrobial strategies. For instance, the thicker and less fragile root canal walls in mature permanent teeth allow for more emphasis on conventional instrumentation and intracanal medicaments, which have *in vivo* shown greater contribution to endodontic disinfection than lower NaOCl concentrations.[138]

Moreover; EDTA, which is currently considered a weak antimicrobial agent, destabilizes the outer cell membranes of gram-negative bacteria and deteriorates the macrostructures of established biofilms.[139] Although these effects alone may not always induce cell death, they could potentially be enhanced enough to do so when combined with mechanical instrumentation and irrigant agitation techniques. The reductions in microbial load achieved through these mechanisms may equate to that of NaOCl treatment and exceed the threshold necessary to control infection while preserving the biological components within dentine.[2] Further investigations are required to test these hypotheses.

Another challenge is that the effectiveness of the proposed strategy has yet to be proven in concept. Although the regenerative potentials of dECMs have been demonstrated in DPSCs, SCAPs, and SHEDs; it is currently unknown whether similar effects are observed in cultures of PL-MSCs. This niche has already demonstrated different stem-like characteristics and thus may yield results at variance to that of other MSCs.[55] Animal studies, utilizing the intentional pulp exposure model of AP, could be employed to further support or challenge the aforementioned hypothesis. They would provide valuable histological and radiographic insight into the periradicular healing process at key time points after dECM exposure, which is data that ethically cannot be attained *in vivo* using human participants.

Rodents such as rats and mice provide researchers endodontic anatomy (i.e., molar teeth), infected root canal microflora, and wound-healing physiology comparable to that of humans and they conform to the public opposition of using larger animals, thus making them the species of choice.[140–142] Overall, these preliminary studies are necessary to justify more time-consuming, labor-intensive, expensive, and appropriately powered prospective randomized controlled trials, which would be the ultimate means of demonstrating the clinical effectiveness of the proposed intervention. Such investigations would also benefit from more sensitive outcome measures that could longitudinally detect biological changes within the periradicular tissues.

Conclusion

The discovery of multipotent stem cells within periapical lesions presents novel opportunities for managing AP by way of harnessing local tissue regeneration. Multiple *in vitro* studies have confirmed their immunomodulatory and stem cell-like characteristics, which implicates them as being key determiners of the periapical healing process and provides the foundations for subsequent *in vivo* investigation. Further, there is extensive evidence demonstrating that components within the dentine's extracellular matrix are capable of upregulating the very regenerative responses within dental MSCs that would otherwise be necessary for periradicular regeneration. This includes the enhancement of cellular proliferation, migration, viability, differentiation, and mineralization.

It is well established that these bioactive properties can be harnessed by clinicians on command with common chelating agents such as EDTA, which provides the theoretical and clinical basis of the proposed protocol. Further *in vitro* and *in vivo* studies, however, are still required to determine the regenerative effects of dECMs in PL-MSC cultures, optimal irrigant regimes for liberating dECMs, and their effects on the clinical success rates of root canal treatment. Such investigations at the very least would improve understanding of the biological mechanisms associated with periradicular healing, which could in future lead to the development of regenerative endodontic treatment strategies for AP.

Authors' Contribution

S.S. contributed to conception, design, data acquisition and interpretation, drafted, and critically revised the article; N.B. contributed to data acquisition and interpretation, drafted, and critically revised the article; J.C. contributed to conception, drafted, and critically revised the article; P.R.C. contributed to conception, drafted, and critically revised the article; P.L.T. contributed to conception, drafted, and critically revised the article.

All authors gave their final approval and agree to be accountable for all aspects of the work.

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Supplementary Material

Supplementary Table S1

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CHAPTER 3

PROOF OF CONCEPT

Publication 2: Virdee, S. S., Grant, M. M., Pai, S., Khandhari, S., Bashir, N., Cooper, P. R., & Tomson, P. L. (2024) Bioavailability of dentine extracellular matrix components and their regenerative effects on periapical lesion-derived mesenchymal stem cells: an in vitro study. *International Endodontic Journal,* (Submitted)

The following chapter consists of a two-part *in vitro* study, which is presented in one manuscript (Publication 2), that aims to demonstrate proof of concept on several key aspects of the clinical protocol outlined in Chapter 1. Briefly, for this initial study, the aim was to determine the effects of clinically recommended irrigation protocols suggested in regenerative endodontic guidelines on solubilisation of dECM components. In addition, determine if there was periradicular bioavailability of dECMs liberated into root canals of mature permanent teeth with a minimally pre-enlarged apical foramen. As much of the previous literature reported on studies using dentine powders or disks and irrigant protocols that could not be implemented chairside, emphasis was placed on the biofidelity and clinical translatability of an experimental model. Findings from the *in vitro* work were used to develop a clinically translatable irrigant regime that could be used during root canal treatment to promote dECM release. In the second part of the study, the ability of dECMs solubilized from the intact tooth model used above to induce regenerative events in PL-MSCs was explored. These primary cells, which have been identified as key determinants of apical healing,

were isolated from periapical granulomas, cultured and exposed to dECMs at concentrations measured in the periradicular bioavailability study. Their ability to increase cellular proliferation, migration, osteogenic gene expression and mineralisation without negatively effecting cell viability, all of which would be necessary for treatment success, were tested to provide proof of concept for the proposed treatment strategy.

CHAPTER 3

PUBLICATION 2

BIOAVAILABILITY OF DENTINE EXTRACELLULAR MATRIX COMPONENT AND THEIR REGENERATIVE EFFECTS ON PERIAPICAL LESION-DERIVED MESENCHYMAL STEM CELLS: AN IN VITRO STUDY

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Abstract

Background: Endogenous dentine extracellular matrix components (dECMs) have been shown to yield positive effects in pulp regeneration. Their potential role in the treatment of apical periodontitis is yet to be considered.

Aims: 1) Determine *in vitro* a clinically relevant irrigant regime to optimise dECM bioavailability**. 2)** Establish if solubilised dECM extracts upregulate regenerative activities in periapical lesion-derived mesenchymal stem cells (PL-MSC).

Methods: 1) Forty extracted mature permanent incisor were decoronated, the root canals prepared to 40/0.06, apical foramen pre-enlarged to 0.2 mm and encased in silicone with apices adapted to absorbent methylcellulose strips. Teeth were randomly divided into 4 groups and irrigated for 5 minutes with 20mL of i) 0.5% NaOCl; ii)1.5% NaOCl; iii) 1.5% NaOCl and re-instrumented; or iv) NIL, which acted as control (*n*=10). dECMs were then solubilised with 17% EDTA at 5, 10, 20 and 60-minutes with 30-seconds ultrasonic activation and 5mL per interval. Solutions were collected, alongside periradicular methylcellulose strips, and centrifuged to 1 mL with 3kD protein concentrators. Samples were subjected to sandwich-ELISA to determine median TGF-β1 concentration (pg/mL). **2)** The irrigant regime giving the greatest dECM yield was characterised via quantitative growth factor array and used to harvest extracts for cellular studies. Samples were applied to PL-MSC cultures at 50-3pg/mL alongside 1% and 10% FBS supplemented media as negative and positive controls respectively. Regenerative effects were evaluated using i) A 14-day WST-1 assay for proliferation; ii) chemotaxis assay for migration; iii) Annexin-V & PI FACS analysis to determine cell viability; iv) alizarin red and real-time-qPCR gene expression analysis for osteogenic differentiation. All experiments were conducted in triplicates with four biological replicates (*n*=12) with 0.05 alpha for all statistical comparisons.

Results: 1) dECMs can be liberated by root canal irrigants and they are bioavailable to periradicular tissues. NaOCl significantly impacted dECM solubilisation at all concentrations used when compared to 17% EDTA alone (p<0.05). **2)** 38 regenerative-related growth factors were detected within 17% EDTA-derived dECM extracts. These extracts significantly stimulated proliferation, migration, osteogenic gene expression and mineralisation in PL-MSCs without negatively effecting cell viability (p<0.05).

Conclusions: Irrigant regimes that enhance dECM release and their periradicular bioavailability during root canal treatment could improve periradicular tissue healing.

Conflicts of Interest: Nil

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Introduction

From a clinical standpoint, dentine has traditionally been perceived as an inert structural tissue that serves to mechanically protect the underlying pulp. Current understanding of its organic composition has however revealed the presence of a plethora of growth factors, cytokines, chemokines and other signalling molecules (Smith et al. 2012). Many of these are closely associated with mediating intrinsic wound healing processes with noteworthy examples including transforming growth factor (TGF-β); insulin-like growth factors (IGF); vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF), amongst many others (Park et al 2009, Jágr et al 2012). These non-collagenous proteins, termed dentine extracellular matrix components (dECM), originate from odontoblastic secretions during dentinogenesis and become sequestered by subsequent mineralisation processes. Interestingly, whilst their bioactive properties remain highly preserved in this quiescent state, owing to proteoglycan bond formation and lack of mineral turnover, this can be immediately reinstated upon liberation from the dentine matrix (Baker et al. 2009). This has been demonstrated multiple times *in vitro*, where solubilised dECM components upregulate cellular proliferation, migration, differentiation and mineralisation events when topically applied to established dental mesenchymal stem cell (MSC) niches (Sadaghiani et al. 2016, Tomson et al. 2017, Widbiller et al. 2018). Dentine should therefore be considered a precious reservoir of bioactive cell-signalling molecules that can be clinically exploited for novel endodontic tissue engineering strategies.

Tissue engineering strategies within endodontics have typically been reserved for pulp regeneration in necrotic teeth with wide open apices. However, there has been growing interest in developing similar approaches in mature permanent teeth with apical periodontitis (Yang et al. 2016, Virdee et al. 2022). This could be attributed to

stagnant success rates when using conventional anti-microbial approaches over the last several decades, which consistently result in one in five teeth failing to heal despite guideline standard treatment (Ng et al., 2011), along with the discovery of a novel dental MSC niche within periapical lesions (PL-MSCs; Liao et al. 2011). These MSCs *in vitro* have exhibited high proliferation rates, self-renewing capabilities and multipotency with commitment towards osteogenic activity (Liao et al. 2011, Dokić et al. 2012, Marrelli et al. 2013). They are now implicated as the key determinants of periradicular healing processes and make ideal candidates for therapeutic targeting. One such approach has recently been hypothesised and involves solubilising endogenous dECM components and facilitating their interaction with the periapical tissues to upregulate the regenerative capacity of local PL-MSCs *in situ* (Virdee et al. 2022).

There are currently several challenges that preclude the clinical translation of this cell-free approach to periradicular tissue healing. Firstly, pre-treating dentine with sodium hypochlorite (NaOCl) is detrimental to dECM bioavailability, which is typically achieved using ethylenediaminetetraacetic acid (EDTA) irrigation (Galler et al. 2015, Tavares et al. 2021). Whilst lower concentrations (1.5%) and limited contact times (5 minutes) have been advocated in regenerative clinical protocols for mitigating these effects, recommendations are based on MSC viability and not the integrity of dECMs (Martin et al. 2014, Galler et al. 2016). Secondly, there are no investigations into dECM bioavailability used for the purposes of treating apical periodontitis. Where similar concepts have been explored for pulp regeneration, experimental conditions and protocols do not reflect *in vivo* conditions well and warrant the need for more clinically representative laboratory models to bridge this knowledge gap (Galler *et al.* 2015, Sadaghiani *et al.* 2016, Widbiller *et al.* 2017). To develop novel periradicular

regenerative treatment strategies, studies would need to demonstrate efficacy of the dECMs released into root canals and periradicular tissues, as this has not previously been investigated. Finally, it is currently unknown if dECMs solubilised with clinically reproducible irrigant regimes enhance regenerative events within the recently discovered PL-MSC niche. Combined, these data would enable the development of irrigant protocols that preserve dECM integrity and promote regenerative events within diseased periradicular tissues during root canal treatment to improve treatment outcomes.

Aims

To identify a clinically relevant irrigant regime that promotes PL-MSC dependant tissue healing events, two linked *in vitro* studies were conducted. The primary aim of the first study was to determine if irrigant regimes recommended in regenerative endodontic protocols, which principally limit NaOCl use, facilitate dECMs bioavailability in comparison to 17% EDTA alone. The second study primarily aimed to establish if solubilised dECM extracts upregulated regenerative events in PL-MSC cultures. Secondary objectives included exploring i) the effects of re-instrumenting dentine following NaOCl pre-treatment; ii) periradicular bioavailability of dECMs; and iii) growth factor profile within solubilised dECMs extracts. The null hypotheses tested were there was no difference in dECM solubilisation between regenerative endodontic irrigant regimes and 17% EDTA, and that topical application of dECM extracts did not induce regenerative events in PL-MSC cultures.

Materials & Methodology

This study was reported in accordance with 2021 Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) guidelines (Nagendrababu et al., 2021;

Figure 1). Ethical approval was obtained from the University of Birmingham's Dentistry Research Tissue Bank (DRTB; 19/SW/0198) with informed consent to retain waste tissue documented in participant's electronic medical records. All procedures were performed by a single endodontically trained operator (SSV).

1. dECM bioavailability with clinically representative irrigation protocols

Sample Selection: Fifty human maxillary mature permanent incisors were randomly selected from a pool of teeth that were extracted for reasons not related to this study, collected in a 200 mL solution of 15 mmol/L sodium azide, washed in distilled water for 5 hours and then stored at -20˚C in the Dentistry Research Tissue Bank. Mesio-distal and bucco-lingual digital radiographs were captured to ensure only teeth with single canals and root lengths of ≥ 16 mm were included. Teeth with caries, root fractures, open apices, root curvatures > 10º calcified canals, resorptive defects, posts and previous root fillings were excluded.

Sample Preparation: The intact tooth model previously described by Virdee et al. (2020) was used with modifications to determine the dECM bioavailability (Figure 2). Briefly, teeth were decoronated and the root adjusted to 15 mm using cooled silicon carbide grinding paper (Struers, Pederstrupvej, Denmark). Residual periodontal and pulp tissues were removed with ultrasonic scalers and 08/.02 K-flex files respectively (Dentsply Sirona, Ballaigues, Switzerland). Canal length was subsequently determined using a 10/.02 tip being barely visible beyond the apex under magnification. To facilitate periradicular passage of dEMCs, the apical foramen was widened by introducing a size 20/.02 K-file to this length. Thereafter, 0.5 mm was subtracted, and all root canals prepared to this working length (WL) up to 40/.10 (ProTaper Gold; Dentsply Sirona, Ballaigues, Switzerland) at recommended speeds and torques with 1 mL deionised distilled water irrigation used between instruments.

External root surfaces were coated with nail polish (Maybelline, New York, USA), to ensure irrigants only interacted with intraradicular dentine, and then sealed in Aquasil soft putty silicone (Dentsply Sirona, Ballaigues, Switzerland) to mimic a closed system.

Sample Size: The lack of prior data with respect to the current experimental model precluded sample size calculations. Therefore, 10 teeth were analysed per group in duplicates based on prior *in vitro* studies investigating dECM solubilisation using different models (*n* = 10; Tomson et al. 2007, Galler et al. 2015, Sadighiani et al 2016, Widbiller et al. 2018).

Groups: In test groups, root canals were irrigated with i) 17% EDTA; ii) 0.5% NaOCl; iii) 1.5% NaOCl; or iii) 1.5% NaOCl followed by mechanical re-instrumentation. Proprietary solutions were diluted with deionised distilled water to achieve these concentrations (Cerkamed, Stalowa Wola, Poland). This was followed with 17% EDTA (Cerkamed) irrigation to solubilise dECMs. The control group consisted of only this latter component with autoclaved teeth.

Irrigation Protocol: A total of 20 mL irrigant solution was delivered into root canals via conventional needle irrigation using 27-gauge side vented tips (Covidien, Dublin, Ireland) positioned 2 mm from WL with light finger pressure. This solution remained *in situ* for 5 minutes, before being irrigated away with 6 mL deionised distilled water. For mechanical re-instrumentation, Sx rotary files (Dentsply Sirona) were advanced apically to WL and withdrawn using brushing actions. This motion was repeated circumferentially in a clockwise direction five times with 1 mL of deionised distilled water irrigation after each cycle. All canals were then dried using 25/0.02 sterile paper points after which teeth were removed from silicone moulds and absorbent methylcellulose papers (Oraflow, New York, USA) closely adapted to the apical foramen prior to reassembly. These strips detected interactions between growth

factors liberated into canals and the periradicular tissues. At this point, dECMs were solubilised with 17% EDTA with conventional needle irrigation. More specifically, 5 mL was deposited into canals and allowed to remain undisturbed for 5 minutes where it was ultrasonically activated in the final 30 seconds with a 20/0.02 Irrisafe tip (Acteon, Norwich, UK) and Satelec (Acteon) at half power 1 mm from WL. This protocol was repeated four times in the same tooth to facilitate EDTA exposures of 5, 10, 20 and 60 minutes, giving a total EDTA volume of 20 mL per tooth.

Sample Collection: For each time point, the EDTA solutions from the canal were collected in 3 kilodalton protein concentrators (Thermofisher, Scientific, MA, USA), centrifuged at 4^0C and 4000 g until 1 mL retentate remained, and transferred into coded 1.5 mL microfuge tubes. Methylcellulose strips were collected after final irrigation and transferred to a separate coded 1.5 mL microfuge tube containing 100 µL phosphate buffered saline (PBS) elution buffer. Samples were stored at -20^0C until further analyses.

Sample Analysis: As TGF-β1 is regarded as the most abundant growth factor in dentine matrix extracts and is frequently used as a representative biomarker for dECM solubilisation (Tomson *et al.* 2017). Intracanal irrigant and periradicular methylcellulose paper samples were thus analysed using a TGF-β1 sandwich-ELISA as per manufacturer's instruction (Quantikine Kit; Bio-Techne, MS, USA). Briefly, 50 µl of activated test, control and standard samples were added in duplicates to 96-well ELISA plates, which were pre-coated with the respective monoclonal capture antibody. After incubation for 2 hours at room temperature, 100 µl of polyclonal antibody conjugated to horseradish peroxidase was added to each well and incubated again for 2 hours at room temperature. Between each step, plates were washed using an automated plate washer (ELx50; Bio-Tek, VM, USA) and the PBS/0.1% Tween20

wash buffer provided. A substrate solution containing hydrogen peroxidase and chromogen was then added and allowed to react for 30 minutes in the dark after which the cytokine concentration in each well was determined using an ELISA plate reader (ELX800; Bio-Tek, VM, USA) at an optical density of 450 and 570 nm. Values for the latter were subtracted from the former to eliminate optical imperfections and the resulting outputs calibrated against a standard curve to determine TGF-β1 concentration (pg/mL).

2. Regenerative effects of solubilised dECMs on PL-MSCs

dECM Isolation & Characterization: The previously described intact tooth model was utilised alongside the irrigant regime yielding the largest abundance of TGF-β1 from which to harvest dECMs. To explore the range of growth factors within the resulting extracts, samples from individual teeth were analysed using the human Quantibody growth factor array as per manufacturer's instructions (RayBio, Norcross, GA, USA; Duncan et al. 2017). This quantitative high-throughput assay simultaneously detected 40 regenerative related-mediators via pre-validated fluorescent multiplex ELISA technology. Briefly, a standard Bradford dye-binding protocol (Bio-Rad, Hemel Hempstead, UK) was utilised in conjunction with bovine serum albumin to normalise total protein concentrations (TPC) in each sample with PBS to 200 µg/mL. Non-specific protein binding on the glass slide was prevented by a blocking step before adding 100 µl of dECM samples and standards for 2-hour incubation at room temperature. Thereafter, wells underwent washing to remove unbound non-specific proteins and were further incubated with a 80 µl biotinylated antibody cocktail for 2 hours. This was proceeded by 1-hour dark incubation at room temperature with 80 µl Cy3 equivalent dye-conjugated streptavidin. Glass slides were then washed, scanned and the data analysed in Quantibody Q-Analyzer software (RayBiotech). This analysis

was conducted on 10 independent samples (n = 10) before pooling and stored at -20°C in batches of 4000 pg/mL TGF-β1.

Participants: Periapical granulomas were collected from consenting and immunocompetent adults (≥ 18 years) attending the Oral Surgery department at the Birmingham Dental Hospital for exodontia of teeth diagnosed with apical periodontitis. Diagnoses were determined using clinical and radiographic examination by clinical staff and defined as teeth displaying frank radiolucencies with potential sensitivity to percussion and palpation testing (Glickman 2009). Only lesions retrievable from the avulsed apices of teeth following exodontia, as opposed to curetted from within alveolar sockets, were included to ensure cells were periapical in origin. Furthermore, combined endodontic-periodontal lesions associated with or without root damage; and teeth exhibiting clinical and radiographic signs of abscesses or cysts (i.e., ≥ 10 mm or well corticated radiolucencies) were excluded.

Sample Collection: Teeth were atraumatically extracted under local anaesthesia. A sterile scalpel blade was used to immediately to dissect the periradicular lesion(s) into 5 mL transport media. This consisted of Minimum Essential Media-Alpha modification (α-MEM; Capricorn Scientific, Germany) supplemented with 2 mM L-glutamine and 1% penicillin (10 000 units/mL), streptomycin (10 mg/mL) and amphotericin B (250 µg/mL; Sigma-Aldrich, UK). Samples were immediately incubated under standard culture conditions (5% CO_2; 37^0C) and processed within 10 minutes.

Cell Isolation & Culture: The enzyme-digestion method was employed to isolate cells from periapical lesions in a laminar flow hood (Tomson et al., 2017). Briefly, tissues were initially washed with PBS, mechanically minced with a sterile scalpel into ≤ 1 mm^3 pieces and digested using 4 mL type 1collagenase (4 mg/mL; Merck, Darmstadt, Germany) for 60 minutes under constant agitation. Enzymatic activity was terminated

by adding equal volume culture media (α-MEM, 2 mM L-glutamine, 1% penicillin [10 000 units/mL], 1% streptomycin [10 mg/mL], 1% amphotericin [250 µg/mL] & 20% foetal bovine serum [FBS; Biowest, Nuaillé, France]). The resulting cell suspension was pelleted and resuspended in 500 µL culture media, filtered through a 70 µm cell strainer (Merck), seeded into a 25 cm^2 flask (ThermoFisher Scientific, Massachusetts, US) and incubated under standard culture conditions. These cultures were defined as being passage 0. Media was changed every 48 hours leaving only adherent cells, which were expanded upon until flasks were 70-90% confluent where they were passaged using Trypsin 1mM EDTA 4Na (Gibco, Paisley, UK). All experiments were conducted in a laminar flow hood under sterile conditions at passage 2 in triplicate with four biological replicates, each representing a different donor [n = 12]).

PL-MSC Immunophenotype Characterisation: Fluorescence activated cell sorting (FACS) analysis was performed as per Liao et al., (2011) to characterise cellular immunophenotype. Briefly, isolated cells were trypsinised, washed twice in PBS and resuspended in 10% FBS supplemented PBS at a 1 x 10^6 cells per mL concentration. Thereafter, 100 µL cell suspension (1 x 10^5) was aliquoted into 1.5 mL micro-centrifuge tubes (Merck) and incubated in the dark for 60 minutes at room temperature, under constant agitation. The monoclonal FITC-conjugated antibodies for anti-human Cluster of Differentiation [CD]-105, CD-90, CD-73, CD-45 and CD-34 at their recommended working concentrations (Abcam, Cambridge, UK). The CD-105, CD-90 and CD-73 were used as mesenchymal markers and CD-34, and CD-45 were erythropoietic markers. A monoclonal FITC-conjugated anti-human IgG1 was used as the isotype control (Abcam). After incubation, labelled cells were washed twice in PBS, resuspended in 1 mL of 10% FBS/PBS and then immediately subjected to FACS analysis using a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose,

California, USA). Excitation was performed at 488 nm alongside a FITC (530/530) emission filter and data from 20,000 events per sample was analysed using FACSuite software (BD Biosciences). Initially, viable and single cells were gated using forward (FSC) vs side scatter (SCC) and FSC-height vs FSC-width density plots, respectively. The isotype control was used to gate and determine the percentage of positive and negative staining. Results were visualised using univariate histograms and presented as the percentage of positively stained cells for each antibody.

Proliferation Assay: To determine the proliferative effects of dECMs on PL-MSCs, a water-soluble tetrazolium [WST]-1 assay (Merck) was performed (Tomson et al. 2017). Briefly, cells were resuspended into concentrations of 1 x 10^6 per mL of 5% FBS culture media, seeded into clear flat bottom 48-well plates (Corning, New York, US) at densities of 1 x 10^4 per well and incubated overnight to facilitate attachment. Cultures were then serum starved for 24 hours. At this time point (Day 0), 180 µL culture media supplemented with 50, 25, 12.5, 6.25 or 3.125 pg/mL of dECM was added to each well, with 1% and 10% FBS serving as negative and positive control, respectively. 500 µL PBS was transferred into any superfluous wells to minimise edge effects. Cultures were maintained for 14 days in 5% CO_2 incubators, with media changes every 48 hours. Cell numbers were determined at day 1, 3, 5, 7, 10 and 14 by adding 20 µL WST-1 solution into wells and then incubating plates for 1 hour under standard conditions. Absorbance of the reduced tetrazolium salt was measured in a plate reader (Tecan Spark, Männedorf, Switzerland) at 450 nm alongside a 630 nm reference filter. Standard curves obtained on Day 0 were used to quantify the median cell numbers per group and time point.

Chemotaxis Assay: The cell homing capabilities of dECMs components on PL-MSCs were investigated using a two chamber 96-well plate assay system as per

manufacturer's instructions (Chemotaxis assay kit, Abcam; Tomson et al. 2017). Briefly, cells were serum starved for 24 hours *a priori* and seeded at densities of 5 x 10^4 onto semi-permeable 8 µm pore size polyester membranes within the top chamber. Assemblies were incubated under standard culture conditions for 72 hours with the bottom chamber containing α-MEM supplements with solubilised dECM components at concentrations of 50, 25, 12.5, 6.25 or 3.125 pg/mL or with only 1% and 10% FBS acting as negative and positive controls, respectively. Subsequently, the top chamber was removed, and the bottom chamber underwent several wash steps alongside 5 minutes centrifugation at 1000 x g at room temperature. The cell dye was then added and the plate incubated for 1 hour under standard culture conditions before fluorescence was measured with a plate reader (Tecan Spark) at excitation and emission wavelengths of 530 nm and 590 nm respectively. Data was presented as median RFU values and percentage migration with respect to positive control.

Cell Viability Assay: Trypan blue exclusion and FACS analysis with Annexin V (AV) and Propidium iodide (PI) staining (Abcam, Cambridge, UK) was performed to determine PL-MSC viability after dECM exposure (Duncan et al. 2013). Briefly, 1 x 10^5 cells per well were seeded into six-well plates and cultured until 70% confluent. At this time point (day 0), media was substituted for dECM supplemented α-MEM at concentrations of 50, 25, 12.5, 6.25 or 3.125 pg/mL, with 1% FBS and 1% saponin supplemented media serving as negative and positive controls, respectively. Media was replenished daily and after 48 hours (day 2), cells were detached, stained with 0.4% trypan blue (Sigma-Aldrich) and counted using a Neubauer haemocytometer (Hausser Scientific, PA, USA). Thereafter, 1 x 10^5 cells from each well were resuspended into 500 µL AV binding buffer and incubated in the dark with 5 µl AV and PI for 15 minutes at room temperature under constant agitation. Fluorescence was

assessed via FITC (530/530) and phycoerythrin (585/545) emission filters to detect AV and PI fluorescence respectively from 20 000 events per group. Gating was determined in AV- vs. PI-fluorescence density plots using controls. Results were visualised in quadrant regions of bivariate density plots and presented as the average median percentage of individual cell states for each dECM concentration.

Osteogenic Differentiation Analysis: To assess the osteogenic potential of solubilised dECMs in PL-MSC cultures, a differentiation and gene expression study was conducted. Differentiation assays were performed in clear flat bottom 48-well plates (Corning) with seeding densities of 1 x 10^4 per well, whereas the gene expression analysis was performed using cultures from 25 cm^2 flasks (Merck) with seeding densities of 5 x 10^5 per flask. All experiments commenced when cells reached 70-90% confluency and assays performed after 21 days incubation with 50 pg/mL dECM or 1% FBS supplemented media, the latter serving as negative control. Osteogenic inductive media served as positive control and consisted of standard culture media supplemented with 10% FBS, 0.2 mmol/mL ascorbic acid (Merck), 0.1 µmol/mL dexamethasone (Merck) and 10 mmol/mL β-glycerophosphate (Merck). The assays detailed below were utilised to determine differentiation and associated gene expression changes.

i) Alizarin Red Assay: To detect extracellular calcified mineral deposits, cells were fixed with 4% paraformaldehyde for 30 minutes, washed in PBS and stained with 40 mmol/L Alizarin Red S (pH 4.1; Merck) for 20 minutes at room temperature. Stain intensity was quantified by removing non-specific dye with three 5-minute distilled water washes, solubilising residual stain in 10% acetic acid (Merck) for 30 minutes on an orbital shaker, and then analysing absorbance at 405 nm via spectrophotometry

(ELx800; Biotek, Vermont, US) against a serial dilution of Alizarin Red solution (Gregory et al., 2004).

ii) Gene Expression Analysis: To determine osteogenic gene expression profiles real-time quantitative polymerise chain reaction (RT-qPCR) was performed in cultures as described below (Widbiller et al. 2018).

RNA Isolation & Purification: Total RNA was extracted using 350 µL of lysis buffer using the Rneasy® minikit (Qiagen, West Sussex, UK), which was then mixed with an equal volume of 70% molecular grade ethanol (Merck). This lysate was homogenised, transferred to a spin column for 30 seconds centrifugation at 8 000 x g and then washed with 350 µL of buffer. DNA digestion was then performed on the spin column membranes by treatment with 80 µL of Dnase solution (Rnase-Free Dnase Set; Qiagen) for 15 minutes at room temperature, washed as per manufacturer's instructions and dried with 2 minutes centrifugation at 13,200 g. The resulting RNA was eluted into 30 µL Rnase free water, by 1 minute centrifugation at 8,000 g, and concentration and purity determined using a spectrophotometer (BioPhotometer, Eppendorf, UK). The former was determined by measuring absorbance at 260 nm and the latter from the 260/280 nm absorbance ratio equating between 1.8 – 2.0.

cDNA Synthesis: Complementary strands of DNA (cDNA) were generated using Oligo(dT)18 primers (Thermo Fischer Scientific) using the Tetra cDNA synthesis kit (Meridian Bioscience, Ohio, USA). As per manufacturer's instructions, each reaction was performed on ice and consisted of 2 µg RNA, which was equilibrated to a final volume of 20 µL with RNase free water. Using a thermocycler (Biometra Tadvanced Twin 48G; Analytik, Jena, Germany), this mixture was maintained at 45^{o}C for 1 hour, terminated at 85^{o}C for 5 minutes and rapidly cooled to 4^{o}C where it was stored at -20^{o}C until further use.

RT-qPCR: Reference and target genes were selected from the relevant literature and human gene specific primers were purchased from ThermoFisher Scientific (Table 1; Marelli et al., 2013). Real-time qPCR was performed in 96-well microtiter plates with a 20 µl total reaction volume, which consisted of 2 µl cDNA, 10 µl Roche SYBR green I Master PCR mix, 0.5 µM of each primer and Rnase free water. For all samples, initial denaturation occurred at 95°C for 5 mins followed by 45 cycles of 95°C for 20 seconds and annealing at 60°C for 1 minute using the LightCycler480 system (Roche Diagnostics, Indiana, US). Two no-template controls per primer pair were included in every run, with their efficiency pre-determined using dilutions (1:1 – 1:1000) of sample cDNA. Expression levels for each target gene were determined by normalizing Cp values to the B2M housekeeper gene and relating them to untreated controls by way of the second derivative max method, as computed by the LightCycler 480 software (V1.5; Roche Diagnostics).

Statistical Analysis

All data was coded, and statistical analyses performed in SPSS software (V.25 IBM, New York, US). A preliminary screen for data normality using the Shapiro-Wilk test revealed a majority non-normally distributed data thus, descriptive statistics were presented as medians ± [interquartile range]. Inter-group unpaired and intra-group paired comparisons were respectively made using the Mann-Whitney U or Wilcoxon Matched Pair tests and Kruskal-Wallis or Friedmans tests with post-hoc pairwise comparisons alongside Bonferonni correction. Initial alpha values were set at 0.05.

Results

1. dECM bioavailability

Effect of clinically representative irrigation protocols: All variations of regenerative irrigant regimes that utilised NaOCl significantly reduced intracanal dECM bioavailability, with 17% EDTA alone consistently yielding the greatest abundance at all time points ($p < 0.05$) (Figure 2). Detrimental effects were more pronounced with higher NaOCl concentrations, which required longer durations of EDTA exposure to enable detection of TGF-β1 above the assay's lower threshold limit of quantification. Mechanically re-instrumenting dentine nevertheless significantly negated this impact when compared to its comparator group however, quantities were always significantly lower than the positive control after 5 minutes ($p > 0.05$). In all groups, EDTA exposure time was proportionate to dECM solubilisation of TGF-β1 with there being a high degree of variability in its release. The control gave nil data as expected.

Periradicular Bioavailability: dECMs were detected in periradicular methylcellulose strips in only the 17% EDTA (33.4 pg/mL [21.41 – 76.20]) and 0.5% NaOCl (6.38 pg/mL [4.27 – 10.72]) groups, with differences being statistically insignificant ($p > 0.05$). These quantities nevertheless were always significantly lower than their intracanal counterparts ($p < 0.01$; Figure 2).

2. Regenerative effects of solubilised dECMs on PL-MSCs

dECM Characterisation: The median TPC within dECM extracts from 60 minute 17% EDTA irrigation was 998.3 µg/mL (871.46 – 1200.33). Multiplex analyses consistently detected 38 of a potential 40 growth factors above the minimum threshold. The most abundant of these being IGFBP-3, -1, TGF-β1 and BMP-4 (Figure 3).

Baseline Characteristics of Donor Patients: Four samples of periapical lesions were collected from patients with a median age of 29 yrs [33.5 – 52.3]. Patients comprised 3 females and 1 male, with ethnicities being Afro-Caribbean (50%), Asian (25%) and Caucasian (25%). The teeth studied were 3 maxillary incisors and 1 mandibular premolar with a median lesion size of 6.5 mm [4.7 – 8.3] (Table 2).

Immunophenotype: Significantly positive expression of mesenchymal markers CD-73 (90.3% [85.07 – 90.46]), CD-90 (97.7% [95.11 – 99.31]) and CD-105 (55.9% [41.34 – 74.57]) and negative expression of haematopoietic markers CD-34 (1.2% [0.83 – 1.42]) and CD-45 (1.0% [0.71 – 1.39]) were identified using FACS analysis of isolated cells ($p < 0.001$; Figure 4).

Proliferation: The WST-1 assay identified increased PL-MSC proliferation in dECM supplemented cultures on day 3, with statistical significance detected for 12.5, 6.25 and 3.125 pg/mL ($p < 0.01$). No similar differences were observed however on days 5, 7 and 10 cell numbers peaked; after which there was a general trend towards negative growth effects amongst all test groups on day 14. However, this was statistically insignificant ($p > 0.05$). The positive control always exceeded the other groups (Figure 5).

Chemotaxis: dECM components induced statistically significant PL-MSC chemotaxis at only the 50 pg/mL concentration when compared to the 1% FBS group ($p < 0.01$). Cell migration amongst lower dECM concentrations however were consistent with that of the control (Figure 5).

Viability: FACS and trypan blue exclusion analysis revealed that dECMs at all concentrations had no significant apoptotic effects on PL-MSCs ($p > 0.05$). Within these test groups, the proportion of unstained viable cells ranged from 50.1% to 62.0%; Annexin-V positive early apoptotic cells ranged from 1.7% to 3.4%; Annexin-V

and PI positive late apoptotic cells ranged from 6.0% to 15.2%; and PI positive necrotic cells ranged from 22.5% to 36.4%. The percentage of dead cells determined by the uptake of trypan blue ranged from 6.7% to 23.5% and 16.9% to 32.7% in PL-MSCs cultured in dECM and 1% FBS supplemented media ($p > 0.05$). Significantly lower apoptotic events were detected in both experiments for 6.25 and 3.125 pg/mL exposure concentrations when compared with the positive control ($p < 0.001$; Figure 6).

Osteogenic Differentiation: Dentine extracellular matrix components induced osteogenic differentiation in PL-MSCs. Alizarin red stain intensity was quantified to be 1.5-fold greater in test wells compared with 1% FBS controls ($p < 0.05$), with central islands of mineralisation evident after only 1 week of culture. These results were supported by gene expression data, where RT-qPCR revealed significant upregulation of DMP-1 (9.3 [6.94 – 13.53]), DSPP (18.0 [14.94 – 22.01]), OPN (16.6 [8.69 – 25.43]) and RUNX2 (13.8 [6.70 – 20.31]) when compared to the 1% FBS group ($p < 0.05$). Conversely, ALP (0.4 [0.27 – 0.52]), COL1A1 (0.3 [0.18 – 0.31]) and OSC (0.6 [0.23 – 2.61]) were all downregulated after 21 days induction, although these were not statistically significant ($p > 0.05$). Osteogenic media serving as the positive control highlighted the significant osteogenic potential of PL-MSCs (Figure 7).

Discussion

The present investigation evaluated dECM bioavailability beyond the apical foramen in the periradicular tissues using clinically representative irrigant protocols and their subsequent regenerative effects on a novel MSC niche retrieved from inflamed human periapical tissues. Any application of NaOCl was found to severely impact the intra-canal and periradicular bioavailability of dECMs, even after mechanically re-instrumenting the dentine in an attempt to remove the dentine matrix affected by

NaOCl. 17% EDTA was the most effective irrigant regime and liberated a wide range of growth factors. Exposing this extract to PL-MSC cultures demonstrated upregulation of functional cellular processes related to tissue regeneration. For these reasons, all null hypotheses have been rejected.

The experimental model utilised to harvest dECMs in the current investigation helps bridge the gap between laboratory-based protocols and therapeutic application. For instance, in this model, teeth remained intact and root canals were prepared to standardised dimensions leaving only a clinically representative surface area from which growth factors could be liberated. Commonly available proprietary solutions that are used clinically were also administered via conventional needle irrigation and were capable of being activated by any means within a closed system. Irrigant exposure times, concentrations and volumes were similar to those recommended under clinical guidelines (Galler et al. 2016). Furthermore, this was the first model to consider the interaction between dECMs solubilised into the canal during irrigation and periradicular tissues. This work provides contrast with previous studies that utilised dentine powders and disks (Tomson *et al.* 2013, Galler *et al.* 2015, Sadaghiani *et al.* 2016, Widbiller *et al.* 2017) or where analyses were performed after many hours of immersing intact prepared teeth in large volumes of media (Zeng *et al.* 2016, Chae *et al.* 2018, Ivica *et al.* 2018). As the methodological approach used closely mimics clinical procedures the results are therefore likely more clinically relevant than those from these previous studies. Caution must still nevertheless be taken when extrapolating results due to them being derived from *in vitro* models.

A notable challenge to clinically implementing the above hypothesis is the ability of dECMs to interact with the periradicular tissues once solubilised into the root canal, particularly in mature permanent teeth. The model used here however demonstrated

proof of concept for adequate periradicular bioavailability of dECMs when the apical foramen was only minimally enlarged to 0.2 mm. At present, this is the first investigation to report such a finding and considerably advances the paradigm shifting notion of cell-free tissue engineering strategies for treating apical periodontitis. This contrasts previous recommendations of pre-enlarging to 0.5 – 1.0 mm, which could be attributed to this guideline being based on the need of sufficient intraradicular influx of blood and cellular components for revascularisation (Fang et al. 2018, Kim et al. 2018). These would invariably require a larger interface than the extraradicular efflux of smaller dECM molecules. Although less than their intracanal counterparts, quantities of dECMs measured within periradicular methylcellulose strips were nevertheless within the picogram range previously shown to induce cellular regenerative events (Galler et al. 2015, Widbiller et al. 2018). The limited absorbance capacity of the methylcellulose strips however could have underestimated bioavailability.

In preliminary methodological studies, concentrations of 3.0% were investigated and confirmed to have nil detection whilst 0.5% was included in the present study to account for the lowest NaOCl concentration reported to be effective at disinfection of the root canal system (Baumgartner & Cuenin 1992). This contrasts Galler et al. (2015) who investigated similar effects of NaOCl on dECMs at concentrations of 5.25% using a less clinically representative model. These pilot data also confirmed that downstream ELISAs were not contaminated with NaOCl following deionised water flushing of root canals (unpublished data). Instead, these findings more likely result from the non-specific proteinaceous properties of this irrigant that not only eliminate endodontic micro-organisms, but also causing degradation of the organic components of the dentine (Galler et al. 2015). This would also explain why higher concentrations, which have greater dentinal tubular penetration, had more pronounced effects and why

reducing surface area of NaOCl affected dentine via mechanical re-instrumentation improved dECM yields (Virdee et al. 2020). These results are consistent with several previous reports (Galler et al. 2015, Gonçalves et al. 2016, Widbiller et al. 2022) however it is acknowledged that they conflict with data reported by Zeng et al. (2016) and Chae et al. (2018). These differences are likely attributable to extended solubilisation periods, which in some experiments was undertaken for up to 24 hours. As demonstrated by the present longitudinal analyses, these longer durations of exposure likely offset the now well-established negative effects of NaOCl treatment.

As per previous reports, exclusively irrigating with 17% EDTA yielded the greatest dECM bioavailability and so this protocol was used in further analyses (Tavares et al. 2021). Quantitative multiplex characterisation of resulting extracts identified a broad growth factor profile. These included members of the TGF-β superfamily (including TGF-α; TGF-β1, -β3; BMP-4, -5, -7; and GDF-15); fibroblast growth factors (bFGF; and FGF-4, -7); neurotrophic growth factors (BDNF; β-NGF; GDNF; NT-3, -4); angiogenic growth factors (EG-VEGF; PDGF-AA; SCF, -R; and VEGF, -R2, -R3, -D), insulin related growth factors and cytokines (IGFBP-1, -2, -3; IGF-I; Insulin); colony-stimulating factors (MCF-R) and others cytokines (AR; EGF, -R; GH; HB-EGF; HGF; NGFR; OPG; and PIGF). These have all previously been associated with periradicular regenerative processes and their presence reported when more broader analyses were conducted (Park et al 2009, Jágr et al 2012, Márton & Kiss 2014). There are however conflicting observations with Duncan et al. (2017), who identified a smaller range of 18 growth factors when applying the same array to lyophilised extracts. The concentrations for some of the more abundant proteins were also considerably greater than those measured in the present investigation. These differences could respectively be explained by differences in sample size and the dentinal surface areas available

for solubilisation. Both reports nevertheless highlight a considerable discrepancy between TGF-β1 measurements from the multiplex array and sandwich-ELISA. As the former assay quantifies analytes from neat samples without the need to activate them via several dilution steps, it is likely to be a more representative reading. The latter technique however was used to aliquot extracts in preparation for cellular experiments due to logistical and pragmatic reason. It is acknowledged the actual quantity of dECMs in each group may therefore be an underestimation.

This is the first investigation to evaluate the regenerative effects of dECM extracts on PL-MSCs. FACS analyses revealed these cells exhibited an immunophenotype that was consistent with a mesenchymal lineage and with the previous reports that further explored their stem-like characteristics (Liao et al., 2011, Đokić et al., 2012, Marelli et al., 2013). When dECMs were topically applied to these cultures, the WST-1 assay largely demonstrated a time-dependant effect on proliferation. More specifically, cell numbers significantly increased in comparison with controls relatively early in the experimental time period however they exhibited a downward trend at later stages. Similar growth patterns have been observed in dental pulp stem cell cultures, although these experiments demonstrated a dose-dependent effect (Tomson et al. 2017, Widbiller et al. 2018). Whilst this may have been indicated at day 3, where lower concentrations proved to be more mitogenic, proliferation rates were relatively consistent across all groups at the other timepoints studied. This may be attributable to the narrower dECM concentration range tested in the present study, as negative effects have been reported to occur at the much higher concentrations of 200 pg/mL (Widbiller et al. 2018). These observations nevertheless, could result from the net biological outcome of the various molecules within dECM extracts, as some constituents, such as TGF-β1, possess anti-mitogenic properties after exceeding a

critical concentration (Massagué et al. 2000). Moreover, dECM-induced terminal differentiation may further contribute to reducing cell numbers over time.

Periapical lesion-derived MSCs have been found to largely reside within the peripheral capsules of granulomas, cysts and abscesses (Estrela et al. 2019). It would thus be highly desirable for prospective periradicular tissue engineering strategies to stimulate their migration to more central regions of the lesion. Chemoattractants known to bring about such a change include HGF, FGF and TGF-β1 (Howard et al. 2010, Mathieu et al. 2013, Tomson et al. 2013). Despite these growth factors being detected in high abundances within dECM extracts, significant chemotaxis of PL-MSCs was observed at only the highest tested concentration of 50 pg/mL. This contrasts with data from Widbiller et al. (2018) who observed notable dose dependant migratory effects at concentrations as low as 10 pg/mL. A potential explanation for this discrepancy is that MSCs isolated from diseased and inflammatory tissues may require a stronger stimulus than their healthy counterparts due to the molecularly complex local milieu. Further studies would be required to confirm this hypothesis.

Flow cytometry with Annexin-V and PI staining confirmed that dECMs did not have a significant detrimental effect on the viability of PL-MSCs. Indeed, it could be suggested there was a trend towards an anti-apoptotic effect, as slightly higher and lower proportions of viable and dead cells, respectively, were observed in test groups when compared with the 1% FBS control. This observation is supported by both mechanistic data, where dECMs are shown to reduce apoptotic caspase-3 activity whilst simultaneously increasing expression of the Ak1 cell survival protein, with similar observations reported in dental pulp stem cell cultures (Lee et al. 2015, Widbiller et al. 2018).

Differentiation analyses confirmed dECMs were capable of committing PL-MSCs down a mineral producing lineage. These data agree with previous *in vitro* studies that explored similar effects in other endodontic stem cell niches (Virdee et al. 2022). Gene expression profiles were highly consistent with mature osteoblastic differentiation after 21 days induction. For example, ALP and COL1A1, which typically achieve their greatest expression during the transformation of precursory cells to pre-osteoblasts soon after induction, were downregulated at this late time-point (Dacic et al. 2001, Lee et al. 2015, Sadiaghiani et al. 2016, Widbiller et al. 2018). Conversely, genes involved in the biomineralization of calcific hard tissues, including DMP-1, DSPP and OPN, were significantly overexpressed whilst inhibitors of bone formation such as OSC was downregulated (Ducy et al. 1996). Interestingly, the expression of RUNX2, a multifunctional transcription factor that regulates osteoblastic differentiation, significantly increased when media was supplemented with dECMs as opposed to osteo-inductive components, where there was little change with respect to the 1% FBS control. Relatively high expression at this same time-point indicate the potent ability of dECMs to terminally differentiate PL-MSCs into osteoblastic as opposed to odontoblastic lineage, which would require RUNX2 to be downregulated (Li et al. 2011). Nevertheless, these transcriptional changes translated to only modest mineral production, with alizarin red stain intensity only marginally exceeding that of the 1% FBS group. This could be attributed to using much lower concentration of dECM extracts when compared with previous studies (Lee et al. 2015, Widbiller et al. 2018).

Conclusions

When 17% EDTA is used as the primary irrigant during orthograde root canal treatment, a broad range of bioactive molecules will be liberated from the dentine matrix which are bioavailable at the periradicular interface. Analyses here has

demonstrated *in vitro*, that these signalling molecules significantly upregulate proliferation, chemotaxis, osteogenic differentiation and mineralisation in PL-MSC without compromising cell viability. This mechanistic data provides proof of concept for more novel periradicular tissue healing strategies where dECMs can be exploited during root canal therapy to treat apical periodontitis. Further interventional investigations that now test the clinical efficacy of such irrigant regimes are now warranted.

Conflicts of Interest

The authors deny any conflicts of interest related to this study

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- 2017 British Endodontic Society Annual Research Grant
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- 2021 RCS Edinburgh Pump Priming Grant

Table 1. Detailed of primers used for real-time quantitative PCR and their efficiencies

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Size (bp)	Efficiency
ALP	GACCCAAGAAACCAAAGTCTGCC	GAGGGAGCAAAGGCTGGAGG	95	1.8
COL1A1	CCTCTGCTCTCCGACCTCTCT	CTTTGTGCTTTGGGAAGTTGTCTCT	128	1.8
DMP-1	GCAGAGAGTCAGAGCGAGGAA	CCGTGGAGTTGCTATCTTCTTTG	91	1.8
DSPP	AGCCACAAACAGAAGCAACAC	GACAACAGCGACATCCTCATT	97	2.0
OSC	GGCACCCTTCTTTCCTCTTCCC	CCCACAGATTCCTCTTCTGGAGTTT	114	1.9
OPN	ATAAGCGGAAAGCCAATGATGAGAG	GGGTCTACAACCAGCATATCTTCA	130	1.8
RunX2	CTCCACCCACCCAAGCAGAA	GTTTGAGAAGGACCAGAGAACAAGG	80	1.8

[ALP] Alkaline Phosphatase; **[COL1A1]** Collagen Type 1; **[DMP-1]** Dentine Matrix Acid Phosphoprotein; **[DSPP]** Dentine Sialophosphoprotein; **[OSC]** Osteocalcin; **[OPN]** Osteopontin; **[RunX2]** Runt-related transcription factor

Table 2: Baseline characteristics of samples

Sample Characteristics		Total (%)
Age (years)	Median [IQR]	29 [33.5 – 52.3]
Sex	Female	3 (75)
	Male	1 (25)
Ethnicity	Afro-Caribbean	2 (50)
	Asian	1 (25)
	Caucasian	1 (25)
Inter-arch position	Mandible	3 (75)
	Maxilla	1 (25)
Intra-arch position	Incisors	3 (75)
	Premolar	1 (25)
Lesion Size (mm)	Median [IQR]	6.5 [4.7 – 8.3]

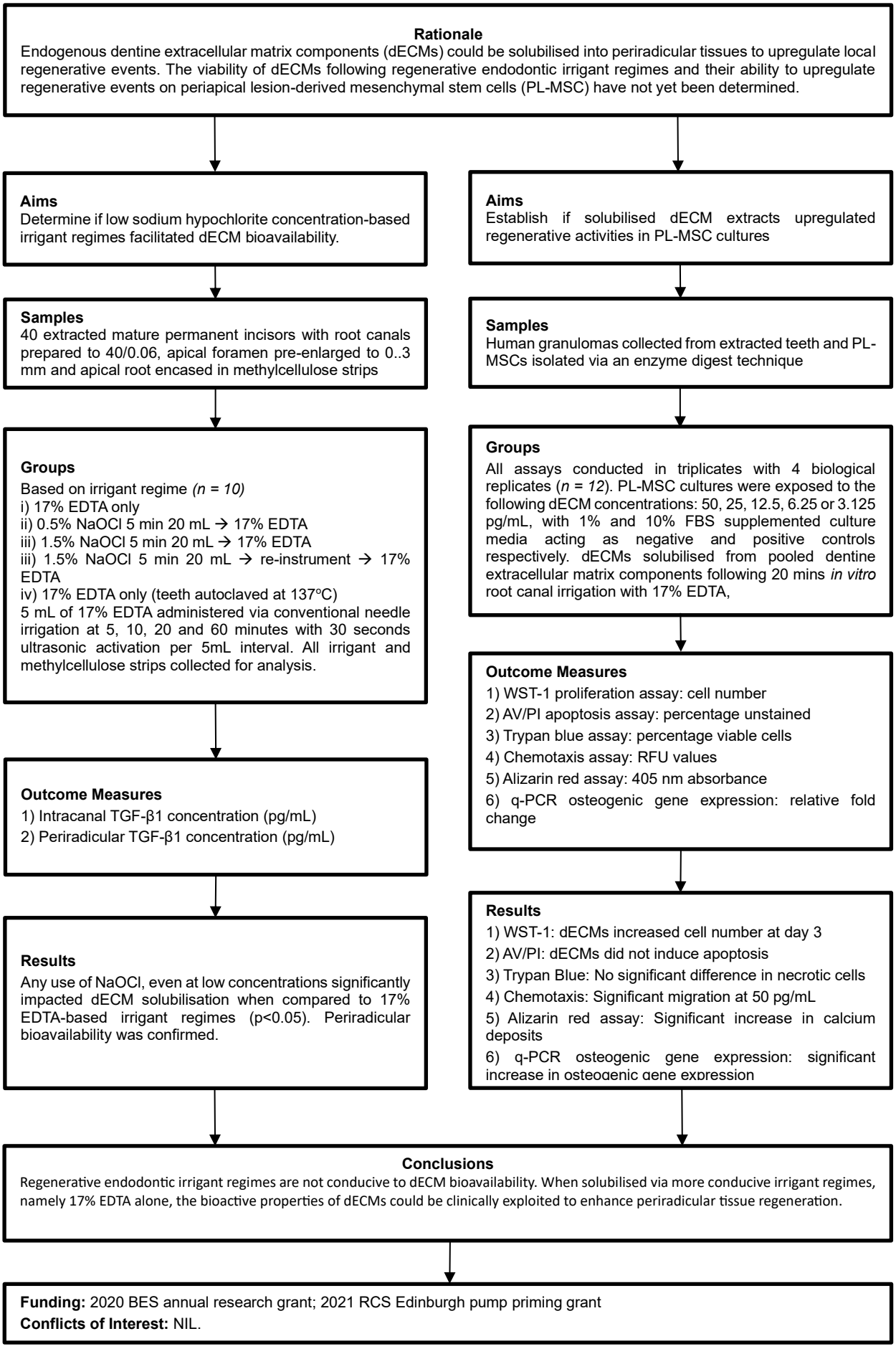

Figure 1: PRILE Flowchart

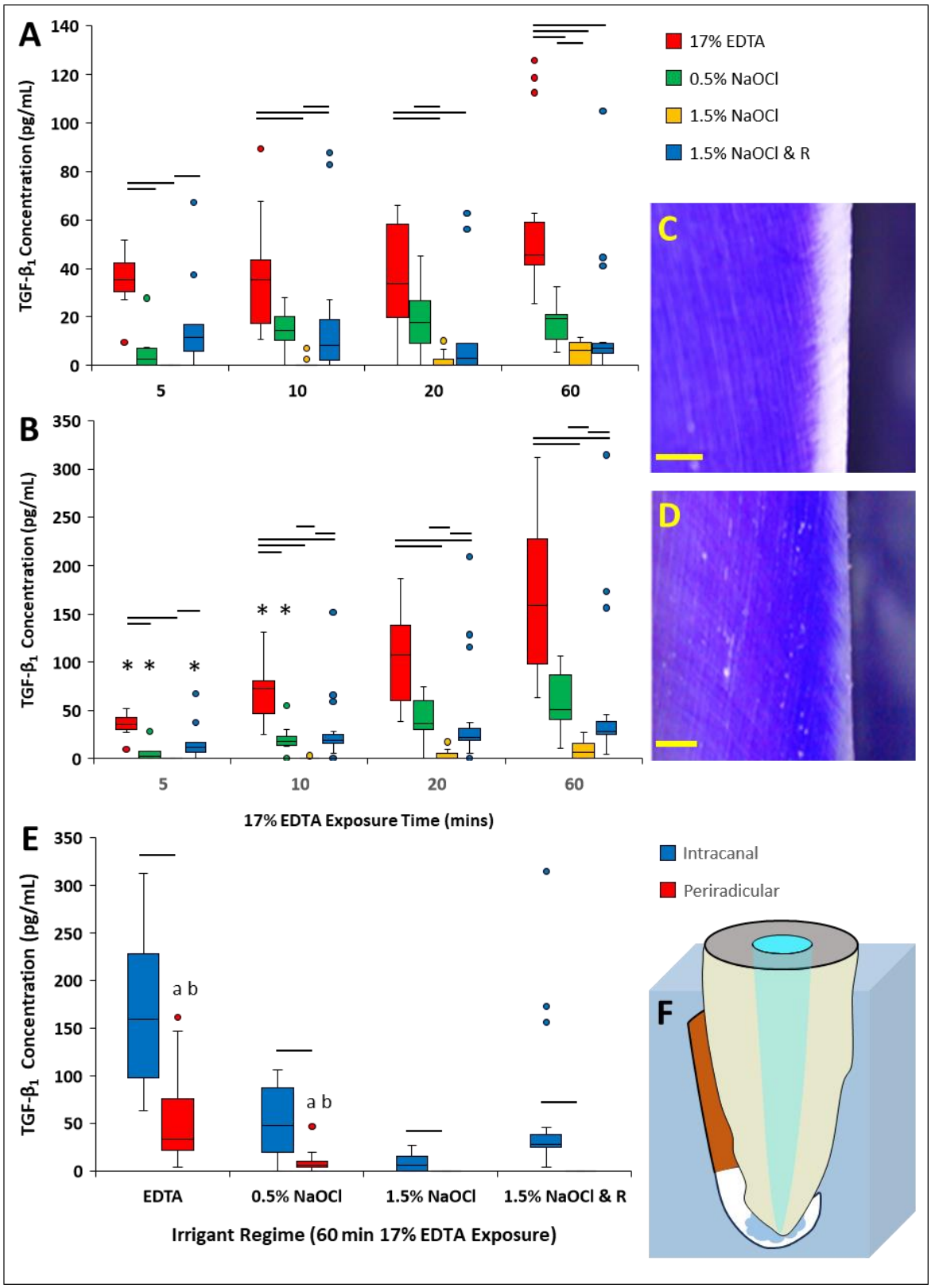
A
TGF-β1 Concentration (pg/mL)
140
120
100
80
60
40
20
0
5
10
20
60
17% EDTA
0.5% NaOCl
1.5% NaOCl
1.5% NaOCl & R
B
TGF-β1 Concentration (pg/mL)
350
300
250
200
150
100
50
0
5
10
20
60
17% EDTA Exposure Time (mins)
C
D
E
TGF-β1 Concentration (pg/mL)
350
300
250
200
150
100
50
0
a b
a b
EDTA
0.5% NaOCl
1.5% NaOCl
1.5% NaOCl & R
Irrigant Regime (60 min 17% EDTA Exposure)
Intracanal
Periradicular
F

Figure 2: Effect of different irrigant regimes on intracanal and periradicular bioavailability of TGF-β_1 (*n* = 10). [A] Isolated and [B] cumulative median intracanal TGF-β_1 concentrations [pg/mL] longitudinally recorded at each 17% EDTA exposure time point. Canals pretreated with 5 minutes i) Nil NaOCl, which was referred to as the 17% EDTA group; 0.5% NaOCl; 1.5% NaOCl; or 1.5% NaOCl with dentine re-instrumentation prior to 5; 10; 20; or 60 minutes 17% EDTA longitudinal exposure with 1 minute passive ultrasonic irrigation within last minute of each time point. Representative light microscopy images of NaOCl affected dentine in the coronal segment of the canal before [C] and after mechanical re-instrumentation of dentine [D] following 5 minutes 1.5% NaOCl irrigation. The white/bleached region of dentine, which was stained with crystal violet, demonstrates that which has been penetrated by NaOCl. Scale bars represent 200 μm. [E] Periradicular bioavailability of TGF-β_1 across the various irrigant regimes following 60 minutes exposure time to 17% EDTA. [F] Schematic diagram representing the closed in vitro system containing tooth with standardised root canal dimensions from which irrigants were retrieved and intracanal bioavailability was determined and an apically positioned paper strip from which periradicular bioavailability was determined. Data presented as box and whisker plots where central bars represent the median alongside upper and lower interquartile ranges at the edge of boxes and minimum and maximum values for the whiskers. Statistically significant comparisons within and between groups *(p < 0.01*; Friedman's & Kruskal-Wallis tests respectively with post-hoc pairwise comparisons) presented as horizontal lines and superscripts ([*] vs. corresponding 60 min group; [a] vs. 1.5% NaOCl; [b] vs. 1.5% NaOCl & R) respectively. [EDTA] Ethylenediaminetetraacetic acid; [R] Re-instrumentation; [NaOCl] Sodium hypochlorite; [TGF-β_1] Transforming growth factor-beta 1.

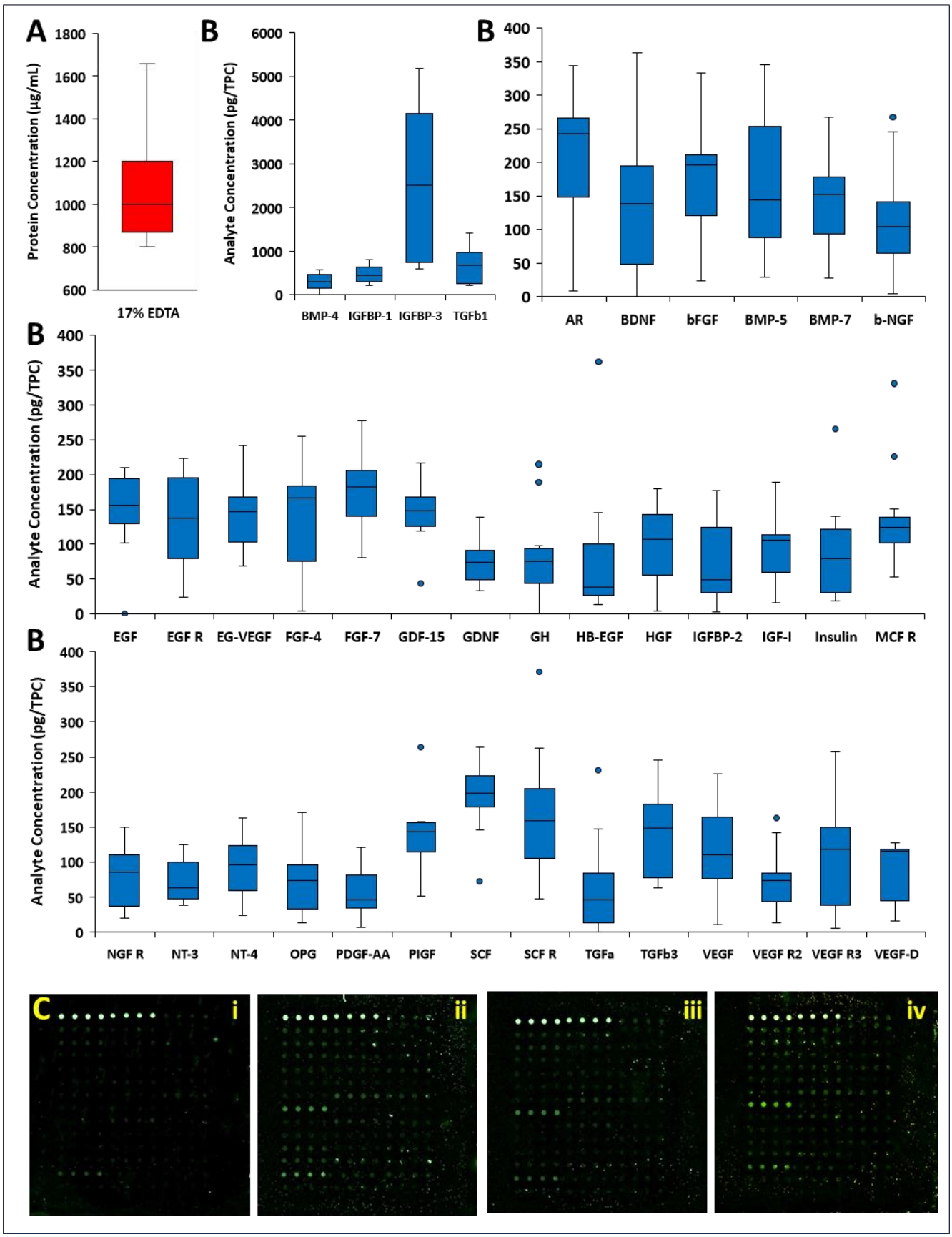
A
Protein Concentration (µg/mL)
1800
1600
1400
1200
1000
800
600
17% EDTA
B
Analyte Concentration (pg/TPC)
6000
5000
4000
3000
2000
1000
0
BMP-4
IGFBP-1
IGFBP-3
TGFb1
B
400
350
300
250
200
150
100
50
0
AR
BDNF
bFGF
BMP-5
BMP-7
b-NGF
B
Analyte Concentration (pg/TPC)
400
350
300
250
200
150
100
50
0
EGF
EGF R
EG-VEGF
FGF-4
FGF-7
GDF-15
GDNF
GH
HB-EGF
HGF
IGFBP-2
IGF-I
Insulin
MCF R
B
Analyte Concentration (pg/TPC)
400
350
300
250
200
150
100
50
0
NGF R
NT-3
NT-4
OPG
PDGF-AA
PIGF
SCF
SCF R
TGFa
TGFb3
VEGF
VEGF R2
VEGF R3
VEGF-D
C
i
ii
iii
iv

Figure 3: Analysis of the presence and concentrations of growth factors within dentine extracellular matrix components solubilised with 17% EDTA irrigant collected from within root canals after 60 minutes exposure time (*n* = 10). [A] Total protein concentration (µg/mL). [B] Presence and concentration (pg/TPC) of growth factors. [C] Representative quantitative antibody array images from [i] negative control and [ii – iv] independent test samples. Data presented as box and whisker plots where central bars represent the median alongside upper and lower interquartile ranges at the edge of boxes and minimum and maximum values for the whiskers.

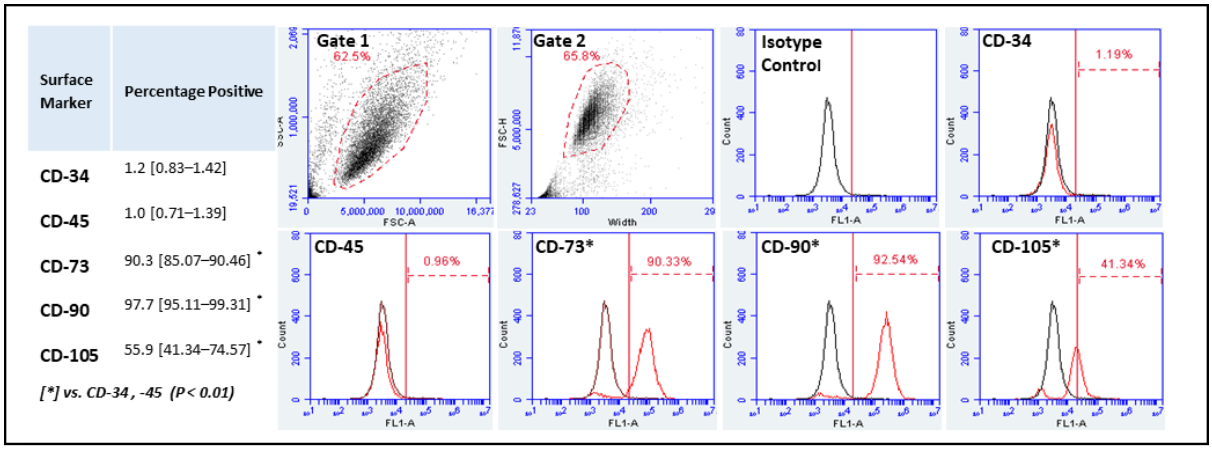

Figure 4: Immunotype characterisation of periapical lesion derived-mesenchymal stem cells. Fluorescence activated cell sorting analysis of cultures for mesenchymal (CD-73; CD-90; CD-105) and haematopoietic (CD34; CD-45) surface markers against isotype controls. Percentage positive cells presented as univariate histograms where isotype control is represented by the black peak, and the tested population by the red peak.

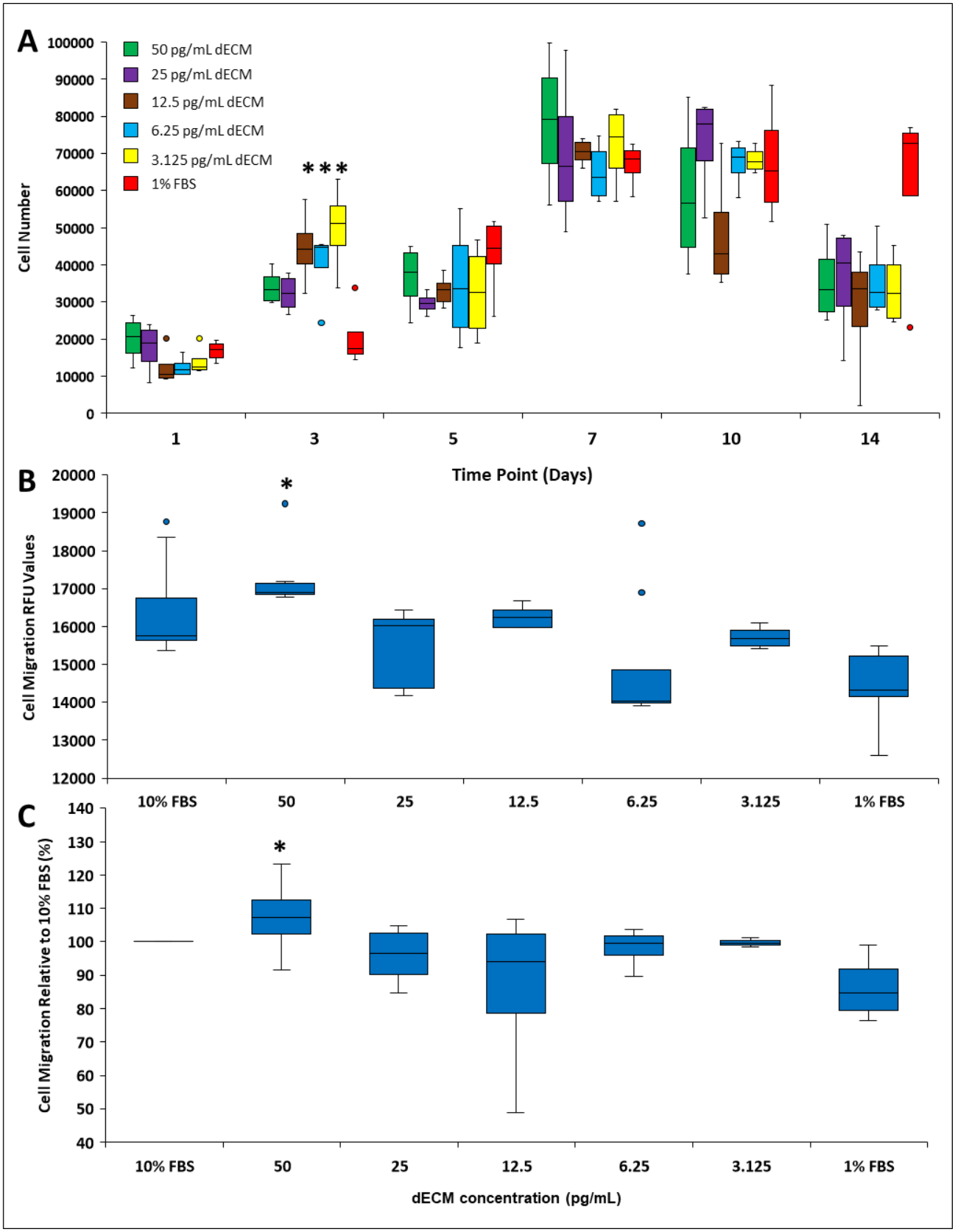
A
50 pg/mL dECM
25 pg/mL dECM
12.5 pg/mL dECM
6.25 pg/mL dECM
3.125 pg/mL dECM
1% FBS
Cell Number
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
***
1
3
5
7
10
14
Time Point (Days)
B
Cell Migration RFU Values
20000
19000
18000
17000
16000
15000
14000
13000
12000
*
10% FBS
50
25
12.5
6.25
3.125
1% FBS
C
Cell Migration Relative to 10% FBS (%)
140
130
120
110
100
90
80
70
60
50
40
*
10% FBS
50
25
12.5
6.25
3.125
1% FBS
dECM concentration (pg/mL)

Figure 5: PL-MSC proliferation and chemotaxis assays following exposure to a range of TGF-β1 normalised dentine extracellular matrix component (dECM) concentrations solubilised from root canals following 20 mins *in vitro* irrigation with 17% EDTA. Cultures were analysed in duplicate over a 14-day period for the proliferation assay and 48 hours for the chemotaxis assay with 10% and 1% FBS supplemented media acting as positive and negative control respectively (n = 8). **[A]** Cell numbers, **[B]** RFU values and **[C]** percentage cell migration relative to 10% FBS data presented as box and whisker plots where central bars represent the median alongside upper and lower interquartile ranges at the edge of boxes and minimum and maximum values for the whiskers. Statistically significant comparisons between groups (p < 0.01; Kruskal-Wallis tests with post-hoc pairwise comparisons) presented as superscripts ([*] vs 1% FBS group).

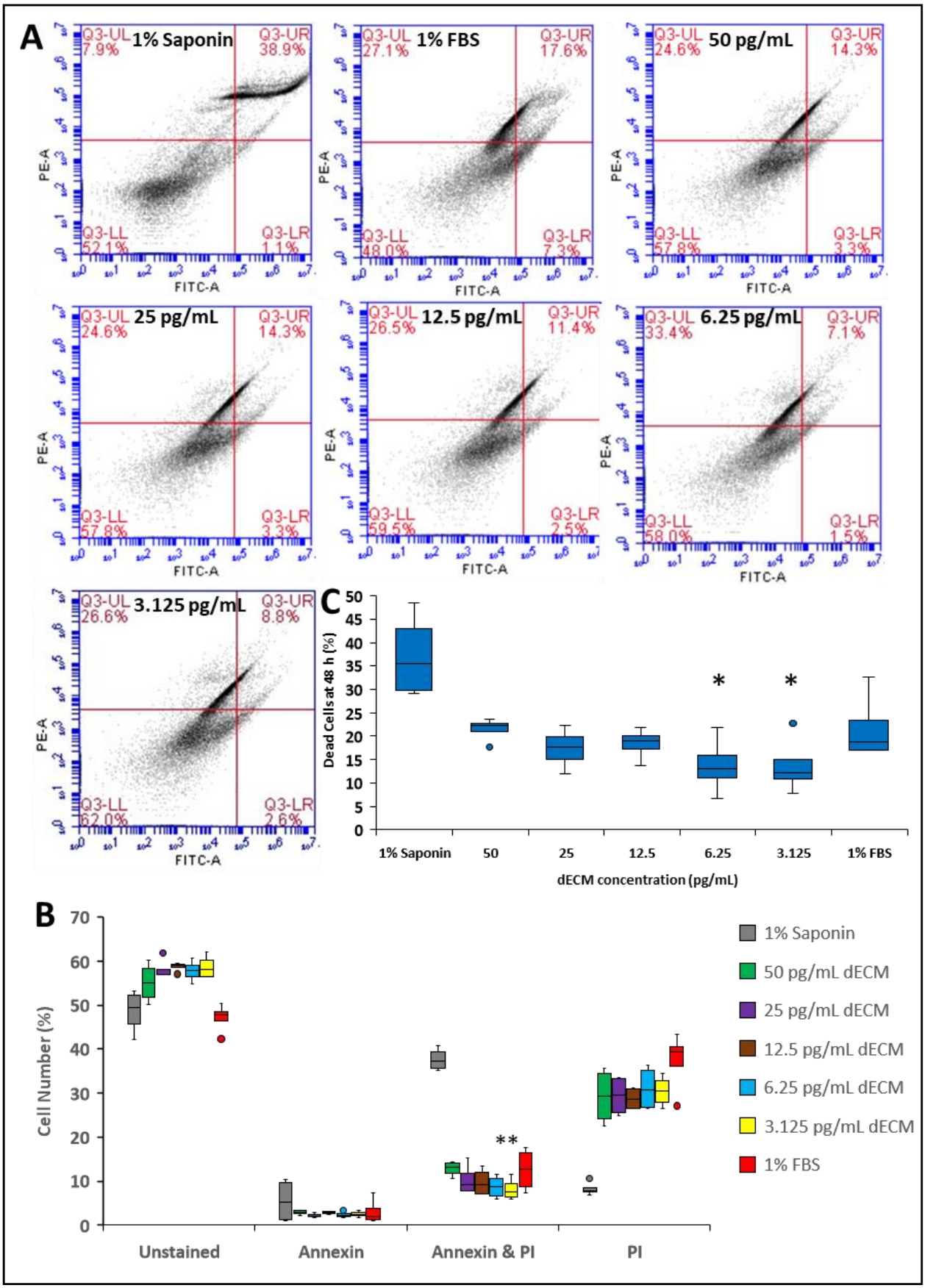
A
Q3-UL 7.9%
1% Saponin
Q3-UR 38.9%
Q3-LL 52.1%
Q3-LR 1.1%
Q3-UL 27.1%
1% FBS
Q3-UR 17.6%
Q3-LL 48.0%
Q3-LR 7.3%
Q3-UL 24.6%
50 pg/mL
Q3-UR 14.3%
Q3-LL 57.8%
Q3-LR 3.3%
Q3-UL 24.6%
25 pg/mL
Q3-UR 14.3%
Q3-LL 57.8%
Q3-LR 3.3%
Q3-UL 26.5%
12.5 pg/mL
Q3-UR 11.4%
Q3-LL 59.5%
Q3-LR 2.5%
Q3-UL 33.4%
6.25 pg/mL
Q3-UR 7.1%
Q3-LL 58.0%
Q3-LR 1.5%
Q3-UL 26.6%
3.125 pg/mL
Q3-UR 8.8%
Q3-LL 62.0%
Q3-LR 2.6%
PE-A
FITC-A
C
Dead Cells at 48 h (%)
1% Saponin
50
25
12.5
6.25
3.125
1% FBS
dECM concentration (pg/mL)
B
Cell Number (%)
Unstained
Annexin
Annexin & PI
PI
1% Saponin
50 pg/mL dECM
25 pg/mL dECM
12.5 pg/mL dECM
6.25 pg/mL dECM
3.125 pg/mL dECM
1% FBS

Figure 6: Determining the apoptotic and necrotic effects on PL-MSCs to TGF-β1 normalised dentine extracellular matrix component (dECM) concentrations solubilised from root canals following 20 mins *in vitro* irrigation with 17% EDTA. Cultures were analysed in duplicates following 48 hours exposure via **[A]** & **[B]** Annexin-V and PI staining and **[C]** Trypan Blue Exclusion with 1% saponin and 1% FBS serving as positive and negative controls respectively (n = 8) . **[A]** Representative density plots where cells in several phases of viability are presented as percentage of gated cells. (Unstained: viable cells – lower right quadrant; Annexin-V & PI: cells in late apoptosis – upper left quadrant; Annexin-V: cells in early apoptosis - lower right quadrant; PI: cells in necrosis – upper left quadrant). Data presented from FACS analysis **[B]** and Trypan Blue Exclusion **[C]** as box and whisker plots where central bars represent the median percentage cell number and dead cells at 48 hours respectively alongside upper and lower interquartile ranges at the edge of boxes and minimum and maximum values for the whiskers. Statistically significant comparisons between groups ($p < 0.01$; Kruskal-Wallis tests with post-hoc pairwise comparisons) presented as superscripts ([*] vs 1% saponin group).

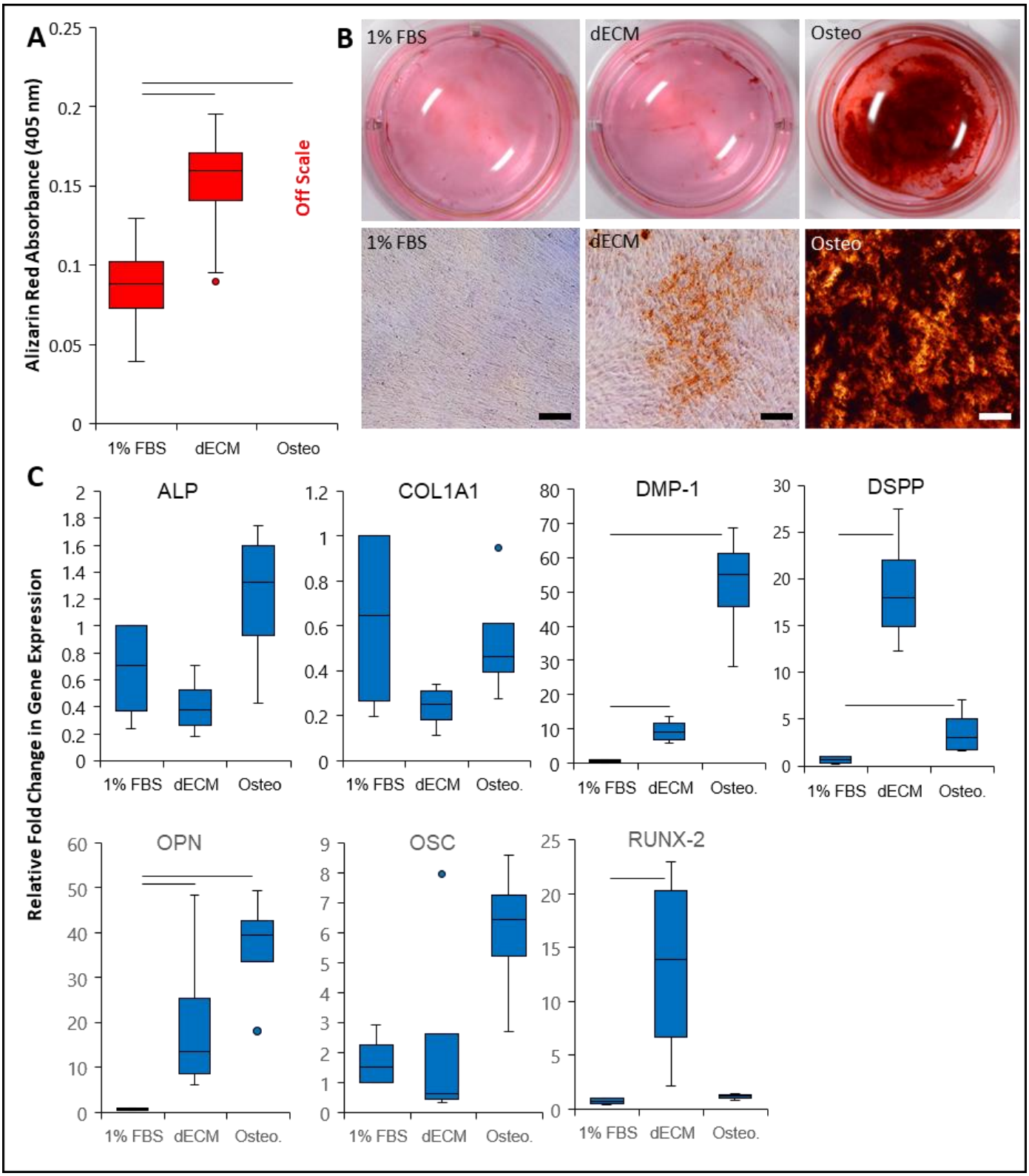

Figure 7: Osteogenic differentiation analysis of PL-MSCs following 21 days culture in media supplemented with 1% FBS (Control), 50 pg/mL dECMs solubilised from pooled dentine extracellular matrix components following 20 mins *in vitro* root canal irrigation with 17% EDTA, or osteogenic inductive media. **[A]** Alizarin red analysis; **[B]** Alizarin

red staining test well; Light microscope image of control and test wells following alizarin red staining taken at 20 x magnification. Scale bars represent 1000 μm; **[F]** Relative fold change in osteogenic gene expression following real-time quantitative PCR. Cultures were analysed in duplicate with data presented as medians alongside upper and lower interquartile ranges, minimum and maximum values, and outliers (*n* = 8). Statistically significant comparisons ($P < 0.05$; *Mann-Whitney U* test) presented as horizontal lines (vs. 1% FBS). **[ALP]** Alkaline Phosphatase; **[COL1A1]** Collagen Type 1; **[DMP-1]** Dentine Matrix Acid Phosphoprotein; **[DSPP]** Dentine Sialophosphoprotein; **[OSC]** Osteocalcin; **[OPN]** Osteopontin; **[RUNX-2]** Runt-related transcription factor.

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CHAPTER 4

SUPPORTING METHODOLOGICAL WORK

Publication 3: Virdee, S. S., Ravaghi, V., Camilleri, J., Cooper, P., & Tomson, P. (2020). Current trends in endodontic irrigation amongst general dental practitioners and dental schools within the United Kingdom and Ireland: a cross-sectional survey. *British Dental Journal*, Online.

Publication 4: Virdee, S. S., Farnell, D. J. J., Silva, M. A., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2020). The influence of irrigant activation, concentration and contact time on sodium hypochlorite penetration into root dentine: an ex vivo experiment. *International Endodontic Journal*, 53(7), 986–997.

Publication 5: Virdee, S. S., Albaaj, F. S., Grant, M. M., Walmsley, D., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2023). Antimicrobial Efficacy of Different Irrigant Solutions Using a Novel Biofilm Model: An In Vitro Confocal Laser Scanning Microscopy Experiment. *The European Journal of Prosthodontics and Restorative Dentistry*, *31*(1), 50–58.

In order to develop a clinical trial to test the hypothesis in this thesis and build on the *in vitro* work outlined in chapter 3, particular methodological work was required. In the first instance, as the proposed clinical protocol relied heavily on alternative endodontic irrigant regimes, a cross-sectional survey was conducted amongst practitioners and teaching institutes to explore current trends in endodontic irrigation (Publication 3).

This would provide an understanding of what could be considered a conventional irrigant regime in *in vitro* and *in vivo* experiments. The *in vitro* methodological studies presented in this chapter were conducted to support the irrigant regime proposed for the clinical trial, which abstained from any use of NaOCl. Firstly, tubular penetration of this solution was examined across several commonly used irrigant regimes in order to gain an appreciation for the potential degree of dentine substrate that is penetrated by NaOCl and therefore may lack any viable dECMs (Publication 4). The methodology was informed by the findings of the survey conducted (Publication 3) and helped determine if the deleterious effects of NaOCl on dECM solubilisation could be overcome by mechanical re-instrumentation (Publication 2). Secondly, conventional understanding of EDTA is that it lacks any antimicrobial action, which is a prerequisite to any endodontic procedure (Publication 5). Much of the previous work used to derive this notion however did not consider the combined effect of chemo-mechanical debridement, as they only topically applied EDTA to biofilms, as well as EDTA's anti-biofilm capabilities. Publication 5 therefore utilised a novel experimental model to determine if 17% EDTA could significantly reduce the presence of *E.faecalis* biofilm during root canal preparation.

CHAPTER 4

PUBLICATION 3

CURRENT TRENDS IN ENDODONTIC IRRIGATION AMONGST GENERAL DENTAL PRACTITIONERS AND DENTAL SCHOOLS WITHIN THE UNITED KINGDOM AND IRELAND: A CROSS-SECTIONAL SURVEY

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Current trends in endodontic irrigation amongst general dental practitioners and dental schools within the United Kingdom and Ireland: a cross-sectional survey

Satnam Singh Virdee,*[1,2] Vahid Ravaghi,[3] Josette Camilleri,[4] Paul Cooper[5] and Phillip Tomson[6]

Key points

- Provides information regarding the current undergraduate teaching practices, in relation to endodontic irrigation, within UK and Ireland dental schools.
- Describes the current trends in endodontic irrigation amongst NHS and private general dental practitioners within the UK.
- Discusses the changes in teaching and usage of endodontic irrigants over the last two decades.

Abstract

Aims To investigate current trends in endodontic irrigation amongst general dental practitioners (GDPs) and dental schools within UK and Ireland. Secondly, to evaluate if significant differences exist between the irrigant practices of National Health Service (NHS) and private GDPs.

Methodology In 2019, an online questionnaire was distributed to the 18 dental schools within the UK and Ireland and 8,568 GDPs. These surveys explored current trends in teaching and usage of endodontic irrigants. Chi-squared tests were performed to make comparisons between NHS and private GDPs ($\alpha < 0.01$).

Results All 18 dental schools (100%) and 495 GDPs (6%) returned valid questionnaires. Three hundred and thirty (66.7%) practitioners were NHS and 165 (33.3%) were private. There was strong consensus on irrigation teaching amongst dental schools. These results aligned with GDP responses in terms of irrigant selection (sodium hypochlorite [NaOCl]); NaOCl concentration (≤3%); ethylenediaminetetraacetic acid (EDTA) contact time (>0–5 minutes); final rinse protocols (penultimate EDTA rinse); irrigant temperature (room); and agitation techniques (manual dynamic activation; >0–60 seconds). There was, however, considerable variation in NaOCl contact time and GDPs infrequently used chelating agents or agitation techniques. Compared with private practitioners, NHS GDPs used significantly lower NaOCl contact times and concentrations, less EDTA and activation techniques, and more chlorhexidine ($P < 0.01$).

Conclusions Overall, irrigation teaching within the UK and Ireland is consistent and evidence-based. Furthermore, trends in irrigant usage amongst UK GDPs are now more aligned with these teaching practices. Significant differences were, however, observed between NHS and private practitioners.

Introduction

Apical periodontitis is an inflammatory condition initiated by pathogenic microorganisms residing within infected root canals.[1] Current treatment strategies focus on reducing the endodontic bacterial load to levels that are compatible with periradicular healing.[2] This is clinically achieved through a process of chemo-mechanical disinfection, whereby hand or rotary instruments widen the root canal to facilitate deeper penetration of antibacterial solutions.[3] Emphasis is placed on the latter irrigation component as a significant portion of the root canal surface can remain uninstrumented following mechanical preparation.[4,5]

As the majority of endodontic treatment is performed by general dental practitioners (GDPs) in primary care, it is important to update our knowledge of how this cohort of dentists chemically disinfects root canals. The last studies on irrigant practices within the United Kingdom (UK) were conducted over ten years ago.[6,7,8] At that time, considerable variation in irrigant selection was reported amongst GDPs who worked predominately in the National Health Service (NHS), with local anaesthetic solution being a popular choice. This contrasts with the irrigant practices of dentists in Australia,[9] USA,[10] Turkey[11] and India,[12] where over 90% reported using sodium hypochlorite (NaOCl). No follow-up investigations have since been conducted and none that examine clinical trends beyond simply the choice of irrigant solution. Moreover, the NHS has undergone significant reform and a considerable amount of research on chemical disinfection has been disseminated during this period.[13,14,15] Whether these factors have had any impact on clinical behaviours of primary care practitioners in the UK, with respect to root canal irrigation, currently remains unknown.

One potential explanation for the

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aforementioned international discrepancies is differences in the irrigation protocols taught at dental schools within and immediately local to the UK (that is, Ireland). Current perceptions amongst experienced UK dentists are that graduates from these institutes enter into dental practice with unsatisfactory endodontic knowledge and skills.[16] It would therefore be of use to also ascertain how the undergraduate curriculum across this region prepares students for general dental practice in relation to root canal irrigation. Once again, previous dental school surveys have only reported on the type of solution advocated and not any other parameter that could potentially enhance the efficacy of irrigants within root canals.[17,18]

The primary aim of this cross-sectional survey was to investigate the current trends in endodontic irrigation amongst GDPs and dental schools within UK and Ireland. Secondly, this study explored if there were any significant differences in the irrigant practices between NHS and private GDPs. The tested null hypothesis was that there were no significant differences between these two GDP cohorts.

Table 1 Demographic characteristics of GDP respondents (the figures in parentheses represent the total number of respondents)

Characteristics	Percentage response (n)		
	GDPs (495)	NHS (330)	Private (165)
Graduated from UK/Ireland			
Yes	85.7 (424)	85.7 (283)	85.5 (141)
No	14.3 (71)	14.3 (47)	14.5 (24)
Years practised in UK			
1–10	48.7 (241)	55.8 (184)	34.5 (57)
11–20	26.3 (130)	25.7(85)	27.3 (45)
21–30	15.3 (76)	10.6 (35)	24.9 (41)
31–40	8.7 (43)	6.7 (22)	12.7 (21)
41–50	1.0 (5)	1.2 (4)	0.6 (1)
Region of practice			
England	84.4 (418)	82.4 (272)	88.5 (146)
Scotland	8.1 (40)	10.9 (36)	2.4 (4)
Wales	5.1 (25)	4.6 (15)	6.1 (10)
Northern Ireland	2.4 (12)	2.1 (7)	3.0 (5)
Proportion of private practice			
0–50%	66.7 (330)	66.7 (330)	-
51–100%	33.3 (165)	-	33.3 (165)

Methodology

Questionnaire design

Following full ethical approval from the University of Birmingham's Research Ethics Committee (Ref: ERN_19-0854), two anonymised questionnaires were designed using the Bristol Online Survey tool (Bristol Online Survey, Bristol, UK). The questionnaire sent to endodontic teaching leads (that is, the dental school survey) consisted of ten questions investigating various aspects of irrigant teaching within the 18 UK and Ireland dental schools with an undergraduate training programme (see online supplementary information 1). The questionnaire sent to primary care practitioners (that is, the GDP survey) consisted of five initial demographic questions and a further ten questions relating to the trends in irrigant usage amongst UK GDPs (see online supplementary information 2). Questions were either open or closed, with some allowing multiple answers, and space was provided after each closed question for respondents to make additional comments in the event that their usual practice was not adequately represented by the available choices. Questions were then independently reviewed by a subject matter (PLT) and survey design (VR) expert to confirm they captured the relevant information without being leading or ambiguous. Thereafter, both surveys were piloted by GDP tutors and clinical lecturers at the University of Birmingham School of Dentistry and revised based on feedback. The final questionnaires were disseminated alongside an explanatory cover letter detailing the aims of the project.

Questionnaire distribution

The dental school questionnaire was e-mailed to the endodontic teaching leads in all UK and Ireland dental schools via the British Endodontic Society Teachers in Endodontology group. Three follow-up e-mails were sent to non-respondent staff members at one-month intervals, after which they were contacted via telephone.

The GDP questionnaire was posted online to 8,568 GDP members of the private social media group 'The Dentist UK' (https://www.facebook.com/groups/738281152968425/), which explicitly required verification of General Dental Council (GDC) registration before membership and participation. This web-based/online survey was re-advertised to practitioners within the forum on three occasions at one-month intervals. In order to reduce the risk of double responses, the cover letter explicitly invited only those GDPs working in primary care who had not already taken part in the survey. In order to ensure the results accurately reflected the irrigant practices of this cohort, a sample size calculation was conducted using an online sample size tool (Roasoft Inc., Washington, USA). Based on the latest data from the Office for National Statistics, there are currently 25,000 self-employed dental practitioners working across the UK.[19] As no prior data exists on many of the questions asked within this survey, an expected outcome of 50% was assumed. Thus, to achieve a confidence level of 95%, with a 5% error margin, a minimum of 379 GDP responses were required.

Both surveys were opened from 1 August to 31 December 2019. Respondents from either group did not receive any incentive to participate and were under no obligation to respond; consent was simply implied by completion and submission of the questionnaire. Only those responses where all questions were answered were considered valid and included in the subsequent analysis. In the GDP survey, practitioners were categorised into those who worked predominantly (>50%) in an NHS or private setting.

Table 2 Trends in endodontic irrigant solution usage for primary root canal treatment (multiple response questions; the figures in parentheses represent the total number of respondents)

Irrigant solutions	Percentage response (n)				P*
	Schools (18)	GDP (495)	NHS (330)	Private (165)	
Chlorhexidine	17.0 (3)	23.0 (113)	27.0 (90)	14.0 (23)	<0.001
Citric acid	11.0 (2)	1.6 (8)	1.2 (4)	2.4 (4)	NS
Dual Rinse HEDP	-	0.2 (1)	-	0.6 (1)	NS
EDTA	72.2 (13)	56.8 (281)	48.2 (159)	73.9 (122)	<0.001
Hydrogen peroxide	-	1.6 (8)	1.2 (4)	2.4 (4)	NS
Hypochlorous acid	-	0.4 (2)	0.3 (1)	0.6 (1)	NS
Iodine	5.6 (1)	0.4 (2)	-	1.2 (2)	NS
Isopropyl alcohol	-	0.2 (1)	-	0.6 (1)	NS
Local anaesthetic	-	9.1 (45)	10.6 (35)	6.1 (10)	NS
Saline	5.6 (1)	6.5 (32)	7.6 (25)	4.2 (7)	NS
Sodium hypochlorite	100.0 (18)	93.7 (464)	92.4 (305)	96.4 (159)	NS
Succinic acid	-	0.2 (1)	0.3 (1)	-	NS
Sodium hypochlorite and chelating agent	83.0 (15)	56.2 (278)	48.8 (161)	73.3 (121)	<0.001

* = statistically significant difference between NHS and private GDPs as per the chi-squared test
NS = non-significant (P >0.01)

Statistical analyses

Descriptive statistics (n; %) were used to describe trends in endodontic irrigation and chi-squared tests were performed with SPSS V.25 software (IBM, New York, USA) to make comparisons between NHS and private GDPs. After Bonferroni correction, the alpha value considered statistically significant for all tests was 0.01.

Results

Response rate

All 18 dental schools provided a valid response and 503 GDPs returned questionnaires, of which 495 were appropriately completed. This gave an overall response rate of 100% and 6%, respectively.

Demographic characteristics of GDP respondents

The demographic characteristics of GDP respondents are summarised in Table 1. Overall, 424 (85.7%) practitioners attained their primary dental qualification from a UK or Ireland tertiary education institute, with 71 (14.3%) qualifying overseas. While respondents graduating from the University of Liverpool (56; 11.3%) formed the largest single number, all dental schools were represented. The greatest proportion of respondents had been practising in the UK for 1–10 years (241; 48.7%) and the least for 31–40 years (5; 1.0%). England was the most represented region (418; 85%), followed by Scotland (40; 8.1%), Wales (25; 5.1%) and Northern Ireland (12; 2.4%). Three hundred and thirty (66.7%) practitioners declared themselves predominantly NHS and 165 (33.3%) private. Similar demographic characteristics were present when these two GDP groups were analysed independently.

Irrigant solution usage

Trends in irrigant selection are summarised in Table 2. All 18 dental schools teach their undergraduates to use NaOCl during root canal treatment, with three (17.0%) advocating its use to the exclusion of other irrigant solutions. The remaining 15 (83.0%) institutes teach NaOCl irrigation alongside a chelating agent, such as ethylenediaminetetraacetic acid (EDTA) (13; 72.2%) or citric acid (2; 11.1%). Relatively few teaching institutes encouraged the use of chlorhexidine (3; 16.7%), iodine (1; 5.6%) or saline (1; 5.6%).

Amongst all GDP respondents, the most widely selected irrigant was NaOCl (464; 93.7%), followed by EDTA (281; 56.8%), chlorhexidine (113; 23.0%), local anaesthetic solution (45; 9.1%) and saline (32; 6.5%). Other less frequently used irrigants included citric acid, hydrogen peroxide, hypochlorous acid, iodine, Dual Rinse HEDP, isopropyl alcohol and succinic acid. One hundred and twenty-nine (26.1%) respondents reported using only NaOCl during root canal treatment and 282 (60.0%) used it in conjunction with a chelating agent such as EDTA (278; 56.2%) and/or citric acid (8; 1.6%). Private GDPs reported more frequently using EDTA and less frequently using chlorhexidine when compared with NHS counterparts (P <0.001).

The majority of practitioners who abstained from using NaOCl (31; 6.3%) stated that they opted for alternative solutions, namely chlorhexidine, as a way of avoiding hypochlorite accidents (12; 2.4%). A smaller proportion (3; 0.6%) reported that they did not have access to NaOCl in their practice and the remaining 16 GDPs offered no explanation. From the 45 (9.1%) respondents who used local anaesthetic solution, of which three (0.6%) reported it as being their sole irrigant, only 17 provided a reason for its use. These included aiding analgesia (6; 1.2%) and haemostasis (3; 0.6%), practice accessibility (3; 0.6%), avoiding hypochlorite accidents (2; 0.4%), solution sterility (2; 0.4%) and ease of needle manipulation when access to the tooth was limited (1; 0.2%).

Sodium hypochlorite irrigation

Trends in the teaching and use of NaOCl are summarised in Table 3. Overall, the NaOCl concentration most frequently advocated by dental schools was >2–3% (8; 44.4%). This was followed by >1–2% (7; 38.9%) and then

Table 3 Trends in NaOCl concentration and contact time usage for primary root canal treatment (the figures in parentheses represent the total number of respondents)

NaOCl	Percentage response (n)				P*
	Schools (18)	GDPs (495)	NHS (330)	Private (165)	
Concentration (%)					
N/A	-	6.3 (31)	7.6 (25)	3.6 (6)	NS
>0.5–1	16.7 (3)	9.9 (49)	12.4 (41)	4.8 (8)	<0.01
>1–2	38.9 (7)	14.9 (74)	16.7 (55)	11.5 (19)	NS
>2–3	44.4 (8)	39.6 (196)	39.7 (131)	39.4 (65)	NS
>3–4	-	11.5 (57)	10.0 (33)	14.7 (24)	NS
>4–5	-	9.3 (46)	7.0 (23)	13.9 (23)	NS
>5–6	-	6.3 (31)	3.6 (12)	11.5 (19)	<0.01
Unknown	-	2.2 (11)	3.0 (10)	0.6 (1)	NS
Contact time (min)					
N/A	-	6.3 (31)	7.6 (25)	3.6 (6)	NS
>0–5	5.6 (1)	16.7 (83)	19.4 (64)	11.5 (19)	NS
>5–10	22.2 (4)	18.5 (92)	22.1 (73)	11.5 (19)	<0.01
>10–15	11.2 (2)	11.0 (54)	10.5 (35)	11.5 (19)	NS
>15–20	5.6 (1)	8.9 (44)	8.8 (29)	9.1 (15)	NS
>20–25	-	5.9 (29)	5.8 (19)	6.1 (10)	NS
>25–30	-	10.5 (52)	8.8 (29)	13.9 (23)	NS
>30–35	5.6 (1)	8.3 (41)	8.2 (27)	8.5 (14)	NS
>35–40	11.2 (2)	12.5 (62)	8.2 (27)	21.3 (35)	<0.0001
Other	38.9 (7)	1.4 (7)	0.6 (2)	3.0 (5)	NS

* = statistically significant difference between NHS and private GDPs as per the chi-squared test
NS = non-significant (P >0.01)

>0.5–1% (3; 16.7%). No institute supported the use of NaOCl solutions greater than 3%. Of those GDPs who routinely used NaOCl, the most frequently selected concentration was also >2–3% (196; 39.6%) and the least >5–6% (31; 6.3%), the latter of which was significantly more popular among private practitioners (P <0.01). Worryingly, a small proportion of this GDP cohort (11; 2.2%) stated they were administering this irrigant at an unknown concentration.

A small group of dental schools (7; 38.9%) did not teach a specific timeframe for NaOCl exposure, but instead encouraged its use throughout the duration of the treatment. The remaining institutes were considerably varied in their responses, as were GDPs. Nevertheless, >5–10 minutes (92; 18.5%) was the most reported contact time amongst practitioners, followed closely by >0–5 minutes (83; 16.7%). Private GDPs, however, were found to use NaOCl for a significantly longer period of time (>35–40 minutes) than NHS respondents (P <0.0001).

EDTA irrigation

Trends in the teaching and use of EDTA are summarised in Table 4. Briefly, the majority of dental schools advocated >0–5 minutes of exposure during root canal treatment (22; 66.7%). Two (11.1%) institutes taught >5–10 minutes of contact; however, time periods greater than this were not supported by teaching in any dental school. Similarly, most GDPs reported using EDTA for >0–5 (197; 39.9%) and >5–10 minutes (51; 10.3%). Most dental schools (10; 55.6%) and GDPs (225; 45.5%) use EDTA as a penultimate rinse to NaOCl during root canal treatment, as opposed to a final rinse.

Irrigant temperature

There was almost universal alignment between dental school (17; 94.4%) and GDP respondents (477; 96.4%), both NHS and private (P >0.01), in that they did not advocate or heat irrigant solutions during root canal treatment.

Irrigant agitation

Trends in the teaching and use of irrigant agitation techniques are summarised in Table 5. Overall, manual dynamic activation was the most widely advocated irrigant agitation method amongst dental schools (13; 72.2%) and was occasionally taught alongside passive ultrasonic and sonic irrigation techniques (3; 16.7%). Similarly, manual dynamic activation was highly popular amongst practitioners (222; 44.8%), followed by passive ultrasonic (99; 20.0%) and sonic irrigation (36; 7.3), the latter of which was used significantly more by private practitioners (P <0.01). Apical negative pressure systems (9; 1.8%) and canal brushes (1; 0.2%) were also utilised in primary care, but to a much lesser degree, and were not taught by any institute. Interestingly, 61 (12.3%) practitioners reported the use of a combination of manual and machine-assisted techniques per canal. Five dental schools (27.8%) and 199 GDPs (40.2%), however, declared that they did not

Table 4 Trends in EDTA contact time and final rinse protocols usage for primary root canal treatment (the figures in parentheses represent the total number of respondents)

EDTA	Percentage response (n)				P*
	Schools (18)	GDPs (495)	NHS (330)	Private (165)	
Contact time (min)					
N/A	22.2 (4)	43.2 (214)	51.8 (171)	26.1 (43)	<0.001
>0–5	66.7 (12)	39.9 (197)	32.1 (106)	55.2 (91)	NS
>5–10	11.1 (2)	10.3 (51)	10.7 (35)	9.6 (16)	NS
>10–15	-	2.6 (13)	0.9 (3)	6.1 (10)	NS
>15–20	-	1.8 (9)	2.1 (7)	1.2 (2)	NS
>20–25	-	0.4 (2)	0.6 (2)	-	NS
>25–30	-	1.0 (5)	1.2 (4)	0.6 (1)	NS
>30–35	-	0.6 (3)	0.6 (2)	0.6 (1)	NS
>35–40	-	0.2 (1)	-	0.6 (1)	NS
Other	-	-	-	-	-
Penultimate [P] and final [F] rinse protocol					
N/A	22.2 (4)	43.2 (214)	51.8 (171)	26.1 (43)	<0.001
NaOCl [P] → EDTA [F]	22.2 (4)	11.3 (56)	10.0 (33)	13.9 (23)	NS
EDTA [P] → NaOCl [F]	55.6 (10)	45.5 (225)	38.2 (126)	60.0 (99)	NS

* = statistically significant difference between NHS and private GDPs as per the chi-squared test
NS = non-significant (P >0.01)

Table 5 Trends in irrigant agitation for primary root canal treatment (multiple responses were allowed for the various techniques; the figures in parentheses represent the total number of respondents)

Irrigant agitation	Percentage response (n)				P*
	Schools (18)	GDPs (495)	NHS (330)	Private (165)	
Techniques					
N/A	27.8 (5)	40.2 (199)	49.1 (162)	22.4 (37)	<0.001
Manual dynamic activation	72.2 (13)	44.8 (222)	140.0 (132)	54.5 (90)	NS
Passive ultrasonic	16.7 (3)	20.0 (99)	15.2 (50)	29.7 (49)	NS
Sonic irrigation	16.7 (3)	7.3 (36)	3.94 (13)	13.9 (23)	<0.01
Apical negative pressure	-	1.8 (9)	1.2 (4)	3.0 (5)	NS
Canal brush	-	0.2 (1)	0.3 (1)	-	NS
Duration (seconds)					
N/A	27.8 (5)	40.2 (199)	49.1 (162)	22.4 (37)	<0.001
>0–30	27.8 (5)	22.2 (110)	21.5 (71)	23.6 (39)	NS
>30–60	22.2 (4)	21.2 (105)	18.2 (60)	27.3 (45)	NS
>60–90	11.1 (2)	8.7 (43)	5.5 (18)	15.2 (25)	NS
>90–120	-	5.5 (27)	4.5 (15)	7.3 (12)	NS
>120–150	-	0.6 (3)	0.3 (1)	1.2 (2)	NS
>150–180	11.1 (2)	1.6 (8)	0.9 (3)	3.1 (5)	NS
Other	-	-	-	-	-

* = statistically significant difference between NHS and private GDPs as per the chi-squared test
NS = non-significant (P >0.01)

teach or use any irrigant agitation technique. The latter cohort consisted of significantly more NHS practitioners (P <0.001).

Most dental schools that taught irrigant agitation techniques advocated durations of >0–30 (5; 27.8%) and >30–60 seconds (4; 22.2%) per canal. A much smaller proportion of institutes opted for more extended time periods, such as >60–90 and >150–180 seconds (2; 11.1%). Similarly, the majority of practitioners who agitated irrigants did so for >0–30 (110; 22.2%) and >30–60 seconds (105; 21.2%). No significant differences were found between NHS and private GDPs (P >0.01).

Discussion

Overall, the results of this study indicate that the teaching practices for irrigant use within UK and Ireland dental schools are consistent and evidence-based. Furthermore, trends in endodontic irrigation usage amongst UK GDPs are now more aligned with these teaching practices. Significant differences were, however, identified between NHS and private practitioners and so the null hypothesis has been rejected.

Valid questionnaire responses were received from the endodontic course leads of every dental school approached to take part in this survey. Thus, the information presented from this portion of the study provides a comprehensive, accurate and representative overview of current undergraduate teaching across the UK and Ireland. For the GDP survey, however, the methods used to achieve high response rates in previous investigations (that is, GDC and Deanery registers) were extensively explored, but unable to be reproduced due to the implementation of General Data Protection Regulations.[6,7,8] Therefore, an online/web-based questionnaire survey design was instead selected due to its ethical fidelity. It is acknowledged, however, that this method carries with it limitations that may affect the ability of the findings to be representative of the entire UK GDP population. This is principally due to the fact that practitioners who had no internet access or were non-members of the forum could not participate. This is further compounded by a low GDP response rate and that the majority of respondents in this study worked in England. Nevertheless, the minimum target determined by the sample size calculation was still superseded and a broader range of age groups, practice types and dental school backgrounds were captured than ever before. Therefore, while caution must be taken when extrapolating results of the present GDP survey to all UK practitioners, they could still provide valuable information to interested dental clinicians, educators, researchers and third-party funders.

This study demonstrated that NaOCl is still advocated as the irrigant of choice by dental schools within the UK and Ireland. However, unlike previous surveys, it is now often taught to be used alongside a chelating agent such as EDTA. The rationale provided for this combination is consistent with the latest European Society of Endodontology Undergraduate Curriculum Guidelines, in that it maximises root canal disinfection by eliminating 'microorganisms, organic tissue and inorganic material' from within infected root canals.[20] The GDP questionnaire also revealed NaOCl as being the most highly administered irrigant amongst NHS and private practitioners. Where previous investigations in the UK reported 19–75% use of this solution, results comparable to international studies were found for the first time in this survey.[6,7,8] This dramatic shift away from the use of local anaesthetic solutions (that is, from 63% to 9%) and towards NaOCl (that is, from 19% to 94%) over the last 20 years demonstrates that fewer dentists are deviating from the irrigant practices taught to them during their undergraduate training. While EDTA was the second most popular irrigant solution, it was only used by approximately half of GDP respondents and was strongly associated with private dentists. This alludes to there being financial barriers to the more widespread usage of this solution; however, variation in undergraduate learning experience could also play a role as several institutes do not include this irrigant as part of their curriculum. The usage of chlorhexidine on the other hand has remained relatively stable amongst approximately a quarter of GDP respondents and several dental institutes.[8] This outcome is despite emerging evidence highlighting its negative effects on periradicular healing and increased incidents of anaphylaxis.[21,22,23] Frequent reasons cited for its use were not related to its substantive antiseptic activity, but instead to eliminating the risks of hypochlorite injury and, more worryingly, when rubber dam was not applied.

In contrast to American practitioners, the current opinion amongst UK GDPs and dental schools favours the use of more diluted NaOCl solutions (≤3%).[10] This is likely due to this irrigant demonstrating similar antimicrobial and tissue-dissolving properties to its higher strength counterparts, while also exhibiting lower periradicular cytotoxicity.[24,25,26] Consequentially, regular replenishing and more contact time would be required as the active chlorine ions that contribute to the NaOCl mechanism of action are spent more rapidly in less concentrated solutions.[27] However, there is currently a lack of evidence-based guidelines on the minimum duration of NaOCl exposure needed for adequate disinfection of root canals. This could explain the considerable variation reported by both GDP and dental school respondents. Of note, less contact time was highly associated with NHS practitioners and longer durations with private GDPs, a finding that further strengthens the association between methods of remuneration and practising behaviours.[28] Nevertheless, there is an emerging trend amid a smaller group of endodontic educators within this study that NaOCl use should span the entire length of the root canal procedure. This was explicitly stated in the additional comments section by those dental school respondents who selected 'other' for this question (7; 38.9%). Unfortunately, such a notion was acknowledged by only seven (1.4%) practitioners, which highlights the need for greater clarification in this area.

When EDTA was administered into root canals, educators and practitioners almost always reported it to be used in conjunction with NaOCl for periods of up to five minutes as a penultimate rinse. This strong consensus could be explained by the fact that both groups cited its use as being solely for removing smear layer. For this purpose, the reported contact time is consistent with the conclusions of several *in vitro* investigations that sought to determine the minimum duration of EDTA exposure needed to achieve this goal.[29,30] However, a final rinse with NaOCl has long been associated with excessive erosion of peri- and inter-tubular dentine,[31,32] a phenomenon proposed but not proven to render endodontically treated teeth more prone to vertical root fracture.[33] Nevertheless, the continued practice of the reported irrigation sequence would suggest that, for dental school and GDP respondents in this survey, the additional disinfection attained by a final NaOCl rinse outweighs these theoretical risks.

In this study, only two-thirds of dental schools and GDP respondents advocated or used irrigant agitation techniques during root canal treatment. The most favoured method amongst both groups was manual dynamic activation for periods of up to 60 seconds per canal. This trend could be attributed to the relatively inexpensive and simple nature of this technique, which makes it widely accessible and easy to teach irrespective of experience level. Furthermore, *in vitro* investigations have demonstrated it as being an effective mechanism for eliminating smear layer and dentinal debris from within all regions of the root canal, in addition to promoting deeper tubular penetration of irrigants.[34,35] Machine-assisted devices, however, were used much less but by a higher proportion of predominantly private GDPs, particularly sonic agitation. This once again highlights the financial barriers associated with their use, which would disproportionately affect NHS practitioners as the appropriate incentive structures are currently not in place.[28,36,37] Another possible explanation for the sparse use of these devices is that they have yet to demonstrate any clinical effectiveness, with respect to periapical bony healing.[38,39]

Conclusion

Within the limitations of this study, the following conclusions can be drawn:

1. The teaching practices for irrigant use within the UK and Ireland are consistent and evidence-based. However, greater consensus is needed for NaOCl contact time and additional teaching is required on machine-assisted agitation techniques
2. Current trends in endodontic irrigation amongst UK GDPs are now more aligned to the aforementioned teaching practices. These include irrigant selection (NaOCl); NaOCl concentration (≤3%); EDTA contact time (>0–5 minutes); final rinse protocols (penultimate EDTA rinse); irrigant temperature (room) and agitation technique (manual dynamic activation; >0–60 seconds). Once again, there is considerable variation in NaOCl contact time and, contrary to contemporary teaching practices, practitioners do not routinely use chelating agents or agitation techniques
3. There are significant differences between the irrigant practices of NHS and private GDPs. NHS practitioners use significantly lower contact times and concentrations for NaOCl, less frequently use EDTA and agitation techniques, and more regularly administer chlorhexidine than private GDPs.

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Conflict of interest

The authors explicitly declare no conflicts of interest.

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CHAPTER 4

PUBLICATION 4

THE INFLUENCE OF IRRIGANT ACTIVATION, CONCENTRATION AND CONTACT TIME ON SODIUM HYPOCHLORITE PENETRATION INTO ROOT DENTINE: AN EX VIVO EXPERIMENT

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The influence of irrigant activation, concentration and contact time on sodium hypochlorite penetration into root dentine: an *ex vivo* experiment

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Abstract

Virdee SS, Farnell DJJ, Silva MA, Camilleri J, Cooper PR, Tomson PL. The influence of irrigant activation, concentration and contact time on sodium hypochlorite penetration into root dentine: an *ex vivo* experiment. *International Endodontic Journal*, **53**, 986–997, 2020.

Aim To establish whether irrigant activation techniques, namely manual dynamic activation (MDA), passive ultrasonic irrigation (PUI) and sonic irrigation (SI), improve the tubular penetration of sodium hypochlorite (NaOCl) into root dentine when compared with conventional needle irrigation (CNI). Secondly, investigate if increasing NaOCl concentration and/or contact time improves the performance of these techniques.

Methodology A total of 83 extracted human maxillary permanent canines were decoronated to 15 mm, and root canals prepared to a size 40, .10 taper. Root dentine was stained with crystal violet for 72 h and embedded in silicone. Eighty specimens were randomly distributed into 16 groups ($n = 5$) according to the irrigant activation technique, NaOCl concentration (2%; 5.25%) and irrigant contact time (10 min; 20 min). All activation techniques were used for 60 s in the last minute of irrigation. Additionally, three teeth were not exposed to NaOCl to confirm adequate dentine staining had occurred (i.e. negative control). All specimens were subsequently dissected, observed under a light microscope and NaOCl penetration depth (μm) determined by measuring the average width of bleached dentine using ImageJ software. Statistical comparisons were made with paired and unpaired *t*-tests, ANOVAs followed by *post hoc* Tukey's and Dunnett's tests, and a general linear model ($\alpha < 0.05$).

Results Overall, NaOCl penetration ranged from 38.8 to 411.0 μm with MDA, PUI and SI consistently resulting in significantly greater tubular infiltration than CNI ($P < 0.05$). The deepest measurements in the coronal, middle and apical segments were all recorded in the MDA; 5.25%; 20 min group and the least in the CNI; 2%; 10 min group. Increasing either irrigant concentration or contact time resulted in significantly greater NaOCl penetration depths for all techniques and segments of the canal ($P < 0.05$). However, when irrigant concentration and contact time were increased together, a significant interaction effect between these two independent variables was observed on overall NaOCl penetration ($P < 0.05$).

Conclusions Agitating irrigants with MDA, PUI or SI, as well as using greater irrigant concentrations or contact times, potentiated NaOCl penetration into root

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dentine. However, longer durations of NaOCl exposure at lower concentrations resulted in similar depths of tubular penetration as those achieved at higher concentrations.

Keywords: irrigant penetration, manual dynamic activation, passive ultrasonic irrigation, root dentine, sonic irrigation.

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Introduction

Microbial infection of the pulp is a prerequisite to the development of apical periodontitis (Kakehashi *et al.* 1965, Möller *et al.* 1981). Current treatment strategies therefore focus largely on reducing the bacterial load within the root canal system to levels that induce periradicular healing (Siqueira & Rôças 2008). Generally, this is achieved through the use of instruments to enlarge the canal and antibacterial solutions (Schilder 1974, Haapasalo *et al.* 2005). Greater emphasis is placed on the latter as up to 35% of the canal wall can remain uninstrumented (Peters *et al.* 2001). Sodium hypochlorite (NaOCl) is currently the irrigant of choice, due to its efficacy against a broad spectrum of microbes and tissue dissolution capabilities (Haapasalo *et al.* 2014). It is routinely deposited into root canals at concentrations of 0.5%–5.25% (Baumgartner & Cuenin 1992), by way of conventional needle irrigation (CNI).

Although NaOCl substantially reduces the number of microorganisms within superficial layers of root dentine via CNI, bacteria more deeply embedded within tubules often remain unaffected (Wong & Cheung 2013, Azim *et al.* 2016, Vatkar *et al.* 2016). This could negatively impact the prognosis of root canal treatment as enduring pathogens, which have been found residing at depths of up to 420 μm in human dentine (Love 1996, Kakoli *et al.* 2009), may later contribute to persistent periradicular disease (Siqueira & Rôças 2008). Deeper irrigant penetration is therefore desirable as some *ex vivo* experiments have demonstrated CNI only allows NaOCl infiltration up to 250 μm into root dentine (Ghorbanzadeh *et al.* 2016, Faria *et al.* 2019).

The aforementioned limitations of CNI could, however, be overcome through the use of irrigant activation techniques. Manual dynamic activation (MDA), passive ultrasonic irrigation (PUI) and sonic irrigation (SI) are currently some of the most widely used and studied methods (Gu *et al.* 2009, Virdee *et al.* 2018). MDA involves repeatedly inserting a well-fitting gutta-percha (GP) cone to the working length of an instrumented canal to produce hydrodynamic displacing forces within irrigants (McGill *et al.* 2008). Passive ultrasonic irrigation uses freely oscillating files at ultrasonic frequencies (25–30 kHz) to generate acoustic cavitation and streaming forces (van der sluis *et al.* 2007). Sonic irrigation devices create hydrodynamic phenomenon within irrigants by oscillating a smooth flexible polymer file at frequencies of 1–10 kHz (Gu *et al.* 2009).

Currently, only a few studies directly compare the NaOCl tubular penetration achieved by MDA, PUI or SI to that of CNI (Ghorbanzadeh *et al.* 2016, Faria *et al.* 2019). Furthermore, even fewer studies report how irrigant concentration or contact time influences the performance of these techniques. In studies which have investigated NaOCl penetration into root dentine, floating dentine segments (Zou *et al.* 2010, Palazzi *et al.* 2016) or irrigant regimes spanning durations of only 1–2 min (Ghorbanzadeh *et al.* 2016, Generali *et al.* 2018, Faria *et al.* 2019) have often been used. These do not mimick *in vivo* conditions or irrigant protocols well (Darcey *et al.* 2016). Therefore, investigating more commonly used irrigant regimes in a more clinically representative model system would better inform clinicians on effective endodontic disinfection strategies.

The primary aim of this *ex vivo* study was to establish whether the use of MDA, PUI and SI significantly improve the tubular penetration of NaOCl into root dentine when compared with CNI. Secondly, this study investigates if increasing NaOCl concentration and/or contact time improves the performance of the aforementioned irrigant activation techniques. The null hypotheses tested were as follows: (i) MDA, PUI or SI do not increase NaOCl tubular penetration when compared with CNI and (ii) raising irrigant concentration and/or contact time does not significantly increase NaOCl tubular penetration.

Material and methods

This study was performed under the ethical approval (14/SW/1148) for the University of Birmingham's Dentistry Research Tissue Bank (DRTB), and the workflow is summarized in Fig. 1.

Specimen selection

A total of 83 human maxillary canines were selected from a pool of teeth that were extracted, for reasons not related to this study and stored under controlled

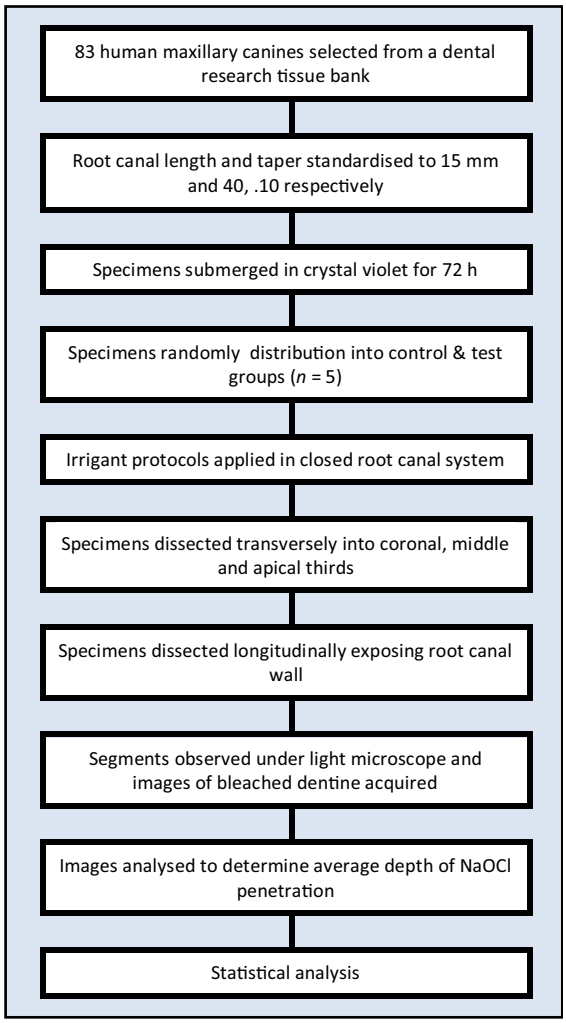

Figure 1 A flow chart depicting the key stages of the experimental protocol.

conditions at −20 °C in the DRTB. Mesio-distal and bucco-lingual digital radiographs were taken to ensure only permanent teeth with single canals and root lengths of ≥16 mm were included. Teeth with caries, root fractures, open apices, root curvatures >10°, calcified canals, resorptive defects, posts and previous root fillings were excluded (Fig. 2a).

Specimen preparation

Tubular penetration of NaOCl was evaluated based on the stained dentine model proposed by Zou *et al.* (2010), but with modifications to better reflect *in vivo* conditions. Briefly, teeth were decoronated with a slow-speed diamond disc (ContacEZ, Vancouver, Washington, USA) and the remaining root adjusted to 15 mm using cooled silicon carbide grinding paper (Struers, Pederstrupvej, Denmark). Residual periodontal tissues were removed using an ultrasonic scaler and the working length (WL) subsequently determined by inserting a size 10 K-File (Dentsply Sirona, Ballaigues, Switzerland) into the root canal until the tip was visible beyond the apex under magnification. One millimetre was then subtracted from this distance, and all root canals were prepared to this WL up to a ProTaper Gold F4 rotary file (size 40, .10 taper) at speeds and torques recommended by the manufacturer (Dentsply Sirona). During instrumentation, 1 mL of 5.25% NaOCl (Cerkamed, Stalowa-Wola, Poland) was administered between files.

A slow-speed diamond disc (ContacEZ) was used to prospectively scribe grooves, without penetrating into the canal space, along the planes at which specimens would later be dissected. First, transverse grooves were made circumferentially at locations of 5 and 10 mm from the anatomical apex so that the root could be divided into coronal, middle and apical thirds. Vertical grooves were then made mesio-distally along the entire length of the tooth so that the longitudinal axis of the root canal could be exposed. Bucco-lingual vertical grooves were also scribed into the coronal and middle segments so that NaOCl penetration could be analysed in two planes (Fig. 2b).

Staining protocol

To remove both organic and inorganic components of the intracanal smear layer, each specimen was immersed in 10 mL of 5.25% NaOCl for 5 min followed by 10 mL in 17% ethylenediaminetetraacetic acid (EDTA; Cerkamed) for a further 5 min. Thereafter, dentine blocks were washed in distilled water for 5 min to remove residual irrigant solutions, dried using paper towels and then immersed in 10 mL of crystal violet (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 72 h at room temperature. To maximize dye penetration, every 24 h the crystal violet was renewed and irrigated into canals via CNI (Fig. 2c). After this period, teeth were rinsed under tap water for 30 min and sealed in Aquasil soft putty silicone (Dentsply Sirona) to simulate a closed root canal system (Tay *et al.* 2010, Fig. 2d).

Control and test groups

To evaluate the efficacy of MDA, PUI and SI in relation to CNI, 80 prepared teeth were randomly distributed into 16 groups ($n = 5$) depending on the

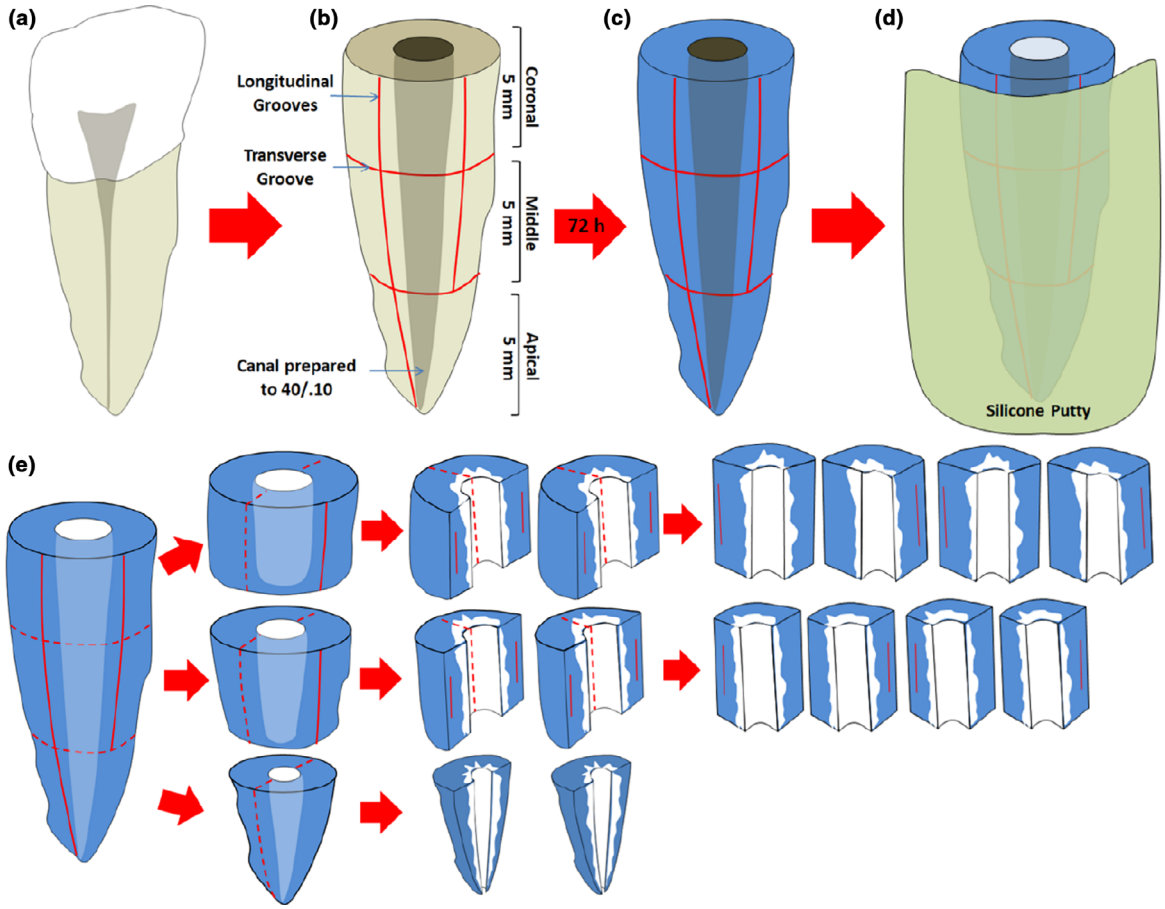

Figure 2 A schematic diagram displaying the stages of preparing root canal blocks used in this study. (a) Maxillary canines selected. (b) Teeth decoronated and splitting grooves included (indicated by solid red lines) using slow speed diamond disc (c) Specimens dyed in crystal violet for 72 h. (d) Specimens sealed in silicone putty and irrigant protocols applied. (e) Transverse and longitudinal dissections made (indicated by broken red lines) as per splitting grooves. Orientation grooves were also scribed on root canal walls in mesio-distal plane.

method of irrigant agitation, NaOCl concentration (%) and irrigant contact time (min). Briefly, each irrigant activation technique was used alongside 2% or 5.25% NaOCl at room temperature for a period of 10 or 20 min (Faria *et al.* 2019). The CNI groups acted as controls and a further three specimens were dyed without being exposed to NaOCl to confirm adequate dentine staining had occurred (i.e. negative control). The individual groups are displayed in Table 1.

Irrigant activation protocols

For all groups, NaOCl was deposited into the canal via CNI with a 27 gauge side vented needle and 3 mL monoject syringe (Covidien, Dublin, Ireland). The needle tip was placed 2 mm short of WL, and the irrigant solution replenished with 1 mL of irrigant every 2 min to prevent depletion of NaOCl activity (Moorer & Wesselink 1982, Boutsioukis *et al.* 2010). For test groups, the irrigant was agitated in the final minute of NaOCl exposure by a single trained operator in accordance with protocols outlined by McGill *et al.* (2008) and Mancini *et al.* (2013). Briefly, for MDA a single Protaper F4 GP cone (Dentsply Sirona) was repeatedly inserted to WL, via short 2–3 mm longitudinal push–pull strokes, for 60 s at a rate of 100 strokes per minute. Passive ultrasonic irrigation was completed using a size 15 K-file (Dentsply Sirona) which was activated via a MiniEndo II device (SybronEndo, Orange County, California, USA) at half power for 1 min at 1 mm from WL. Finally, for SI an Endoactivator device (Dentsply

Sirona) was used for 1 min with a size 15/ .02 point 1 mm from the WL. Following irrigation, canals were flushed with 5 mL distilled water and then dried with sterile paper points.

Specimen dissection

Dentine blocks were removed from the silicone putty and a chisel and hammer used to transversely separate roots into coronal, middle and apical sections using the aforementioned splitting grooves as guides. Sections were further divided mesio-distally, and the coronal and middle regions once again bucco-lingually, to expose the longitudinal axis of the root canal which created four coronal, four middle and two apical segments per tooth. Thereafter, the internal surfaces were grooved mesio-distally for orientation purposes and then polished to remove topographical irregularities using a medium-grit abrasive polishing disc (3M, St. Paul, Minnesota, USA; Fig. 2e). With five teeth per group and three negative controls, a total of 830 sections were generated for experimental study.

Evaluating NaOCl penetration

Using a custom silicone jig, specimens were positioned under a light microscope (Zeiss Axiophot, Carl Zeiss, Oberkochen, Germany) so that the longitudinal plane of the root canal wall could be observed. Two images at random locations either side of the canal were captured, alongside a calibrated scale bar, for coronal and middle segments using a digital camera (Apple, Cupertino, California, USA). Only a single image was acquired for apical segments, due to reduced dye penetration in this region (Paqué *et al.* 2006, Russell *et al.* 2013). For the purposes of standardization, all images were captured using the same objective at a fixed resolution and optimal focus, saved in.tiff format and then uploaded onto ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Quantitative morphometric image analysis was conducted to calculate the average NaOCl penetration depth for each image (Fig. 3a). Briefly, the 'polygon' and 'clear outside' tools were used to outline and isolate the internal canal wall. The image was then separated via 'split channel' function and a manual 'threshold' applied to the green image, which provided the greatest contrasts between bleached and dyed dentine (Fig. 3b). A calibrated 'straight' line, spanning perpendicularly from the inner canal wall towards the periphery, was then used to obtain 10 measurements from each image at 150 μm vertical increments (Fig. 3c). The average NaOCl penetration depth for the coronal, middle and apical segments of each group was then calculated in micrometres (μm), and the results presented as averages (μm) ± standard deviations alongside 95% confidence intervals for the mean.

Statistical analyses

All statistical analyses were performed using SPSS (V.25) software (IBM, New York, USA). Initially, a preliminary screen for data normality was conducted using histograms and normal plots. A majority

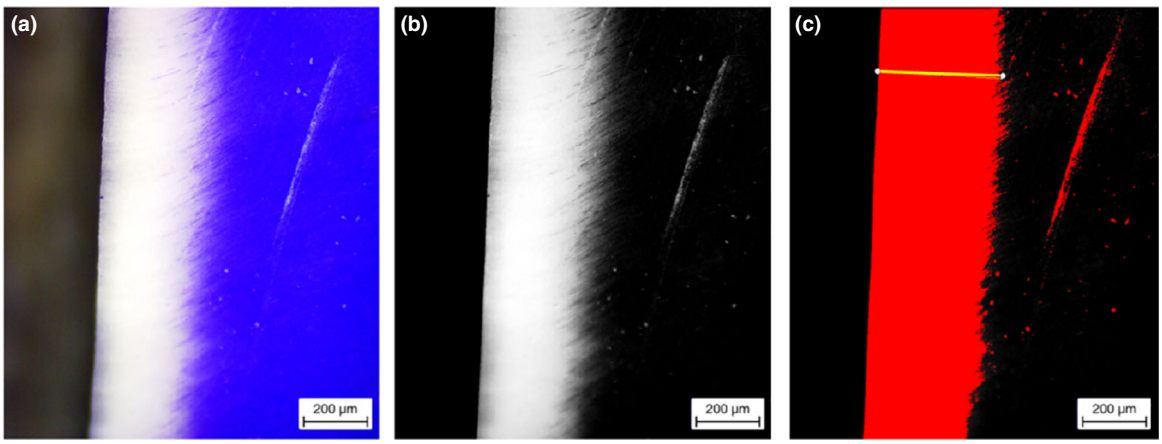

Figure 3 Morphometric analysis calculating average depth of NaOCl penetration (a) Original image. (b) Green image following 'split channel' function. (c) Manual 'threshold' applied and calibrated 'straight line' tool used 10 times at 150 μm vertical increments to calculate average NaOCl penetration per images.

normal distribution was revealed, and subsequent comparisons between groups were made using unpaired *t*-tests and ANOVAs followed by *post hoc* Tukey's and Dunnett's tests. Comparisons within groups (intra-tooth) were made using paired *t*-tests.

The effects of irrigant concentration and contact time were explored using a general linear model. The 'main effects' included in this model were (i) irrigant concentration and (ii) irrigant contact time, and the 'interaction effect' was considered the simultaneous effect of increasing both of these independent variables (i.e. irrigant concentration and contact time) on NaOCl penetration. These main and interaction effects were explored for the overall NaOCl penetration (averaged across all techniques) as well as for the individual methods of irrigant agitation (CNI, MDA, PUI and SI) across all thirds of the canal. In this model, comparisons were made only between groups (inter-tooth) and not within (intra-tooth).

The intra-rater reliability for image analysis was conducted in each group using the intra-class correlation coefficient, and the alpha value for all tests was set at a 5% level of significance ($\alpha = 0.05$).

Results

The intra-class correlation coefficient demonstrated 'excellent' intra-rater agreement with coefficients

Table 1 Average NaOCl penetration into root dentine following various irrigation protocols

	NaOCl irrigation protocol			Average depth of tubular penetration (μm)		
Group	IAT	Conc. (%)	Time (m)	Coronal	Middle	Apical
1	CNI	2	10	179.4 ± 67.2[a,b,c] [172.8–186.0]	158.7 ± 70.3[a,b,c,d] [151.8–165.5]	38.8 ± 38.1[a,b,c,d] [31.3–46.2]
2	CNI	2	20	265.2 ± 71.1[a,b,c] [258.2–272.1]	246.5 ± 82.1[a,b,c,d] [238.4–254.5]	124.5 ± 47.2[a,b,c,d] [115.2–133.7]
3	CNI	5.25	10	255.4 ± 72.0[a,b,c] [248.4–262.5]	226.8 ± 62.9[a,b,c,d] [220.5–233.0]	113.1 ± 49.6[a,b,c,d] [103.4–122.9]
4	CNI	5.25	20	272.6 ± 76.1[a,b,c] [265.1–280.0]	259.8 ± 71.2[a,b,c,d] [252.8–266.8]	171.2 ± 54.9[a,b,d] [160.5–182.0]
5	MDA	2	10	240.6 ± 71.9 [b,c] [233.5–247.7]	237.8 ± 87.1[c] [229.2–246.3]	159.9 ± 63.5[c,d] [147.5–172.4]
6	MDA	2	20	300.4 ± 100.8[c] [290.6–310.1]	294.7 ± 96.7[b] [285.7–304.3]	162.2 ± 75.7[d] [147.4–177.0]
7	MDA	5.25	10	279.9 ± 65.4[b,c] [273.4–286.3]	325.6 ± 108.8[b,c,d] [314.7–336.4]	212.9 ± 76.0[b,c,d] [198.1–227.8]
8	MDA	5.25	20	379.0 ± 110.8[b,c] [368.0–390.0]	411.0 ± 132.3[b,c,d] [398.2–423.8]	232.7 ± 78.2 [c,d] [217.3–248.0]
9	PUI	2	10	258.5 ± 78.7[a,c] [250.9–265.6]	241.9 ± 69.9 [c,d] [235.0–248.9]	172.3 ± 51.3 [c,d] [162.3–182.9]
10	PUI	2	20	298.3 ± 64.9[c] [294.6–308.2]	322.1 ± 87.1[a,d] [313.5–330.7]	169.2 ± 55.8[d] [158.3–180.1]
11	PUI	5.25	10	306.5 ± 85.2[a] [298.1–314.9]	303.5 ± 83.4[a] [295.4–311.7]	154.9 ± 40.4[a,d] [147.0–162.9]
12	PUI	5.25	20	300.0 ± 69.5[a,c] [293.2–306.8]	296.5 ± 77.3[a] [289.1–303.9]	216.3 ± 92.3 [c,d] [198.3–234.4]
13	SI	2	10	280.3 ± 72.8[a,b] [273.1–287.4]	275.3 ± 85.6[a,b] [267.1–284.0]	121.0 ± 51.6[a,b,d] [110.9–131.2]
14	SI	2	20	326.8 ± 83.2[a,b] [318.5–335.1]	310.1 ± 80.1[d] [302.3–317.9]	149.5 ± 37.4[d] [142.2–156.9]
15	SI	5.25	10	302.4 ± 93.3[a] [293.2–311.6]	296.1 ± 86.5[a] [287.8–304.5]	165.7 ± 66.9[a,d] [152.6–178.8]
16	SI	5.25	20	329.2 ± 81.2[a,b] [321.5–337.0]	306.3 ± 83.0[a,d] [298.2–314.5]	161.9 ± 82.5[a,b,d] [145.7–178.1]

Results are presented as means (μm) ± standard deviations and [95% confidence intervals] for the mean.
[a]Versus corresponding MDA segment ($P < 0.05$).
[b]Versus corresponding PUI segment ($P < 0.05$).
[c]Versus corresponding SI segment ($P < 0.05$).
[d]Versus preceding coronal segment ($P < 0.05$).

spanning upwards of 0.87. Results in NaOCl penetration for the different irrigant protocols are summarized in Table 1 and Figure 4, with representative light microscopy images for each group displayed in Fig. 5.

The overall NaOCl dentine penetration ranged from 38.8 to 411.0 μm. With exception to the apical third in group 16 ($P > 0.05$), CNI displayed significantly less NaOCl penetration than MDA, PUI and SI in all locations for any given irrigant protocol ($P < 0.05$). The deepest measurements across the entire canal were all recorded in group 8 (MDA; 5.25%; 20 min) and the least in group 1 (CNI; 2%; 10 min). Individual comparisons between MDA, PUI and SI revealed no single superior method of irrigant agitation across all protocols and locations, however; SI consistently performed the poorest apically after CNI.

For all irrigant protocols, the apical region exhibited significantly lower NaOCl penetration compared with the respective coronal and middle sections ($P < 0.05$). By contrast, differences between coronal and middle regions were varied with groups exhibiting positive, negative or no significant differences. Furthermore, there were no significant differences in NaOCl penetration between mesio-distal and buccolingual planes for the coronal or middle segments of dentine ($P > 0.05$).

The general linear model indicated significantly deeper NaOCl penetration for each technique across all regions of the canal when the main effect of either concentration or contact time was increased ($P < 0.05$). A more pronounced effect was demonstrated for CNI and MDA compared with PUI or SI. Additionally, when increasing both irrigant concentration and contact time, a highly significant interaction effect was observed in the coronal and middle segments ($P < 0.05$), but not apically ($P > 0.05$).

Discussion

This study demonstrates that MDA, PUI and SI substantially improve NaOCl penetration across all regions of the canal for any given irrigant regime compared with CNI. Additionally, when NaOCl concentration or contact time is increased, the performance of these techniques is further improved. Finally, longer periods of exposure to lower concentrations of NaOCl result in similar depths of tubular penetration as those achieved by higher concentrations. Both null hypotheses have therefore been rejected.

A broad range of methodologies have been used previously to determine NaOCl penetration into root dentine. Several studies have added fluorescent dyes to irrigants for subsequent confocal laser scanning microscopy analysis (Llena *et al.* 2015, Vadhana *et al.* 2015, Gu *et al.* 2017). Others have substituted NaOCl completely for fluorescent solutions, which have then been viewed under a light microscope (Galler *et al.* 2019). These approaches were not employed here as the oxidizing nature of NaOCl could affect the fluorescent capacity of the dye and surrogate solutions may not have the same penetrative properties as the test irrigant. Alternatively, the stained dentine model proposed by Zou *et al.* (2010) was used in this study as it overcame the aforementioned limitations. Whilst this experimental model has been used in several previous investigations, significant efforts were made in this study to improve its biofidelity so that it better mimicked *in vivo* conditions. For instance, human teeth were used instead of bovine (Faria *et al.* 2019), irrigants were deposited into intact root canals in a clinically representative manner rather than dentine blocks being submerged in pools of NaOCl (Zou *et al.* 2010, Palazzi *et al.* 2016) and the durations of disinfection were more akin to those delivered throughout root canal treatment (Ghorbanzadeh *et al.* 2016, Generali *et al.* 2018). Thus, this model allowed for more robust analysis of the various irrigant protocols, including the complex interaction effects between NaOCl concentration and contact time which at the time of this study have not been previously reported. Furthermore, the number of segments (10), images (18) and data points (180) per tooth, the multiple planes analysed and the highly reproducible image threshold techniques applied in the present study also allowed for more accurate and sensitive evaluation of NaOCl penetration than any preceding investigation. Nevertheless, several limitations of this methodology are still present and acknowledged. For example, the method for selecting teeth did not allow the baseline conditions of root dentine (i.e. age, tubular density or size) to be standardized and further investigations would be required to establish whether the reported findings are applicable to curved canals where irrigant flow dynamics are different (Nguy & Sedgley 2006). Additionally, the limited sample size in this study, which was based on previous experiments by Zou *et al.* (2010) and Palazzi *et al.* (2016), may also increase the risk of type 2 errors (false negatives). It is important to note, however, highly significant differences were consistently identified between the various irrigant regimes indicating

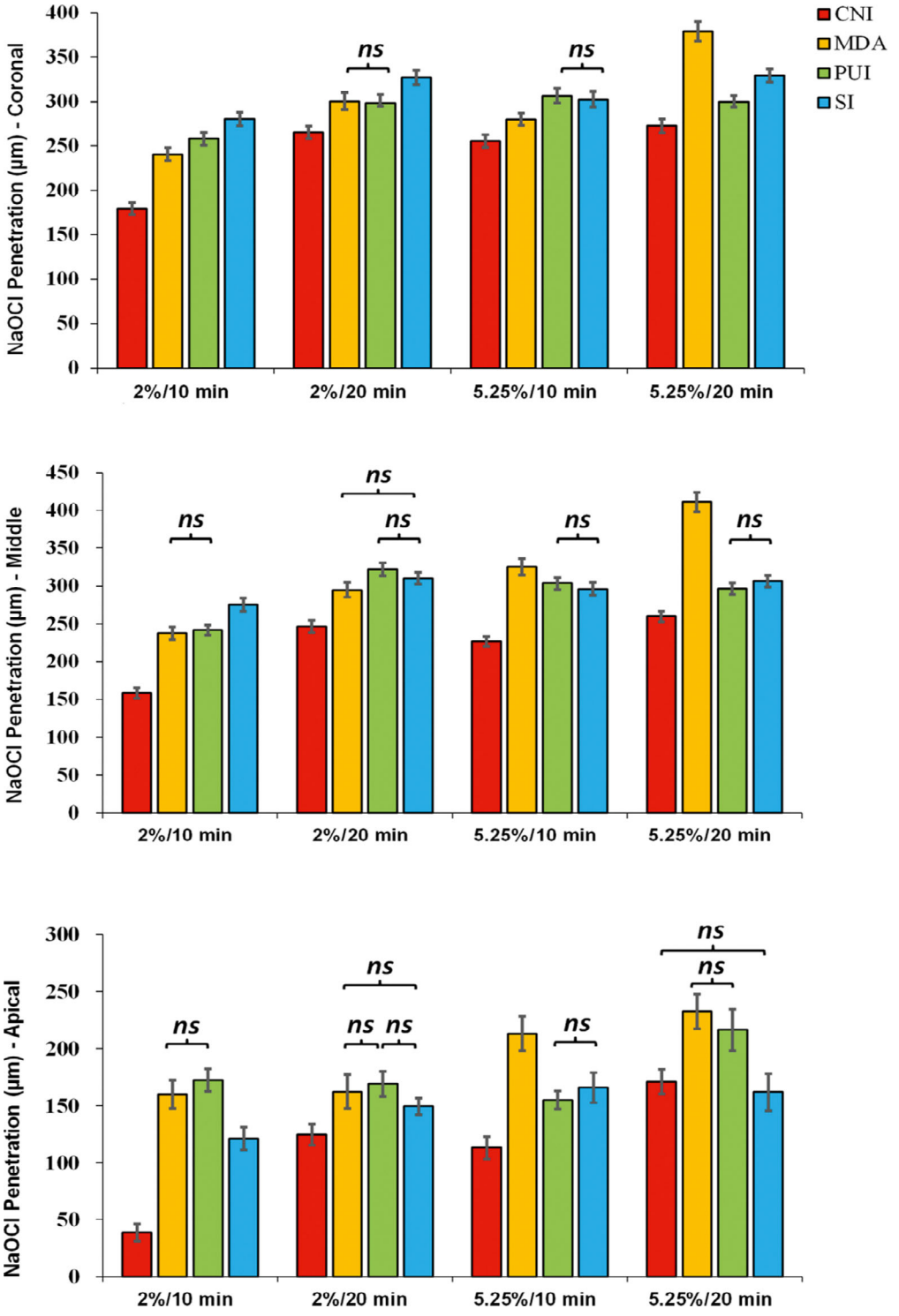

Figure 4 Average NaOCl penetration (µm) in the coronal, middle and apical regions of the canal for each agitation technique compared with of CNI. The error bars indicate the 95% confidence intervals for the mean. Those comparisons with respect to technique (CNI versus MDA versus PUI versus SI) in each group that are not significant are indicated by '*ns.*' All other comparisons within each group are significant ($P < 0.05$).

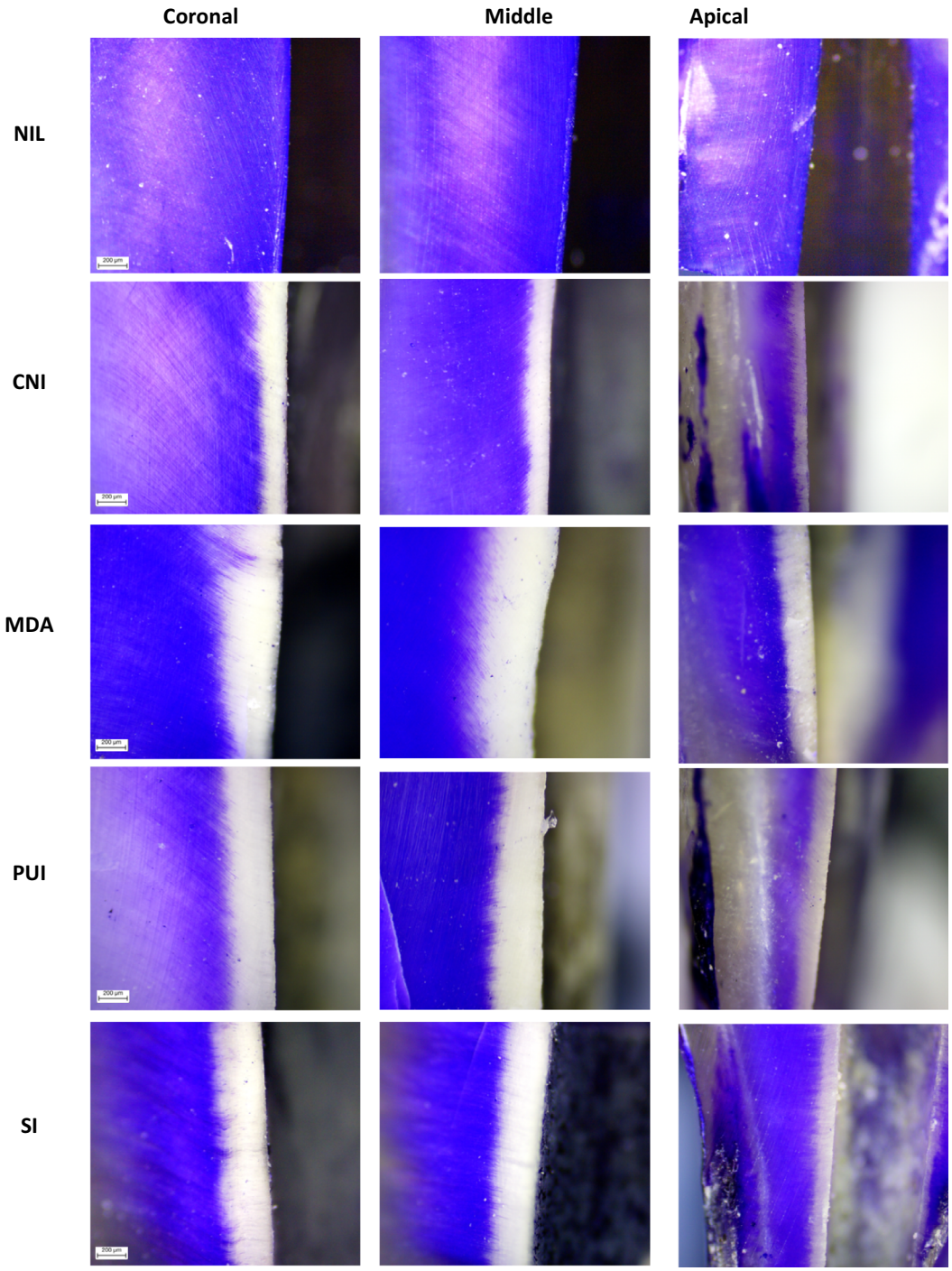

Figure 5 Representative light microscopy images of dentinal tubular penetration in the coronal, middle and apical segments of the root canal following use of various agitation techniques with 5.25% NaOCl for 20 min. The white/bleached region of dentine, which was stained with crystal violet, demonstrates that which has been penetrated by NaOCl. CNI, conventional needle irrigation; MDA, manual dynamic activation; Nil, negative control; PUI, passive ultrasonic irrigation and SI, sonic irrigation. Scale bar represents 200 μm.

high statistical power. Nevertheless for the reasons described above, caution must still be taken when extrapolating outcomes of this laboratory experiment into the clinical setting.

The results of this study demonstrate that the tubular infiltration of NaOCl was considerably improved in all segments of the canal when irrigants were agitated with MDA, PUI or SI. Similar trends are present in other investigations that have made comparisons in irrigant penetration between CNI and any of the aforementioned agitation techniques (Ghorbanzadeh *et al.* 2016, Faria *et al.* 2019). This discrepancy could be attributed to the superior irrigant flow dynamics of the latter which help promote more intimate interactions between solutions and the internal surfaces of the root canal wall. For instance, Chen *et al.* (2014) reported the shear stresses and hydrodynamic pressures generated within irrigants during PUI were significantly greater and more evenly distributed across a larger area of the canal wall than those produced by CNI. Conversely, Munoz & Camacho-Cuadra (2012) demonstrated that irrigants delivered via conventional syringes passively backflow towards the pulp chamber soon after they have been expressed into canals, thus limiting their penetrative potential. The use of these agitation techniques is therefore likely to assist clinicians achieve optimum disinfection throughout the entirety of the canal during treatment.

In this study, NaOCl infiltration was consistently lower apically when compared to the corresponding coronal and middle segments, where there was relatively even penetration in both bucco-lingual and mesio-distal planes. Despite the use of different methodologies, similar observations have also been reported by Paqué *et al.* (2006), Giardino *et al.* (2017) and Galler *et al.* (2019). This regional variation can be attributed to characteristic features of apical dentine, which include increased peritubular sclerosis that advances in a coronal direction from 30 years of age, as well as reduced tubular density (Mjör & Nordahl 1996, Mjör *et al.* 2001, Paqué *et al.* 2006). Additionally, the dentinal sclerosis that physiologically develops along the mesio-distal direction of teeth has been shown to be more pronounced apically than in any other segment of the root canal (Russell *et al.* 2013, Giardino *et al.* 2017, Generali *et al.* 2018). This 'butterfly effect' is also likely to have contributed significantly to the reduced penetration observed in this region.

No single technique consistently achieved the greatest effect across the full length of the root canal. However, SI generally exhibited the highest NaOCl penetration coronally, all techniques performed relatively equally in the middle third and MDA and PUI outperformed SI apically. These regional differences in penetrative efficacy can likely be attributed to the agitating mechanisms of these techniques. For example, with MDA the simultaneous poor coronal and tight apical adaption of a single GP cone when placed within an instrumented canal would lead to greater differences in irrigant penetration apically, with respect to CNI, compared with any other region. This would suggest that each technique is suited for a specific region of the canal and a combination of agitation methods may be required to achieve the maximum irrigant penetration across the entire length of the root canal. Data reported by Spoorthy *et al.* (2013) and Ismail *et al.* (2016) support this hypothesis as they found deeper infiltration of irrigants and sealers, respectively, when several agitation techniques were used in a single canal. Further investigations are, however, required to identify the most efficacious combination of agitation techniques.

Highly significant increases in tubular penetration were measured across all irrigant activation techniques and canal locations when either NaOCl concentration or contact time was exclusively increased from 2% to 5.25% and from 10 min to 20 min, respectively. Despite methodological variations, these trends are consistent with findings from other studies that have investigated similar variables (Zou *et al.* 2010, Palazzi *et al.* 2016, Faria *et al.* 2019). These outcomes would also be expected as the amount of freely available chlorine increases when the NaOCl concentration is raised or when the solution is repeatedly replenished over a longer period of time (Siqueira *et al.* 2000). Interestingly, these effects were found to be much more pronounced for CNI and MDA than PUI or SI. This could be attributed to the fact that these are manually operated techniques and so greater time may be required for maximum effect on tubular penetration to be achieved, which in turn could be compensated for by using greater concentrations of NaOCl. There was also a highly significant interaction between irrigant concentration and contact time when both were increased in the coronal and middle thirds of canals. Therefore, the additional effect of raising either irrigant concentration or contact time will be of less magnitude than the initial effect of raising one of these parameters alone. For example, in this experiment, raising the irrigant contact time from 10 to 20 min with 2% NaOCl produced an estimated marginal mean increases in penetration of 58.2 μm coronally and 64.7 μm in the middle third. However,

when raising the irrigant contact time with 5.25% NaOCl, estimated marginal mean increases in penetration of only 34.1 and 30.4 μm were observed in the coronal and middle regions, respectively. This could be explained by the fact that NaOCl penetration occurs more quickly at higher concentrations and consequentially the results saturate at a given depth earlier. Therefore, whilst clinicians can use higher concentrations of NaOCl during root canal treatment to improve tubular penetration, the findings suggest similar outcomes can be achieved when lower concentrations are administered over longer periods of time. Given the harmful cytotoxic and caustic properties of more concentrated NaOCl solutions (Kleier *et al.* 2008, Martin *et al.* 2014), it may be more desirable to adopt this strategy for the purposes of patient safety.

Conclusions

Within the limitation of this study, three main conclusions can be drawn:

1. Manual dynamic activation, passive ultrasonic irrigation and sonic irrigation significantly improve tubular penetration of sodium hypochlorite throughout the root canal, including apically, when compared to conventional needle irrigation.
2. Increasing sodium hypochlorite concentration or contact time further improves tubular penetration for each of the aforementioned techniques.
3. Longer durations of sodium hypochlorite exposure at lower concentrations result in similar depths of tubular penetration as those achieved at higher concentrations.

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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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CHAPTER 4

PUBLICATION 5

ANTIMICROBIAL EFFICACY OF DIFFERENT IRRIGANT SOLUTIONS USING A NOVEL BIOFILM MODEL: AN IN VITRO CONFOCAL LASER SCANNING MICROSCOPY EXPERIMENT

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ABSTRACT

Aim: To determine the ability of different irrigation solutions to biomechanically remove Enterococcus faecalis biofilm from a novel artificial root canal model during chemomechanical preparation. Methods: High resolution micro-computer-tomography scans of a mandibular molar's mesial root were used to produce 50 identical 3D-printed resin root canal models. These were cultured with E.faecalis over seven days to generate biofilm and subjected to chemomechanical preparation using: saline; 17% ethylenediaminetetraacetic acid (EDTA) or 2% sodium hypochlorite (NaOCl) alongside positive/negative controls (n = 10). Canals were prepared to 40/.06 taper, with 1 mL irrigation between instruments, followed by 5 mL penultimate rinse, 30 s ultrasonic activation and 5 mL final rinse. Residual biofilm volume (pixels) was determined following immunofluorescent staining and confocal-laser-scanning-microscopy imaging. Statistical comparisons were made using Kruskal-Wallis with post-hoc Dunn's tests (α <0.05). Results: In all canal thirds, the greatest biofilm removal was observed with NaOCl, followed by EDTA and saline. The latter had significantly higher E.faecalis counts than NaOCl and EDTA (P <0.01). However, no statistical differences were found between EDTA and NaOCl or saline and positive controls (P >0.05). Conclusions: Within limitations of this model, 17% EDTA was found to be as effective as 2% NaOCl at eradicating E.faecalis biofilm following chemomechanical preparation. Further investigations with multi-species biofilms are encouraged.

INTRODUCTION

Putative endodontic microbial communities, and their by-products, are the causative agents of pulp and periapical diseases.[1,2] Current therapeutic strategies therefore aim to disrupt these biofilms and disinfect root canals so that the bacterial load is below the critical threshold required for periradicular healing.[3] This is typically achieved by using a combination of hand or rotary instruments alongside constant irrigation with antibacterial solutions. Greater emphasis is placed on cleaning the canal as opposed to shaping it due to the inherently complex nature of the endodontic anatomy.[4] For these reasons, irrigant selection plays a critical role in determining the success of root canal treatment.

Currently, the most commonly used endodontic irrigants are sodium hypochlorite (NaOCl) and ethylenediaminetetraacetic acid (EDTA).[5,6] Whilst the former possesses potent antimicrobial and tissue dissolving properties,[7] it is also a caustic agent that when used incorrectly has the capability to inflict diffuse soft tissue swelling, bruising, ulceration and in severe cases, necrosis and neurological damage.[8] This is compounded by its cytotoxic potential,[9] inability to remove infected inorganic debris from within the surgical smear layer [7] and detrimental effects to the dentine's flexural strength.[10,11] For these reasons, NaOCl is often administered at concentrations less than 3%, with strengths of 2% still being considered an effective dose for disinfection.[5] Conversely, EDTA, which is widely available at 17% concentration and is conventionally used for its chelating action on the smear layer,[12] overcomes many of these limitations but at present has questionable antimicrobial properties. For instance, there are numerous studies that have reported this solution as being effective against a broad spectrum of endodontic bacteria,[13-18] including *Enterococcus faecalis* which is known for its invasive, adherent and pH resistant properties.[19] However, almost an equal number of studies have observed limited or no antimicrobial effect despite using similar analytical methodologies.[20-27] The results of these studies must also be interpreted with caution as they were conducted using relatively simple *in vitro* experimental model systems in addition to planktonic forms of bacteria rather than biofilms.[28] Such investigations do not reflect *in vivo* conditions well.

In recent years, technological advancements have allowed for the development of resin-based materials that facilitate growth of microbial biofilms. These have been found to possess similar properties to dentine, with respect to bacterial attachment,[29] and through rapid processing techniques can be manufactured into three-dimensional (3D) models that accurately mimic the intricate anatomy and unique environment of the root canal system.[30] These experimental models show promise in overcoming many of the limitations of those that have been previously used. They also allow for the antimicrobial activity of irrigant solutions to be evaluated throughout the course of both chemical and mechanical preparation of root canals, a feature which is difficult replicate with extracted teeth. At the same time, highly sensitive methods of microbial analysis are now available to quantify viable micro-organisms within biofilms by way of fluorescent staining and confocal laser scanning microscopy (CLSM).[23,31,32] These offer more careful morphological observation through higher resolution imaging, the possibility of 3D reconstructions and overcoming many of the limitations associated with dentine demineralisation and microtome sectioning. As very few studies have employed such techniques in relation to EDTA, further and more sophisticated investigations into the antimicrobial capabilities of this solution are warranted.[23] The resulting information could contribute to the development of more biocompatible irrigant regimes and an improved understanding of the potential mechanisms in which these solutions interact with microbes.

The aim of this *in vitro* experiment was to determine the ability of saline, 17% EDTA and 2% NaOCl to biomechanically remove an *E. faecalis* biofilm from a novel root canal model during chemomechanical preparation. The tested null hypothesis was that there were no significant differences between the different irrigant solutions.

MATERIALS AND METHODS

ROOT CANAL MODEL

Following ethical approval (REC Ref: 14/SW/1148), 20 extracted human mandibular molars were randomly selected from the University of Birmingham's Dentistry Research Tissue Bank. Teeth with extensive caries and restorations, root fractures, open apices, resorptive defects, previous root fillings and fused roots were excluded. A high-resolution micro–computed tomographic scan (μCT; 13.6 μm/pixel; Skyscan 1172; Chelmsford, UK) was subsequently obtained for each tooth. The resulting image slices were uploaded onto ImageJ software (National Institutes of Health, Bethesda, USA) and reconstructed into 3D.

The μCT series of a mandibular molar's mesial root, which contained two distinct unprepared canals and complete isthmus, was selected to generate the model as demonstrated in Figure 1 due to its anatomical complexity. Initially, the mesial root was segmented at the point of furcation and the curvature straightened so the endodontic system could be centrally bounded in rectangular geometry and subsequently divided into two equal halves. Three-dimensional replicas were then printed from a transparent auto-fluorescent resin material (Accura; 3D Alchemy, Shropshire, UK), via stereolithographic rapid processing technology at 50 μm/layer resolution and ± 0.2 mm surface accuracy (3D Alchemy). A precisely fitting white polypropylene cubic frame was also fabricated so the rectangular halves of each model could be firmly approximated during chemomechanical preparation to mimic a closed root canal system with standardised dimensions. More specifically the canal length, inter-canal distance and range of isthmus width in each assembled model were measured as being 9.5 mm, 3.6 mm, and 0.08 to 0.31 mm respectively using the calibrated line tool on ImageJ software.

BIOFILM CULTIVATION

Under aseptic condition in a laminar flow hood, the NCTC 12697 strain of *E. faecalis* (Public health England, Wiltshire, UK) was cultivated on Brain Heart Infusion (BHI) agar (Sigma-Aldrich, Gillingham, UK) for 24 h at 37°C in a 5% CO_2 incubator.

Thereafter, a single colony was transferred into 10 mL BHI broth and incubated under the same conditions alongside a sterile control. The resulting bacterial suspension was diluted 100-fold in fresh BHI broth and adjusted to an optical density of 1 using a flow cytometer (BD accuri, California, USA). This standardised the bacterial concentration to 1.6 x 10^6 CFU/mL.

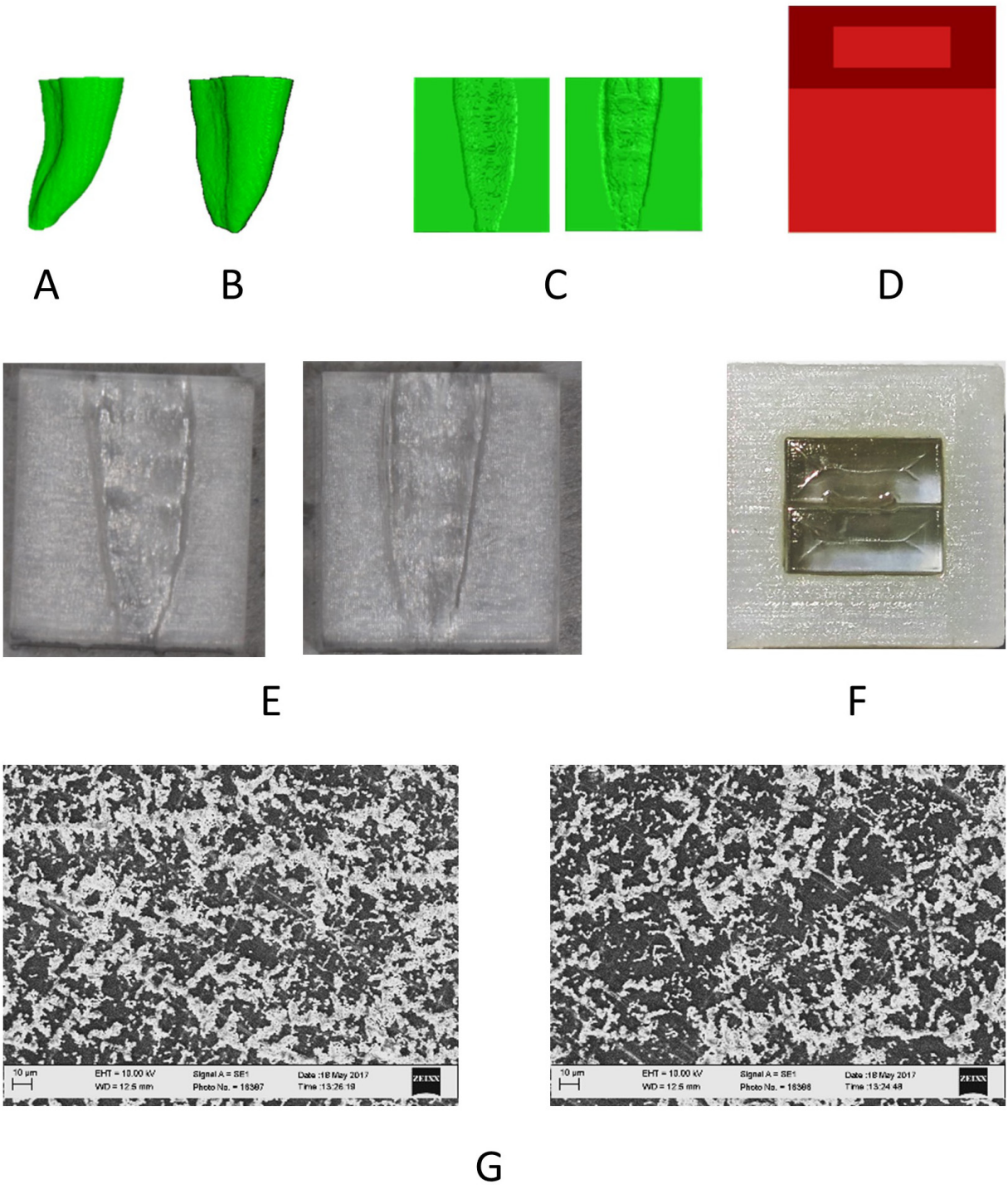

Figure 1: The stages involved in developing the 3D printed root canal model: (A) 3D image of a segmented mesial root of a mandibular molar, (B) root curvature straightened, (C) the root canal divided into two equal rectangular halves, (D) 3D image of the cubic frame, (E) model parts following 3D printing, (F) an assembled model before root canal preparation, (G) Field emission scanning electron micrographs of *E. faecalis* biofilms grown on Accura resin after seven days incubation (magnification x 1200, scale bars represent 10 µm).

Prior to inoculation, all blocks and frames were autoclaved for 30 minutes at 121°C. The models were then positioned into a 24-well tissue culture plate, so the internal canal surface faced towards the plate cover. Two millilitres of bacterial suspension were subsequently added into each well, after which plates were cultured for 7 days in a 5% CO_2 incubator. The BHI growth medium was replenished every 48 h and following this period, biofilms were washed with Phosphate Buffered Saline (PBS) and fixed for 10 minutes with 2.5% glutaraldehyde (Sigma-Aldrich).

CONTROL & TEST GROUPS

Infected root canal blocks were inserted into the polypropylene cubic frame and then randomly distributed into 3 groups (n = 10) according to irrigant solution. These included i) Saline (CD Medical, Bolton, UK), ii) 17% EDTA (Cerkamed, Stalowa-Wola, Poland) and iii) 2% NaOCl (Cerkamed). To confirm adequate biofilm growth (positive control), 10 blocks were contaminated but not chemomechanically prepared and a further 10 samples were cultured in sterile BHI broth to determine background staining (negative controls). As a sample size calculation could not be conducted, due to the lack of prior data, the sample size was determined using previous studies investigating similar hypotheses.[20,21,23]

ROOT CANAL PREPARATION

Root canal preparation was performed by a single blinded operator (SSV) to a pre-determined working length (WL) of 9 mm. After assembled blocks were firmly clamped to the bench top, a glide path was established using a stainless-steel size 10 K-file (Dentsply Sirona, Ballaigues, Switzerland) in a watch winding motion. Root canals were then prepared up to a ProTaper Gold F4 (size 40/.06 taper) rotary file at speeds and torques recommended by the manufacturer (Dentsply Sirona). Between instruments, 1 mL of irrigant was expressed into each canal with the tip of a 27 gauge side vented needle positioned 2 mm short of the WL (Monoject, Covidien, Mansfield, USA). A 5 mL penultimate rinse was then administered followed by 30 seconds passive ultrasonic irrigation, with an ISO size 20 Irrisafe tip (Acteon, Norwich, UK) activated half power 1 mm from WL (MiniEndo II; SybronEndo, California, USA). To terminate the irrigation sequence, a final 5 mL rinse was performed as above followed 5 mL sodium thiosulphate or 5 mL saline to arrest NaOCl and EDTA activity respectively. Root canal blocks were then disassembled and washed with PBS prior to immunofluorescent staining.

BIOFILM STAINING

To label the residual *E. faecalis* biofilm, samples were incubated for 24 h at 4°C with 50 µl of a primary non-conjugated polyclonal antibody (Rabbit anti-*Enterococcus* species; MyBioSource, San Diego, USA). Blocks were then washed in PBS, incubated in a dark environment for 24 h at 4°C with 50 µl of a secondary tetramethyl rhodamine-isothiocyanate (TRITC) conjugated polyclonal antibody (Donkey Anti-Rabbit IgG H&L; Abcam, Cambridge, UK) and then washed again in PBS. Both antibodies were diluted 300-fold with 3% w/v bovine serum albumin.

CONFOCAL LASER SCANNING MICROSCOPY

Labelled models were mounted onto a customised glass slide and viewed under a CLSM at 5 x magnification (Carl Zeiss, Oberkochen, Germany). A plastic seating jig standardised the position of each block and a copper grid (TedPella, California, USA) with unique patterns allowed images to be captured between samples at reproducible positions *(Figure 2*). Multi-track lasers, set at 488 nm and 555 nm, were used to reduce cross talk between the green auto-fluorescence inherently emitted by the Accura resin material and the red fluorescence emitted by the TRITC labelled biofilm. Sixteen images (seven coronal, six middle and three apical) were captured per model at optimal focus and fixed resolution (512 x 512 pixels) with the same objective and laser settings *(Table 1*). All images were saved in .tiff format, coded and then analysed in ImageJ software by a blinded assessor (SSV).

A semi-automated method was used to quantify the remaining biofilm.[29] For each image, the four central squares were isolated ("clear outside") to standardise the area of analysis and split into individual colour channels ("split channel"). The resulting green and red grey-scale images represented the Accura material and residual biofilm respectively, the latter of which was used in subsequent analyses. Background fluorescence was removed ("subtract background") and an "auto-threshold" applied to allow the residual biofilm to be quantified via a calibrated "voxel counter" tool.

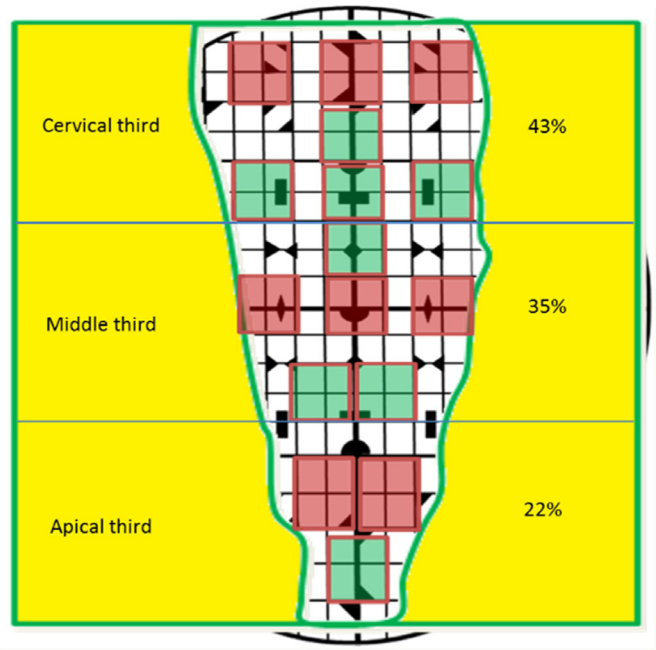

Figure 2: A schematic diagram illustrating the positions of images acquired for the first (red) and second half (green) of the root canal model when disassembled and positioned in the jig. The number of images per canal segment were distributed in relation to the percentage volume of each canal third. The unique patterns of the superimposed copper grid facilitated reproducible positioning of images between samples.

STATISTICAL ANALYSIS

Statistical tests were performed using SPSS software (V.25; IBM, New York, USA). The Shapiro-Wilk test revealed data to be non-normally distributed and therefore, comparisons between groups were made using Kruskal-Wallis and *post-hoc* Dunn's

Table 1. Laser settings used during confocal laser scanning microscopy imaging.

	Scanning speed	Range (Frame)	Pin hole size	Gain master	Digital offset	Colour	Wavelength (nm)
Track 1	3	2	54.2	732	0	Green (AF)	488
Track 2	3	2	54.2	732	0	Red (TRITC)	555

AF: Alexa fluor 488; TRTIC: Tetramethyl rhodamine-isothiocyanate

tests with the initial alpha values set at 0.05. Additionally, 10 randomly selected images from each group were analysed one month apart to determine intra-rater reliability via the intra-class correlation coefficient (ICC). Data was presented as medians and means alongside the interquartile range and standard deviation respectively.

RESULTS

The volume of residual biofilm for each group is summarised in Table 2 and Figure 3 with representative CLSM images displayed in Figure 4. The ICC demonstrated intra-rater agreement at greater than 0.95.

In all canal thirds, the greatest biomechanical removal of biofilm following chemomechanical preparation was found in the 2% NaOCl group, followed by 17% EDTA and then saline. However, no solutions were able to eradicate the entirety of the biofilm. Nevertheless, when compared to the positive control group, the greatest percentage reductions for any given regime were observed in a coronal to apical direction. The positive controls presented the largest *E. faecalis* counts whereas negative controls showed zero *E. faecalis* presence.

Highly significant differences were identified between irrigant solutions in all canal thirds ($P < 0.001$). Root canals prepared with 17% EDTA and 2% NaOCl resulted in significantly less residual *E. faecalis* biofilm than saline ($P < 0.01$). However, no statistical differences were found between 17% EDTA and 2% NaOCl or between saline and positive control groups ($P > 0.05$).

DISCUSSION

The current study used a novel model for testing the antimicrobial efficacy of several commonly used irrigants throughout chemomechanical preparation of artificial root canals. Under these parameters, 17% EDTA was found to be comparable to 2% NaOCl at biomechanically removing *E. faecalis* biofilms in all canal thirds. However, saline was significantly less effective and so the null hypothesis was rejected.

Table 2. Residual volume of *E. faecalis* biofilm following chemomechanical preparation of root canals with different irrigant solutions.

		Volume of residual *E. faecalis* biofilm (pixels)			
Group		**Total**	**Coronal**	**Middle**	**Apical**
Control	**Median ± IQR**	280 109 ± 40 972	111 364 ± 45 868	97 956 ± 48 872	78 273 ± 40 101
	Mean ± SD	277 626 ± 73 590	110 323 ± 23 575	102 076 ± 37 211	65 227 ± 27 220
Saline	**Median ± IQR**	244 572 ± 96 201 [13%]	89 124 ± 43 171 [20%]	84 519 ± 31 410 [14%]	57 625 ± 28 682 [26%]
	Mean ± SD	250 670 ± 53 865 [10%]	93 827 ± 25 333 [15%]	94 203 ± 24 443 [8%]	62 640 ± 17 922 [4%]
17% EDTA*	**Median ± IQR**	87 063 ± 85 255 [69%]	28 856 ± 20 159 [74%]	37 170 ± 36 316 [62%]	26 212 ± 22 165 [67%]
	Mean ± SD	114 412 ± 65 344 [59%]	40 285 ± 28 606 [63%]	47 813 ± 26 382 [53%]	26 315 ± 13 348 [60%]
2% NaOCl*	**Median ± IQR**	83 590 ± 39 465 [70%]	23 686 ± 16 606 [79%]	35 056 ± 17 593 [64%]	29 185 ± 14 131 [63%]
	Mean ± SD	99 194 ± 37 669 [64%]	24 785 ± 9 189 [78%]	42 336 ± 17 207 [59%]	32 073 ± 14 766 [52%]

EDTA: ethylenediaminetetraacetic acid; IQR; interquartile range; NaOCl: sodium hypochlorite; SD: standard deviation; [%]: percentage change in point median or mean value with respect to control group.
*** vs. control and saline group ($P < 0.01$) [Kruskal-Wallis & *post-hoc* Dunn's test]**

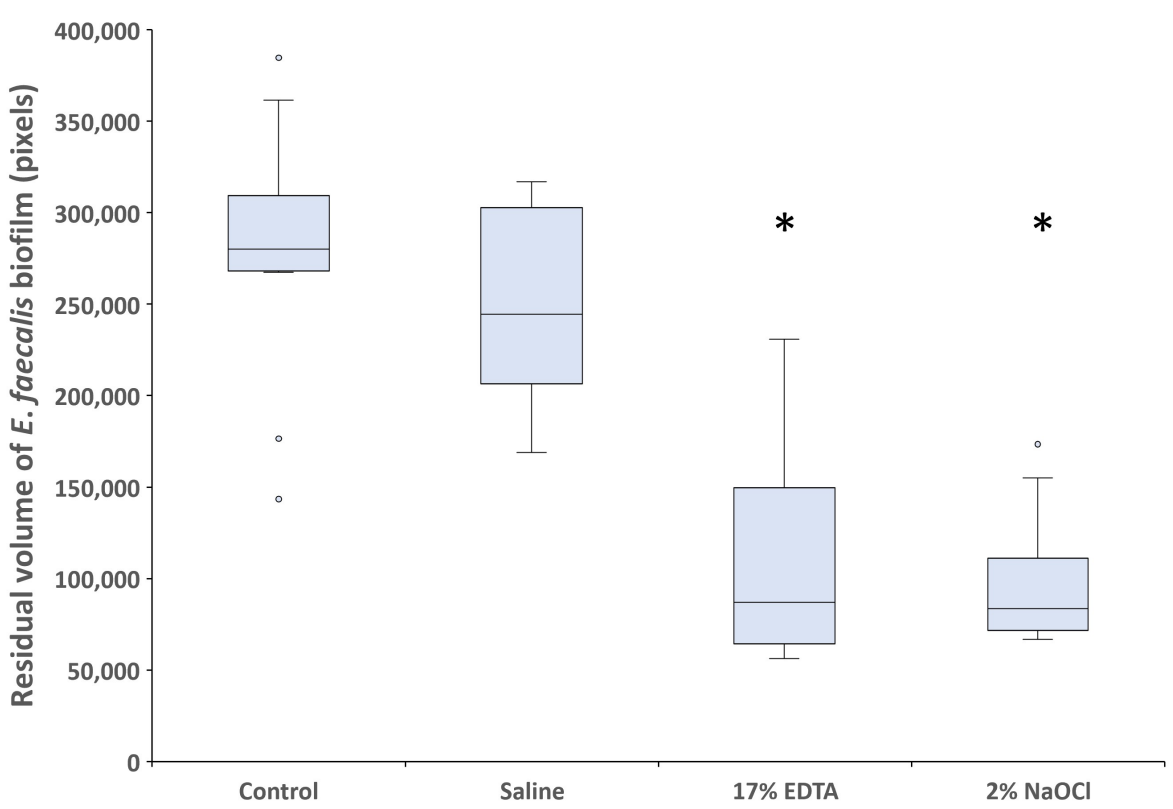

Figure 3: Volume of residual *E. faecalis* biofilm following root canal preparation with different irrigant solutions. Data presented as box and whisker plots where the central bar represents the median alongside upper and lower interquartile ranges at the edge of boxes, minimum and maximum values for the whiskers, and outliers shown as open circles. Statistically significant comparisons ($P < 0.01$; Dunn's test) between groups are presented as superscripts (* vs. control and saline groups).

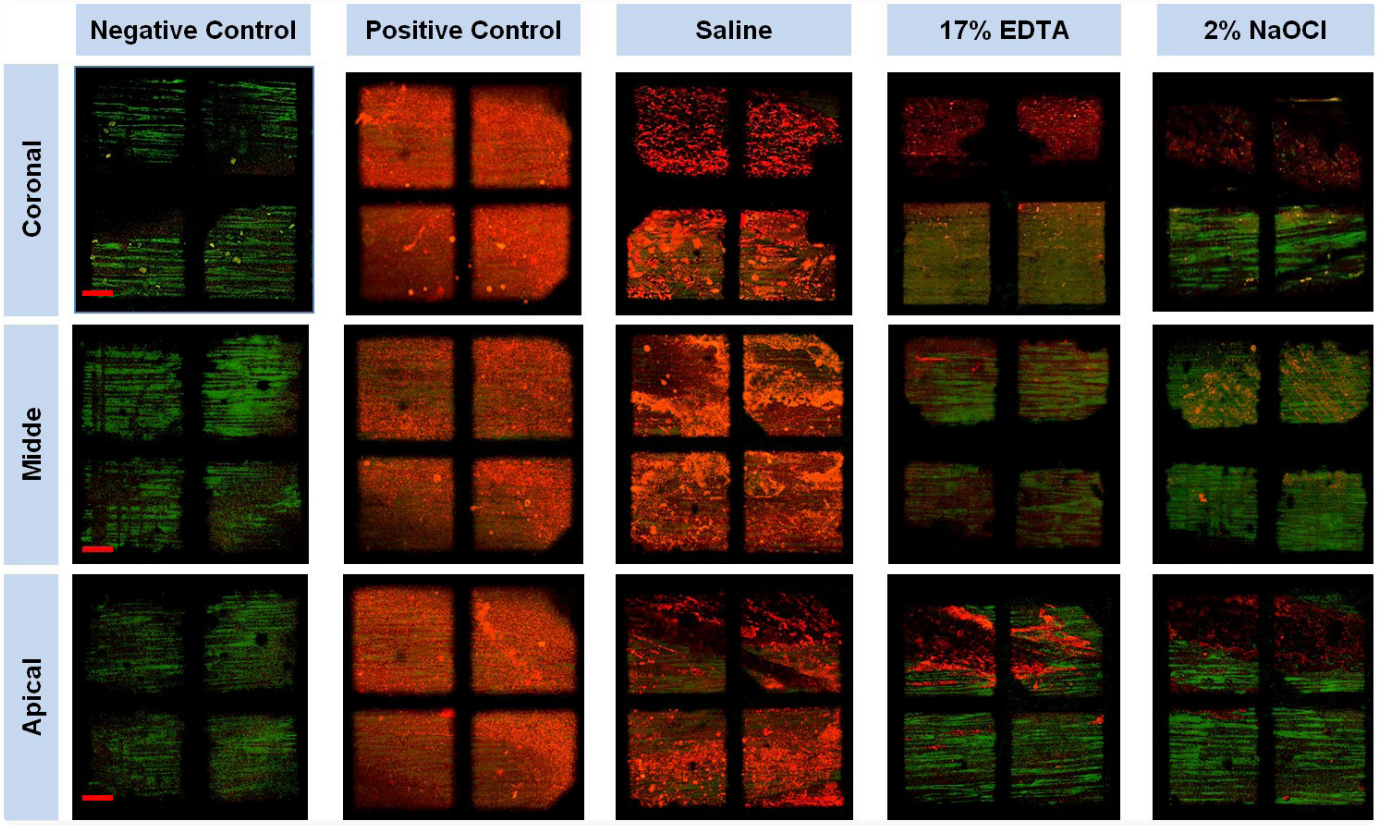

Figure 4: Representative confocal laser scanning microscopy images of residual *E. faecalis* biofilm following root canal preparation with different irrigant solutions. The green fluorescence represents the transparent auto-fluorescent resin Accura material (i.e. eradicated biofilm) and the red fluorescence represents the residual TRITC labelled biofilm (scale bars represents 50 µm).

Endodontic disinfection involves both chemical and mechanical debridement within a closed root canal system. However, previous investigations into the antimicrobial efficacy of EDTA have seldom been conducted under such conditions. More than often, test solutions have been administered onto infected agar plates,[14,17,22] cover slips,[24] cell suspensions[15,20] and dentine disks.[23] Whilst extracted teeth were used in more recent experiments,[25,26] the root canals in these samples were inoculated only after instrumentation, where they then underwent a distinct chemical disinfection protocol. The resin model employed in the present study however overcame these limitations by allowing solutions to be delivered into an infected and closed endodontic system prior to and throughout the entire chemomechanical debridement process. This is more akin to how root canal treatment is performed clinically and at present is difficult to test with an *ex vivo* or intratubular infection approach. The precise manufacturing process employed in this study also offered a degree of anatomical replication that far surpassed prior synthetic models,[33-35] giving way for more representative irrigant flow dynamics and methodological standardisation that cannot be achieved with extracted teeth.[36] Additionally, the model could be longitudinally split on demand without disturbing the residual biofilm, which would allow continued analysis following chemomechanical debridement. This feature would be particularly useful for investigating bacteria that have *in vitro* demonstrated a potential for regrowth, such as *E.faecalis*, after NaOCl administration.[37] Furthermore, the use of the copper ring depicted in Figure 2 to select specific regions of the canal to image and the semi-automated quantitative method of analysis offers a more reproducible and accurate approach to investigating endodontic biofilm removal than many preceding studies. The most apparent limitations of this study however arise from the model's material composition and structure, in that there is no peri-, intra- and inter-tubular dentine or tubules for micro-organisms to penetrate into. This non-biological substrate could alter the inherent mechanisms of bacterial surface adherence thus, affecting subsequent biofilm formation. There would also be more freely available chlorine ions for NaOCl disinfection due to the absence of collagen, fluid and necrotic debris.[38] Additionally, single-species biofilms exhibit less biomass and resistance to endodontic irrigants than their more representative multi-species counterparts and those made of *E.faecalis* in particular have demonstrated the potential to re-establish themselves following exposure to common endodontic irrigants.[37,39,40] Collectively, these limitations could overestimate the antimicrobial efficacy of the tested irrigant solutions; however, attempts were made to reduce the impact of these confounding variables. For instance, Accura resin was selected as *E. faecalis* attached to its surface at a force comparable to that of dentine, which precluded the need for any prior collagen coating.[29] Similarly, this species was used as the test micro-organism due to its ability to rapidly form biofilms on resin materials and remain within root canals even after thorough chemomechanical disinfection protocols.[18] Furthermore, lower NaOCl concentrations were administered to compensate for the lack of organic matter and biofilms were cultured until they reached peak biomass at seven days, which is significantly longer than other simulated root canal studies.[33-35] The disinfection challenge these measures created was further potentiated by the complicated endodontic anatomy of the model, as evidenced by high treatment failure rates associated with the tooth it was based on.[41] However, despite these endeavours, some caution must still be taken when extrapolating the present results into the clinical setting and further investigations using multi-species biofilm models are encouraged to reinforce these findings.

In the present study, 17% EDTA and 2% NaOCl were found to be equally as effective at eradicating *E. faecalis* biofilm from within artificial root canals. These findings contrast previous studies that reported the antimicrobial efficacy of the former to be absent, limited or vastly inferior to NaOCl even after prolonged periods of exposure.[20-27] This discrepancy could be attributed to methodological heterogeneity, varying irrigation protocols and the differing mechanisms in which these solutions interact with micro-organisms. For instance, NaOCl disassociates into its bactericidal hydroxyl and chlorine ion derivatives and then rapidly eliminates microbes by disrupting enzymatic processes essential to their physiology.[42] Conversely, EDTA only destabilises gram-negative bacteria by chelating cations from within their outer cell membranes.[43] Whilst this effect alone may not always induce cell death, it could potentially be sufficiently enhanced enough to do so when combined with mechanical instrumentation, a feature which has only been tested in the current study. This chelating action has also shown to promote cellular detachment and weaken the macrostructures of established biofilms, which can then be more easily flushed from root canals via the mechanical shearing forces created by conventional irrigant flow dynamics and agitation techniques.[24] Furthermore, higher EDTA concentrations and exposure times, as used in this experiment, have demonstrated greater disinfection capabilities.[15,16] Collectively, these mechanisms could potentially equate to the antimicrobial activity of 2% NaOCl and would explain why 17% EDTA was found to be comparable to this solution post root canal preparation but significantly more effective than saline, the latter of which possesses no antibacterial properties and was also used to provide additional validation of the experimental model.

Whilst 2% chlorhexidine has previously been considered an alternative endodontic irrigant to NaOCl, evidence highlighting its negative association with periradicular healing and increased incidents of anaphylaxis has recently emerged.[44,45] Consequently, this has deterred its use as demonstrated by a recent national survey which found 15 of the 18 undergraduate dental schools across the UK & Ireland abstaining from teaching its use during root canal treatment.[5] For these reasons, it was not included as an additional test group.

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CONCLUSION

Based on the results of this study, which were derived from a novel biofilm model, it can be proposed that 17% EDTA is as effective as 2% NaOCl at biomechanically removing *E. faecalis* biofilm following chemomechanical preparation of a complex root canal system. Further investigations however with a multi-species biofilm model are encouraged to reinforce the promising results observed within the present study.

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CHAPTER 5

PERIRADICULAR TISSUE FLUID – A TOOL TO DETERMINE THE PERIRADICULAR CONDITION

Publication 6: Virdee, S. S., Butt, K., Grant, M., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2019). A systematic review of methods used to sample and analyse periradicular tissue fluid during root canal treatment. *International Endodontic Journal*, *52*(8), 1108–1127.

Publication 7: Virdee, S. S., Bashir, N. Z., Krstic, M., Camilleri, J., Grant, M. M., Cooper, P. R., & Tomson, P. L. (2023). Periradicular tissue fluid-derived biomarkers for apical periodontitis: An in vitro methodological and in vivo cross-sectional study. *International Endodontic Journal*, 56(10), 1222–1240.

When solubilised into the canal it is hypothesised that efflux of bioactive dECMs into the periradicular tissues will alter cytokine activity. However, these changes will likely be too subtle to be detected using current clinical and radiographic tools. As such, the prospect of using PTF-derived biomarkers of apical periodontitis as a surrogate marker for healing during orthograde root canal treatment was explored in this chapter. Initially, a systematic review was conducted to identify the methods currently used to

sample and analyse PTF during root canal treatment (Publication 6). This body of work clearly identified a lack of standardisation in such methods and the broad range of analytes explored. Consequently, an *in vitro* study was performed to optimise the PTF sampling and analysis protocols. This was applied *in vivo* to mature permanent teeth diagnosed with apical periodontitis in a cross-sectional manner to provide proof of concept of downstream multiplex analysis and identify a reliable profile of biomarkers for apical periodontitis that could be used to monitor disease progression in a subsequent interventional study (Publication 7). This chapter thus consists of two publications with the first being a systematic review, and the second a combined *in vitro* methodological optimisation and *in vivo* cross-sectional study.

CHAPTER 5

PUBLICATION 6

A SYSTEMATIC REVIEW OF METHODS USED TO SAMPLE AND ANALYSE PERIRADICULAR TISSUE FLUID DURING ROOT CANAL TREATMENT

Satnam Singh Virdee, Kasim Butt, Melissa Gant, Josette Camilleri, Paul Cooper & Phillip Tomson

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REVIEW

A systematic review of methods used to sample and analyse periradicular tissue fluid during root canal treatment

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Abstract

Virdee SS, Butt K, Grant M, Camilleri J, Cooper PR, Tomson PL. A systematic review of methods used to sample and analyse periradicular tissue fluid during root canal treatment. *International Endodontic Journal*.

Aim The primary aim was to identify techniques used to sample and analyse periradicular tissue fluid (PTF) in permanent teeth diagnosed with apical disease during root canal treatment. Secondly, to identify the types of inflammatory mediators studied using this approach.

Methodology *Data Sources:* PubMed, EMBASE, Cochrane Library, Science Direct, Web of Science and OpenGrey. *Eligibility Criteria*: Clinical studies published until 1 June 2018 which utilized orthograde techniques to sample and analyse PTF were included. Cell culture, laboratory or animal studies and those concerned with investigating inflammatory mediator activity from within healthy or diseased pulp tissue, and not periradicular tissues, were excluded. *Study appraisal and methods:* In accordance with PRISMA guidelines, data were extracted on study characteristics, target mediators, sampling and assay techniques and the parameters associated with the PTF sampling and eluting protocol. A qualitative synthesis was conducted, and studies were critically appraised using a modified version of the Cochrane risk of bias tool.

Results *Study Characteristics:* From 251 studies, 33 were eligible for inclusion. Sampling techniques included the use of paper points (n = 27), fine needle aspiration (n = 4) and filter strips (n = 2). Assay techniques included enzyme-linked immunosorbent assay (n = 18), quantitative polymerase chain reaction (n = 9), radioimmunoassay (n = 4), colorimetric assay (n = 2), immunofluorometric assay (n = 1) and cytometric bead array (n = 1). Forty-five different inflammatory mediators were targeted at the proteomic/metabolomic (n = 25) or transcriptomic level (n = 9). *Limitations:* Significant heterogeneity exists within the methodology, and only 5 studies disclosed unambiguous information about their PTF sampling and eluting protocols.

Conclusions Paper points and proteomic/metabolomic analysis are currently the preferred methods for studying and analysing PTF during root canal treatment. The most studied analytes were IL-1β and TNF-α. *Implications:* Further research is required to develop an optimized PTF sampling and eluting protocol to overcome methodological heterogeneity, and future studies are advised to follow a standardized approach to reporting their methodology.

Keywords: Endodontics, periradicular tissue fluid, cytokine, inflammation, sampling, systematic review.

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Introduction

Apical periodontitis is an inflammatory reaction of the periradicular tissues induced predominantly by

complex interactions between the host's immune system and pathogenic bacterial communities of endodontic origin (Nair 1997). Although this process is initiated by invading microorganisms and their by-products, the destructive effects are the result of a localized inflammatory response (Yamasaki *et al.* 1994, Stashenko *et al.* 1995). This reaction consists of various leucocytes (i.e. macrophages and lymphocytes) which in turn produce a myriad of soluble inflammatory mediators (Graunaite *et al.* 2011). These host-derived auto- and para-crine signalling molecules coordinate overlapping destructive and regenerative inflammatory processes (i.e. apical bone resorption and deposition respectively) to facilitate the formation of a granuloma (Márton & Kiss 2014). This organized collection of leucocytes is a defensive response which acts to restrain endodontic pathogens inside the infected root canal (Metzger 2000, Márton & Kiss 2014). The vascular nature of this tissue results in production of an inflammatory exudate, which becomes enriched with key components of the immune response as the disease progresses (Nair 2004). This extracellular fluid is commonly referred to as the periradicular tissue fluid (PTF) or periapical exudate.

The concentrations of known inflammatory mediators found within periradicular lesions have been linked to specific states of disease activity (Kawashima & Stashenko 1999). For example, interleukin [IL]1-α, IL-1β, IL-2, prostaglandin [PGE]-2, tumour necrosis factor [TNF]-α, interferon [IFN]-ɣ and macrophage inflammatory protein [MIP]-1β are considered potent stimulators of osteoclastic activity (Márton & Kiss 2000, Metzger 2000) whereas high concentrations of IL-4, IL-5, IL-6, IL-10 and IL-13 are reported to antagonistically suppress apical bone resorption (Stashenko *et al.* 1987, Fukada *et al.* 2009, Popovska *et al.* 2017). Furthermore, increased levels of IL-17A have been associated with the development of radicular cysts and abscesses (Ajuz *et al.* 2014, Ferreira *et al.* 2016). As described above, it is evident these molecular changes orchestrate the inflammatory process once it has been initiated and precede the presentation of clinical symptoms. It would therefore be highly informative to have the ability to study levels of these mediators within infected periradicular tissues. The precise information attained from a simple, non-invasive and accurate sampling procedure could help clinicians determine disease states, inform prognosis and establish a point at which treatment should be concluded to enable predictable outcomes. It could also provide researchers with more objective tools to investigate the biological processes involved in periradicular disease and their response to novel therapeutic interventions.

Unfortunately, traditional methods used to sample periradicular inflammatory mediators (i.e. direct surgical access) are invasive, technique sensitive and do not permit longitudinal analyses (Torabinejad *et al.* 1992, Ajuz *et al.* 2014, Popovska *et al.* 2017). More recently, less invasive orthograde approaches have been developed. Consequently, an increasing number of clinical studies are sampling PTF via the root canal, during root canal treatment, and subsequently analysing levels of inflammatory mediators to inform clinicians of best-practice approaches (Matsuo *et al.* 1994, Shimauchi *et al.* 1996, Kuo *et al.* 1998a). Although this demonstrates proof of concept, very little is known about these techniques or whether they have been optimized, through methodology work-up experiments, to serve this important function. This contrasts with intricate sampling procedures in other areas of dentistry (i.e. collection of periodontal pathogens from subgingival plaque) where the influences of several basic parameters have been investigated in depth (Hartroth *et al.* 1999). Additionally, conflicting findings are often reported from studies with similar objectives and designs, which further warrant the need for investigating how these methods are currently being employed. For instance, Alptekin *et al.* (2005a) found no difference in PTF levels of PGE_2 in patients with acute apical periodontitis following endodontic treatment whereas Liu *et al.* (2003) identified a significant reduction. Therefore, the relevance of this review is that it would for the first time clarify the overall picture of how inflammatory mediators are currently being sampled and analysed from PTF during root canal treatment, as well as highlighting areas where strategies can be improved and informing the methodologies of future studies investigating molecular activity in diseased apical tissues.

Objectives

The primary aim of this study was to systematically review the literature to identify qualitative evidence to answer the following question: 'what techniques have been used to sample and analyse inflammatory mediator activity from the PTF of permanent

teeth diagnosed with apical disease during root canal treatment?' Secondly, this review aims to identify 'what types of periradicular inflammatory mediators had been studied using these methodologies'.

Methodology

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Preregistration

A protocol was preregistered with the International Prospective Register of Systematic Reviews (PROSPERO) on 17th of July 2018 (record number: CRD42018100351).

Eligibility criteria

A comprehensive search was carried out on all *in vivo* studies investigating the expression of inflammatory mediators from within periradicular tissues in human permanent teeth diagnosed with symptomatic/acute or asymptomatic/chronic apical periodontitis, acute or chronic apical abscess or condensing osteitis with or without vital pulp tissue (Glickman 2009). Permanent teeth with normal apical tissues, undergoing elective root canal treatment, were also included if mediators were being sampled from within the periradicular tissues. Only studies utilizing orthograde sampling techniques to retrieve PTF through the root canal during root canal treatment were included whilst those utilizing retrograde surgical approaches were not. Cell culture, laboratory or animal studies and those concerned with investigating inflammatory mediator activity from within healthy or diseased pulp tissue only, and not periradicular tissues, were also excluded. To prevent errors when interpreting data, searches were limited to articles available in languages that could be translated by the research team (i.e. English or Chinese). Furthermore, all studies published prior to the commencement date of the searches (i.e. 1 June 2018), as well as grey literature, were included in this review.

Information sources

From the 1st of June 2018, six electronic databases were searched independently by SSV and KB. These included PubMed, EMBASE, Cochrane Library, Science Direct, Web of Science and OpenGrey. Supplemental search methods included reference list follow-up at the full-text evaluation stage, expert contact and hand searched the contents pages and abstracts of articles published in the 2016–2018 editions of the International Endodontic Journal and Journal of Endodontics.

Search strategy

An electronic search strategy was developed based on the primary research questions of this review which was constructed using the Population, Intervention, Comparison and Outcome (PICO) framework. The strategy comprised of key terms relevant to the research topic and included both British and American spellings with subject headings of 'apical periodontitis', 'sampling', 'periradicular tissue fluid' and 'cytokine expression'. These headings were expanded upon through synonyms, key phrases and indexed terms (i.e. MeSH) identified using the knowledge of the authors, existing literature and indexed databases. A search strategy was then developed using truncations and Boolean operators ('OR', 'AND') and adapted for each database. Once completed, it was modified based on recommendations from an independent clinical lecturer who peer-reviewed it against PRESS guidelines for quality assurance purposes (Sampson *et al.* 2009). An example of the PubMed search can be found in Table 1.

Study selection

After duplicates were removed, screening of titles/abstracts and full-text evaluation was performed independently by SSV and KB using the above eligibility criteria. Any disagreements were resolved through discussion with a third reviewer (PLT). Once selected for inclusion, the same reviewers used a standardized prepiloted form to extract data items for evidence synthesis and quality assessment.

Data items

Predetermined information was extracted from published studies and organized as follows: 1. Study objectives, 2. Sample characteristics, 3. Mediators studied, 4. PTF Sampling method, 5. Laboratory assay technique and 6. Results. For each sampling method, information around the parameters used to retrieve

Table 1 PubMed search strategy

Search	Input Query	Items
#1	((((((((((((((“apical periodontitis”[Title/Abstract] OR “periapical periodontitis”[MeSH Terms]) OR “acute apical periodontitis”[Title/Abstract]) OR “chronic apical periodontitis”[Title/Abstract]) OR “apical abscess*”[Title/Abstract]) OR “acute apical abscess*”[Title/Abstract]) OR “chronic apical abscess*”[Title/Abstract]) OR “periradicular abscess*”[Title/Abstract]) OR “periapical granuloma”[MeSH Terms]) OR “periapical granuloma*”[Title/Abstract]) OR “apical granuloma*”[Title/Abstract]) OR “periapical lesion*”[Title/Abstract]) OR “apical lesion*”[Title/Abstract]) OR “endodontic lesion*”[Title/Abstract]) OR “endodontic infection*”[Title/Abstract]) OR “periradicular disease*”[Title/Abstract]) OR “infected root canal*”[Title/Abstract] AND “humans”[MeSH Terms]	
#2	(“sampling”[Title/Abstract] OR “sampling studies”[MeSH Terms]) OR “sampling studies/methods”[MeSH Terms] AND “humans”[MeSH Terms]	
#3	((((((periradicular[All Fields] AND tissue fluid[Title/Abstract]) OR (periapical[All Fields] AND (tissue exudate[Title/Abstract] OR tissue exudates[Title/Abstract])))) OR “periapical exudate*”[Title/Abstract]) OR “periapical exudate samples”[Title/Abstract]) OR “inflammatory exudate”[Title/Abstract]) OR “exudate”[Title/Abstract]) OR “exudates and transudates”[MeSH Terms] AND “humans”[MeSH Terms]	
#4	((((((((((((((((((((((((“cytokines”[MeSH Terms] OR “cytokine expression”[Title/Abstract]) OR “cytokine profile”[Title/Abstract]) OR “cytokine analysis”[Title/Abstract]) OR “chemokine”[Title/Abstract]) OR “inflammation mediators”[MeSH Terms]) OR “interleukin 1”[MeSH Terms]) OR “interleukin*”[Title/Abstract]) OR “il 1 alpha”[Title/Abstract]) OR “il 1 beta”[Title/Abstract]) OR “il 2”[Title/Abstract]) OR “il 4”[Title/Abstract]) OR “il 6”[Title/Abstract]) OR “il 8”[Title/Abstract]) OR “il 10”[Title/Abstract]) OR “il 12”[Title/Abstract]) OR “il 13”[Title/Abstract]) OR “il 17”[Title/Abstract]) OR “il 23”[Title/Abstract]) OR “tumour necrosis factor”[Title/Abstract]) OR “tnf alpha”[Title/Abstract]) OR “interferon gamma”[MeSH Terms]) OR “ifn gamma”[Title/Abstract]) OR “prostaglandins”[MeSH Terms]) OR “pge2”[Title/Abstract]) OR “leukotriene b4”[MeSH Terms]) OR “ltb4”[Title/Abstract] AND “humans”[MeSH Terms]	
#5	(#1) AND #2	
#6	(#1) AND #3	
#7	(#1) AND #4	
#8	(#2) AND #3	
#9	(#2) AND #4	
#10	(#3) AND #4	
#11	((#1) AND #2) AND #3	
#12	((#1) AND #2) AND #4	
#13	((#1) AND #3) AND #4	
#14	((#2) AND #3) AND #4	
#15	(((#1) AND #2) AND #3) AND #4	

* = Truncation term.

PTF (i.e. brand of device, size, insertion depth, sampling duration, how PTF volume was measured and how a dry, bleeding or suppurative canal was managed) and the sampling regime (i.e. samples per tooth and when baseline and subsequent samples were taken) was recorded. Additionally, information around the parameters used to prepare samples for laboratory analysis was collected with studies grouped according to whether proteomic/metabolomic or transcriptomic level analysis was performed. When data were unattainable, it was coded as being not reported.

Data synthesis and outcome measures

A qualitative synthesis was conducted on all studies that met the inclusion criteria. Briefly, key characteristics of each study were initially summarized and presented in text and table format. These data were then explored to determine the number and frequency of the different types of 1. PTF sampling techniques, 2. laboratory assay techniques and 3. targeted inflammatory mediators. These categories acted as the outcome measures in this review and were descriptive in nature.

Risk of bias assessment

Bias was assessed independently by SSV and KB using the risk of bias tool proposed by Viswanathan *et al.* (2012), which accounted for different designs of clinical studies. Briefly, the design of each study was initially determined, and then, design-specific criteria for randomized and non-randomized controlled trials, cohort studies and cross-sectional studies were

applied respectively to assess for bias in 5 domains. These included selection, performance, attrition, detection and reporting bias (Appendix S1). For each criterion, the risk of bias was deemed as being 'low' when details were mentioned with no ambiguity, 'high' if no evidence was presented or 'unclear' if insufficient information was provided. If there were several criterions for an individual bias domain (i.e. selection, performance and detection bias), the total risk for that individual domain was considered high if 2 or more criterion were scored as being 'high' or 'unclear'. Upon completion, the overall risk of bias for each study was then considered as being low, medium or high if ≤ 2, 3–4 or ≥ 5 bias domains respectively were deemed as having 'high' or 'unclear' risks of bias. Disputes were resolved through discussion with a third author (PLT), and outcomes were presented textually and graphically. Studies were not excluded based on the bias assessment as it would have no impact on the descriptive outcome measures of this review; however, this information was used to highlight areas of improvement in studies investigating periradicular inflammatory mediator activity.

Inter-rater reliability

Cohen's Kappa statistical analysis was performed to evaluate the extent of inter-rater reliability in the process of extracting data from studies that met the inclusion criteria. The SPSS (V25) software was used to conduct this analysis, and a score of 0.76 was achieved, which demonstrates 'excellent' inter-rater agreement according to Cicchetti (1994).

Results

Study selection

In total, 251 citations were identified from the initial database search. Eighty-four publications were eliminated due to duplications, and the remaining 167 were reviewed against the inclusion criteria. Following title and abstract screening, 60 citations were eligible for full-text evaluation of which 33 qualified for inclusion in the qualitative synthesis, all of which were published between 1991 and 2016 (Figure 1). Reasons for rejecting the 27 studies at the full-text evaluation stage are provided in Appendix S2.

Study characteristics and qualitative synthesis

Study objectives

Of the 33 evaluated studies, 11 sampled PTF from teeth with apically infected lesions with the sole aim to determine the concentration of specific mediators. Four studies compared the analyte concentrations of diseased and normal apical tissues in medically fit patients and 2 studies compared the PTF cytokine profiles in patients with co-morbidities such as HIV and sickle cell anaemia (SCA) to that of healthy controls. Ten studies explored correlations between the concentration of specific inflammatory mediators and clinical/radiographic signs of apical disease, 8 studies monitored changes in cytokine levels at different stages of root canal treatment and six evaluated the impact of clinical interventions on periradicular inflammatory mediator activity. Twenty-seven studies were observational in their design, and six were interventional clinical trials, of which four were randomized. The objectives, design and results for individual studies can be found in Table 2.

Teeth sampled and disease state studied

Sample size ranged from 16 to 77 mature permanent teeth. Ten studies sampled from single-rooted teeth, 12 studies sampled from single/multirooted teeth, and 11 studies did not disclose this information. Chronic apical periodontitis was the most frequently studied diagnosis ($n = 14$) followed by acute apical periodontitis ($n = 6$), acute apical abscess ($n = 1$) and normal apical tissues ($n = 1$). Four studies collected PTF from teeth with both chronic and acute apical periodontitis, and 9 did not declare a specific diagnosis (Table 2).

PTF sampling methods

It was determined that a wide range of sampling techniques were used, namely:

1. Paper points ($n = 27$): Four brands were used including Kerr (Kerr Manufacturing Co., Romulus, MI, USA [$n = 10$]), Dentsply Maillefer (Dentsply Maillefer Co., Ballaigues, Switzerland [$n = 3$]), Ariadent (Ariadent Co., Tehran, Iran [$n = 1$]) and Orbis Dental (Orbis Dental Co., Münster, Germany [$n = 1$]). The size used included size 40 ($n = 11$), 30 ($n = 3$) and 15 ($n = 2$), and these were inserted into the canal to working length ($n = 16$) or 2 mm past the apex ($n = 11$). Sampling time lasted for 30

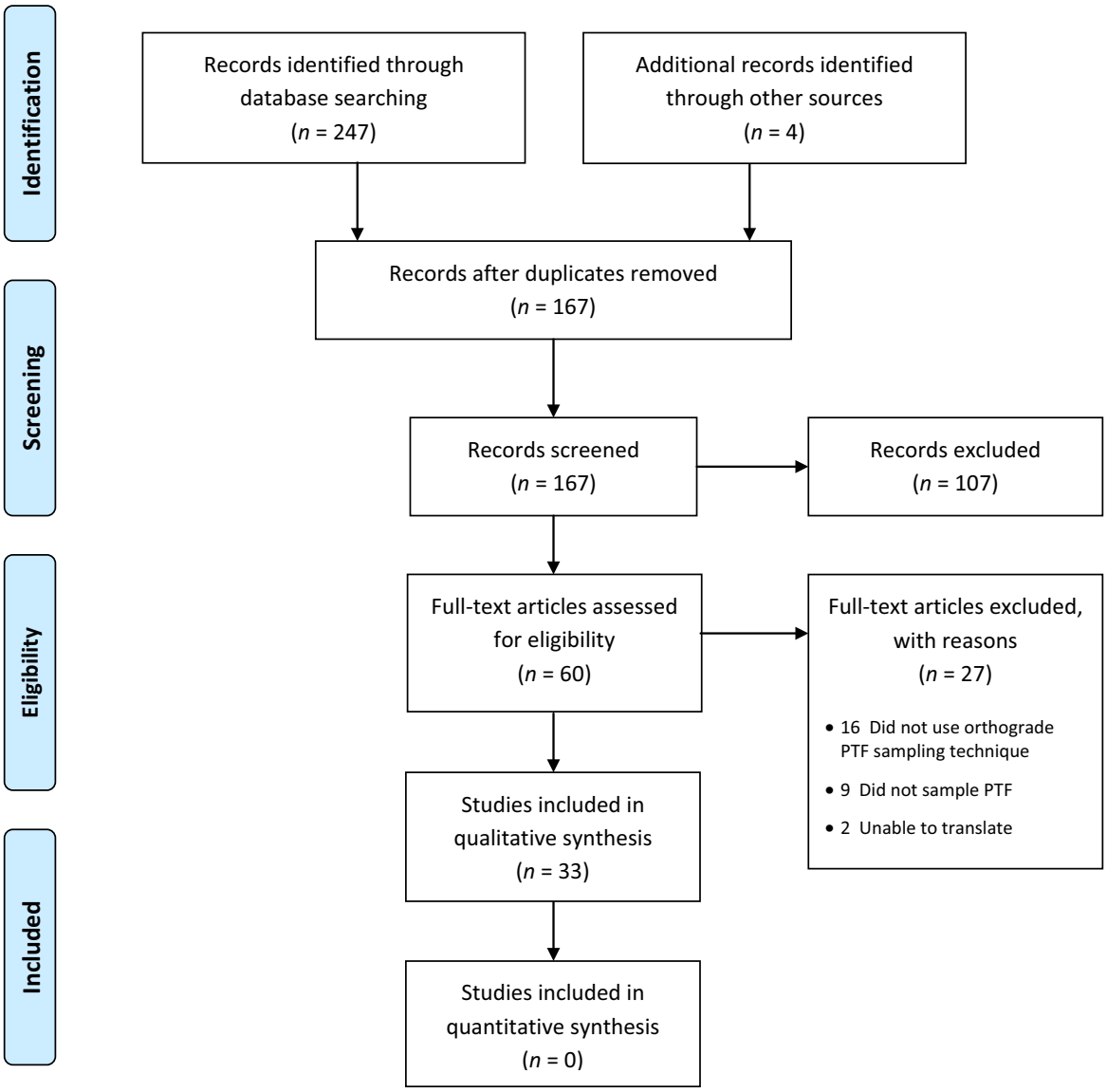

Figure 1 PRISMA flow diagram depicting the flow of information through the phases of the systematic review.

(n = 14), 60 (n = 12) or 120 (n = 1) seconds. Four studies used 1 point per sample (Wahlgren *et al.* 2002, Liu *et al.* 2003, Pezelj-Ribarić *et al.* 2007, Shahriari *et al.* 2011) whereas five used 2 (Ataoğlu *et al.* 2002, Alptekin *et al.* 2005a,b, Ehsani *et al.* 2012, Grga *et al.* 2013), eleven used 3 (Henriques *et al.* 2011, de Brito *et al.* 2012, 2015, Tavares *et al.* 2012, 2013, Rechenberg *et al.* 2014, Bambirra *et al.* 2015, Ferreira *et al.* 2015, Martinho *et al.* 2015, 2016, Sette-Dias *et al.* 2016), four used ≤ 5 (Takayama *et al.* 1996, Shimauchi *et al.* 1997, 1998, 2001) and two continued sampling until canals were dry (Safavi & Rossomando 1991, Shimauchi *et al.* 1996). In thirteen studies, modifications were made to paper points either before or after sampling which included precoating with an eluting buffer (Safavi & Rossomando 1991) or cutting the tip from the wet portion (Liu *et al.* 2003, Zhi *et al.* 2017) or a fixed 3 – 4 mm (Shahriari *et al.* 2011, de Brito *et al.* 2012, 2015, Tavares *et al.* 2012, 2013, Bambirra *et al.* 2015, Ferreira *et al.* 2015, Martinho *et al.* 2015, 2016, Sette-Dias *et al.* 2016).

Table 2 Characteristics of all studies that met the inclusion criteria

Study			Sample			Methodology		
Author	Objectives	Design	Diagnosis/ size (n)	Tooth type	Mediators studied	PTF sampling	Assay technique	Results
Safavi & Rossomando (1991)	Compare PTF TNF-α levels in teeth with and without apical periodontitis	Cross-sectional	CAP (6) NAT (5)	Single/ multiroot	TNF-α	Paper points	ELISA	Elevated TNF-α levels in teeth with apical lesions compared to those without.
Matsuo *et al.* (1994)	Explore correlations between PTF IL-1α and IL-1β levels and clinical findings in teeth with apical lesions	Cohort	'Apical lesion' (69)	Single-root	IL-1α, IL-1β	Fine needle aspiration	ELISA	Pus containing PTF had higher IL-1α ($P < 0.01$) and larger lesions showed higher IL-1β than IL-1α ($P < 0.05$). After treatment, IL-1α levels increased whilst IL-1β decreased.
Matsuo *et al.* (1995)	Explore correlations between PTF IgG and IgA levels and clinical findings in teeth with apical lesions	Cohort	'Apical lesion' (69)	Single-root	IgG, IgA	Fine needle aspiration	ELISA	IgG levels higher than IgA ($P < 0.01$). Larger lesions showed higher IgG and IgA levels ($P < 0.01$). Throughout treatment, IgG and IgA decreased.
Shimauchi *et al.* (1996)	Develop a quantitative method for collecting and quantifying biomarkers in PTF	Cross-sectional	CAP/AAP (29)	-	IL-1β	Paper points	ELISA	There is a curvilinear relationship between absorbed PTF and paper point wetted length ($P < 0.0001$). There is a linear relationship between absorbed and eluted IL-1β ($P < 0.05$).
Takayama *et al.* (1996)	Explore correlations between PTF PGE_2 levels and clinical findings of teeth with apical periodontitis	Cross-sectional	'Apical lesion' (77)	-	PGE_2	Paper points	RIA	Higher PGE_2 levels in teeth with radiolucent areas ($P < 0.05$) and acute clinical symptoms ($P < 0.05$). As lesion size increased, PGE_2 levels decreased ($P < 0.05$).
Takeichi *et al.* (1996)	Quantify PTF levels of PMN derived inflammatory cytokines in teeth with apical periodontitis	Cross-sectional	CAP (16)	-	IL-1α, IL-1β, IL-6, TNF-α	Fine needle aspiration	ELISA, qPCR	Purified PMNs from PTF expressed high levels of IL-1α, IL-1β and TNF-α mRNA ($P < 0.05$). Although IL-6 mRNA was not detected, high IL-6 levels of protein were present.
Shimauchi *et al.* (1997)	Monitor longitudinal changes in PTF PGE_2 levels in teeth with apical lesions during RCT	Cohort	'Apical lesion' (20)	-	PGE_2	Paper points	RIA	PGE_2 levels decreased after treatment ($P < 0.01$). Remission of disease associated with reduction in PGE_2 levels ($P < 0.05$).
Shimauchi *et al.* (1998)	Quantify PTF IL-1β and IL-1ra levels in teeth with apical lesions	Cross-sectional	'Apical lesion' (29)	-	IL-1β, IL-1ra	Paper points	ELISA	High levels of IL-1ra, compared with IL-1β, and positive correlations between IL-1ra and IL-1β levels found ($P < 0.05$). IL-1ra:IL-1β ratio from symptomatic lesions lower than asymptomatic lesions ($P < 0.05$).

Table 2 Continued

Study			Sample			Methodology		
Author	Objectives	Design	Diagnosis/ size (n)	Tooth type	Mediators studied	PTF sampling	Assay technique	Results
Takeichi *et al.* (1998)	Quantify PTF NO levels in teeth with apical periodontitis	Cross-sectional	CAP/AAP (30)	-	NO	Fine needle aspiration	Colorimetric	PMNs can spontaneously produce NO at the site of chronic infection
Kuo *et al.* (1998a)	Quantify PTF βG, IL-1β, IgG, IgA and IgM levels in teeth with apical lesions and explore correlations with clinical findings	Cohort	'Apical lesion' (32)	Single/ multiroot	βG, IL-1β, IgG, IgA, IgM	Methylcellulose filter paper strips	ELISA	Higher βG and IL-1β levels in pus containing PTF ($P < 0.05$) and larger lesions ($P < 0.05$). Higher IgM in lesions with a sinus tract or swelling (IgM).
Kuo *et al.* (1998b)	Monitor longitudinal changes in PTF βG, IL-1β, IgG, IgA and IgM levels when accessing the root canal of teeth with apical lesions	Cohort	'Apical lesion' (32)	Single/ multiroot	βG, IL-1β, IgG, IgA, IgM	Methylcellulose filter paper strips	ELISA	Mediator activity in less involved teeth did not change after treatment; however, βG and IL-1β levels in teeth with pus containing PTF decreased after treatment whereas IgA and IgM increased ($P < 0.05$).
Shimauchi *et al.* (2001)	Quantify PTF IL-8 and NO levels in teeth with apical lesions	Cross-sectional	'Apical lesion' (27)	-	IL-8, NO	Paper points	ELISA, colorimetric	IL-8 levels higher in teeth with pus containing PTF ($P < 0.01$) and clinical symptoms ($P < 0.05$). A positive correlation was found between IL-8 and NO ($P < 0.001$).
Ataoğlu *et al.* (2002)	Quantify PTF IL-1β and TNF-α levels in teeth with apical lesions and explore correlation with clinical findings	Cross-sectional	'Apical lesion' (35)	Single-root	IL-1β, TNF-α	Paper points	ELISA	IL-1β levels 12-fold higher than TNF-α however no significant correlation found between these mediators ($P > 0.05$). High IL-1β levels associated with large lesions ($P < 0.05$).
Wahlgren *et al.* (2002)	Quantify PTF and pulp MMPP-8 levels in teeth with necrotic pulps and apical lesions and monitor longitudinal changes during RCT	Cohort	CAP/AAP (11) NAT (10)	Single-root	MMP-8	Paper points	IFMA	MMP-8 levels decreased after treatment ($P < 0.05$)
Liu *et al.* (2003)	Monitor longitudinal changes in PTF PGE_2 levels in teeth with AAP during RCT	Cohort	AAP (25)	Single/ multiroot	PGE_2	Paper points	RIA	PGE_2 levels decreased after treatment ($P < 0.0001$)
Alptekin *et al.* (2005a)	Explore correlations between PTF NE and PGE_2 levels and clinical findings in teeth with AAP and monitor longitudinal changes during RCT	Cohort	AAP (31)	Single-root	PGE_2, NE	Paper points	ELISA	PGE2 and NE levels higher in teeth with clinical symptoms ($P < 0.05$) however their levels did not change after treatment ($P > 0.05$)

Table 2 Continued

Study			Sample			Methodology		
Author	Objectives	Design	Diagnosis/ size (n)	Tooth type	Mediators studied	PTF sampling	Assay technique	Results
Alptekin *et al.* (2005b)	Explore correlations between PTF NE levels and clinical findings in teeth with AAP	Cross-sectional	AAP (31)	Single-root	NE	Paper points	ELISA	Higher NE levels in teeth with clinical symptoms ($P < 0.05$)
Pezelj-Ribarić *et al.* (2007)	Quantify PTF TNF-α levels in teeth with apical periodontitis and explore correlations with clinical findings	Cross-sectional	AAP (20) CAP (40)	Single-root	TNF-α	Paper points	ELISA	Higher TNF-α levels in teeth with larger lesions ($P < 0.05$)
Henriques *et al.* (2011)	Compare PTF inflammatory cytokine levels in teeth with and without apical lesions refractory to endodontic treatment	Cross-sectional	CAP (20) NAT (20)	-	IL-1β, IL-17A, IFN-γ, IL-10, TNF-α, MCP-1	Paper points	qPCR	Higher IFN-γ, TNF-α, IL-17A and MCP-1 mRNA expression in secondary lesions ($P < 0.05$). No difference in IL-1β mRNA expression ($P > 0.05$) between groups and IL-10 was insignificant in both groups ($P > 0.05$).
Shahriari *et al.* (2011)	Evaluate impact of Ibuprofen on PTF IL-1β, TNF-α and PGE_2 levels in teeth with apical lesions undergoing RCT	Randomized controlled trial	CAP/AAP (30)	-	IL-1β, TNF-α, PGE_2	Paper points	ELISA	PGE_2 levels reduced after treatment in Ibuprofen group only ($P < 0.05$). No significant difference in IL-1β and TNF-α reduction between groups before and after treatment ($P > 0.05$).
de Brito *et al.* (2012)	Quantify PTF CD4+CD28+ and CD8+ derived inflammatory cytokine mRNA expression in teeth with CAP and monitor longitudinal changes during RCT	Cohort	CAP (20)	Single/ multiroot	IL-1β, IL-10, IL-17A, TNF-α, IFN-γ, MCP-1, MIP-1β, RANTES, RANKL, CXCR4, CCR5	Paper points	qPCR	IFN-γ, IL-1β, RANKL and RANTES mRNA expression reduced ($P < 0.05$) and increase in IL-10 and CxCR4 mRNA expression after treatment ($P < 0.05$). No difference in TNF-α, IL-17A, MCP-1, MIP-1β and CCR5 levels after treatment.
Ehsani *et al.* (2012)	Evaluate impact of Ibuprofen and N-acetylcysteine on PTF TNF- α, IL-6 and IL-17A levels in teeth with CAP and explore correlations to clinical findings	Randomized controlled trial	CAP (80)	Single/ multiroot	TNF-α, IL-6, IL-17	Paper points	ELISA	IL-6 levels reduced in Ibuprofen group ($P < 0.05$) and IL-17A levels reduced in ibuprofen/NAC group ($P < 0.05$). No difference in detected in TNF-α
Tavares *et al.* (2012)	Evaluate impact of calcium hydroxide dressing on PTF inflammatory cytokine mRNA expression in teeth with CAP	Non-randomized controlled trial	CAP (20)	Single/ multiroot	IL-1β, IL-10 IL-17A, TNF-α, IFN-γ, MCP-1	Paper points	qPCR	IL-1β, IL-10, IFN-γ mRNA expression in teeth receiving a calcium hydroxide dressing lower than in those that did not ($P < 0.05$).

Table 2 Continued

Study			Sample			Methodology		
Author	Objectives	Design	Diagnosis/ size (n)	Tooth type	Mediators studied	PTF sampling	Assay technique	Results
Grga *et al.* (2013)	Monitor longitudinal changes in PTF PGE2 levels during RCT in vital teeth with or without a large restorations	Cohort	NAT (47)	Single-root	PGE_2	Paper points	RIA	PGE_2 levels in teeth with large restorations higher after treatment than in intact teeth ($P < 0.05$)
Tavares *et al.* (2013)	Evaluate impact of chlorhexidine dressing on PTF inflammatory cytokine mRNA expression in teeth with CAP	Non-randomized controlled trial	CAP (20)	Single/ multiroot	IL-1β, IL-17A, IL-10, TNF-α, IFN-ɣ, MCP-1	Paper points	qPCR	IL-1β, IL-10, MCP-1 and IFN-ɣ mRNA expression increased after treatment in teeth with no dressing than in those which received a chlorhexidine dressing ($P < 0.05$).
Rechenberg *et al.* (2014)	Compare PTF RANKL, OPG and IL-8 levels in teeth with irreversible pulpitis to those with AAP	Cross-sectional	AAP (27) NAT (21)	Single/ multiroot	IL-8, RANKL, OPG	Paper points	ELISA	Higher RANKL in irreversible pulpitis than apical periodontitis ($P < 0.05$) and lower IL-8 levels in irreversible pulpits than apical periodontitis ($P < 0.05$)
Bambirra *et al.* (2015)	Monitor longitudinal changes in PTF inflammatory mediator mRNA expression in teeth with CAP during RCT	Cohort	CAP (20)	Single/ multiroot	IL-1β, IL-8, IL-10, IL-17A, TNF-α, IFN-ɣ, MCP-1, MIP-1β, ITGA2, HSP47, OPN, FAK	Paper points	qPCR	Reduction in mRNA expression of all inflammatory mediators after treatment ($P < 0.05$)
de Brito *et al.* (2015)	Compare PTF CD4+CD28+ and CD8+ derived cytokine mRNA expression in patients with and without HIV	Cohort	CAP (53)	Single/ multiroot	IL-1β, IL-10, IL-17A, TNF-α, IFN-ɣ, MCP-1, MIP-1β, RANKL, RANTES, CXCR4, CCR5	Paper points	qPCR	Increased IL-10 and CXCR4 mRNA expression and reduced RANKL, IFN-ɣ, IL-1β and RANTES after treatment in HIV-negative patients ($P < 0.05$). Increased RANKL, IFN-ɣ, IL-1β, TNF-α, IL-17A, RANTES and CXCR4 mRNA expression after treatment in HIV-positive patients ($P < 0.05$).
Ferreira *et al.* (2015)	Compare PTF inflammatory cytokine mRNA expression in patients with and without SCA	Cross-sectional	CAP (36)	-	IL-1β, IL-10, IL-17A, TNF-α, IFN-ɣ, MCP-1, MIP-1β, RANKL	Paper points	qPCR	No significant difference observed in inflammatory mRNA expression in SCA positive and SCA-negative patients after treatment ($P > 0.05$)
Martinho *et al.* (2015)	Evaluate impact of different interappointment dressing materials on PTF levels of Th1-type and Th2-type cytokines in teeth with CAP	Randomized controlled trial	CAP (30)	Single-root	TNF-α, IFN-ɣ, IL-2, IL-4, IL-5, IL-13	Paper points	ELISA	Lower IL-2, TNF-α, IFN-ɣ levels and higher IL-4, IL-5 and IL-13 levels after use of dressing ($P < 0.05$). No difference observed between types of dressings ($P > 0.05$).

Table 2 Continued

Study			Sample			Methodology		
Author	Objectives	Design	Diagnosis/ size (n)	Tooth type	Mediators studied	PTF sampling	Assay technique	Results
Martinho *et al.* (2016)	Quantify PTF levels of MMPs, TIMPs and MMP/TIMP complexes in teeth with CAP and explore their correlation to clinical findings	Cross-sectional	CAP (20)	Single-root	MMP1, MMP2, MMP9, TIMP1, TIMP2, MMP1,2,9/ TIMP1,2	Paper points	ELISA	Higher MMP1, -2 and -9 in teeth with larger lesions ($P < 0.05$). Higher MMP-1 levels decreased chances of TTP, whereas MMP-9 increased chances of TTP ($P < 0.05$).
Sette-Dias *et al.* (2016)	Compare PTF levels of inflammatory cytokines in teeth with AAA to those with NAT	Cross-sectional	AAA (12) NAT (12)	Single/ multiroot	IL-1β, IL-8, IL-10, IL-17A, TNF-α, IFN-γ, MCP-1, MIP-1β, TGF-β	Paper points	qPCR	Higher IFN-γ, IL-1β, TNF-α, IL-17A, IL-8, MCP-1 mRNA expression in odontogenic infections ($P < 0.05$)
Zhi *et al.* (2017)	Evaluate impact of minocycline interappointment dressing on PTF levels of IL-7A in teeth with AAP	Randomized controlled trial	AAP (16) NAT (16)	-	IL-17A	Paper points	CBA	Lower IL-17A levels in calcium hydroxide and minocycline groups after treatment ($P < 0.05$)

AAP, acute apical periodontitis; CAP, chronic apical periodontitis; CBA, cytometric bead array; CCR5, chemokine receptor type 5; CXCR4, chemokine receptor type 4; ELISA, enzyme-linked immunosorbent assay; FAK, focal adhesion kinase; HSP, heat-shock protein; IFMA, immunofluorometric assay; IFN, interferon; Ig, immunoglobulin; IL, interleukin; Il-1ra, interleukin-1 receptor agonist; ITGA2, alpha-2-integrin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein ; MMP, matrix metalloproteinase; NAT, normal apical tissues; NE, neutrophil elastase; NO, nitrous oxide; OPG, osteoprotegerin; OPN, osteopontin; PGE_2, prostaglandin-E2; PMN, polymorphonucleocyte; PTF, periradicular tissue fluid; qPCR, quantitative polymerase chain reaction; RANKL, receptor activator of nuclear factor kappa-B ligand; RANTES, regulated on activation normal T cell expressed and secreted; RCT, root canal treatment; RIA, radioimmunoassay; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumour necrosis factor; βG, β-glucuronidase and -, not reported.)

If PTF volume was measured, only a wetted length (mm): volume (μL) calibration curve was used to determine this. Twelve studies did not disclose details on brand of paper point, eleven studies did not disclose details on size, one study did not indicate the sampling duration and number of points per sample and fourteen studies did not report on how the PTF volume was measured (Table 3).

2. Fine needle aspiration (n = 4): Two studies used a Neo Dental syringe (Neo Dental Chemical Products Co. Ltd, Tokyo, Japan), one used a Hamilton microsyringe (Hamilton Co., NE, USA) and one study used Drummond Scientific Microdispenser replacement tubes (Drummond Scientific Co., Broomall, PA, USA). Information regarding needle gauge, length, insertion depth, sampling duration or PTF volume measurements was not disclosed (Table 3).
3. Methylcellulose filter paper strips (n = 2): Two strips of the Interstate brand (Interstate Drug Exchange, Amityville, NY, USA) were used per sample for a 'few seconds' and a Periotron (Harco Electronics, Tustin, CA, USA) subsequently measured PTF volume. No further information was disclosed (Table 3).

PTF sampling regime

Throughout treatment, 16 studies sampled PTF only once from each tooth, twelve sampled twice, one sampled three times, two studies sampled six times and another two studies repeated sampling '7–14' times. Baseline samples were taken either before (n = 5) or after (n = 25) root canal instrumentation. Of those sampling longitudinally (n = 17), the timing of subsequent samples ranged from 3-min to 15-day intervals after baseline. Three studies did not disclose details on when the baseline sample was taken (Table 3).

Managing dry, bleeding and suppurative canals

Where the sampling protocol resulted in no retrieval of PTF (i.e. dry canal), one study (Safavi & Rossomando 1991) excluded samples whereas four others stated they used patency filing to draw PTF into the canal and then proceed with re-sampling (Ataoğlu *et al.* 2002, Alptekin *et al.* 2005a,b, Ehsani *et al.* 2012). Seven studies excluded samples when 'more than a small amount of blood' was retrieved (Kuo *et al.* 1998a,b, Ataoğlu *et al.* 2002, Alptekin *et al.* 2005a,b, Ehsani *et al.* 2012, Rechenberg *et al.* 2014). Samples from discharging canals were included following drainage in five studies (Matsuo *et al.* 1994, 1995, Ataoğlu *et al.* 2002, Alptekin *et al.* 2005a,b) but excluded in two other studies (Ehsani *et al.* 2012, Rechenberg *et al.* 2014). Twenty-eight, 26 and a further 26 studies did not disclose information on how they managed dry, bleeding or suppurative canals, respectively.

Laboratory assay techniques

1. Proteomic/Metabolomic level (n = 25): Enzyme-linked immunosorbent assay [ELISA] (n = 18), radioimmunoassay [RIA] (n = 4), colorimetric assay [CA] (n = 2), immunofluorometric assay [IMFA] (n = 1) and cytometric bead array [CBA] (n = 1) were all techniques used to quantify the inflammatory mediators found within PTF, at the proteomic/metabolomic level (Table 3). In preparing samples, 50–300 μL of the elution buffers of phosphate-buffered saline [PBS] (n = 11), PBS bovine serum albumin [PBS-BSA] (n = 2), PBS Tween-20 (n = 4), PBS Tween-20 + foetal calf serum (n = 1) and trisaminomethane–hydrochloric acid [Tris–HCl] (n = 1) were used. Two studies incubated samples for 60–180 min, five vortexed samples for 60 s, five centrifuged samples for 10–30 min at 4–15 000 ***g***, seven vortexed samples for 30 s and then centrifuged for 10 min at 5000 ***g*** and one incubated samples for 300 min, vortexed for 30 s and then centrifuged for 10 min at an unspecified gravity force. Further details on how samples were processed, including unspecified information, can be found in Table 3.
2. Transcriptomic level (n = 9): Quantitative polymerase chain reaction (qPCR) was the only technique used to quantify the inflammatory mediators found within PTF at the transcriptomic level. All the samples were prepared in a consistent manner using TRIzol reagent for RNA isolation. Briefly, samples underwent phase separation centrifugation for 15 min at 12 000 ***g***, precipitation centrifugation for 10 min at 12 000 ***g*** and then incubation for 10 min at 55°C.

Inflammatory mediators analysed

Forty-five different mediators were reported as being studied with IL-1β being the most frequent analyte (n = 17). Interleukin-1α, IL-1β, IL-6, IL-8, IL-17A, TNF-α, IFN-γ and receptor activator of nuclear factor kappa-B ligand [RANKL] were studied independently at a proteomic/metabolomic and transcriptomic level. Interleukin-1 receptor antagonist [IL-1ra], IL-2, IL-4,

Table 3 Basic parameters of the sampling methods used to retrieve periradicular tissue fluid via the root canal.

		Parameters used to sample PTF via root canals				Timing of PTF samples			Parameters used to elute and prepare PTF samples				
Technique	Study	Brand	Size (ISO)	Insertion depth	Time (s)	Samples/ Tooth	Baseline	Subsequent	Assay tech.	Elution buffer	Incubated (mins)	Vortexed (secs)	Centrifuged (mins)
Paper Point	Safavi & Rossomando (1991)	–	–	WL	–	1	Before Instrumenting	–	ELISA	PBS + Tween–20 and FCS (100 μL)	60	–	–
	Shimauchi *et al.* (1996)	Kerr	40	WL	30	1	Before Instrumenting	–	ELISA	PBS + Tween–20 (–μL)	–	–	10 at 10 000 ***g***
	Takayama *et al.* (1996)	Kerr	40	WL	30	1	After Instrumenting	–	RAI	PBS (150 μL)	–	30	10 at 5000 ***g***
	Shimauchi *et al.* (1997)	Kerr	40	WL	30	2	After Instrumenting	7–10 days	RAI	PBS (150 μL)	–	30	10 at 5000 ***g***
	Shimauchi *et al.* (1998)	Kerr	40	WL	30	1	After Instrumenting	–	ELISA	PBS (150 μL)	–	30	10 at 5000 ***g***
	Shimauchi *et al.* (2001)	Kerr	40	WL	30	1	After Instrumenting	–	CA	PBS (150 μL)	–	30	10 at 5000 ***g***
	Ataoğlu *et al.* (2002)	Kerr	40	WL	30	1	After Instrumenting	–	ELISA	PBS (250 μL)	–	60	–
	Wahlgren *et al.* (2002)	–	–	WL	120	3	After Instrumenting	14 days	IFMA	Tris + HCl (50 μL)	180	–	–
	Liu *et al.* (2003)	Dentsply–Mai.	30	WL	30	2	After Instrumenting	10–12 days	RIA	–	–	–	30 at 4000 ***g***
	Alptekin *et al.* (2005a)	Kerr	40	WL	30	2	After Instrumenting	7–10 days	ELISA	PBS – BSA (250 μL)	–	60	–
	Alptekin *et al.* (2005b)	Kerr	40	WL	30	1	After Instrumenting	–	ELISA	PBS – BSA (250 μL)	–	60	–
	Pezelj–Ribarić *et al.* (2007)	–	–	WL	60	1	After Instrumenting	–	ELISA	PBS (–μL)	–	–	–
	Henriques *et al.* (2011)	–	40	2 mm past WL	60	1	After Instrumenting	–	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	Shahriari *et al.* (2011)	Ariadent	30	WL	30	2	After Instrumenting	4 days	ELISA	PBS (250 μL)	–	60	–
	de Brito *et al.* (2012)	–	–	2 mm past WL	60	2	After Instrumenting	7 days	qPCR	TRIzol (–μl)	–	–	–
	Ehsani *et al.* (2012)	Kerr	40	WL	30	1	After Instrumenting	–	ELISA	PBS (300 μL)	–	60	–
	Tavares *et al.* (2012)	–	–	2 mm past WL	60	2	After Instrumenting	15 days	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	Grga *et al.* (2013)	Kerr	40	WL	30	2	After Instrumenting	3 days	RIA	PBS (150 μL)	-	30	10 at 5000 ***g***
	Tavares *et al.* (2013)	—	–	2 mm past WL	60	2	After Instrumenting	15 days	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	Rechenberg *et al.* (2014)	Orbis	–	2 mm past WL	30	1	After Instrumenting	–	ELISA	PBS (300 μL)	300	30	10 at – ***g***
	Bambirra *et al.* (2015)	–	–	2 mm past WL	60	2	After Instrumenting	7 days	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	de Brito *et al.* (2015)	–	–	2 mm past WL	60	2	After Instrumenting	7 days	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	Ferreira *et al.* (2015)	–	–	2 mm past WL	60	1	After Instrumenting	–	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	Martinho *et al.* (2015)	Dentsply–Mai.	15	2 mm past WL	60	2	After Instrumenting	14 days	ELISA	–	–	–	–
	Martinho *et al.* (2016)	Dentsply-Mai.	15	2 mm past WL	60	1	After Instrumenting	–	ELISA	–	–	–	–
	Sette-Dias *et al.* (2016)	–	–	2 mm past WL	60	2	After Instrumenting	14 days	qPCR	TRIzol (–μL)	10	–	25 at 12 000 ***g***
	Zhi *et al.* (2017)	–	30	WL	30	2	After Instrumenting	7 days	CBA	–	–	–	30 at 4000 ***g***
Filter Paper Strip	Kuo *et al.* (1998a)	Interstate	–	–	'few sec.'	7–14	Before Instrumenting	3 min	ELISA	PBS + Tween-20 (50 μL)	–	–	–
	Kuo *et al.* (1998b)	Interstate	–	–	'few sec.'	7–14	Before Instrumenting	3 min	ELISA	PBS + Tween-20 (50 μL)	–	–	–

Table 3 Continued

		Brand	Gauge	Insertion depth	Time (s)				Assay tech.	Elution buffer	Incubated (mins)	Vortexed (secs)	Centrifuged (mins)
Fine Needle Aspiration	Matsuo *et al.* (1994)	Neo Dental	–	–	–	6	–	14 days	ELISA	PBS + Tween-20 (–μL)	–	–	10 at 15 000 ***g***
	Matsuo *et al.* (1995)	Neo Dental	–	–	–	6	–	14 days	ELISA	PBS + Tween-20 (–μL)	–	–	10 at 15 000 ***g***
	Takeichi *et al.* (1996)	Hamilton	–	–	–	1	Before Instrumenting	–	ELISA	–	–	–	10 at 10 000 ***g***
	Takeichi *et al.* (1998)	Drummond Sci.	–	–	–	1	–	–	CA	–	–	–	–

–, not reported; WL, working length; BSA, bovine serum albumin; FCS, foetal calf serum; HCl, hydrochloric acid; PBS, phosphate-buffered solution; PTF, periradicular tissue fluid; Tris, trisaminomethane; g, gravity force; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; CA, colorimetric assay; IMFA, immunofluorometric assay; CBA, cytometric bead array; qPCR, quantitative polymerise chain reaction.

IL-5, IL-13, Immunoglobulin [Ig]-A, IgG, IgM, PGE_2, matrix metalloproteinases [MMP]-1, MMP-2, MMP-8, MMP-9, tissue inhibitor of metalloproteinase [TIMP]-1, TIMP-2, MMP-1, 2, 9/TIMP-1, 2 complexes, osteoprotegerin [OPG], neutrophil elastase [NE], nitrous oxide [NO] and β-glucuronidase [βG] were studied exclusively at a proteomic/metabolomic level. Interleukin-10, monocyte chemoattractant protein [MCP]-1, MIP-1β, regulated on activation normal T cell expressed and secreted [RANTES], chemokine receptor [CXCR]-4, CCR5, transforming growth factor [TGF]-β, osteopontin [OPN], alpha-2-integrin [ITGA2], heat-shock protein [HSP]-47 and focal adhesion kinase [FAK] were studied exclusively at a transcriptomic level. Only 1 study assayed the same analytes at both proteomic/metabolomic and transcriptomic levels (Takeichi *et al.* 1996). The types and frequency of targeted mediators analysed are presented in Table 2 and Figure 2.

Bias assessment

An overall risk of bias was deemed 'high' in 4 studies, 'medium' in 23 and 'low' in 6. A 'high' or 'unclear' risk of bias was found in 12 studies for the selection domain, 15 for the performance domain, 3 for the attrition domain, 24 for the detection domain and 32 for the reporting domain. Only 11 studies disclosed information on all the parameters associated with sampling and eluting periradicular inflammatory mediators (Shimauchi *et al.* 1996, 1997, 1998, 2001, Takayama *et al.* 1996, Ataoğlu *et al.* 2002, Alptekin *et al.* 2005a,b, Shahriari *et al.* 2011, Ehsani *et al.* 2012, Grga *et al.* 2013). However, only 5 of these studies reported unambiguous and precise information (Alptekin *et al.* 2005a,b, Shahriari *et al.* 2011, Ehsani *et al.* 2012, Grga *et al.* 2013). The results of the bias assessment for individual studies are presented in Appendix S3.

Discussion

Summary of findings

Thirty-three studies met the inclusion criteria and were generally deemed to have a 'medium' risk of bias due to lack of reporting and heterogeneous methodology (Appendix S3). However, the studies suggest paper points and proteomic/metabolomic analyses are the most common approaches used to sample and quantify analytes respectively from diseased apical tissues

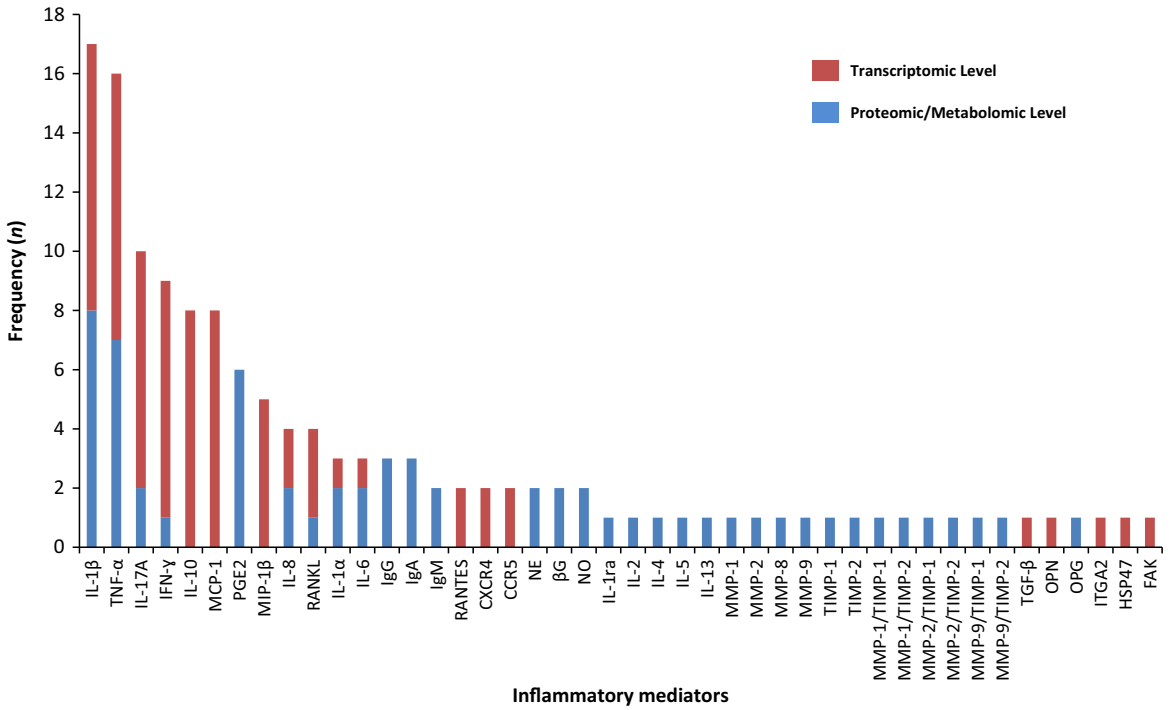

Figure 2 The types of inflammatory mediators targeted and the frequency at which they were studied at a proteomic/metabolomic and transcriptomic level (CCR5: chemokine receptor type 5, CXCR4: chemokine receptor type 4, FAK: focal adhesion kinase, HSP: heat-shock protein, IFN: interferon, Ig: immunoglobulin, IL: interleukin, IL-1ra: interleukin-1 receptor agonist, ITGA2: alpha-2-integrin, MCP: monocyte chemoattractant protein, MIP: macrophage inflammatory protein , MMP: matrix metalloproteinase, NE: neutrophil elastase, NO: nitrous oxide, OPG: osteoprotegerin, OPN: osteopontin, PGE_2: prostaglandin-E2, RANKL: receptor activator of nuclear factor kappa-B ligand, RANTES: regulated on activation normal T cell expressed and secreted, TGF: transforming growth factor, TIMP: tissue inhibitor of metalloproteinase, TNF: tumour necrosis factor, βG: β-glucuronidase and -: not reported).

during root canal treatment (Table 3). Furthermore, a broad range of inflammatory mediators have been subjected to analysis with IL-1β and TNF-α being the most studied (Figure 2). These findings are discussed in more detail below.

PTF sampling method

According to the outcomes from this review, Safavi & Rossomando (1991) were the first identified study to sample PTF and determine expression of inflammatory mediators when sampled through the root canal using paper points. Whilst several other methods such as fine needle aspiration and absorption with methylcellulose filter paper strips have since been explored, paper points remain the most commonly used approach. This could be because, unlike filter strips, their length, shape and taper readily conform to the shape of the root canal and therefore allow for more accurate and controlled sampling within the periradicular region. Clinical operators would also be familiar with their use. This was acknowledged by Kuo *et al.* (1998a), who highlighted the need for filter strips to be made longer as well as their limited absorbance capacity. Furthermore, paper points are also very efficient at absorbing small volumes of fluid, as evident by their application in other disciplines within (Hartroth *et al.* 1999) and outside dentistry (Lima *et al.* 2015). This property is particularly favourable for longitudinal sampling of PTF, as tissue fluid volume decreases over the course of root canal treatment due to healing (Matsuo *et al.* 1994). Conversely, syringes are not well adapted for retrieving such small volumes of fluid as was reported by Matsuo *et al.* (1995), who experienced challenges in attaining adequate amounts of PTF in the latter stages of treatment. Additionally, small amounts of fluid will also be lost in the lumen of the syringe and needle. Therefore,

it appears paper points are the most well-established approach for sampling PTF and subsequently analysing the concentration of single or multiple analytes during root canal treatment. Nevertheless, this method is not without its limitations as reliable periradicular sampling requires a patent root canal, which is not always predictably achievable due to calcifications, curvatures or procedural errors. Furthermore, the paper point could become contaminated with blood or pus, originating from the infected periradicular tissues, which may eventually interfere with the assay procedure.

PTF sampling protocol

The findings of this review confirm that within any given approach to sampling PTF (i.e. paper points, fine needle aspiration and filter strips), there is an absence of standardization within the protocol (Table 3). This variation in basic parameters (i.e. brand, duration of sampling, insertion depth and size of device) can explain the conflicting outcomes reported by some studies. For example, Liu *et al.* (2003) found PTF levels of PGE_2 significantly reduced in patients with acute apical periodontitis following root canal treatment; however, Alptekin *et al.* (2005a) found no difference. Both studied a population with similar characteristics and used paper points to retrieve periapical exudate; however, their sampling protocol varied in that different brands, sizes and number of points per sample were used (Table 3). This contrasts sampling procedures in other areas of dentistry (i.e. collection of periodontal pathogens from subgingival plaque) where these parameters have been investigated in depth and an optimized protocol developed (Hartroth *et al.* 1999). On the other hand, it is currently not known how these factors would influence PTF sampling.

Eluting protocol

This review highlights a significant variation in how samples are being prepared for proteomic/metabolomic analysis. Several elution methods including vortex, centrifugation and incubation have been used alongside numerous buffers to elute inflammatory mediators from paper points (Table 3). However, it is not known how these differing strategies would influence the percentage recovery of analytes. Such an *in vitro* investigation has been carried out in the field of ophthalmology where sponges were spiked with known concentrations of 25 different recombinant pro-inflammatory analytes, and then eluted using various buffers and techniques prior to being assayed (Inic-Kanada *et al.* 2012). Significant variation in the percentage recovery was noted between different eluting buffers and inflammatory mediators, which are not isolated findings (VanDerMeid *et al.* 2011). Conversely, Shimauchi *et al.*'s (1996) study was the only article identified in this review to carry out a similar experiment; however, only IL-1β was assayed and the influence of various eluting buffers and techniques on the recovery of other periradicular inflammatory analytes has not yet been investigated.

Laboratory assay techniques

A wide group of periradicular inflammatory mediators have been studied at either the proteomic/metabolomic level or the transcriptomic level (Figure 2). Assay techniques for the latter use transcribed mRNA sequences as biomarkers whereas the former target the actual secreted protein/metabolite (Vogel & Marcotte 2012). As mRNA is translated into its respective protein, it is assumed there should be a strong correlation between the two, and therefore, both can be used to quantify the presence of a specific mediator. However in human cells, a weak correlation between concentrations of protein and its respective mRNA abundances has been observed (Vogel *et al.* 2010), which could be attributed to various post-transcriptional or translational mechanisms (i.e. controls/checkpoints) (Maier *et al.* 2009). These findings are further supported by Takeichi *et al.* (1996), which provided the only study in this review to assay the same biomarkers at both the gene and protein level. They reported that although the mRNA for IL-6 was not detected, a significant amount of its respective protein was present in the sampled PTF. These data imply that evaluating protein/metabolite expression is likely to be more representative of actual periradicular inflammatory mediator activity than mRNA expression and potentially explains why it is the preferred approach amongst the studies in this review.

Target analytes

Interleukin-1β and TNF-α were the most frequently studied analytes according to this review (Figure 2). This may be for several reasons, first, their role in the pathophysiology of periradicular disease has been previously well reviewed (Nair 2004); secondly, they are considered the most relevant to human osteoclastic

activity (Stashenko *et al.* 1987); and thirdly, their presence in apical lesions has been repeatedly demonstrated with their concentrations being proportionate to the size of lesions (Safavi & Rossomando 1991, Matsuo *et al.* 1994). This currently makes them ideal biomarker for periradicular disease activity; however, growing research into the role of other analytes is likely to give rise to alternative targets.

Quality of included studies

The studies included in this review were generally of medium to low quality according to the aforementioned risk of bias tool (Appendix S3). The source of bias in interventional studies (i.e. RCTs and CCTs) originated from the lack of clarity on the randomization and concealment process and absence of any power calculations. No study reported on using a blinded assessor or analytical techniques (i.e. stratification or multivariate analysis) to control confounding factors, and only one study (Ehsani *et al.* 2012) referenced a preregistered protocol in their text. Furthermore, this review confirms a lack of reporting and high levels of heterogeneity in the sampling and eluting protocols, which would make it difficult to pursue any quantitative synthesis of data. This lack of standardization could be attributed to the absence of any existing evidence-based guidelines on how to apply these techniques in the context of root canal treatment. For these reasons, there is a degree of uncertainty around the conclusions drawn from these studies, which should be taken with caution when applying them to a clinical setting.

Recommendations for future studies

In terms of paper point sampling, basic parameters such as (i) manufacturer (Pumarola-Suñé *et al.* 1998), (ii) ISO size (Hartroth *et al.* 1999), (iii) duration of sampling (Hartroth *et al.* 1999) and (iv) insertion depth need to be studied to develop an optimized protocol that allows for maximum PTF absorbance. To attain maximum mediator recovery, factors such as different (i) buffer types (Inic-Kanada *et al.* 2012) and (ii) elution methods (i.e. incubation, vortex, centrifugation and combinations) need to be investigated to develop an optimized elution protocol.

Finally, key information needs to be explicitly and unambiguously provided in the methodology of studies investigating periradicular inflammatory mediator activity so that a meta-analysis can be pursued in the future. This includes (i) sample characteristics: an explicit diagnosis and tooth type; (ii) parameters of PTF sampling: method, number of operators, manufacturer, ISO size, insertion depth, sampling duration, number of points per sample, if any modifications were made to the point, how PTF volume was measured, number of samples per tooth, timing of the baseline and subsequent samples in relation, and the management of a dry, bleeding and suppurative canal; and (iii) parameters of PTF elution: assay technique, buffer type and volume, duration and temperature of incubation if used, duration of vortex if used and duration, force and temperature of centrifugation if used.

Conclusions

Within the limitations of the studies included in this review, which were of medium to low quality, two main conclusions can be drawn regarding how periradicular inflammatory mediators are currently being studied during root canal treatment:

1. Paper points and proteomic/metabolomic level assays are currently the most commonly used methods to sample and analyse inflammatory mediators within PTF, respectively.
2. The most targeted analytes are currently IL-1β and TNF-α.

This review also highlights the need for the development an optimized sampling and eluting protocol and a standardized approach to reporting by future studies.

Conflicts 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:

Appendix S1. Design specific criteria used for assessing the bias in studies which met the inclusion criteria in this systematic review.

Appendix S2. Excluded articles at full-text evaluation with reason.

Appendix S3. Results table for the risk of bias assessment.

CHAPTER 5

PUBLICATION 7

PERIRADICULAR TISSUE FLUID-DERIVED BIOMARKERS FOR APICAL PERIODONTITIS: AN IN VITRO METHODOLOGICAL AND IN VIVO CROSS-SECTIONAL STUDY

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Periradicular tissue fluid-derived biomarkers for apical periodontitis: An *in vitro* methodological and *in vivo* cross-sectional study

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Abstract

Background: Periradicular tissue fluid (PTF) offers a source of diagnostic, prognostic and predictive biomarkers for endodontic disease.

Aims: (1) To optimize basic parameters for PTF paper point sampling *in vitro* for subsequent *in vivo* application. (2) To compare proteomes of PTF from teeth with normal apical tissues (NAT) and asymptomatic apical periodontitis (AAP) using high-throughput panels.

Methodology: (1) To assess volume absorbance, paper points ($n = 20$) of multiple brands, sizes and sampling durations were inserted into PBS/1%BSA at several depths. Wetted lengths (mm) were measured against standard curves to determine volume absorbance (μL). To assess analyte recovery, paper points ($n = 6$) loaded with 2 μL recombinant IL-1β (15.6 ng/mL) were eluted into 250 μL: (i) PBS; (ii) PBS/1% BSA; (iii) PBS/0.1% Tween20; (iv) PBS/0.25 M NaCl. These then underwent: (i) vortexing; (ii) vortexing/centrifugation; (iii) centrifugation; (iv) incubation/vortexing/centrifugation. Sandwich-ELISAs determined analyte recovery (%) against positive controls.

(2) Using optimized protocols, PTF was retrieved from permanent teeth with NAT or AAP after accessing root canals. Samples, normalized to total fluid volume (TFV), were analysed to determine proteomic profiles (pg/TFV) of NAT and AAP via O-link Target-48 panel. Correlations between AAP and diagnostic accuracy were explored using principal-component analysis (PCA) and area under receive-operating-characteristic curves (AUC [95% CI]), respectively. Statistical comparisons were made using Mann–Whitney *U*, ANOVA and *post hoc* Bonferonni tests ($\alpha < .01$).

Results: (1) UnoDent's 'Classic' points facilitated maximum volume absorbance ($p < .05$), with no significant differences after 60 s (1.6 μL [1.30–1.73]), 1 mm depth and up to 40/0.02 (2.2 μL [1.98–2.20]). For elution, vortexing (89.3%) and PBS/1% BSA (86.9%) yielded the largest IL-1β recovery ($p < .05$).

(2) 41 (NAT: 13; AAP: 31) PTF samples proceeded to analysis. The panel detected 18 analytes (CCL-2, -3, -4; CSF-1; CXCL-8, -9; HGF; IL-1β, -6, -17A, -18; MMP-1,

-12; OLR-1; OSM; TNFSF-10, -12; VEGF-A) in ≥75% of AAP samples at statistically higher concentrations ($p < .01$). CXCL-8, IL-1β, OLR-1, OSM and TNFSF-12 were strongly correlated to AAP. 'Excellent' diagnostic performance was observed for TNFSF-12 (AUC: 0.94 [95% CI: 0.86–1.00]) and the PCA-derived cluster (AUC: 0.96 [95% CI: 0.89–1.00]).

Conclusions: Optimized PTF sampling parameters were identified in this study. When applied clinically, high-throughput proteomic analyses revealed complex interconnected networks of potential biomarkers. TNFSF-12 discriminated periradicular disease from health the greatest; however, clustering analytes further improved diagnostic accuracy. Additional independent investigations are required to validate these findings.

KEYWORDS

absorbance, apical periodontitis, biomarkers, elution, inflammation, periradicular tissue fluid

INTRODUCTION

Periradicular tissue fluid (PTF) is an interstitial fluid derived from the vasculature surrounding the root of a tooth (Nair, 2004). In normal apical tissues (NAT), this serum is transudative and accumulates within extracellular spaces via passive diffusion across osmotic gradients. Stimulation from endodontic pathogens, however, initiates a localized inflammatory response that encourages fluid extravasation by increasing capillary membrane permeability (Hama et al., 2006; Ricucci & Bergenholtz, 2004). In this diseased state, such as asymptomatic apical periodontitis (AAP), PTF is considered an inflammatory exudate that progressively becomes enriched with autocrine and paracrine signalling molecules (Márton & Kiss, 2000). These pro- and anti-inflammatory peptides are responsible for orchestrating a myriad of cellular events involved in local tissue destruction and the development of clinical symptoms (Márton & Kiss, 2014).

In recent years, PTF has been retrieved clinically through root canals using paper points and its composition investigated at the proteomic and transcriptomic levels (Virdee et al., 2019). These experiments have quantified analytes (Martinho et al., 2016; Safavi & Rossomando, 1991; Shimauchi et al., 1996), correlated them with acute endodontic symptoms (Alptekin et al., 2005; Martinho et al., 2016; Pezelj-Ribarić et al., 2007), longitudinally monitored changes throughout treatment (Alptekin et al., 2005; Grga et al., 2013; Liu et al., 2003) and utilized several markers as surrogate outcome measures in clinical trials (Corazza et al., 2021; Teixeira et al., 2022; Zhi et al., 2017). This body of evidence demonstrates PTF's ability to be harvested for local biomarker molecular analysis and proof-of-concept for their diagnostic, prognostic and predictive potential. Thus, a noninvasive chairside sampling procedure targeting individual or groups of analytes could provide clinicians with more sensitive and specific information regarding periradicular disease status than currently available diagnostic techniques (Dummer et al., 1980; Klausen et al., 1985; Lofthag-Hansen et al., 2007). Similar studies have already identified biomarkers within other oral exudates, notably Matrix Metallopeptidase [MMP]-8 in gingival crevicular fluid (GCF) for use in periodontal disease diagnosis (Sorsa et al., 2020).

Despite recent progress, there still remains an absence of universal protocols for sampling PTF. This could be due to a lack of methodological work-up data, which contrasts other intricate diagnostic procedures where basic parameters have been first optimized *in vitro* (Hartroth et al., 1999; Inic-Kanada et al., 2012; Zehnder et al., 2014). Additionally, existing PTF studies have limited their investigations to a small array of cytokines using conventional enzyme-linked immunosorbent assays (ELISA; Rechenberg et al., 2014; Teixeira et al., 2022; Zhi et al., 2017). It thus remains one of the only oral exudates that has not been characterized using more contemporary high-throughput methods of proteomic analysis, an approach that better reflects the complex multifaceted nature of periradicular pathophysiology. Furthermore, whilst 45 PTF-derived mediators have been detected across 33 studies (Virdee et al., 2019), data pertaining to their diagnostic performance has yet to be reported. This analysis would be required for determining those individual or molecular groups that could reliably serve as biomarkers for different periradicular disease states, for which there is currently no consensus.

AIMS

In order to enhance PTF retrieval for the *in vivo* cross-sectional study an *in vitro* methodological study was

conducted. The primary aims of the *in vitro* study were to determine the basic parameters facilitating (1) maximum volume absorbance and (2) analyte recovery during paper point sampling of PTF. The primary aim of the *in vivo* cross-sectional study was to use the optimized sampling protocol to identify potential biomarkers for AAP by comparing the proteome of PTF from teeth diagnosed with NAT or AAP. Secondary *in vivo* objectives included (1) exploring associations between peptides and disease states to identify distinct clusters of biomarkers, or biosignatures, for AAP and (2) determining the diagnostic performance of PTF-derived mediators in discriminating periradicular disease from health.

The null hypotheses tested were: (i) different paper point brand, size and sampling durations do not significantly influence absorbed volume; (ii) eluting buffer and technique do not significantly influence analyte recovery; (iii) there are no significant differences in PTF analyte concentrations between NAT and AAP.

MATERIALS AND METHODOLOGY

This study was performed in accordance with 2021 Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) and 2023 Preferred Reporting items for Observational studies in Endodontics (PROBE) guidelines (Nagendrababu et al., 2021, 2023; Figure 1). Ethical approval was attained from the University of Birmingham's Dentistry Research Tissue Bank (19/SW/0198) for obtaining PTF as part of routine treatment. Informed consent to retain waste tissue was documented in the participant's electronic medical records.

In vitro methodological studies

Volume absorbance

The laboratory model previously described by Shimauchi et al. (1996), which explored the relationship between fluid volume and paper point wetted length, was adapted to determine the optimal parameters for PTF absorbance. Briefly, paper points from five independent batches were pooled into a polythene bag in accordance with their size and brand and subsequently coded for operator blinding. They were then randomly selected from each bag and with locking college tweezers, perpendicularly clasped at prespecified lengths from their tips depending on the desired insertion depth. These were then suspended within 1.5 mL microfuge tubes (Thermo Fisher Scientific) containing 500 μL phosphate-buffered saline (PBS) and 1% bovine serum albumin (BSA; Sigma-Aldrich), which acted as an *in vitro* substitute for PTF. To improve stability, the gripping component of tweezers was firmly rested upon the coronal aspect of the open microfuge tube, which in itself was supported in a microtube rack. Absorbed volume (μL) was then immediately calculated using a predetermined wetted length (mm) to volume (μL) standard curve. All measurements, including those for testing the parameters below, were taken under magnification using a 0.5 mm graduated stainless-steel ruler. The following parameters, all selected for their use in prior PTF studies, were evaluated using sample sizes consistent with other similar investigations ($n = 20$; Pumarola-Suñé et al., 1998; Shimauchi et al., 1996):

(i) Brand: Classic (UnoDent), DeTrey (Densply Sirona), DiaDent, HS Maxima (Henry Schein), Maillefer, Orbis, Panadent, QED, Roeko Colour, SybronEndo (Kerr Dental) and VDW tested using 15/0.02 cones for 60 s and 1 mm insertion depth. The brand yielding the greatest absorbance was used in subsequent investigations.
(ii) Insertion depth: 1-, 2-, 3- and 4-mm insertion depths tested using 15/0.02 cones for 60 s.
(iii) ISO size: 15/0.02, 20/0.02, 30/0.02 and 40/0.02 cones tested for 60 s at 1 mm insertion depth.
(iv) Sampling duration: 30, 60, 90 and 120 s tested using 15/0.02 cones at 1 mm insertion depth.

Analyte recovery

The laboratory model previously described by Inic-Kanada et al. (2012), which compared cytokine recovery of different ophthalmic sponges and solutions, was adapted to determine eluting efficacy of several buffers and techniques employed in prior PTF studies. Briefly, recombinant Interleukin [IL]-1β (Bio-Techne), used as a representative analyte (Shimauchi et al., 1996), was reconstituted to 15.6 ng/mL in PBS/1% BSA (Sigma-Aldrich). Two microlitres were loaded onto a single 15/0.02 cone via calibrated pipettor (Gilson), which was then inserted into a 1.5 mL microfuge tube containing 250 μL of: (i) PBS; (ii) PBS/1% BSA; (iii) PBS/0.1% Tween20; (iv) PBS/0.25 M Sodium Chloride (NaCl). These then underwent elution via:

(i) 1 min vortex (Starlab Ltd)
(ii) 1 min vortex (Starlab Ltd) and 10 min centrifugation (Eppendorf) at 5000 *g* at 4°C
(iii) 10 min centrifugation (Eppendorf) at 5000 *g* at 4°C
(iv) 300 min incubation at room temperature, 1 min vortex and 10 min centrifugation (Eppendorf) at 5000 *g* at 4°C

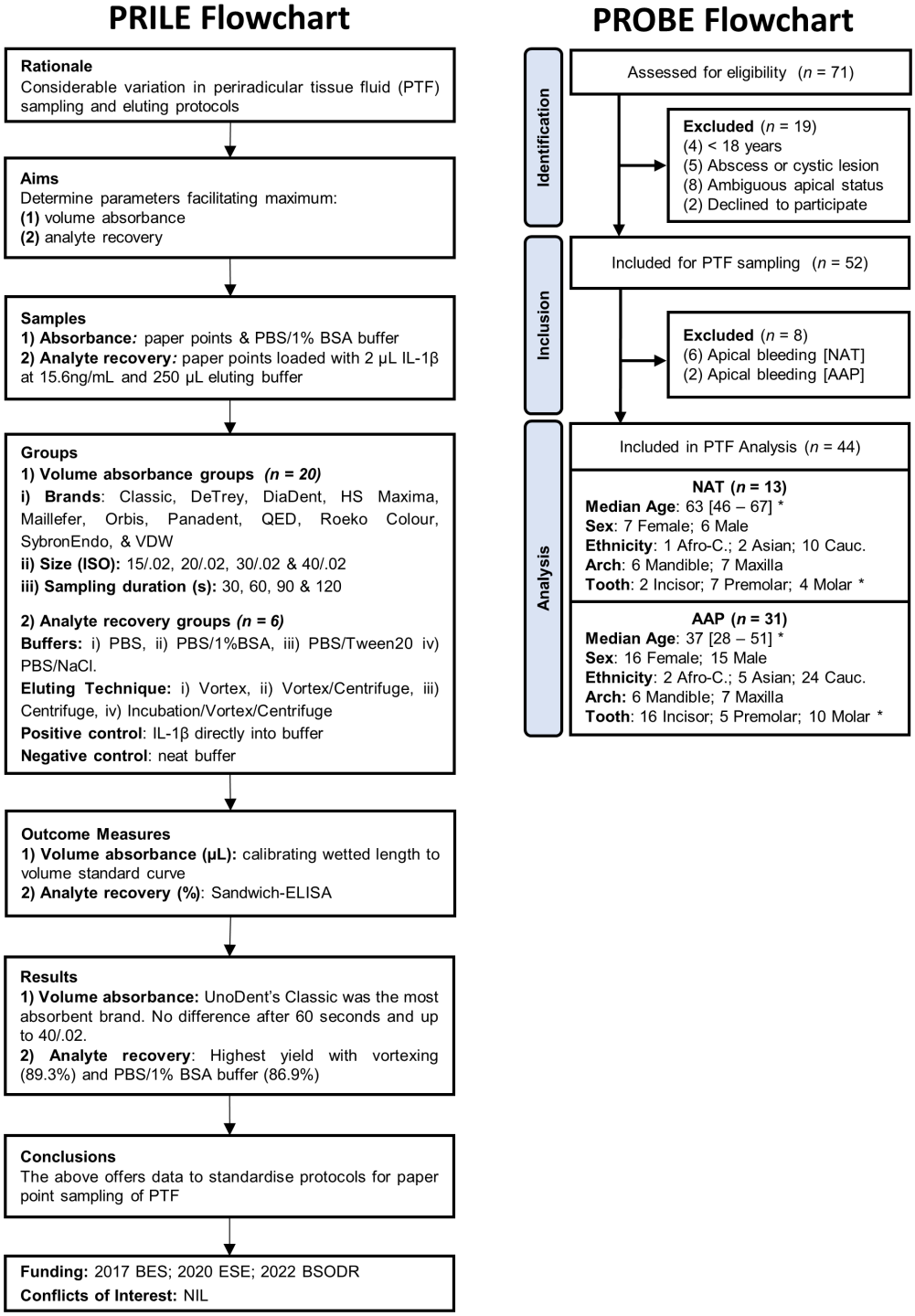

FIGURE 1 PRILE and PROBE flowcharts. *$p < .05$.

Under these conditions, 100% analyte recovery equated to 125 pg/mL and the median of the standard curve. For positive controls, 2 µL reconstituted IL-1β was inserted directly into buffers whereas neat solutions served as negative controls. Each group had sample sizes consistent with other experiments investigating analyte recovery ($n = 6$; Inic-Kanada et al., 2012). The resulting buffer containing the single loaded cone was then collected, coded for operator blinding and stored at −20°C until analysis. This was performed using a standard commercial IL-1β sandwich-ELISA kit (Bio-Techne) in accordance with the manufacturer's instructions. Briefly, 100 µL test, control and standard samples were added in duplicates to a clear flat bottom 96-well plate after coating with IL-1β specific monoclonal capture antibody and blocking with the reagent diluent provided. After 2 h of incubation at room temperature,

100 μL IL-1β polyclonal antibody conjugated to horseradish peroxidase was added to each well and incubated again for 2 h at room temperature. Between each step, plates were washed using an automated plate washer (ELx50, Bio-Tek) and the PBS/0.1% Tween20 wash buffer provided. One hundred microlitres hydrogen peroxidase and chromogen substrate solution were then added for 20 min in the dark followed by 50 μL sulfuric acid stop solution. The cytokine concentration in each well was then analysed using the plate reader (ELX800, Bio-Tek) at an optical density of 450 nm and 570 nm, with latter values subtracted from the former to eliminate optical imperfections. The resulting outputs were then calibrated against a standard curve to determine concentration and IL-1β, with percentage recovery (%) calculated by comparing concentrations expressed in test samples with those of positive controls. Pilot investigations revealed that the above-mentioned buffer solutions did not interfere with the assay (data not shown).

In vivo cross-sectional study

Setting

A cross-sectional study was conducted on the undergraduate endodontic specialty teaching clinics at Birmingham Dental Hospital between September 2021 and June 2022. All procedures were performed under a dental operating microscope (Global Surgical Corp.), by the lead investigator (SSV).

Participants

Medically fit consenting adults (≥18 years) undergoing root canal treatment in mature permanent teeth diagnosed with NAT or AAP were consecutively recruited. The exclusion criteria consisted of those who had undergone antimicrobial therapy 3 months prior to screening or were immunocompromised. Teeth with previously initiated endodontic treatment or existing root fillings; periodontal pocketing ≥5 mm; that were unable to retain a rubber dam; had apices closely associated with the maxillary sinus; exhibited clinical or radiographic signs of a periradicular abscess or cyst (i.e. ≥10 mm or corticated radiolucencies), root resorption or fracture were also excluded, alongside whole PTF samples where paper points were contaminated with profuse apical bleeding.

Sample size

Statistical methods for determining sample size could not be performed due to lack of prior data. Therefore, researchers collected as many samples as possible during the nine-month study period with minimum of 12 per group, as reported by (Sette-Dias et al., 2016).

Diagnostic reference standard

Diagnoses were made by two independent staff with postgraduate endodontic qualifications. Teeth were subjected to clinical (percussion and palpation) and plain-film radiographic examination in conjunction with thermal (Endo-Frost, Roeko) and electric pulp testing. Normal apical tissues were defined as teeth that clinically were not sensitive to percussion or palpation testing and radiographically exhibited an intact laminar dura with uniform periodontal ligament space. Conversely, AAP was defined as teeth that were also clinically not sensitive to percussion or palpation testing but radiographically demonstrated a periradicular radiolucency (Glickman, 2009).

Clinical protocol

Upon attaining local anaesthesia with 2% lidocaine and 1:80 000 adrenaline solution (Dentsply Sirona), teeth were isolated using rubber and liquid dam (Liquidam, Cerkamed). A traditional stable four-walled access cavity to the pulp chamber was then created with cooled diamond burs under high-volume aspiration. After gently washing and air drying away gross dentinal debris and residual moisture with a triple air syringe, a 10/0.02 K-Flex file (Dentsply Sirona) was advanced slowly down the canal whilst connected to a Dentaport ZX electronic apex locator (Morita). Initially, patency was confirmed when only the first red display bar became visible, after which files were retracted to the zero- (i.e. terminal green display bar) and 0.5-reading markers (i.e. fifth green display bar) to determine positions of the apical foramen and constriction, respectively (Connert et al., 2018). All sampling protocols were respective to the former whereas the latter was considered the working length for subsequent root canal preparation and obturation procedures. To improve accuracy, this process was repeated with a 15/0.02 alongside a periapical radiograph taken at the zero-reading via a paralleling technique (ESE, 2006). When file tips were ≥2 mm from the radiographic apex or extruded, length modifications were informed by a third electronic apex locator reading. Canals were then pre-flared by watch-winding a 20/0.02 file to the zero-reading in a crown-down manner, which also standardized the apical constrictions' diameter; dried using 25/0.02 paper points set 2 mm short of this measurement; and patency filed with a 10/0.02 to disrupt dentinal debris at the apices and encourage intraradicular influx of tissue fluid.

PTF sampling

Prior to any irrigation, PTF samples were collected with three paper points per tooth using an optimized protocol determined *in vitro*. Briefly, each cone was gripped with locking college tweezers at the length equating to the zero-reading plus the optimal insertion depth. It was then slowly advanced into the pre-flared canal until reaching this measurement, where the tweezers were unclasped and a digital countdown timer initiated. The exact parameters including brand, insertion depth relative to the zero-reading, ISO size and sampling duration are outlined in the 'volume absorbance' subheading of the results section. If any of the three cones became contaminated by profuse apical bleeding, the entire sample was abandoned. Nevertheless, once sampled, cones were immediately transferred into sterile microfuge tubes, normalized according to total fluid volume (TFV) in 250 μL of sterile buffer solution, eluted using optimized parameters determined *in vitro* and stored at -80°C until analysis. The exact eluting buffer and technique used can be found in the 'analyte recovery' subheading of the results section. In posterior teeth, samples were retrieved from maxillary palatal and mandibular distal root canals.

PTF analyses

Wetted lengths (mm) of paper points were measured immediately after sampling and calibrated as per *in vitro* methods to calculate total absorbed PTF volume (μL) per sample.

The Bradford dye-binding assay (Thermo Fisher Scientific) was performed to determine the total protein concentration (TPC; μg/mL). Briefly, 5 μL of standards and samples were added to a clear flat bottom 96-well plate in duplicates followed by 250 μL of Bradford reagent. Plates were incubated for 20 min at room temperature with the optical density determined using a plate reader at 595 nm (Tecan Spark). Values were subtracted from those attained from a negative control (i.e. buffer solution only). The TPC was then calculated against a standard series of BSA.

The Target-48 Panel (O-link) was used to quantify the concentration (pg/mL) of 45 different proteins within each sample. Briefly, 1 μL of samples, standards and controls were transferred into a 96 well polymerase chain reaction (PCR) plate and incubated overnight at 4°C with 3 μL of incubation mix consisting of protein binding antibody pairs alongside conjugated DNA tags. Thereafter, the unique DNA reporting sequences generated were amplified for each target protein by adding 96 μL of extension mix to wells and a PCR thermocycler. Thereafter, 2.8 μL extension PCR products and 7.2 μL of detection mix was added to a new 96-well PCR plate, with the DNA reported for each target protein being quantified via high-throughput microfluidics real-time quantitative PCR. All outputs were normalized to TFV and presented as pg/TFV (Mente et al., 2016). Cytokines were considered absent, and excluded from analyses, when concentrations fell below the lower limit of detection in >25% of test PTF samples. Data points were assigned zero and maximum values when readings were below or above the respective limits of detection. All assays were performed in duplicates as per manufacturer's instructions.

Statistical analyses and data presentation

All data were coded by SSV for assessor blinding and statistically analysed per protocol in 'R' (V.4.1.0) software by NZB. Descriptive statistics were presented as medians with [interquartile range] in dot plots using GraphPad Prism software (V.8.0.2). For group comparisons, normality screening was conducted using the Shapiro-Wilks test. Normally distributed groups were compared using one- and two-way ANOVAs with *post hoc* Bonferonni correction tests and skewed groups with Mann–Whitney *U* tests. Independent-samples *t*- and chi-squared tests were used to compare baseline characteristics between groups. Initial alpha values for the *in vitro* and *in vivo* studies were set at .05 and .01, respectively.

Principal component analysis (PCA) was performed to identify clusters of analytes highly associated with AAP. Briefly, Target-48 data was dimensionally reduced into linear variables termed principal components (PC) with those possessing eigenvalues >1 included in further analyses. Loadings were computed against PCs to determine correlations, which were visualized in PC biplots. These were supplemented with a seriated heat map and network analysis graphs depicting only significant ($p < .05$) interactions ($r > .75$) using Gephi (V.0.9.7) software.

Diagnostic performance was determined using receiver operator characteristic (ROC) curves and presented as area under the curve (AUC) with [95% confidence intervals (95%CI)]. Diagnostic accuracy was then classified according to criteria outlined by Šimundić (2009). For individual analytes, optimal cut-offs, and sensitivity and specificity at that concentration, were identified by maximizing the Youden's *J* Index ([sensitivity + specificity] -1; Youden, 1950). For the PCA-derived cluster, continuous data for each constituent peptide was dichotomised using the respective diagnostic thresholds and subsequently entered into a multivariable logistic regression model to calculate AUC. All ROC curves were generated using a leave-one-out cross-validation (LOOCV) approach (Grant et al., 2022).

RESULTS

Results are summarized in Tables 1 and 2 and Figure 2 for the *in vitro* studies and Tables 3 and 4 and Figures 3–6 for the *in vivo* study. Raw data supporting the findings of this study are available from the corresponding author upon reasonable request.

In vitro methodological studies

Volume absorbance

Classic (UnoDent) was the most absorbent brand of paper point ($p < .05$), with no significant differences occurring after 60 s (1.6 μL [1.30–1.73]), 1 mm insertion depth, which would be relative to the EAL's zero-reading (1.6 μL [1.43–1.71]), and up to a size 40/0.02 (2.2 μL [1.98–2.20]).

Analyte recovery

IL-1β recovery ranged from 67.6% to 98.3% with significant differences amongst eluting buffers and techniques ($p < .05$). Overall yields were highest following vortexing (89.3% [82.28–96.41]) and PBS/1%BSA buffer (86.9% [81.44–110.47]) and lowest following incubation, vortexing and centrifugation (80.3% [75.05–84.95]) and PBS (79.9% [73.48–86.80]).

In vivo cross-sectional study

Participant characteristics

Fifty-two PTF samples (NAT: 19; AAP: 33) were retrieved after screening 71 individuals; however, only 44 (NAT: 13; AAP: 31) proceeded to analysis due to profuse apical bleeding. The median patient age was 44 [29–55] (NAT: 63 [46–67]; AAP: 37 [28–51]) with an approximate 1:1 male-to-female ratio. Three participants (NAT: 1; AAP: 2) were of Afro-Caribbean descent, 7 were Asian (NAT: 2; AAP: 5) and 34 were Caucasian (NAT: 10; AAP: 24). Eighteen (NAT: 6; AAP: 12) samples were collected from mandibles and 26 (NAT: 7; AAP: 19) maxilla with incisors/canines being the commonest tooth (18; NAT: 2; AAP: 16) followed by molars (14; NAT: 4; AAP: 10) and premolars (12; NAT: 7; AAP: 5). Groups were matched by gender, ethnicity and interarch tooth position.

PTF characteristics

Wetted lengths were significantly greater in AAP samples ($p < .001$). This translated into larger PTF volumes (1.7 μL [0.70–2.59]) when compared with the NAT group (0.2 μL [0.10–1.53]; $p < .01$). Additionally, AAP samples exhibited 249.0 μg/mL [63.25–373.42] TPC as opposed to 3.8 μg/mL [0–13.75] in controls ($p < .01$).

TABLE 1 Optimizing parameters for maximum paper point absorbance (μL) following *in vitro* PTF sampling.

Parameter	Absorbed volume (μL)			
Paper point brand	**Classic***	**DeTrey**	**DiaDent**	**HS Maxima**
	1.6 [1.30–1.73]	0.6 [0.40–0.80]	0.7 [0.58–0.83]	0.9 [0.70–1.10]
	Kerr	**Maillefer**	**Orbis**	**Panadent**
	0.9 [0.64–0.10]	0.7 [0.45–0.83]	0.9 [0.60–1.23]	1.0 [0.80–1.30]
	QED DiaDent	**Roeka Colour**	**SybronEndo**	**VDW**
	0.6 [0.40–0.63]	0.7 [0.58–0.90]	1.1 [0.80–1.53]	0.5 [0.40–0.70]
Paper point size (ISO)	**15/0.02**	**20/0.02**	**25/0.02**	**30/0.02**
	1.6 [1.30–1.73]	1.6 [1.30–1.90]	1.9 [1.55–2.25]	1.9 [1.68–2.43]
	35/0.02	**40/0.02**[a,b]		
	1.7 [1.40–2.25]	2.2 [1.98–2.20]		
Sampling duration (s)	**30***	**60**	**90**	**120**
	0.7 [0.60–0.83]	1.5 [1.30–1.73]	1.5 [1.30–1.73]	1.6 [1.50–1.73]
	150	180		
	1.5 [1.30–1.70]	1.6 [1.35–1.90]		
Insertion depth (mm)	**1**	**2**	**3**	**4**
	1.6 [1.30–1.73]	1.6 [1.38–1.80]	1.4 [1.20–1.63]	1.4 [1.40–1.63]

Note: Results for each group ($n = 20$) presented as medians and [interquartile ranges]. [*] versus all other groups; [a] versus ISO # 15; [b] versus ISO # 20 ($p < .05$ – One-way ANOVA and *post-hoc* Bonferroni test).

TABLE 2 Optimizing parameters for maximum analyte recovery (%) following *in vitro* PTF elution.

	IL-1β recovery (%)				
Buffer	**Vortex[a]**	**Vortex, centrifuge[b]**	**Centrifuge[c]**	**Incubate, vortex, centrifuge[d]**	**Overall Buffer**
PBS[i]	74.7[b–d] [70.24–78.06]	76.9[b,c] [74.20–80.94]	81.0 [80.66–83.81]	79.2 [73.48–82.91]	79.9[b,c] [73.62–86.80]
PBS & BSA[ii]	94.1[a,iii] [87.74–100.44]	98.3[a,d,iii,iv] [88.74–106.70]	81.8[i,ii] [80.40–83.79]	86.1[ii] [81.57–86.68]	86.9[a,d] [81.44–110.47]
PBS & Tween20[iii]	95.4[a,iv] [93.72–97.27]	94.9[a,d,iii,iv] [92.85–96.91]	84.0[i] [80.92–84.54]	73.7[i,ii] [72.95–77.42]	85.5[a,d] [79.52–104.17]
PBS & NaCl[iv]	92.5[a,ii,iii] [86.81–95.42]	67.6[b,c,i,iv] [59.27–77.70]	80.0[i] [73.17–84.69]	84.5[ii] [79.27–85.12]	83.9[b,c] [74.34–99.98]
Overall Technique	89.3[iii,iv] [82.28–96.41]	84.9 [74.48–94.7]	81.8[i] [80.00–84.65]	80.5[i] [75.05–84.95]	

Note: Results for each group ($n = 6$) presented as medians and [interquartile ranges]. [[a]] versus corresponding PBS; [[b]] versus corresponding PBS and BSA; [[c]] versus corresponding PBS and Tween20; [[d]] versus corresponding PBS and NaCl; [[i]] versus corresponding vortex; [[ii]] versus corresponding vortex and centrifuge; [[iii]] versus corresponding centrifuge; [[iv]] versus corresponding incubate, vortex and centrifuge ($p < .05$ – Two-way ANOVA and *post hoc* Bonferroni test).

Abbreviations: BSA, bovine serum albumin; NaCl, sodium chloride; PBS, phosphate-buffered saline; IL-1β, interleukin 1 beta.

Proteomic analysis

The Target-48 panel consistently detected 18, of a potential 45, analytes. These included Chemokine Ligand [CCL]-2, -3 and -4; Colony Stimulating Factor [CSF]-1; Chemokine Ligand [CXCL]-9; Hepatocyte Growth Factor [HGF]; Interleukin [IL]-1β, -6, -8 [CXCL-8], -17A and -18; MMP-1 and -12; Oxidized Low Density Lipoprotein Receptor [OLR]-1; Oncostatin M [OSM]; Tumour Necrosis Factor Superfamily [TNFSF]-10 and 12 and Vascular Endothelial Growth Factor [VEGF]-A. Concentrations were significantly greater ($p < .001$) in AAP samples, with the most abundant cytokines detected being ORL-1 (567.4 pg/TFV [257.98–874.21]), MMP-12 (264.5 pg/TFV [107.80–1314.25]), CXCL-8 (206.8 pg/TFV [54.18–760.58]) and IL-1β (92.6 pg/TFV [23.24–211.80]). The 27 less frequently detected analytes are detailed in Appendix S1.

Correlations with disease state

The first three PCs, representing 61.6%, 8.5% and 6.3% variance, respectively, were included in PCA. Loadings revealed PC1 to be positively correlated to AAP with higher expressions of all 18 analytes. The second PC exhibited minimal correlations with AAP, lower expression of CCL-3, -4, IL-1β, -6, -17A, -18 and higher expressions of MMP-12, TNFSF-10, VEGF-A. PC3 was negatively correlated with AAP with downregulation of CXCL-8, IL-1β, OLR-1 and upregulation of CXCL-9, IL-6, -17A, MMP-1. The PC biplots and seriated heat maps identified strong positive correlations between AAP and an analyte cluster consisting of CXCL-8, IL-1β, OLR-1, OSM, TNFSF-12. Network analysis graphs illustrate that analyte composition and number of associations are substantially greater and more complex in diseased tissues. Only 13 analytes formed the NAT network with exclusions of CSF-1, CXCL-9, IL-17A, TNFSF-10, -12. Conversely, all 18 cytokines formed the AAP network and contributed to a greater abundance. Heat maps revealed positive associations between all analytes.

Diagnostic performance

Individually, TNFSF-12 exhibited the highest diagnostic performance (AUC: 0.94 [0.86–1.00]) and sensitivity (1.00 [1.00–1.00]) and specificity (0.87 [0.77–0.97]) at its diagnostic threshold (0.90 pg/TFV). It was thus considered an 'Excellent' (AUC: >0.9–1.0) discriminator between AAP and NATs. The remaining analytes were deemed 'Very Good' (AUC: >0.8–0.9; HGF, IL-17A, MMP-1, OLR-1, TNFSF-10, VEGF-A), 'Good' (AUC: >0.7–0.8; CCL-3, −4, CSF-1, IL-18, MMP-12, OSM), 'Sufficient' (AUC: >0.6–0.7; CCL-2, IL-1β, IL-6, CXCL-8) or 'Bad' (AUC: >0.5–0.6; CXCL-9). With exception to TNFSF-12, they all generally exhibited higher sensitivity (0.87–0.1) than specificity (0.55–0.8) at their respective cut-offs. The PCA-derived cluster, however, increased diagnostic accuracy and precision (AUC: 0.96 [0.89–1.00]). Supplementary diagnostic performance data is detailed in Appendix S2.

DISCUSSION

The *in vitro* studies investigated several basic parameters associated with PTF sampling. Unodent's 'Classic' 15/0.02

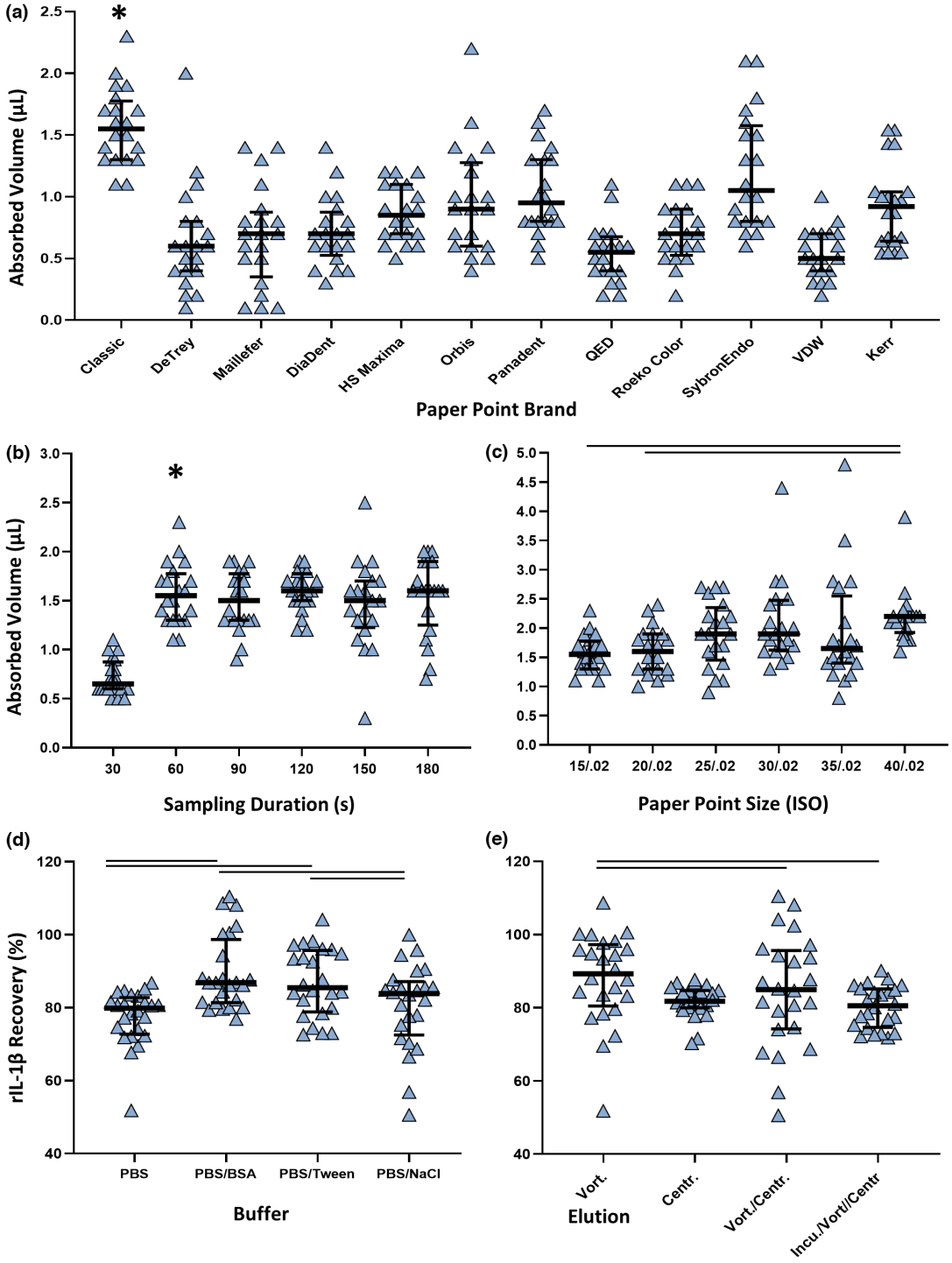

FIGURE 2 *In vitro* optimization of PTF during the sampling ($n = 20$) and eluting process ($n = 6$). Mock PTF absorbed volume (µL) with different: (a) brands of paper points; (b) sampling duration and (c) ISO size. Percentage recovery (%) of IL-1β following use of various (d) buffers and (e) eluting techniques. Data presented as dot plots where central bars represent the median alongside interquartile range for whiskers. Significant differences ($p < .05$; one- and two-way ANOVAs with *post hoc* Bonferroni tests) represented by [horizontal lines] = versus individual groups; [*] = versus all corresponding groups. BSA, bovine serum albumin; IL-1β, interleukin 1 beta; NaCl, sodium chloride; PBS, phosphate-buffered saline.

paper points inserted for 60 s at 1 mm depth relative to the zero-reading, followed by 60 s vortexing into 250 µL PBS/1% BSA facilitated the largest volume absorbance and IL-1β recovery, respectively. When applied clinically to identify potential biomarkers of endodontic disease, AAP samples had greater paper point wetted length, absorbed volume and TPC than their NAT counterparts. Proteomic analysis consistently identified 18 peptides, all

TABLE 3 Baseline characteristics for samples of PTF.

Sample characteristics		Diagnosis [*n* (%)]			
		Total (*n* = 44)	**NAT (*n* = 13)**	**AAP (*n* = 31)**	***p* Value**
Age (years)	Median [IQR]	44 [29–55]	63 [46–67]	37 [28–51]	<.05[a]
Sex	Female	22 (50.0)	7 (53.8)	15 (46.9)	>.05[b]
	Male	22 (50.0)	6 (46.2)	16 (53.1)	
Ethnicity	Afro-Caribbean	3 (6.8)	1 (7.7)	2 (6.4)	<.05[b]
	Asian	7 (16.0)	2 (15.4)	5 (16.1)	
	Caucasian	34 (77.2)	10 (76.9)	24 (77.5)	
Inter-arch position	Mandible	18 (40.9)	6 (46.2)	12 (38.7)	>.05[b]
	Maxilla	26 (59.1)	7 (53.8)	19 (61.3)	
Intra-arch position	Incisor/Canine	18 (40.9)	2 (15.4)	16 (51.6)	<.05[b]
	Premolar	12 (27.3)	7 (53.9)	5 (16.1)	
	Molar	14 (31.8)	4 (30.7)	10 (32.3)	

[a]Independent-samples *t*-test.

[b]Chi-squared test.

Abbreviations: AAP, asymptomatic apical periodontitis; NAT, normal apical tissues.

of which were highly expressed in disease and associated with AAP, particularly CXCL-8, IL-1β, OLR-1, OSM and TNFSF-12. Whilst the latter individually demonstrated excellent diagnostic accuracy, the overall PCA cluster exhibited the highest discriminatory power and thus may serve as a reliable biosignature for AAP. All null hypotheses were, therefore, rejected.

In vitro methodological studies

There are several limitations associated with the present *in vitro* experiments. The volume absorbance model for instance lacks biofidelity, as it does not take into account surface tensions generated by root canal walls (Karamifar et al., 2012). This would influence fluid movement and could explain the extreme wetted length values observed *in vivo*. Further support for this phenomenon is derived from a pilot investigation into volume absorbance that utilized an extracted tooth model. When the tips of paper points were inserted into buffer solution via the pre-flared canal of a central incisor, capillary action was consistently observed which oversaturated the cone. As this gave a false representation of paper point absorbance capacity under various parameters, the approach was abandoned, Digital scales would have also been more precise determinants of volume absorbance as opposed to wetted length, which is subject to human measurements of uneven paper point saturation. It was, however, not adopted as both methods have been previously correlated to each other and balances accurate to 1 nanogram are not readily accessible in the clinical environment (da Cunha et al., 2008). For the eluting experiment, IL-1β was the only cytokine used to determine the effectiveness of several extraction buffers and techniques. Caution should, therefore, be taken when extrapolating these recovery rates to other analytes (Inic-Kanada et al., 2012).

Classic (Unodent) was identified to be the most absorbent paper point brand. This could be attributed to larger pore sizes within its cellulose membrane (Zehnder et al., 2014), which may also explain their less ridged and fibre-shedding properties (Brown, 2017). Surprisingly, no meaningful volume could be further attained after 60 s sampling duration, a curvilinear relationship also observed by Shimauchi et al. (1996), and a 1 mm insertion depth. The latter would be advantageous *in vivo* as there would be less risk of cellulose fibres shedding into the periradicular tissues and inducing a persistent foreign body reaction (Nair, 2004). Whilst 40/0.02 s were significantly more absorbent, they were impractical for *in vivo* application as they could not pass through apical constrictions without erroneous enlargement. Instead, 15/0.02 s were selected as they demonstrated as much absorbency as 35/0.02 s whilst also conforming to the physiological diameter of the minor apical foramen (Chapman, 1969; Dummer et al., 1984). For IL-1β elution, retrieval rates were consistent with those reported by Inic-Kanada et al. (2012), but higher than those found by Shimauchi et al. (1996; 56%–67%). Methodological differences such as more porous paper point material, which has been proposed to lead to less physical entrapment of cytokines (Inic-Kanada et al., 2012), and larger initial loading doses may explain these discrepancies. Nevertheless, when the overall buffer effectiveness was considered, PBS achieved the lowest yields whereas the remaining solutions were relatively interchangeable. These findings are consistent

TABLE 4 Analysis of PTF collected from *in vivo* sampling.

	Diagnosis		PC loadings			Diagnostic performance [95% CI]			
PTF characteristics	**NAT ($n=13$)**	**AAP ($n=31$)**	**PC1**	**PC2**	**PC3**	**AUC**	**Cut-off**	**Sensitivity**	**Specificity**
Total absorbed volume (μL)	0.2 [0.10–1.53]	1.7 [0.70–2.59]*							
Total protein concentration (μg/mL)	3.8 [0–13.75]	249.0 [63.25–373.42]*							
Analyte concentration (pg/TFV)									
Chemokine ligand [CCL]-2	0.1 [0.06–0.96]	7.8 [1.87–26.10]*	0.21	0.02	0.15	0.69 [0.56–0.83]	1.12	0.87 [0.77–0.83]	0.69 [0.56–0.83]
Chemokine ligand [CCL]-3	0.2 [0.06–0.61]	7.4 [1.74–21.15]*	0.25	−0.32	0.05	0.80 [0.69–0.92]	1.13	0.93 [0.85–1.00]	0.69 [0.55–0.82]
Chemokine ligand [CCL]-4	0.8 [0.055–1.67]	25.5 [8.35–68.10]*	0.26	−0.20	0.12	0.74 [0.62–0.87]	4.38	0.92 [0.84–1.00]	0.58 [0.43–0.72]
Colony stimulating factor [CSF]-1	0.1 [0.06–0.18]	1.3 [0.59–3.12]*	0.28	0.10	0.02	0.80 [0.69–0.92]	0.34	0.93 [0.85–1.00]	0.69 [0.55–0.82]
Chemokine ligand [CXCL]-9	1.0 [0.57–1.37]	2.7 [0.36–5.78]*	0.16	0.17	0.34	0.60 [0.46–0.75]	0.10	0.92 [0.84–1.00]	0.55 [0.40–0.70]
Hepatocyte growth factor [HGF]	0.8 [0.31–1.54]	57.4 [27.73–113.93]*	0.28	0.09	0.07	0.86 [0.76–0.96]	5.56	0.96 [0.91–1.00]	0.75 [0.62–0.88]
Interleukin [IL]-1β	0.9 [0.16–2.17]	92.6 [23.24–211.80]*	0.18	−0.31	0.23	0.69 [0.54–0.83]	10.03	0.93 [0.85–1.00]	0.69 [0.55–0.82]
Interleukin [IL]-6	0.3 [0.028–0.63]	3.2 [0.88–9.69]*	0.24	−0.25	−0.05	0.68 [0.56–0.83]	1.14	0.95 [0.89–1.00]	0.55 [0.40–0.69]
Interleukin [CXCL]-8	4.6 [0.28–40.85]	206.8 [54.18–760.58]*	0.24	0.05	−0.33	0.68 [0.54–0.82]	200.17	0.91 [0.82–0.99]	0.50 [0.35–0.65]
Interleukin [IL]-17A	0.1 ± [0.08–0.12]	0.8 [0.41–2.78]*	0.19	−0.19	−0.50	0.84 [0.73–0.95]	0.23	1.00 [1.00–1.00]	0.68 [0.55–0.82]
Interleukin [IL]-18	0.4 [0.26–1.11]	46.6 [28.32–93.75]*	0.16	−0.44	0.22	0.79 [0.67–0.91]	4.05	0.93 [0.86–1.00]	0.79 [0.66–0.91]
Matrix metallopeptidase [MMP]-1	0.2 [0.034–0.837]	58.2 [12.19–177.26]*	0.22	−0.06	0.31	0.87 [0.77–0.97]	2.02	1.00 [1.00–1.00]	0.76 [0.64–0.89]
Matrix metallopeptidase [MMP]-12	0.3 [0.11–6.18]	264.5 [107.80–1314.25]*	0.20	0.50	0.16	0.74 [0.62–0.87]	37.98	0.88 [0.79–0.98]	0.56 [0.41–0.70]
Oxidized low density lipoprotein receptor [OLR]-1	13.6 [2.36–36.44]	567.4 [257.98–874.21]*	0.25	0.08	−0.24	0.83 [0.72–0.94]	91.76	0.97 [0.91–1.00]	0.80 [0.68–0.92]
Oncostatin M [OSM]	0.7 [0.17–1.62]	14.5 [4.01–37.87]*	0.24	−0.09	−0.12	0.74 [0.61–0.87]	3.88	1.00 [1.00–1.00]	0.62 [0.48–0.76]
Tumour necrosis factor superfamily [TNFSF]-10	2.1 [1.69–2.60]	18.3 [7.16–46.38]*	0.26	0.28	0.02	0.87 [0.77–0.97]	3.54	0.93 [0.85–1.00]	0.69 [0.55–0.82]
Tumour necrosis factor superfamily [TNFSF]-12	0.6 [0.40–0.75]	12.5 [5.18–26.55]*	0.25	0.12	−0.07	0.94 [0.86–1.00]	0.90	1.00 [1.00–1.00]	0.87 [0.77–0.97]
Vascular endothelial growth factor [VEGF]-A	2.1 [0.35–4.35]	47.3 [23.79–93.37]*	0.27	0.25	0.06	0.83 [0.72–0.94]	8.37	0.97 [0.91–1.00]	0.80 [0.68–0.92]
Presence of AAP	—	—	0.16	0.03	−0.41	—	—	—	—
PCA cluster (CXCL—8; IL-1β; OLR-1; OSM; TNFSF-12)	—	—	—	—	—	0.96 [0.89–1.00]	—	1.00 [1.00–1.00]	0.87 [0.77–0.97]

Note: Results presented as median and [interquartile range].

Abbreviations: 95% CI, confidence interval; AAP, asymptomatic apical periodontitis; AUC, area under curve; NAT, normal apical tissues; PC, principal component; PCA, principal component analysis; ROC, receiver operator characteristic; TFV, total fluid volume.

*$p < .01$ Mann–Whitney *U* test.

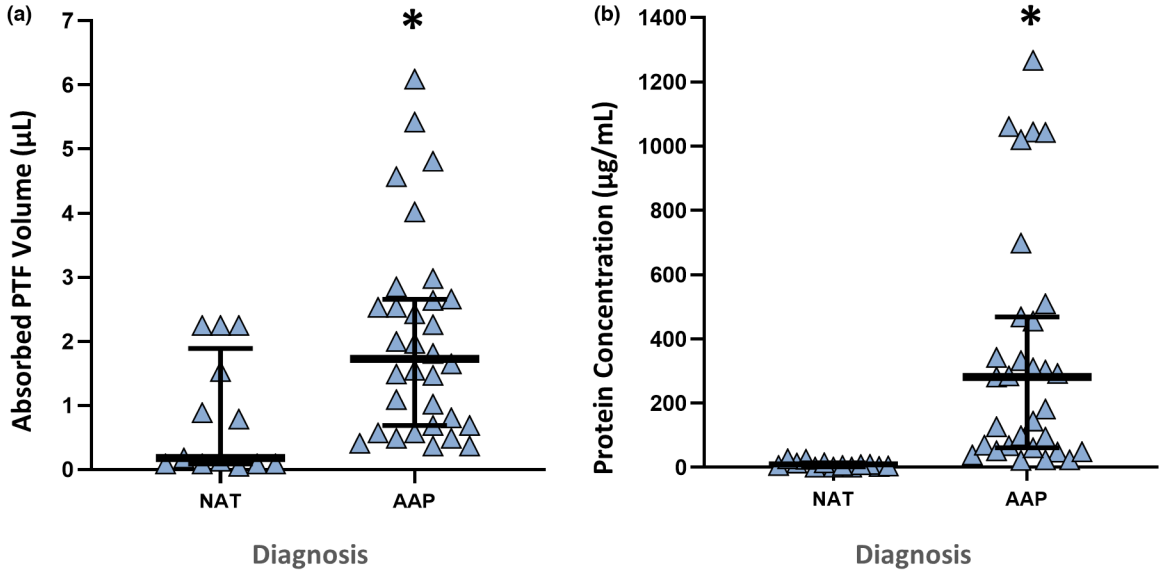

FIGURE 3 Characteristic of PTF following *in vivo* sampling. (a) absorbed PTF volume (µL); (b) total protein concentration (µg/mL). Data presented as dot plots where central bars represent the median alongside interquartile range for whiskers. Significant differences ($p < .05$; Mann–Whitney *U* tests) represented by [*]. AAP, asymptomatic apical periodontitis ($n = 31$); NAT, normal apical tissues ($n = 13$); PTF, periradicular tissue fluid.

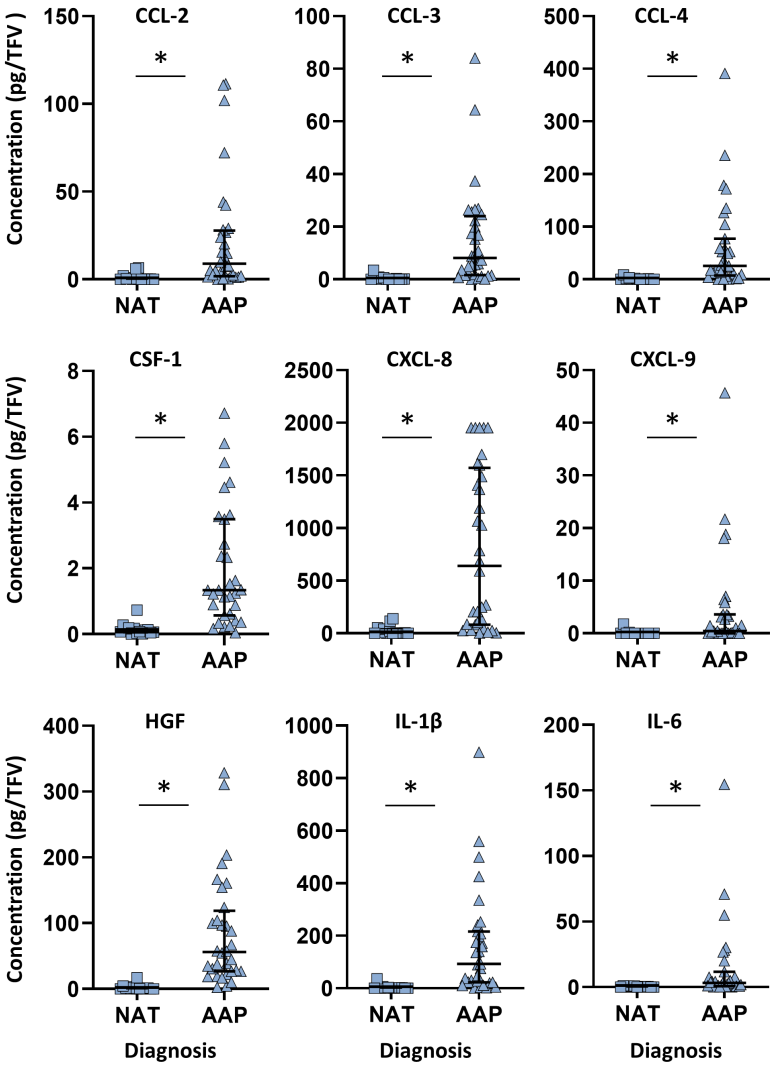

FIGURE 4 Concentration (pg/TFV) of analytes detected within PTF following *in vivo* sampling. Data presented as dot plots where central bars represent the median alongside interquartile range for whiskers. Significant differences ($p < .001$; Mann–Whitney *U* tests) represented by [*]. AAP, asymptomatic apical periodontitis ($n = 31$); NAT, normal apical tissues ($n = 13$). CCL-2, chemokine ligand-2; CCL-3, chemokine ligand-3; CCL-4, chemokine ligand-4; CSF-1, colony stimulating factor-1; CXCL-8, interleukin/chemokine ligand-8; CXCL-9, chemokine ligand-9; HGF, hepatocyte growth factor; IL-17A, interleukin-17A; IL-18, interleukin-18; IL-1β, interleukin-1β; IL-6, interleukin-6; MMP-1, matrix metallopeptidase-1; MMP-12, matrix metallopeptidase-12; OLR-1, oxidized low density lipoprotein receptor-1; OSM, oncostatin M; TNFSF-10, tumour necrosis factor superfamily-10; TNFSF-12, tumour necrosis factor superfamily-12; VEGF-A, vascular endothelial growth factor-A.

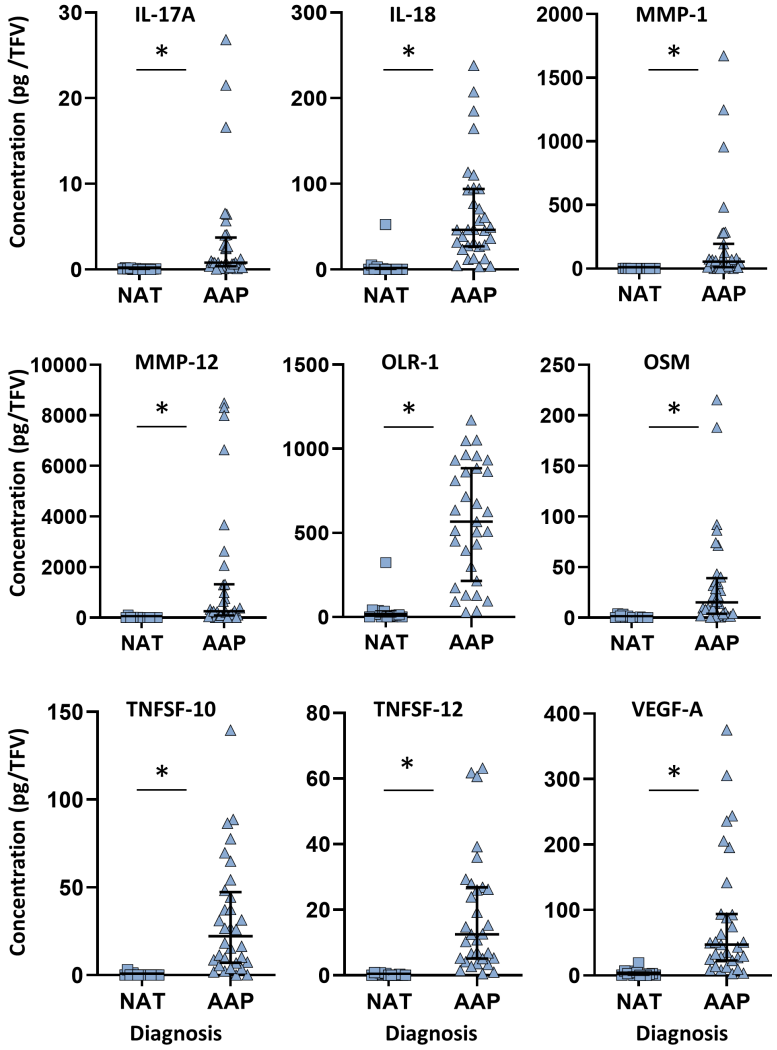

FIGURE 4 (Continued)

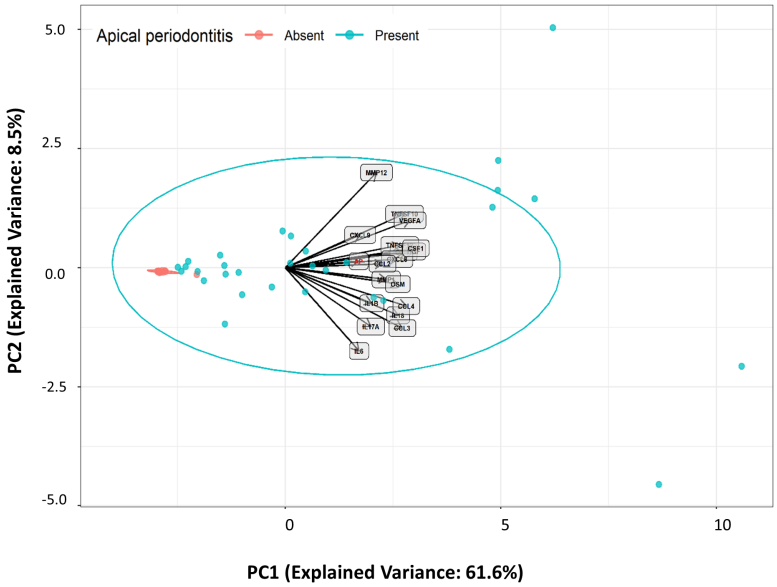

FIGURE 5 Principal component analysis evaluating the correlation of various analytes to each other and disease states. PC1 versus PC2 biplot.

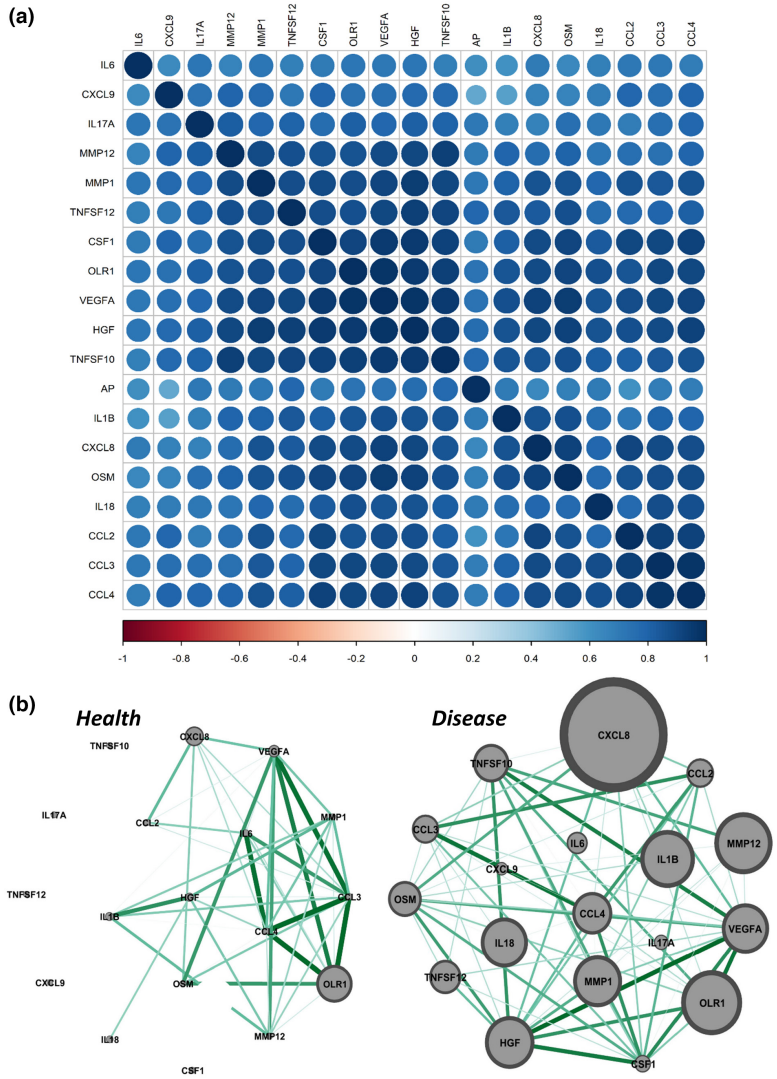

FIGURE 6 Heat map and network analysis graphs. (a) Seriated heat map demonstrating positive (blue) and negative (orange) correlations between analytes and presence of asymptomatic apical periodontitis. Variables plotted closer together were more similar. (b) Network analysis graphs demonstrating positive (green) and negative (red) correlations between analytes relative to disease state. Cytokines and significant correlations ($r > .75$; $p < .05$) were represented as nodes and connections, respectively. Node size was relative to the median concentration of analytes, and their degree of connectivity, with stronger associations depicted as darker and wider connections.

with the favourable properties of BSA, Tween20 and NaCl in that they preserve macromolecular integrity and reduce nonspecific protein binding (Mao et al., 2007; Steinitz, 2000). With respect to eluting techniques, vortexing alone revealed the greatest percentage of recovery. The high velocities of oscillating forces generated may thus be more suited to shedding proteins entrapped in the paper point meshwork or bound to plastic column walls than centrifugal forces (Zhou et al., 2006).

In vivo cross-sectional study

The Target-48 panel was selected due to several advantages over other arrays. Only 1 μL of sample was required to simultaneously quantify 45 cytokines, the proximity extension technology offered exceptionally high sensitivity and specificity and prior q-values demonstrate low false positive rates of analyte detection (Katz et al., 2022; Wik et al., 2021). Furthermore, pilot investigations confirmed that the eluting buffer or exudate matrix did not interfere with internal assay controls, as was observed with other immunoassays.

The present results nevertheless, still need to be interpreted with caution due to several methodological limitations. Most notably, it was not possible to normalize crude analyte values to TPC due to low yields found in NATs, an issue similarly observed by Zehnder et al. (2014) in dentinal tubular fluid. Normalizing to TFV, an alternative strategy utilized in several prior investigations (Ballal et al., 2017; Mente et al., 2016; Teixeira et al., 2022; Wahlgren et al., 2002;

Zehnder & Belibasakis, 2022) and suggested by Zehnder and Belibasakis (2022), may thus be necessary when comparing transudates to exudates. This approach, however, increases risk of type 1 errors as differences in analyte concentration may be overestimated, which is why initial alpha values were set to 0.01. The apical constrictions physiological diameter may also limit the volume of PTF that can enter into the canal and thus, be absorbed. Whilst teeth with a minor apical foramen width of ≤0.2 mm will have been standardized via the above protocol (Chapman, 1969), those naturally larger than this may, therefore, have introduced a sample bias. This could have contributed to the substantial variations observed in total absorbed volume. Attempting to standardize to diameters larger than this, however, may inadvertently introduce noxious insults to the periradicular tissues during root canal preparation and obturation. Other limitations include a small sample size, uneven recruitment rate and several unmatched baseline characteristics; all of which introduce selection bias. Contributing factors include convenient sampling and difficulties in attaining PTF from healthy teeth, as they were less frequently encountered and more prone to profuse apical bleeding. Cytokines demonstrating only modest concentration increases (i.e. IL-6, -17A and CSF-1) or a broad spread of data (i.e. CXCL-8 and MMP-12) may, therefore, be underpowered and their significance should be interpreted with caution. This, however, will not be applicable to all mediators with many exhibited highly significant differences.

The objective reference gold standard for confirming endodontic diagnosis is histological examination. As this is not clinically applicable, the presence or absence of periradicular disease in the current study was based on clinical and radiographic signs and symptoms outlined in the American Association of Endodontist guidelines (Glickman 2009). It is acknowledged greater accuracy could have been achieved with cone-beam computed tomography (Patel et al., 2009); however, this facility was not readily accessible. Therefore, there may have been PTF samples obtained from diseased teeth perceived to have NATs and to a lesser extent, vice versa. This was, however, unlikely as most control samples were retrieved from teeth undergoing elective root canal treatment and only unambiguous radiographic lesions were included in the AAP group. Moreover, to improve generalizability of the diagnostic data, it would have been ideal to perform the current investigation in a similar but independent cohort across several sites as per Grant et al. (2022). The present funding could not permit this and so a LOOCV statistical method was used to reduce overlap between training and test data during performance analyses. Each sample would have, therefore, mimicked an externally validating observation, ultimately leading to more robust and less bias AUC values.

The *in vivo* study consistently identified 18 analytes in PTF samples from teeth with AAP. These comprised of chemokines (CCL2, -3, -4, CXCL-8, -9) cytokines (CSF-1, IL-1β, -6, -17A, -18, OSM, TNFSF-10, -12), growth factors (HGF, VEGF-A), enzymes (MMP-1, -12) and receptors (OLR-1), all of which are of macrophage and/or T cell origin (Marton & Kiss, 1993; Silva et al., 2007). Their detection is supported by numerous immunohistochemical investigations into periapical lesions that have previously identified 12 of these biomarkers (Hasegawa et al., 2021; Kabashima et al., 2001; Nonaka et al., 2008; Tsai et al., 2008; Weber et al., 2019). Interleukin-1β, -6, -8, -17A and MMP-1 specifically have already been quantified at comparable levels in PTF (Virdee et al., 2019), with this study being the first to report on the remaining 13. Amongst this panel were several analytes with little prior association with apical periodontitis, including the pro-angiogenic and -regenerative HGF (Grant et al., 1993); IL-18, a modulator of lymphocytic activity (Nakanishi et al., 2001); MMP-12, otherwise known as elastase; OLR-1, an up-regulator of osteoclastogenesis (Ohgi et al., 2018); and TNFSF-10 and -12, both of which induce apoptosis (Kataria et al., 2010). Interestingly, several well-established pro-inflammatory mediators, namely Tumour Necrosis Factor [TNF]-α and Interferon [IFN]-γ, were undetected. These data conflicts with prior PTF studies and the high concentrations of CXCL-9 and IL-18 observed in this investigation, as the former upregulates expressions of both whilst the latter stimulates IFN-γ production (Kwak et al., 2005; Martinho et al., 2015; Nakanishi et al., 2001; Pezelj-Ribarić et al., 2007). One explanation for this may be that these analytes are more active in acute, as opposed to quiescent, phases of apical disease (Ferreira et al., 2016; Rechenberg et al., 2014).

When data were analysed for correlations, CXCL-8, IL-1β, OLR-1, OSM and TNFSF-12 were found to be highly associated with the presence of AAP. PTF-derived IL-1β has previously been correlated to several clinical symptoms characteristic of periradicular disease (Ataoğlu et al., 2002; Matsuo et al., 1994; Shimauchi et al., 1998); whilst GCF studies reported similar relationships for the remaining molecules; with exception of OLR-1 for which there is limited literature (Majeed et al., 2016). Moreover, network analyses revealed the diseased state to be significantly more complex than health. Numerous connections were present for any individual mediator, suggesting a capacity for the periradicular inflammatory response to adapt and persist. This reflects the complexity of the periapical disease process, which is not yet fully understood, and makes it difficult to identify an individual analyte to therapeutically target. Notable correlations that add validity to the data were found between CCL-2, -3 and -4, all of which share similar receptors and have demonstrated

cross-functionality (Repeke et al., 2010); and HGF and VEGF-A, with the former known to upregulate expression of the latter during wound healing (Reisinger et al., 2003). Similar associations requiring further investigation include VEGF-A and TNFSF-10; VEGF-A and OLR-1; and HGF and CSF-1.

The present diagnostic analysis reveals PTF as being a valid source of biomarkers for distinguishing periradicular disease from health. This objective information would be clinically useful for confirming the resolution of periapical inflammation immediately prior to obturation, particularly considering treatment outcomes are currently determined via temporal analysis of plain-film radiographs (ESE, 2006). Specific clinical situations that would benefit include orthograde treatment of larger lesions where root end surgery may be anticipated or assisting diagnosis of nonspecific orofacial pain in patients with previously initiated root canal treatment. Generally, PTF-derived analytes independently exhibited excellent sensitivity, but it is acknowledged that they lacked the same levels of specificity thus clinically risking over treatment. This was, however, abated when a combination of consistently present biomarkers were used, as the PCA-derived cluster increased diagnostic accuracy and precision (AUC: 0.96 [95% CI: 0.89–1.00]). These observations were previously observed by Grant et al. (2022) for combinations of GCF-derived peptides and also conform to the recently published guidelines for biomarker analysis which promotes the notion of biosignatures (Zehnder & Belibasakis, 2022). Of note, when analytes were evaluated individually, TNFSF-12 possessed the most discriminatory power and so according to this study is considered the most reliable individual biomarker for AAP. This reflects other diagnostic reports for marginal periodontal and peri-implant diseases (Yakar et al., 2019). Further independent analyses, however, need to be conducted to externally validate this data.

CONCLUSION

This two-part study optimized PTF sampling protocols *in vitro* and applied them clinically, alongside a high-throughput panel, to characterize the proteome of healthy and inflamed periapical tissues. A complex interconnected network of 18 potential biomarkers were identified, with CXCL-8, IL-1β, OLR-1, OSM and TNFSF-12 being most associated with the presence of AAP. Both TNFSF-12 and this overall cluster exhibited an excellent diagnostic ability to discriminate periradicular disease from health. The latter could thus be considered a biosignature for AAP. Longitudinal investigations with larger sample sizes are warranted to validate findings and correlate these mediators with long-term treatment outcomes. Furthermore, cell culture experiments to determine the exact roles of OLR-1, OSM, TNFSF-10 and -12 in periradicular pathophysiology are also required.

AUTHOR CONTRIBUTIONS

S. S. Virdee involved in conceptualization, data curation, funding acquisition, investigation, visualization, writing the original draft preparation, review and editing. **N. Z. Bashir** involved in formal analysis, investigation, visualization, writing the original draft preparation, review and editing. **M. Krstic** involved in data curation, investigation, visualization, review and editing. **M. M. Grant** involved in conceptualization, formal analysis, investigation, methodology, resources, supervision, review and editing. **J. Camilleri** involved in conceptualization, funding acquisition, supervision, review and editing. **P. R. Cooper** involved in conceptualization, funding acquisition, methodology, supervision, review and editing. **P. L. Tomson** involved in conceptualization, funding acquisition, supervision, review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors deny any conflicts of interest related to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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CHAPTER 6

THERAPEUTIC IRRIGATION PROTOCOLS FOR TREATING APICAL PERIODONTITIS (TIPTAP)

Publication 8: Virdee, S. S., Bashir, N. Z.., Grant, M. M., Cooper, P. R., & Tomson, P. L. (2024). Therapeutic Irrigant Procedures for Treating Apical Periodontitis (TIPTAP): a triple blinded parallel group randomised controlled phase I/II trial. *International Endodontic Journal*, (Submitted).

The body of work presented in this chapter presents a clinical randomised controlled trial that was aimed at investigating the main hypothesis presented in this thesis implementing all of the aforementioned findings. More specifically, the clinical protocol introduced in Chapter 1, which promotes dECMs solubilisation was tested against a conventional antimicrobial only irrigant regime during root canal treatment of mature permanent teeth diagnosed with a necrotic pulp and asymptomatic apical periodontitis. Proof of concept for this approach was demonstrated in Chapter 2, which also largely informed the irrigation protocol. Whilst the absence of NaOCl was initially concerning, findings from Chapter 3 provided evidence that chemomechanically debriding root canals with 17% EDTA had antimicrobial capability. Nevertheless, patient reported pain scores were recorded as a secondary outcome measure. The longitudinal

analyses of PTF that was used to detect potential subclinical changes in periradicular inflammation was optimised in Chapter 4. The findings of this study (Publication 8) could have significant implications on regenerative endodontic protocols.

CHAPTER 6

PUBLICATION 8

THERAPEUTIC IRRIGANT PROCEDURES FOR TREATING APICAL PERIODONTITIS (TIPTAP): A TRIPLE BLINDED PARALLEL GROUP RANDOMISED CONTROLLED PHASE I/II TRIAL

Satnam Singh Virdee, Nasir Bashir, Melissa Gant, Paul Cooper & Phillip Tomson

A version of this manuscript has been submitted for publication in the

International Endodontic Journal

Abstract

Background: Solubilised endogenous dentine extracellular matrix components (dECMs) are potent mediators in pulp regeneration and could potentially promote similar healing effects in diseased periradicular tissues by upregulating local mesenchymal stem cell-derived regenerative events.

Aims: 1) Determine if endodontic treatment outcomes with irrigation regimes promoting dECM release (17% ethylenediaminetetraacetic acid [EDTA]) are equivalent to conventional regimes (5.25% sodium hypochlorite [NaOCl]) in mature permanent teeth with asymptomatic apical periodontitis. 2) Explore changes in pain scores; expressions of periradicular tissue fluid (PTF) derived-inflammatory mediators; and lesion volume between the different irrigant regimes.

Methods Forty single rooted teeth, from 37 healthy adults, were block randomised into parallel groups of irrigation with either 17% EDTA, optimised for dECM solubilisation, or 5.25% NaOCl (*n*=20). All other endodontic procedure were standardised over two-visits with 14-days calcium hydroxide intracanal medicament. Patient reported pain scores were recorded at six-hours and then daily for one-week post-instrumentation and post-obturation. PTF samples were collected pre-instrumentation and pre-obturation, where analyte profiles (pg/TPC) were determined using O-link Target-48 cytokine array. Treatment outcomes were clinically and radiographically assessed with cone-beam-computer-tomography at one year using dichotomous criteria (favourable/unfavourable) based on volumetric change in lesion size. Participants, operators and assessors were blinded, and per-protocol analyses conducted using binary logistic regression models with initial alpha values for statistical comparisons set at $p<0.05$.

Results: A 90% recall rate was achieved at one-year (NaOCl:19; EDTA:17). Favourable outcomes were observed in 89.5% of treatments using NaOCl and 94.1% of treatments using EDTA irrigation, with median lesion volume reductions of 92.5%(IQR:67.33-99.13) and 95.84%(IQR:78.81-100), respectively (p>0.05). Odds of unfavourable periradicular healing with EDTA irrigation was 0.53 [95%CI:0.04-6.44; p>0.05]. No serious adverse effects or atypical pain patterns were reported, although two acute exacerbations occurred post-instrumentation with NaOCl irrigation (p>0.05). Target-48 panels consistently detected 15 inflammatory analytes in both groups (CCL-2,-3,-4; CSF-1; CXCL-8; HGF; IL-1β,-6,-18; MMP-1,-12; OLR-1; OSM; TNFSF-10; VEGF-A), all of which reduced pre-obturation. At this stage, IL-6 and -18 were significantly more abundant in the intervention group (p<0.05).

Conclusions: Therapeutic irrigant regimes promoting dECM solubilisation resulted in one-year treatment outcomes equivalent to conventional irrigant protocols with no serious adverse effects reported.

Conflicts of Interest: Nil

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Introduction

Apical periodontitis is an inflammatory condition of the periodontium affecting 52% of the global population (Tibúrcio-Machado et al. 2021). It is initiated when endodontic micro-organisms stimulate the host response, a process mediated by numerous auto- and para-crine signalling molecules that are detectable within periradicular tissue fluid (PTF; Nair 2004, Márton & Kiss, 2014, Virdee et al. 2019). Current therapeutic strategies focus solely on the microbial component of this dynamic equilibrium, where a pro-healing micro-environment is established via chemomechanical debridement. Whilst this antimicrobial strategy may enable a highly co-ordinated sequence of periradicular regenerative events, which are driven by local mesenchymal stem cell (MSC) responses, this approach provides no direct biological stimulus (Lin & Rosenberg 2011). This could explain why although there have been advances in technology and materials, clinical outcomes for root canal treatment have remained stagnant for five decades. More specifically, one in five infected teeth consistently fail to heal (Ng et al. 2007). This could also account for why success rates decrease in larger lesions but remain static despite increasingly aggressive disinfection protocols (Ng et al. 2011, Paredes-Vieyra et al. 2012, Liang et al. 2013, Verma et al. 2019). Therefore, persistent periapical lesions may not only represent an inability to reduce the endodontic microbial load below an unknown critical threshold, but also an inadequate capacity to stimulate physiological regeneration (Siqueira & Rôças 2008). A failure to improve predictability in treatment of periradicular disease has resulted in significant interest in the development of more biologically driven treatment strategies for apical periodontitis, where greater emphasis is placed on utilising local MSC-based regenerative events that lead to periradicular tissue healing (Kim et al. 2018).

In recent years, a novel niche of dental MSCs has been identified from within human periradicular granulation tissue (Liao et al. 2011). *In vitro,* these periapical lesion-derived MSCs (PL-MSC) have demonstrated tremendous immunosuppressive and regenerative capabilities with strong commitment towards an osteogenic lineage (Liao et al. 2011, Dokić et al. 2012, Marrelli et al. 2013). For these reasons, these cells have been implicated as the key determinants of the periapical healing process following root canal treatment. Their presence therefore provides clinical opportunities to directly enhance local tissue regenerative events in mature permanent teeth via a cell-free homing approach (Chrepa et al. 2015, Virdee et al. 2022, Lyu et al. 2022). This highly translatable approach, thus far only utilised in pulp regeneration of immature teeth, would involve upregulating PL-MSC *in situ* by way of stimulating damaged periradicular tissues with signalling molecules responsible for co-ordinating physiological healing events (Kim et al. 2018).

Notably, there is an abundant reservoir of odontoblast-derived growth factors, cytokines, chemokines and other signalling molecules locally sequestered within the dentine's extracellular matrix during tooth development (Smith et al. 2012). Key examples include transforming growth factor-beta 1 [TGF-β1], often found at the highest abundance and used as a surrogate quantitative marker for this cocktail of molecules and their inherent bioactivity (Graham et al 2006, Tomson et al 2007 & 2017); as well as bone morphogenetic protein [BMP]; vascular endothelial growth factor [VEGF]; and insulin growth factor [IGF] amongst many others (Park et al 2009, Jágr et al 2012). Proteoglycan bonds formed following dentinogenesis preserve these fossilised morphogens within the dentine however, they can be released under appropriate local tissue conditions and their bioactive properties clinically harnessed using common endodontic irrigants (Baker et al 2009, Galler et al. 2015). Of the

various solutions thus far investigated, the chelating action, neutral pH and protease inhibiting properties of ethylenediaminetetraacetic acid (EDTA) currently make it the gold standard for releasing dentine matrix protein bioactivity (Tavares et al. 2021). The yield can be further enhanced when EDTA is ultrasonically activated however associated bioactivity becomes significantly reduced or even completely eliminated when dentine is pre-treated by even relatively weak concentrations of sodium hypochlorite (NaOCl; Galler at al. 2015, Widbiller et al. 2017). Whilst re-instrumentation could debride some of the dentine penetrated by NaOCl, which can range from 38 - 411 μm (Virdee et al. 2020), this can further weaken tooth structure and still results in considerably less dentine matrix components being solubilised than when EDTA is exclusively used (Widbiller et al. 2022). This outcome can be attributed to its non-specific proteinaceous properties that make the most commonly administered irrigant in endodontics, incompatible with the integrity of local tissue bioactive molecules. The resulting EDTA-derived dentine tissue extracts nevertheless, termed dentine extracellular matrix components (dECM), demonstrate a potent capacity to promote regenerative activities within multiple endodontic MSC niches (Virdee et al 2021). Similar effects were also recently demonstrated in PL-MSCs (unpublished data). Furthermore, the synergistic activity of this cocktail exhibits a greater potency than application of single recombinant morphogens; with their endogenous nature also overcoming many ethical issues associated with clinically using exogenous substitutes (Bègue-Kirn et al 1992, Lee et al. 2015, Widbiller et al. 2018).

To date, these previous findings have translated into the development of novel therapies for managing deep carious lesions and the exposed pulp as well as providing proof of concept for the cell-free homing approaches to treating apical periodontitis

(Duncan et al. 2019, Smith et al. 2016, Murray et al. 2002). Consequently, it has been hypothesised that therapeutic irrigant regimes optimised to liberate dECMs into root canals, and the periradicular tissues thereafter, could upregulate PL-MSCs bioactivity to promote tissue healing in mature permanent teeth diagnosed with apical periodontitis (Virdee et al. 2022). At the time of conducting the present investigation, there were no prior interventional clinical studies which had tested this hypothesis.

Aims

The primary aim of this triple blinded parallel group randomised controlled phase I/II trial was to determine if the treatment outcomes (O) of root canal therapy using conventional irrigant regimes (C; 5.25% NaOCl) were equivalent to those promoting release of dECMs (I; 17% EDTA) in mature permanent teeth diagnosed with a necrotic pulp and asymptomatic apical periodontitis (P). Secondary objectives included exploring the longitudinal differences in (1) patient reported pain scores post-instrumentation and post-obturation; (2) PTF-derived inflammatory mediator concentrations; and (3) periapical lesion volume change between irrigant regimes. The null hypothesis tested was that there were no significant differences in endodontic success rates between conventional irrigant regimes and those that promote release of dECM.

Material & Methods

Ethics: This interventional study was granted ethical approval from the National Health Service West Midlands Research Ethics Committee (reference no. 19/WM/0149) and reported in accordance with the 2019 Preferred Reporting Items for Randomized Trials in Endodontics (PRIRATE) guidelines (Nagendrababu et al. 2019;

Figure 1). The protocol was registered *a priori* on the International Standard Randomized Controlled Trial Number (ISRCTN) Registry (ISRCTN93101288). Informed written consent was obtained from volunteering patients at their initial consultation following provision of detailed verbal and written information on the purposes, methods and risks. This was confirmed again via a telephone conversation one week later after which consent forms were stored in the site file and the referring practitioner informed of participation by written correspondence.

Study design and setting: A triple blinded parallel group randomised controlled phase I/II trial of equivalence with a 1:1 allocation ratio was conducted in the Endodontic Department at the Birmingham Dental Hospital between October 2019 and March 2024. A public and patient involvement group consisting of five non-dental members who had undergone root canal treatment provided input into the planning and design of the study. There were no methodological amendments after the trial commenced.

Participant selection criteria: Medically fit consenting adults (≥ 18 years) referred to the Endodontic Department at the Birmingham Dental Hospital for root canal treatment in mature permanent single rooted teeth diagnosed with a necrotic pulp and asymptomatic apical periodontitis were consecutively recruited. The exclusion criteria consisted of individuals who were: unwilling or unable to consent, pregnant or breast feeding; had undergone antimicrobial therapy 3 months prior to screening; immunocompromised (i.e. diabetes mellitus, human immunodeficiency virus, leukaemia, neutropenia, undergoing chemo- or systemic corticosteroid therapy); had altered bone metabolism (i.e. bisphosphonate or monoclonal antibody therapy, head and neck radiotherapy). Teeth that were multirooted; symptomatic; previously accessed or root filled; exhibited clinical signs and symptoms consistent with pulpitis

or periapical abscess; had periodontal pocketing ≥ 5 mm within the same sextant; unable to retain a rubber dam; had apices that were immature or closely associated with the maxillary sinus; presented evidence of internal or external root resorption or vertical and horizontal root fractures, were also excluded.

Pre-operative assessment: All diagnostic assessments were performed by a single operator (SSV). Briefly, a comprehensive pain, medical and dental history was taken to confirm absence of symptoms, aetiology of disease and potential medical contra-indications to participation. This was followed by a systematic extra- and intra-oral hard and soft tissue examination where the selected tooth was subjected to a focused visual (restorability; restoration presence, type and quality; presence of caries, cracks and fractures), periapical (tenderness to percussion or palpation; presence of sinus or swelling), periodontal (six-point probing depths, mobility) and occlusal (static and dynamic) assessment. This was supplemented with thermal (Endo-Frost; Roeko, Maribor, Slovenia) and electric (SybronEndo; Sybron Dental Specialities Inc., CA, USA) pulp testing with reference to a healthy contralateral tooth.

All potentially eligible teeth underwent digital long cone periapical (LCPA) radiographic assessment. These were obtained via a paralleling technique with rectangular collimation using phosphor plate sensors (Dürr Dental, Bietigheim-Bissingen, Germany) positioned intra-orally with Rinn short arm (Dentsply Sirona) aiming devices to improve standardisation. Phosphor plates were then exposed at 70 kV, 7.0 mA and 0.08 sec. exposure time with a wall mounted intra-oral x-ray unit (Heliodent Plus; Dentsply Sirona) prior to being processed using the VistaScan system (Dürr Dental). Teeth with a necrotic pulp and asymptomatic apical periodontitis were defined as those that were clinically unresponsive to pulp, percussion or palpation testing but

radiographically demonstrated a periradicular radiolucency with no associated sinus (Glickman, 2009).

After confirming eligibility and attaining informed consent, a baseline limited field of view (4 x 4 cm) cone beam computer tomograph (CBCT; 3D Accuitomo 170; J Morita, Kyoto, Japan) was acquired. Operating parameters were set at 85 kV, 4.5 mA and 17.5 sec. exposure time with a 360^{o} arc of rotation and 250 µm voxel size. Volumes were reconstructed to slice thicknesses of 1 mm and reformatted for clinical use on Enterprise Imaging Software (AGFA Healthcare; Mortsel, Belgium).

Sample size: A sample size of 40 teeth (n = 20 per group) was selected based on that of similarly designed studies published within the fields of endodontic tissue engineering (Xuan et al. 2018, Arslan et al. 2019, Jha et al. 2019, Brizuela et al. 2020). This is also consistent with the minimum criteria specified for inclusion within the European society of Endodontology (ESE) S3-level clinical practice guidelines (Duncan et al. 2023).

Randomisation, concealment and blinding: Block randomisation was performed at the tooth level using the online tool, Sealed Envelope ((https://www.sealedenvelope.com/). A computer-generated code list consisting of random permutated block sizes of four or six with a 1:1 allocation ratio was utilised and concealed from researchers. At the time of randomisation, immediately prior to root canal treatment, the lead investigator (SSV) entered the participant identification number into the Sealed Envelope tool. The coded group allocation was then returned. The nature of the intervention was such that the operator, participant and assessors could all be blinded. More specifically, participants received root canal treatment with one of two clear irrigant solutions. These were coded from the operator and assessors,

the former of who delivered them into root canals via an identical regime with respect to volume, duration, activation and vehicle. In addition, a coded opaque bottle was used to store these irrigants, operator masks were fragranced with eucalyptus oil (Cerkamed, Stalowa Wola, Poland) to disguise odours and plastic patient and bracket table coverings were used to prevent accidental sodium hypochlorite bleaching spots.

Groups: The control group, which followed principles of a conventional anti-microbial approach, consisted of performing root canal treatment exclusively using the 5.25% NaOCl (Cerkamed) irrigant solution. Conversely, the intervention group comprised of solely administering 17% EDTA (Cerkamed) throughout the procedure. This regime was informed by precursory *in vitro* methodological work up investigations that demonstrated intracanal solubilisation and subsequent periradicular bioavailability of dentine extracellular matrix proteins (Virdee et al. [submitted]).

Clinical procedure: All root canal procedures were performed by a single endodontically trained operator (SSV) under local anaesthesia (2% xylocaine and 1:80 000 adrenaline; Dentsply Sirona) using a dental operating microscope (Global Surgical Corporation, SL, USA) and over two-visits in accordance with ESE (2006) quality guidelines.

Visit 1: Crowns were initially stabilised by removing caries and any defective restorations were replaced (definitive direct restorations - Clearfil Majesty.; Kuraray Dental, Okayama, Japan / temporary indirect restorations - Protemp; 3M, Minesota, USA) when indicated. Teeth were subsequently isolated using rubber and liquid dam (Liquidam, Cerkamed) and traditional stable four-walled access cavities were created using sterile cooled diamond burs under high-volume aspiration. A 10/0.02 K-Flex file (Dentsply Sirona) was then advanced down canals whilst connected to a Dentaport

ZX electronic apex locator (J Morita, Osaka, Japan) to confirm patency and determine positions of the apical foramen and constriction as per the zero- (i.e. terminal green bar) and 0.5-markings (i.e. fifth green display bar), respectively (Connert et al. 2018). These were supplemented with LCPA radiographs and when file tips were ≥ 2 mm from the radiographic apex or extruded, length modifications were informed by a third electronic apex locator reading. Canals were then pre-flared via crown-down watch winding of 20/0.02 K-flex files to the zero-reading, dried using sterile 25/0.02 paper points set 2 mm short of this length, and patency filed with a 10/0.02 to disrupt dentinal debris apically and encourage intraradicular influx of tissue fluid. Baseline (S1) samples of PTF were then collected prior to any irrigation using an optimised paper point sampling protocol described previously (Virdee et al. 2023).

Root canals were chemomechanically prepared in a crown-down manner up to 40/.06 (Protaper Gold; Dentsply Sirona) at 300 rpm and 4 N torque. During instrumentation, 2 mL of 5.25% NaOCl (control) or 17% EDTA (intervention) solution were administered at room temperature between files using light steady finger pressure on a 27-gauge side vented needle (Covidien, Dublin, Ireland) set 2 mm short of working length (WL). Thereafter, 5 mL of irrigant was deposited into canals and allowed to remain undisturbed for 4 minutes and 30 seconds before 30 seconds passive ultrasonic irrigation with a 20/0.02 Irrisafe tip (Acteon, Norwich, UK) and Satelec (Acteon) at half power 1 mm from WL. This 5-minute irrigant regime was repeated twice more prior to administering calcium hydroxide (UltraCal XS; Ultradent Products, SJ, USA) intracanal medicament via 27-gauge side vented Navitip needle (Ultradent) set 2 mm from WL and temporising the access cavity with Kalzinol (Dentsply Sirona). If experienced, acute exacerbations of pain were managed by re-irrigating canals with 15 mL of the assigned irrigant on a separate emergency visit.

Visit 2: After 14-days, teeth were isolated, re-accessed and the calcium hydroxide intra-canal medicament wicked away with sterile 25/0.02 paper points. The previously described 5-minute irrigant regime was repeated three times prior to drying canals, collecting a second sample of PTF (S2), radiographically confirming master cone length and obturating with gutta-percha and an epoxy resin-based sealer (AH plus; Dentsply Sirona) via a continuous wave warm vertical condensation technique (B&L BioTech, VA, USA). Access cavities were immediately restored with a 2 mm subseal of bulk-fill resin composite (SDR; Dentsply) followed by hybrid composite (Clearfil Majesty; Kuraray Dental). An immediate post-operative LCPA was taken and where necessary, the patient's referring practitioner was asked to provide a definitive indirect cuspal coverage restoration.

Treatment review: A 12-month post-treatment recall was conducted where teeth were clinically assessed and radiographically exposed to LCPA and CBCT's to review for signs and symptoms of active periradicular pathology. These procedures were conducted using the same methods and parameters described as the initial consultation.

Primary outcome measure:

Endodontic Treatment Outcome (n; %): Outcomes for endodontic treatment were clinically and radiographically determined for both LCPA and CBCT imaging techniques using dichotomised loose [favourable vs. unfavourable] and strict [success vs. unsuccessful] criteria (Ng et al. 2011). Favourable outcomes were defined as those where teeth survived the recall interval and presented with an absence of symptoms and clinical signs of inflammation including tenderness to percussion, palpation, swellings or sinus tracts alongside radiographic evidence of complete or incomplete

healing, which represented normal periodontal ligament space around the root or a reduction in lesion size, respectively. Success was defined as per the above description but exclusively with radiographic evidence of complete healing. Extractions for any reason following the initial treatment visit were considered unfavourable and unsuccessful in the final analyses.

Analyses were conducted by a concensus panel consisting of two endodontically trained assessors (SSV & NZB) that were experienced in interpreting LCPA and CBCT images over two face-to-face sessions. One appointment was dedicated to evaluating treatment outcomes based on LCPA radiographs and the second, taking place four weeks later, on CBCT scans which were available in their entirety to align the vertical plane of roots centrally and parallel to the sagittal and coronal views. All images were randomised and viewed at optimal contrast and brightness via Enterprise Imaging Software (AGFA Healthcare) in a dimly lit room. Both assessors were blinded to treatment protocols and pre-calibrated using 20 LCPA and 20 CBCT reference radiographs that were not part of the present study. These were identified by an oral and maxillofacial radiologist, represented teeth with or without periapical lesions and used to confirm inter-rater reliability of > 90% prior to participant data analysis.

Follow up radiographs were scored against four possible periapical healing outcomes as described by Ng et al. (2011; complete, incomplete, uncertain and failure). Periapical lesions were defined as present when periapical radiolucencies were at least twice the width of the periodontal ligament space and considered reduced or increased when lesion length (mm) across the longest diameter or volume (mm^3) changed by at least 20% (Low et al. 2008, Liang et al 2014). The panel discussed discrepancies to arrive at a concensus and in cases of disagreement, a third

endodontically trained assessor (PLT) was consulted and arbitrated over the final decision.

Secondary outcome measures:

i) Patient reported pain scores (n): To explore any differences in patient reported outcomes between irrigant regimes, a numerical rating scale (NRS) was used to evaluate the level of pain participants experienced at 6 hours and then every 24 hours up to 7 days post-instrumentation (visit 1) and -obturation (visit 2). After visual and verbal explanation to facilitate use, individuals were instructed to report their discomfort by marking a point on a 10 cm line, anchored in two opposing extremes of “no pain” [0] and “pain as bad as it could be” [10], at the pre-specified times. This was supplemented with a binary question relating to analgesic use. These numerical scores were then categorised into none [0]; mild [1–3]; moderate [4–6]; and severe [7–10], which was presented as cumulative frequencies for each stratum per irrigant regime (Jalalzadeh et al.2010).

ii) Periradicular tissue fluid analysis (pg/TPC): Coded baseline and pre-obturation PTF samples were analysed using standard protocols previously described by Virdee et al. (2023) to explore if irrigant regimes differentially influenced periradicular inflammatory mediator profiles. Briefly, total volume (µL) was initially calculated via pre-determined wetted length (mm) to volume (µL) standard curves followed by total protein concentration (TPC; µg/mL) using the Bradford dye-binding assay (Thermo Fisher Scientific, MA, USA) against a standard series of bovine serum albumin with a plate reader set at a wavelength of 595 nm (Tecan Spark; Lifesciences, Switzerland). The Target-48 Panel (O-link, Uppsala, Sweden) was then utilised to quantify the concentration (pg/mL) of 45 different proteins related to the immune and inflammatory

response within each sample. All assays were performed in duplicate as per manufacturer's instructions with all outputs normalized to TPC and presented as pg/TPC. Inflammatory markers were considered absent, and excluded from analyses, when concentrations were below the lower limit of detection in > 25% of test PTF samples (Virdee et al. 2023). Data points were assigned zero and maximum values when readings were below or above the respective limits of detection.

iii) Percentage change in periapical lesion volume (mm^3; %): To explore differential rates of healing between irrigant regimes, three-dimensional (3D) changes in periapical lesion volume (mm^3) were determined independently by two pre-calibrated assessors (SSV & NZB) using publicly available ITK-Snap (V4.0.0) software and the semi-automated methods described by Saini et al. (2022). Firstly, periapical regions of test teeth within coded CBCT reconstructions were segmented across the axial, coronal and sagittal planes to encompass lesions. Secondly, upper and lower threshold limits were agreed amongst assessors and applied, after which the automated spherical filler “bubble” function was utilised to initialise the contour. Thirdly, this “bubble” was evolved repeatedly until the lesion was filled with manual adjustments made via the “paintbrush” function to eliminate over and under extensions. Thereafter, the software allowed this volume to be rendered in 3D and calculated in mm^3 units. Resolved lesions were assigned a zero count with values from the two assessors averaged when within 2 mm^3. Larger discrepancies however were resolved through discussion and combined re-analysis until readings fell within this range. Percentage change in periapical lesion volume was then calculated for each case (i.e. percentage change in lesion volume [%] = [pre-operative lesion volume – post-operative lesion volume] / pre-operative lesion volume × 100).

Statistical analysis: All coded data were statistically analysed per protocol in 'R' software (V.4.1.0; R Foundation for Statistical Computing, Vienna, Austria) by NZB. Interclass correlation coefficients (ICC) and Cohen's Kappa statistic were conducted to assess intra- and inter-rater reliability for determining treatment outcome and periapical lesion volume, respectively. All paired and non-paired categorical data comparisons were made with *McNemar's* and *Chi Squared* tests with data presented as frequencies alongside percentages. For quantitative data, an initial normality screen conducted via the Shapiro-Wilk test revealed a majority non-normal distribution. Outcomes were thus presented as medians [interquartile range] with paired and non-paired comparisons made with independent samples *Mann-Whitney U, Wilcoxon Matched Pair* and *Kruskal-Wallis* tests followed by *post-hoc* pairwise comparison and Bonferonni family wise error rate correction with initial alpha values set at 0.05. Associations between the interventional protocol and an unfavourable treatment outcome using strict and loose criteria were investigated using generalised linear mixed models. These binary logistic regression models were fit using random effects to account for patient-level clustering where more than one tooth was included per patient. Coefficients were exponentiated to obtain odds ratios (ORs), with respect to the conventional protocol, alongside corresponding 95% confidence intervals (CIs) and *p* values. Only after completion of all statistical analyses was data uncoded.

Results

Study characteristics: The present study spanned the period from October 2019 to March 2024 with participant recruitment conducted between October 2019 and February 2023 and reviews between January 2021 and March 2024. Participant enrolment, allocation, follow-up and analyses are summarised in Figure 1. Overall, 40

teeth from 37 participants were enrolled into the trial after screening 48 potential candidates, of which 20 were randomised into the NaOCl and EDTA treatment groups with one patient having two teeth enrolled into the two separate arms. All individuals were treated as intended however, 4 teeth (NaOCl: 1; EDTA: 3) were lost to follow up as 1 patient deceased and 3 failed to return leaving a recall rate of 90% (NaOCl:19; EDTA:17) for per protocol analyses.

Participant characteristics: The participant demographic and clinical characteristics at recall are summarised in Table 1. In brief, and at the tooth level, the median age was 36 years [25-43] (NaOCl: 37 [29-47]; EDTA: 36 [23-45]) with a 1:1 male-to-female ratio per group. Seven participants (NaOCl: 2; EDTA: 5) were of Afro-Caribbean descent, 9 Asian (NaOCl: 4; EDTA: 5) and 20 Caucasian (NaOCl: 13; EDTA: 7). Seven (NaOCl: 5; EDTA: 2) teeth were from the mandible and 29 (NaOCl: 14; EDTA: 15) maxilla teeth with incisors being the commonest tooth (33; NaOCl: 18; EDTA: 15) followed by canines (2; NaOCl: 1; EDTA: 1) and premolars (1; NaOCl: 0; EDTA: 1). Seven teeth presented coronally with direct restorations (NaOCl: 3; EDTA: 4) and 6 being indirect (NaOCl: 4; EDTA: 2) with the remainder being unrestored (23; NaOCl: 12; EDTA: 11). Traumatic dental injury was the most common indication for root canal treatment (16; NaOCl: 8; EDTA: 8) followed by iatrogenic injury (8; NaOCl: 4; EDTA: 4), idiopathicity (8; NaOCl: 4; EDTA: 4), deep carious lesions (2; NaOCl: 1; EDTA: 1) and Ohler's class 1 dens-in-dente congenital abnormality (2; NaOCl: 2; EDTA: 0) with a median pre-operative lesion volume of 74.6 mm^3 (NaOCl: 78.9 [46.51 – 131.28]; EDTA: 66.8 [47.21 – 116.35]. Peri-operatively, patency was achieved in all canals without perforations and all root fillings were of adequate quality and length however, two teeth in the NaOCl group experienced an intra-operative flare-up and had to be scheduled for emergency appointments. At review, the median recall interval was 13

[12.0 – 14.0] months (NaOCl: 13 [12.0 – 13.5]; EDTA: 13 [12.0 – 14.0]) with the coronal seal in all teeth remaining intact. Both groups were statistically matched across all clinical and demographic characteristics ($p > 0.05$).

Root canal treatment outcome: The intra- and inter-rater agreements for determining treatment outcome using LCPAs and CBCTs exceeded 0.90 for both categorical and dichotomous criteria, with results summarised in Table 2. All participants were clinically asymptomatic at recall. Based on CBCT imaging and loose criteria, favourable outcomes were observed across 16 (94.1%) test and 17 (89.5%) control single rooted teeth, with odds of an unfavourable outcome in the former being 0.53 [95%CI: 0.04 – 6.44]. For strict criteria, success from CBCT analysis was observed in 8 (47.1%) test and 7 (36.8%) control teeth with odds of failure for the intervention being 0.66 [95% CI: 0.17 – 2.49] for 17% EDTA. When considering LCPAs, favourable outcomes were observed in 16 (94.1%) test and 18 (94.7%) control teeth with odds of an unfavourable outcome in the former being 1.13 [0.06 – 19.50]. Finally, for strict criteria in LCPAs, success was observed in 9 (52.9%) test and 11 (57.9%) control teeth with odds of failure being 1.22 [95%CI: 0.33-4.56] for 17% EDTA. No significant differences were detected between the two irrigant regimes for LCPAs or CBCTs ($p > 0.05$).

Patient reported pain scores: Post-instrumentation, 19 (95%) and 20 (100%) participants within NaOCl and EDTA groups, respectively returned valid NRS pain scores, whereas data from 19 (95%) control and 18 (90%) test patients were available post-obturation with results summarised in Figure 2. No inter-group comparisons detected statistically significant differences at either treatment stage with pain incidence in the former (NaOCl: 9 [47.4%]; EDTA: 11 [55.0%)] and latter (NaOCl: 5 [26.3%]; EDTA: 4 [22.2%]; $p < 0.05$). Notably, pain severity post-instrumentation was

milder to moderate in the EDTA group (8 [40.0%]) than NaOCl (4 [21.0%]), which was characterised by more severe discomfort (NaOCl: 5 [26.3%]; EDTA: 1 [5.3%]) for longer durations (NaOCl: 7 days; EDTA: 5 days) and greater use of analgesics (NaOCl: 8 [40.0%]; EDTA: 4 [20.0%]). This contrasted post-obturation NRS scores for both groups where there was less incidence (NaOCl: 5 [26.3%]; EDTA: 4 [22.2%]), milder severity (mild-moderate NaOCl: 3 [15.7%]; EDTA: 4 [22.2%]), shorter duration (NaOCl: 2 days; EDTA: 1 day) and less analgesic intake (NaOCl: 2 [10.5%]; EDTA: 2 [11.1%]). Participants most commonly experienced pain 6 hours after each treatment stage.

Periradicular tissue fluid analysis: PTF samples were collected from all participants at each stage of treatment (100%) with results summarised in Table 3 and Appendix 1. Whilst intra-group comparisons revealed significant reductions in paper point wetted length, total absorbed volume and TPC from baseline to pre-obturation ($p < 0.001$), there were no significant differences in these characteristics between groups ($p > 0.05$). The Target-48 panel consistently detected 15 of a potential 45 analytes including Chemokine Ligand [CCL]-2, -3 and -4; Colony Stimulating Factor [CSF]-1; Hepatocyte Growth Factor [HGF]; Interleukin [IL]-1β, -6, -8 [CXCL-8] and -18; Matrix Metallopeptidase [MMP]-1 and -12; Oxidized Low Density Lipoprotein Receptor [OLR]-1; Oncostatin M [OSM]; Tumour Necrosis Factor Superfamily [TNFSF]-10 and Vascular Endothelial Growth Factor [VEGF]-A, all of which were similar in abundance between groups at baseline ($p > 0.05$). Nevertheless, whilst all analyte concentrations reduced prior to obturation, there were a greater number of significant reductions within the EDTA group (CCL-2; -4; CSF-1; HGF; IL-1β; CXCL-8; OLR-1; OSM; TNFSF-10; VEGF-A] compared with the NaOCl group (HGF; IL-1β;.-18; OLR-1; OSM; TNFSF-10; $p > 0.05$). Conversely, the EDTA group had a statistically higher concentration of

IL-6 and IL-18 than the control samples at this stage ($p < 0.05$), where the most abundant cytokines for both groups were MMP-12 (NaOCl: 261.8 [22.00 – 520.40]; EDTA: 111.0 [12.36 – 460.78]), CXCL-8 (NaOCl: 105.2 [7.36 – 982.28]; EDTA: 84.69 [24.95 – 1845.91]) and OLR-1 (NaOCl: 43.0 [4.37 – 779.27]; EDTA: 57.3 [1.45 – 1614.18]).

Percentage change in periapical lesion volume: The intra- and inter-rater agreements for determining periapical lesion volume exceeded 0.90, with results summarised in Table 4 and Figure 3 with representative examples of healed, healing and progressing lesions depicted in Figures 4, 5 and 6, respectively. For the NaOCl group, the median pre- and post-operative periapical lesion volume was 78.9 mm^3 [49.81 – 183.30] and 5.8 [2.75 – 32.70] respectively resulting in a statistically significant percentage change of 92.50% [67.33 – 99.13] ($p < 0.05$). Similarly, the median pre- and post-operative lesion volume for the EDTA group was 66.8 [47.21 – 116.35] and 2.6 [0 – 13.17] respectively, giving a statistically significant percentage change of 95.84% [78.81 – 100] ($p < 0.05$). No significant differences were observed in these outcomes between groups ($p > 0.05$).

Discussion

This is the first triple blinded parallel group randomised controlled phase I/II trial assessing the clinical effectiveness of endodontic irrigant regimes that enhance dECMs release. When compared with conventional antimicrobial approaches that utilise NaOCl, no significant difference in treatment outcomes could be observed at the 12 months recall in single rooted teeth diagnosed with a necrotic pulp and asymptomatic apical periodontitis. This was based on loose (NaOCl:89.5%; EDTA: 94.1%) and strict (NaOCl: 26.3%; EDTA: 47.1%) criteria. Moreover, no statistical

differences between groups were identified for patient reported pain scores; changes in periapical lesion volume; and with exception to IL-6 and IL-18, which were more abundant in the intervention group at the point of obturation.Irrigant regimes promoting dECM release thus demonstrated equivalence to conventional protocols with no adverse effects and for this reason, the null hypothesis was accepted.

The present study was designed to overcome many of the biases typically associated with interventional studies. More specifically, the robust randomisation and concealment process facilitated statistical matching of participants across every demographic and clinical characteristic investigated. Patients, operators and assessors were all blinded, a feature that has inherently been difficult to implement in endodontic studies (Yi et al. 2020). Each of the prespecified outcomes in the protocol published *a priori* were reported and recall rates of 90% and greater were achieved for the primary and secondary outcomes respectively, which compares favourably with clinical studies with similar sample sizes and review intervals (Martin et al. 2014, Xuan et al. 2018, Arslan et al. 2019). Notably, the most common reason provided by those failing to attend, one of whom had been randomised into the test arm twice, was COVID-19 travel restrictions, a factor that also led to a protracted recruitment and study period. Nevertheless, all patients barring the one deceased were contactable at the time of review and confirmed the teeth treated were still present, asymptomatic and functional reducing the likelihood these would have contributed to failing outcomes. This rationalised the per protocol analysis that is commonly utilised in most other endodontic clinical trials and studies (Martin et al. 2014, Xuan et al. 2018, Arslan et al. 2019, Saini et al. 2022).

Results of the reported clinical trial however still need to be interpreted with caution due to several methodological limitations. Most notably, the limited sample size may

result in an over- or under-estimation of the effects. A smaller cohort was selected here as the safety and efficacy of the proposed irrigant regime has not been established in combination with the conventional understanding that EDTA, predominantly used for its chelating action on smear layers, lacks robust antibacterial activity (Siqueira et al. 1998, Arias-Moliz et al. 2008 & 2009, Ordinola-Zapata et al. 2012, Mohammadi et al. 2013, de Almeida et al. 2016). Whilst limited evidence pertains to an antibiofilm effect, it is currently unknown if this is sufficient to clinically promote the endodontic micro-environment necessary to facilitate periradicular tissue healing (Siqueira & Rôças 2008, Giardino et al. 2020, Virdee et al. 2023b). Consequentially, this underpinned the rationale for a two-visit approach; as the inter-appointment calcium hydroxide period, which aimed to compensate for an absence of NaOCl via its organic solvent and antimicrobial properties, gave researchers the opportunity to revert back to conventional irrigant regimes if severe side effects were encountered. Furthermore, this medicament provided the added benefit of not deleteriously effecting the integrity of dECMs (Graham et al. 2006, Tomson et al. 2007 and 2017). Nevertheless, future studies with increased sample sizes are now required to strengthen the reported findings.

Single rooted teeth diagnosed with a necrotic pulp and asymptomatic apical periodontitis were selected as they improved standardisation of endodontic anatomy and in turn the contact surface area available for dECM solubilisation, reducing operator error risk and simplifying the PTF sampling process (Vertucci 1984, Virdee et al. 2023). Furthermore, root canals with previously initiated treatment will have been subjected to unknown irrigant regimes that could have inadvertently reduced dECM bioavailability (Virdee et al. [submitted for publication]), whereas those that were root treated or symptomatic are associated with differing endodontic microbiomes,

inflammatory pathophysiology and treatment outcomes; all of which may have influenced results (Ng et al. 2011, Galler et al. 2015, Martinho et al. 2016, Siqueira & Rôças 2022). Whilst this may have improved internal validity, it will have limited generalisability particularly in a secondary care setting where these teeth are seldom encountered. Further investigations are required to determine if similar outcomes are observed in multi-rooted teeth, where irrigation protocols may play a greater role in treatment success, or those that present with more acute clinical signs and symptoms of periradicular disease (Laukkanen et al. 2021).

In the current study, favourable outcomes were observed in 89.5% of participants that underwent conventional irrigation, which is consistent with many other analogous reports (Ng et al. 2011, Liang et al. 2013, Yildiz et al. 2024). Surprisingly, a slightly higher but statistically insignificant result of 94.1%, was observed in the EDTA group, for which there are currently no comparable clinical investigations. These outcomes were derived from robust measures that included a thorough clinical examination, highly sensitive volumetric imaging techniques and guideline standard recall intervals (ESE 2006, Lofthag-Hansen et al. 2007, Duncan et al 2021). It is acknowledged however that a 12-month review period may be too short to adequately assess for complete periradicular healing, a process that whilst occurs over two years in most cases can reportedly take up to four (Ørstavik 1996, Ng et al. 2011). This would account for the considerably lower success rates found in both arms, that were still nevertheless statistically comparable.

The high rates of favourable healing in the intervention group could be explained by the mechanisms proposed in the original hypothesis. For instance, a recent *in vivo* observational study confirmed endogenous TGF-β1 within the root canals of mature permanent teeth following ultrasonically activated EDTA irrigation, following use of a

similar regime that was applied in the present study (Widbiller et al. 2022). Moreover, these were detected at quantities previously shown to elicit tissue healing responses, however NaOCl irrigation significantly hampered these processes (Galler et al. 2015). Although a narrow minor apical foramen may limit the periapical interface, *in vitro* experiments conducted by the authors provide proof of concept for adequate periradicular bioavailability of dECMs when this structure was only minimally enlarged as per the above *in vivo* protocol (i.e. 0.2 mm; Virdee et al. [submitted]). More specifically, methylcellulose strips were externally adapted to the apical thirds of intact extracted teeth during EDTA irrigation of standardised root canals and found to have absorbed bioactive quantities of TGF-β1 when analysed using enzyme-linked immunosorbant assays (Virdee et al. [submitted for publication]). This contrasts previous recommendations of pre-enlarging the apical foramen to 0.5 – 1.0 mm and can be attributed to this size being based on the need for intraradicular influx of sufficient blood and cellular components, which require a larger interface than for the dECMs molecules (Fang et al. 2018, Kim et al. 2018). Finally, the regenerative effects dECMs have on the various endodontic MSC niches are well documented (Virdee et al. 2022), with the authors being able to also demonstrate upregulation of proliferation, chemotaxis, osteogenic differentiation and calcific mineralisation in human PL-MSC cultures (Virdee et al. [submitted for publication]). This collective body of evidence corroborates the proposed hypothesis.

Another implication of this work is that chemomechanical preparation with 17% EDTA provided the endodontic disinfection necessary to initiate periradicular regeneration on par with 5.25% NaOCl irrigation. This outcome could be explained by its chelating properties that destabilises the outer cell membranes of gram-negative bacteria (Finnegan et al. 2015). Whilst this effect alone may not always induce bacterial death

it could be sufficient when combined with mechanical instrumentation (Virdee et al. 2023b). This chelating action has also been shown to promote cellular detachment and weaken the macrostructures of established biofilms, which can then be more easily flushed from root canals via the mechanical shearing forces created by conventional irrigant flow dynamics and agitation techniques (Bryce et al. 2009, de Almeida et al. 2016). Other explanations that require further investigation could be attributed to the anatomical simplicity of single rooted teeth that may not thoroughly test the efficacy of irrigants in all tooth types; the two-week intracanal-medicament period with calcium hydroxide; or an underpowered sample size (Laukkanen et al. 2021).

The Target-48 panel consistently detected 15 PTF-derived cytokines (CCL-2, -3, -4; CSF-1; CXCL-8; HGF; IL-1β, -6, -18; MMP-1, -12; OLR-1; OSM; TNFSF-10; VEGF-A) all of which have previously been associated with periradicular inflammation (Márton & Kiss, 2014). Indeed, these were detected in several immunohistochemical investigations and a cross-sectional PTF study that through similar methodologies, also identified CXCL-9, IL-17A and TNSF-12 (Kabashima et al. 2001; Nonaka et al. 2008 Tsai et al. 2008, Virdee et al. 2023a). In the current analyses, these three molecules were marginally below the 75% inclusion threshold, which was unexpected for the former as this was deemed the most diagnostically distinguishable biomarker for asymptomatic apical periodontitis (Appendix 1; Virdee et al. 2023a). As this discrepancy is likely due to the relatively smaller sample size, there was reluctance to proceed with further receiver operating characteristic analyses with respect to treatment outcomes. These data nevertheless provide useful insight into periradicular pathophysiology as future investigations can focus on the roles these cytokines play in the disease process. Particular attention should be given to IL-6 and IL-18, which

although reduced in concentration throughout treatment were still significantly more abundant in the test group prior to obturation. Interestingly, whilst the former cytokine is considered pro-inflammatory, it has been associated with inhibiting IL-1β and TNF-α induced bone resorption, which results in larger periapical lesions, and has potent regenerative activities in the liver and kidneys (Balto et al. 2001, Kwan Tat et al. 2004, Galun & Rose-John 2013).

Given the lack of prior safety data on the proposed irrigant regime, patient reported pain scores were considered an important outcome measure by the public involvement group. The NRS was thus used as it is significantly simpler and more sensitive than its visual analogue and verbal rating scale counterparts (Hjermstad et al. 2011). For post-obturation, this tool revealed similar results for both groups with pain profiles being highly consistent with those typically described in multiple analogous reports. More specifically, it was of mild severity, peaking within 24 hours and lasting only one to two days where it would then taper down to negligible levels by one week (Sathorne et al. 2008, Pak & White 2011, Ballal et al. 2019). For post-instrumentation, whilst the incidences of discomfort were comparable between groups, it was conventional irrigant regimes that were associated with notably more severe pain profiles and greater use of analgesics, albeit this outcome was not statistically significant. Additionally, the only two acute exacerbations, which nevertheless went on to experience favourable healing outcomes, occurred in this group. Notably, similar characteristics have been observed in the relatively few investigations that have explored inter-appointment pain in teeth with a necrotic pulp (Ng et al. 2011, Mostafa et al. 2020). This finding can be attributed to NaOCls cytotoxic nature that is a function of its concentration, with inflammatory effects on periapical tissues lasting up to 30 days following administration (Gomes-Filho et al. 2008). Furthermore, the efflux of

highly concentrated NaOCl into the periradicular region is likely to have been exacerbated by the intentional pre-enlargement of the apical foramen (Tinaz et al. 2005). Therefore, based on the present findings, no adverse effects or pain profiles were observed for the aforementioned EDTA-derived irrigant regime.

Clinical implications: This triple blinded randomised controlled clinical study provides preliminary data suggesting the healing rates of periapical lesions with irrigation regimes aimed at releasing bioactive dECMs are comparable to conventional disinfection protocols. This is supported by *in vitro* mechanistic data, that demonstrates efficacy of this irrigant regime to release dECMs and their subsequent bioavailability to periradicular tissues in mature permanent teeth and regenerative effects to local MSCs (Virdee et al. [submitted for publication]). As favourable outcomes were observed on par with 5.25% NaOCl treatment, it can be inferred the bacterial load was reduced beyond the critical threshold necessary to facilitate periradicular healing (Siqueira & Rôças 2008). This brings into question the accepted approach of solely disinfecting the root canal system to treat apical periodontitis and not including other therapeutic strategies and mechanisms to enhance healing of diseased periradicular tissues. The reliance of NaOCl treatment may have thus masked a previously undetected biological mechanism to treat apical periodontitis, given its deleterious effects on dECMs (Galler at al. 2015, Virdee et al. [submitted for publication]). Strategies that go beyond simply disinfecting root canals and which exploit innate healing mechanisms as proposed may thus improve treatment outcomes and reduce reliance on NaOCl, which when extrudes can lead to severe swellings or neuroanatomical damage (Farook et al. 2014). Such approaches may lead to a paradigm shift for treating apical periodontitis. The present findings do however require

further exploration via appropriately powered phase III trials with longer review intervals and wider inclusion criteria.

Conclusion

In this triple blinded randomised controlled phase I/II trial of single rooted necrotic mature permanent teeth, therapeutic irrigant regimes promoting dECMs solubilisation exclusively with 17% EDTA resulted in favourable one-year treatment outcomes that were equivalent to conventional 5.25% NaOCl-based irrigant regimes. Moreover, no severe adverse effects or atypical pain profiles were observed in the former, demonstrating the clinical safety of 17% EDTA. Longitudinal analysis of PTF-derived biomarkers revealed significant reductions in the various inflammatory cytokines that were common for both groups, although interestingly IL-6 and IL-18 were significantly more abundant in the intervention group. Further investigations with a larger sample size and wider inclusion criteria are nevertheless warranted to strengthen findings of this clinical approach and delve deeper into the profile of inflammatory biomarkers found in PTF which will lead to better understanding of prognostic indicators for treatment of apical periodontitis.

Conflicts of Interest:

The authors deny any conflicts of interest related to this study.

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Table 1: Demographic and clinical characteristics

Characteristics		Irrigant Regime [n(%)]			
		Total (*n* = 36)	**5% NaOCl** (*n* = 19)	**17% EDTA** (*n* = 17)	***P* Value** [a b]
Demographics					
Age (years)	Median [IQR]	36 [25 – 43]	37 [29 – 47]	36 [23 – 45]	0.754 [a]
Sex	Female	18 (50.0)	10 (52.6)	8 (47.1)	0.738 [b]
	Male	18 (50.0)	9 (47.4)	9 (52.9)	
Ethnicity	Afro-Caribbean	7 (19.4)	2 (10.5)	5 (29.4)	0.213 [b]
	Asian	9 (25.0)	4 (21.0)	5 (29.4)	
	Caucasian	20 (55.6)	13 (68.5)	7 (41.2)	
Pre-Operative Clinical Factors					
Inter-arch position	Mandible	7 (19.4)	5 (26.3)	2 (11.8)	0.271 [b]
	Maxilla	29 (80.6)	14 (73.7)	15 (88.2)	
Intra-arch position	Incisors	33 (91.7)	18 (94.7)	15 (88.3)	0.558 [b]
	Canine	2 (5.6)	1 (5.3)	1 (5.9)	
	Premolar	1 (2.8)	0 (0)	1 (5.9)	
Coronal Restorations	NIL	23 (63.9)	12 (63.2)	11 (64.7)	0.689 [b]
	Direct	7 (19.4)	3 (15.8)	4 (23.5)	
	Indirect	6 (16.7)	4 (21.0)	2 (11.8)	
Disease Aetiology	Deep Carious Lesion	2 (5.6)	1 (5.3)	1 (5.9)	0.755 [b]
	Congenital Abnormality	2 (5.6)	2 (10.5)	0 (0)	
	Iatrogenic	8 (22.2)	4 (21.0)	4 (23.5)	
	Traumatic Dental Injury	16 (44.4)	8 (42.1)	8 (47.1)	
	Idiopathic	8 (22.2)	4 (21.0)	4 (23.5)	
Periapical Lesion Volume (mm^3)	Median [IQR]	74.6 [46.51 – 131.28]	78.9 [49.81 – 183.30]	66.8 [47.21 – 116.35]	0.363 [a]
Peri-Operative Clinical Factors					
Patency Achieved	Yes	36 (100)	19 (100)	17 (100)	1.00 [b]
	No	0 (0)	0 (0)	0 (0)	
Perforations	Yes	0 (0)	0 (0)	0 (0)	1.00 [b]
	No	36 (100)	19 (100)	17 (100)	
Inter-Appointment Flare Up	Yes	2 (5.6)	2 (10.5)	0 (0)	0.169 [b]
	No	34 (94.4)	17 (89.5)	17 (100)	
Root Filling Quality	Adequate	36 (100)	19 (100)	17 (100)	1.00 [b]
	Inadequate	0 (0)	0 (0)	0 (0)	
Root Filling Length	> 2 mm short	36 (100)	19 (100)	17 (100)	1.00 [b]
	≤ 2 mm within	0 (0)	0 (0)	0 (0)	
	Extruded	0 (0)	0 (0)	0 (0)	
Post Operative Clinical Factors					
Review Interval (Months)	Median [IQR]	13 [12.0 – 14.0]	13 [12.0 – 13.5]	13 [12.0 – 14.0]	0.933 [a]
Intact Coronal Seal	Yes	36 (100)	19 (100)	17 (100)	1.00 [b]
	No	0 (0)	0 (0)	0 (0)	

[a] Independent-samples Mann-Whitney U; [b] Chi Squared test

Table 2: Outcome of root canal treatment in teeth diagnosed with asymptomatic apical periodontitis with conventional irrigant regimes and those that promote release of dentine extracellular matrix components.

		Loose Criteria				Strict Criteria			
Imaging	**Group**	**Favorable**	**Unfavorable**	**OR [95%CI]**	**P Value [a]**	**Success**	**Failure**	**OR [95%CI]**	**P Value [a]**
LCPA	**5.25% NaOCl**	18 (94.7)	1 (5.2)	1	0.936	11 (57.9)	8 (42.1)	1	0.765
	17% EDTA	16 (94.1)	1 (5.9)	1.13 [0.06-19.50]		9 (52.9)	8 (47.1)	1.22 [0.33 – 4.56]	
CBCT	**5.25% NaOCl**	17 (89.5)	2 (10.5)	1	0.619	5 (26.3)	14 (73.7)	1	0.536
	17% EDTA	16 (94.1)	1 (5.9)	0.53 [0.04-6.44]		8 (47.1)	9 (52.9)	0.66 [0.17 – 2.49]	

[a] 5% NaOCl success/favorable vs. 17% EDTA success/favorable [Chi Squared Test]

Table 3: Longitudinal analysis of periradicular tissue fluid sampled from teeth diagnosed with asymptomatic apical periodontitis undergoing root canal treatment with conventional irrigant regimes and those that promote release of dentine extracellular matrix components

PTF Characteristics	Baseline (S1)			Pre-obturation (S2)		
	5% NaOCl	17% EDTA	*P* Value [a]	5% NaOCl	17% EDTA	*P* Value [a]
Total Absorbed Volume (µL)	1.8 [0.77 – 2.87]	2.0 [1.18 – 2.97]	0.541	0.4 [0.26 – 0.83] [B]	0.5 [0.26 – 0.89] [b]	0.698
Total Protein Concentration (µg/mL)	322.0 [243.23 – 719.08]	295.0 [255.39 – 501.74]	0.841	183.6 [141.85 – 221.51] [B]	180.5 [131.20 – 255.07] [b]	0.862
Analyte / Total Protein (pg/mg)						
Chemokine Ligand [CCL]-2	14.7 [5.35 – 43.86]	48.8 [12.11 – 230.61]	0.134	3.9 [0.97 – 31.90]	8.0 [2.85 – 59.97]	0.231
Chemokine Ligand [CCL]-3	12.1 [2.45 – 58.17]	18.9 [9.42 – 70.37]	0.512	2.3 [0.55 – 11.69]	2.9 [0.64 – 15.31] [b]	1
Chemokine Ligand [CCL]-4	31.60 [5.59 – 165.31]	77.0 [18.73 – 267.87]	0.289	5.16 [1.32 – 30.90]	6.9 [0.62 – 59.49] [B]	0.947
Colony Stimulating Factor [CSF]-1	2.9 [1.63 – 6.77]	4.5 [2.24 – 12.04]	0.265	0.7 [0.02 – 5.19]	1.4 [0.57 – 4.96] [b]	0.231
Hepatocyte Growth Factor [HGF]	119.6 [24.60 – 204.72]	221.4 [92.63 – 436.65]	0.121	13.54 [2.32 – 35.32] [b]	35.6 [1.93 – 210.34] [b]	0.265
Interleukin [IL]-1β	148.7 [48.33 – 560.90]	550.8 [57.78 – 972.01]	0.231	3.0 [0.256 – 7.68] [b]	4.7 [2.08 – 21.91] [B]	0.142
Interleukin [IL]-6	5.7 [2.75 – 18.39]	8.3 [3.88 – 15.04]	0.779	0.2 [0.16 – 1.87]	3.8 [0.58 -30.69]	0.017*
Interleukin [CXCL]-8	491.6 [73.74 – 3547.34]	1316.4 [222.77 – 6040.90]	0.414	105.2 [7.36 – 982.28]	84.69 [24.95 – 1845.91] [b]	0.799
Interleukin [IL]-18	109.1 [29.2 – 202.75]	184.4 [102.81 – 394.74]	0.277	1.4 [1.01 – 15.92] [b]	43.9 [10.80 – 223.81]	0.030*
Matrix Metallopeptidase [MMP]-1	41.9 [6.46 – 143.86]	28.6 [9.14 – 136.87]	0.947	2.5 [0.03 – 72.29]	10.16 [0.19 – 110.73]	0.820
Matrix Metallopeptidase [MMP]-12	708.4 [43.10 – 4695.83]	380.8 [118.02 – 1320.01]	0.718	261.8 [22.00 – 520.40]	111.0 [12.36 – 460.78]	0.478
Oxidised Low Density Lipoprotein Receptor [OLR]-1	1190.8 [330.96 – 2647.97]	2237.8 [1347.68 – 3061.87]	0.201	43.0 [4.37 – 779.27] [b]	57.3 [1.45 – 1614.18] [b]	0.862
Oncostatin M [OSM]	25.4 [3.89 – 61.38]	51.86 [24.01 – 157.49]	0.165	1.1 [0.21 – 11.61] [b]	0.9 [0.18 – 22.06] [b]	0.947
Tumour Necrosis Factor Superfamily [TNFSF]-10	42.9 [10.01 – 88.10]	59.09 [37.53 – 205.17]	0.134	0 [0 – 2.65] [b]	0 [0 – 17.49] [B]	0.968
Vascular Endothelial Growth Factor [VEGF]-A	118.0 [31.34 – 190.53]	207.0 [88.86 – 408.76]	0.091	23.3 [6.07 – 91.67]	22.6 [10.09 – 147.96] [b]	0.512

[a] Inter-group comparison (NaOCl vs. EDTA); results outlined in *P* Value subsections of baseline and pre-obturation columns; *Independent Samples Mann-Whitney U* test

[b] Intra-group comparison (Baseline vs. Pre-obturation); [b] and [B] indicate *p* values of < 0.05 and < 0.01 respectively; results outlined in pre-obturation columns for each irrigant; *Wilcoxon Matched Paired* test

Table 4: Volumetric and percentage changes in periapical lesion size based on cone beam computer tomography scans of teeth diagnosed with asymptomatic apical periodontitis undergoing root canal treatment with conventional irrigant regimes and those that promote release of dentine extracellular matrix proteins

	5% NaOCl			17% EDTA			
n	**Pre-operative lesion volume (mm^3)**	**Post-operative lesion volume (mm^3)**	**Percentage change in lesion volume (%)**	**Pre-operative lesion volume (mm^3)**	**Post-operative lesion volume (mm^3)**	**Percentage change in lesion volume (%)**	***P* Value [a]**
1	22.85	0	100.00	6.90	2.63	95.84	
2	14.10	0	100.00	187.10	0	100.00	
3	7.34	0	100.00	45.22	75.82	78.81	
4	186.65	4.77	97.45	184.70	0	100.00	
5	44.415	15.05	66.12	188.10	0	100.00	
6	183.80	63.97	65.20	689.70	8.03	95.23	
7	187.10	0	100.00	74.20	0	100.00	
8	690.70	22.67	96.72	183.20	0	100.00	
9	75.55	74.64	1.21	26.56	0	100.00	
10	182.80	79.89	56.30	55.28	24.83	78.66	
11	25.86	4.51	82.54	333.60	10.82	90.88	
12	55.21	9.49	82.82	79.60	7.03	-138.63	
13	333.70	5.75	98.28	73.20	18.55	74.42	
14	78.93	24.83	68.54	81.50	0	100.00	
15	73.65	150.00	-103.67	160.70	13.17	80.27	
16	82.42	0	100.00	120.20	0	100.00	
17	161.55	40.57	74.89	63.30	24.32	50.93	
18	120.90	4.39	96.37				
19	64.20	4.81	92.50				
Total	78.9 [49.81 – 183.30]	5.8 [2.75 – 32.70]	92.50 [67.33 – 99.13]	66.8 [47.21 – 116.35][b]	2.6 [0 – 13.17][b]	95.84 [78.81 – 100]	0.379

[*a*] NaOCl vs EDTA percentage change in lesion volume (*Mann-Whitney U* Test); [b] $P < 0.05$: pre-operative vs. post-operative intra-group comparison (*Wilcoxon Matched Paired* test Test);

Total volumes and percentage changes represented as medians [interquartile range].

PRIRATE 2020 Flowchart

Aims: Determine if success rates **(O)** of root canal treatment with conventional irrigant regimes **(C)** were equivalent to those promoting release of dentine extracellular matrix proteins **(I)** in mature permanent teeth diagnosed with asymptomatic apical periodontitis **(P)**

Enrolment

Ethics #: 19/WM/0149
Pre-registry #: ISRCTN93101288

Assessed for eligibility via clinical & IOPA examination ***(n = 48; 45)***

Excluded **(n = 8; 8)**
Did not meet criteria: 6; 6
Declined to participate: 2; 2

Consented; Concealed; Pre-op CBCT scans taken; Randomized (n = 40; 37)

Allocation

Allocated to control group (n = 20; 20)
Two visit root canal treatment with 2 weeks calcium hydroxide ICM with 5.25% NaOCl irrigant regime

Allocated to intervention group (n = 20; 18)
Two visit root canal treatment with 2 weeks calcium hydroxide ICM with 17% EDTA irrigant regime

Follow Up

Did not receive allocated control (n = 0)

Did not receive allocated intervention (n = 0)

Received allocated control (n = 20; 20)

Received allocated intervention (n = 20; 18)

VAS pain scored returned
- Post instrument. (n = 19; 19)
- Post obturation (n = 19; 19)

VAS pain scored returned
- Post instrument. (n = 20; 18)
- Post obturation (n = 18; 17)

Valid PTF samples collected
- Baseline (S1) (n = 20)
- Pre-obturation (S2) (n = 20)

Valid PTF samples collected
- Baseline (S1) (n = 20; 18)
- Pre-obturation (S2) (n = 20; 18)

Analyses

Lost to 12-month follow-up (n = 1; 1)
Post-op CBCT scans taken (n = 19; 19)
- Deceased: 1; 1

Lost to 12-month follow-up (n = 3; 2)
Post-op CBCT scans taken (n = 17; 16)
- Failed to attend: 3; 2

CBCT Periapical lesion volume analysis (n = 19; 19)

CBCT Periapical lesion volume analysis (n = 17; 16)

Allocated controls analysed for endodontic success (n = 19; 19)
Excluded from per protocol analysis: 1; 1

Allocated interventions analysed for endodontic success (n = 17; 16)
Excluded from per protocol analysis: 3; 2

Conclusions: There were no significant differences in success rates of root canal treatment between conventional irrigant regimes and those that promote dentine extracellular matrix protein release in teeth diagnosed with asymptomatic apical periodontitis.

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Figure 1: PRIRATE Flow Chart. Sample sizes represent participants at the tooth and patient level (t; p).

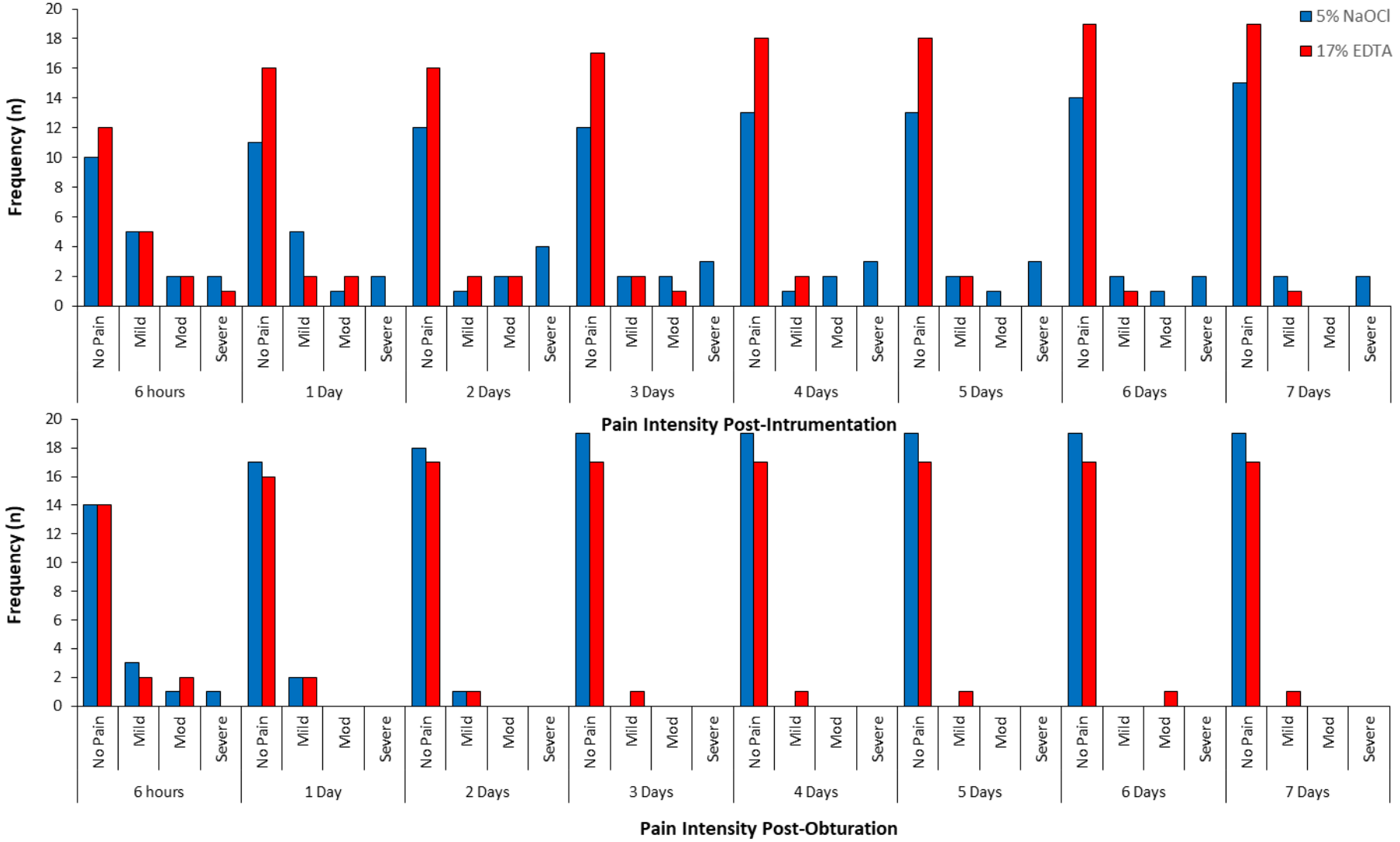

Figure 2: Pain intensity scores at several time points post-instrumentation [A; 5.25% NaOCl *n* = 19; 17% EDTA *n* = 20] and -obturation [B; 5.25% NaOCl *n* = 19; 17% EDTA *n* = 18] following irrigation using conventional irrigant regimes (5.25% NaOCl) and those that promote release of dentine extracellular matrix proteins (17% EDTA). Pain intensity categorized as No Pain = VAS 0; Mild = VAS 1-3; Mod = VAS 4-6; and Severe = VAS 7-10.

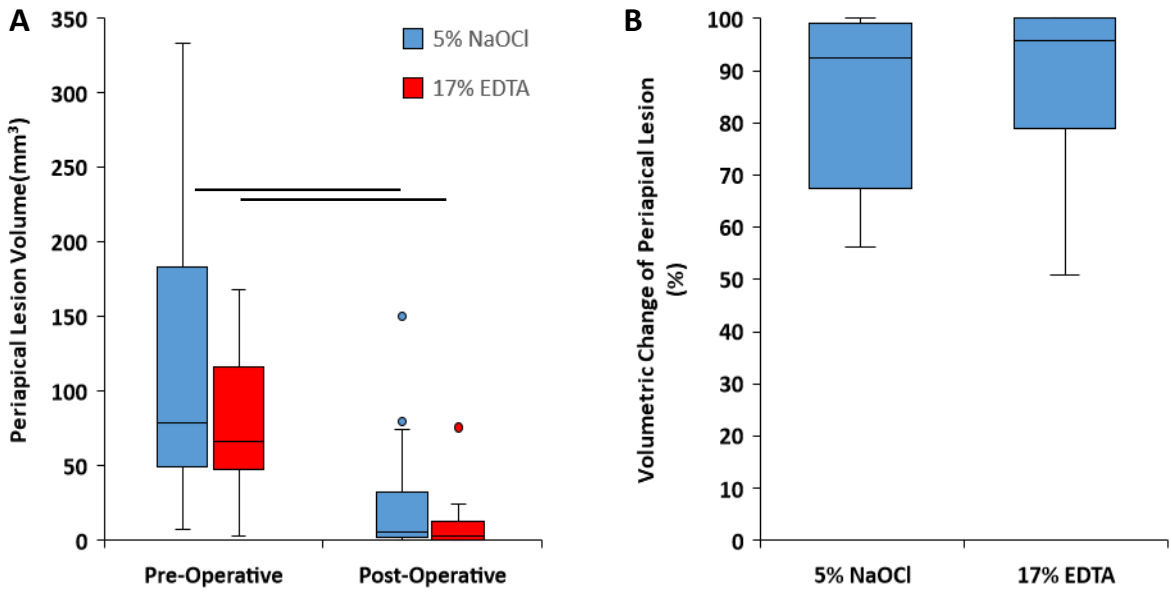

Figure 3: Volumetric (A) and percentage change (B) in periapical lesion size based on cone beam computer tomography scans of teeth diagnosed with asymptomatic apical periodontitis undergoing root canal treatment with conventional irrigant regimes and those that promote release of dentine extracellular matrix proteins. Data presented as box and whisker plots where central bars represent the median alongside upper and lower interquartile ranges at the edge of boxes and minimum and maximum values for the whiskers. Statistically significant comparisons within groups *($p < 0.01$; Wilcoxon Matched Paired tests*) presented as horizontal lines.

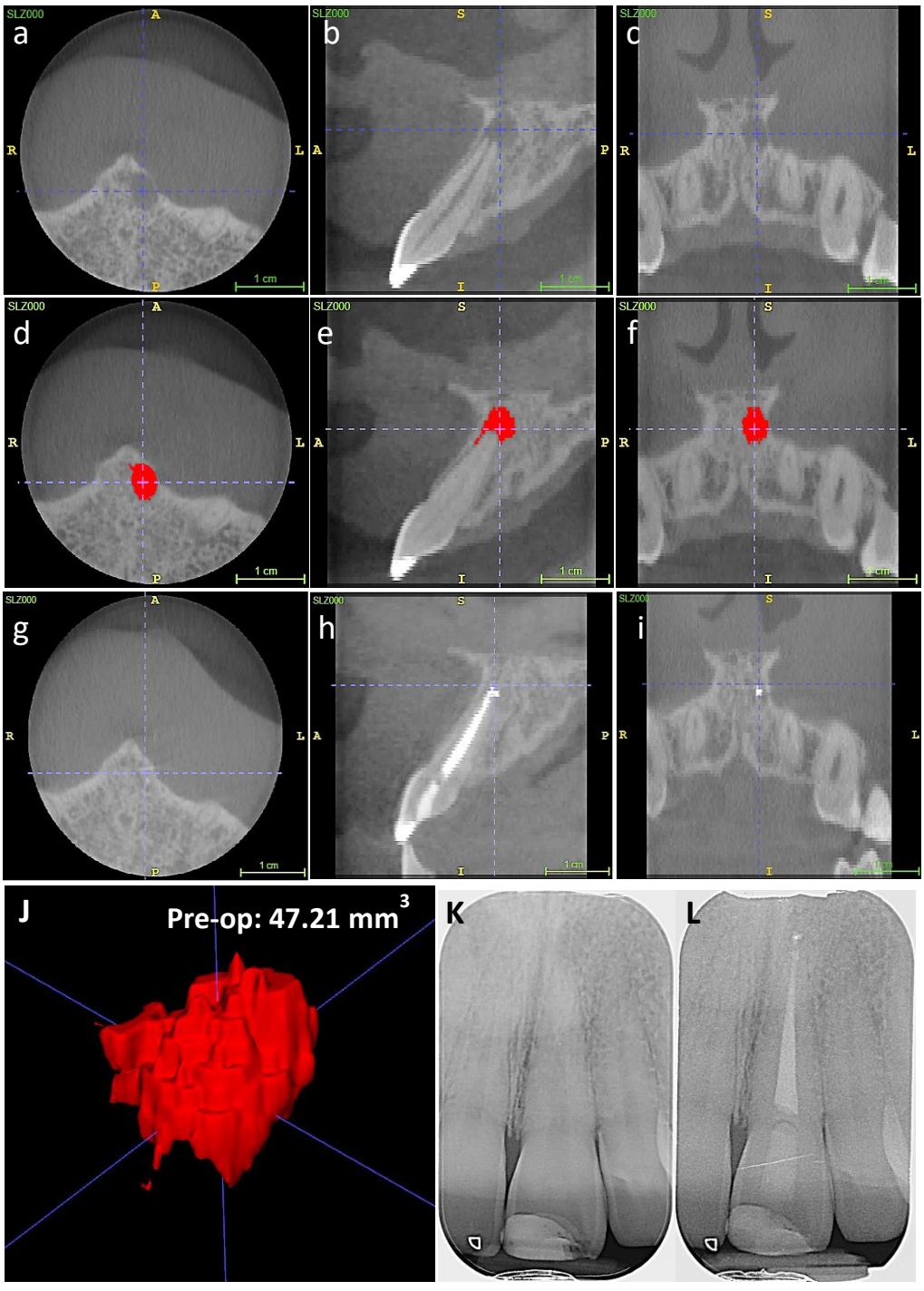

Figure 4: Longitudinal volumetric cone beam computer tomography analysis of periapical lesions following root canal treatment of the UL1. Example represents the axial [a, d, g], sagittal [b, e, h] and coronal [c, f, i] views alongside the pre-operative volumetric analysis [j] of a lesion that has completely resolved following 17% EDTA irrigation at the 12-month review interval [g, h, i]. The corresponding pre- and post-operative periapical radiographs are depicted in [K] and [L] respectively. Scale bars represent 1 cm.

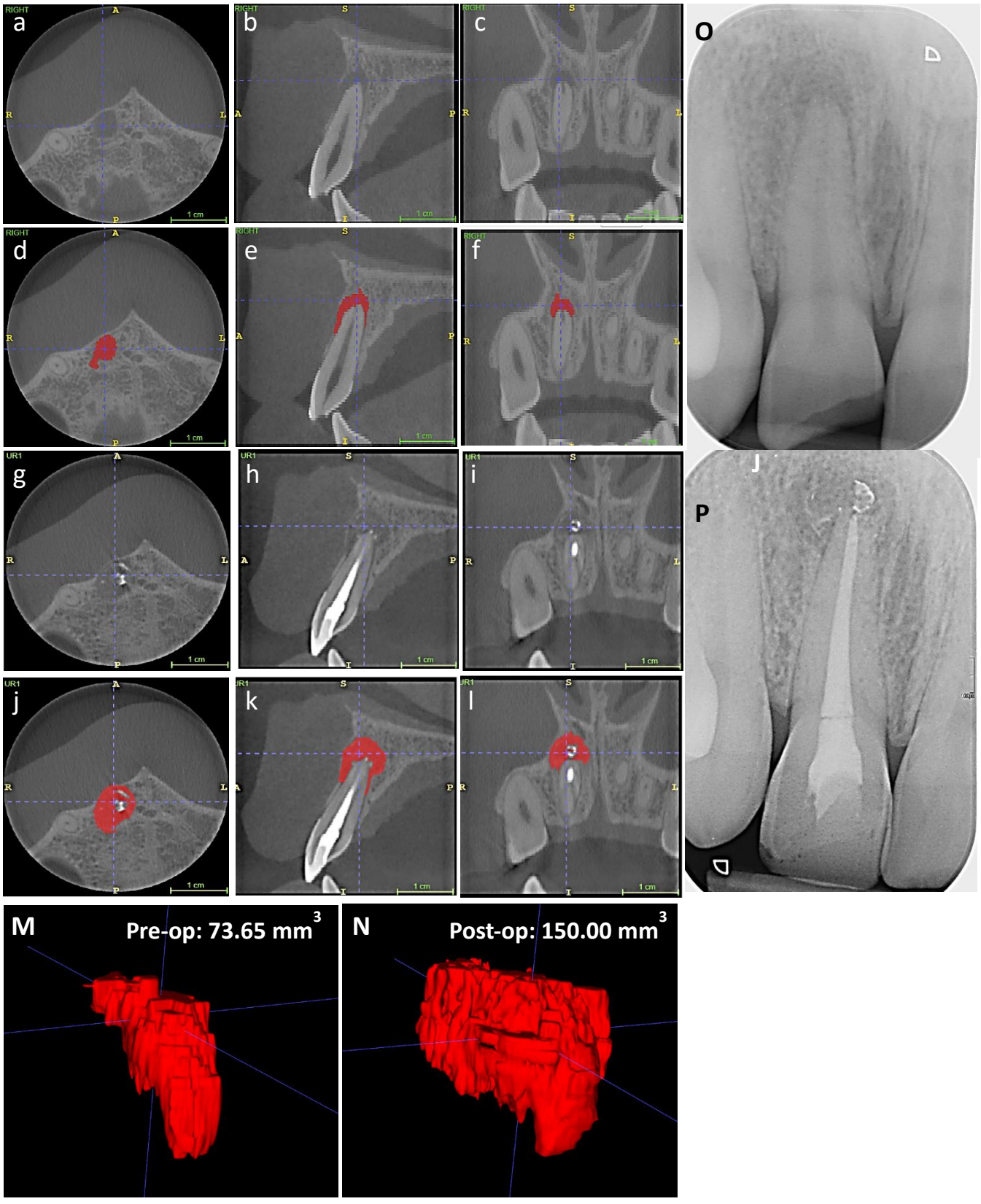

Figure 5: Longitudinal volumetric cone beam computer tomography analysis of periapical lesions following root canal treatment of the UR1. Example represents the axial [a, d, g, j], sagittal [b, e, h, k] and coronal [c, f, I, l] views alongside the pre- [M] and post-operative [N] volumetric analysis of a lesion that has increased in size following 5% NaOCl irrigation at the 12-month review interval [g-l]. The corresponding pre- and post-operative periapical radiographs are depicted in [O] and [P], respectively. Scale bars represent 1 cm.

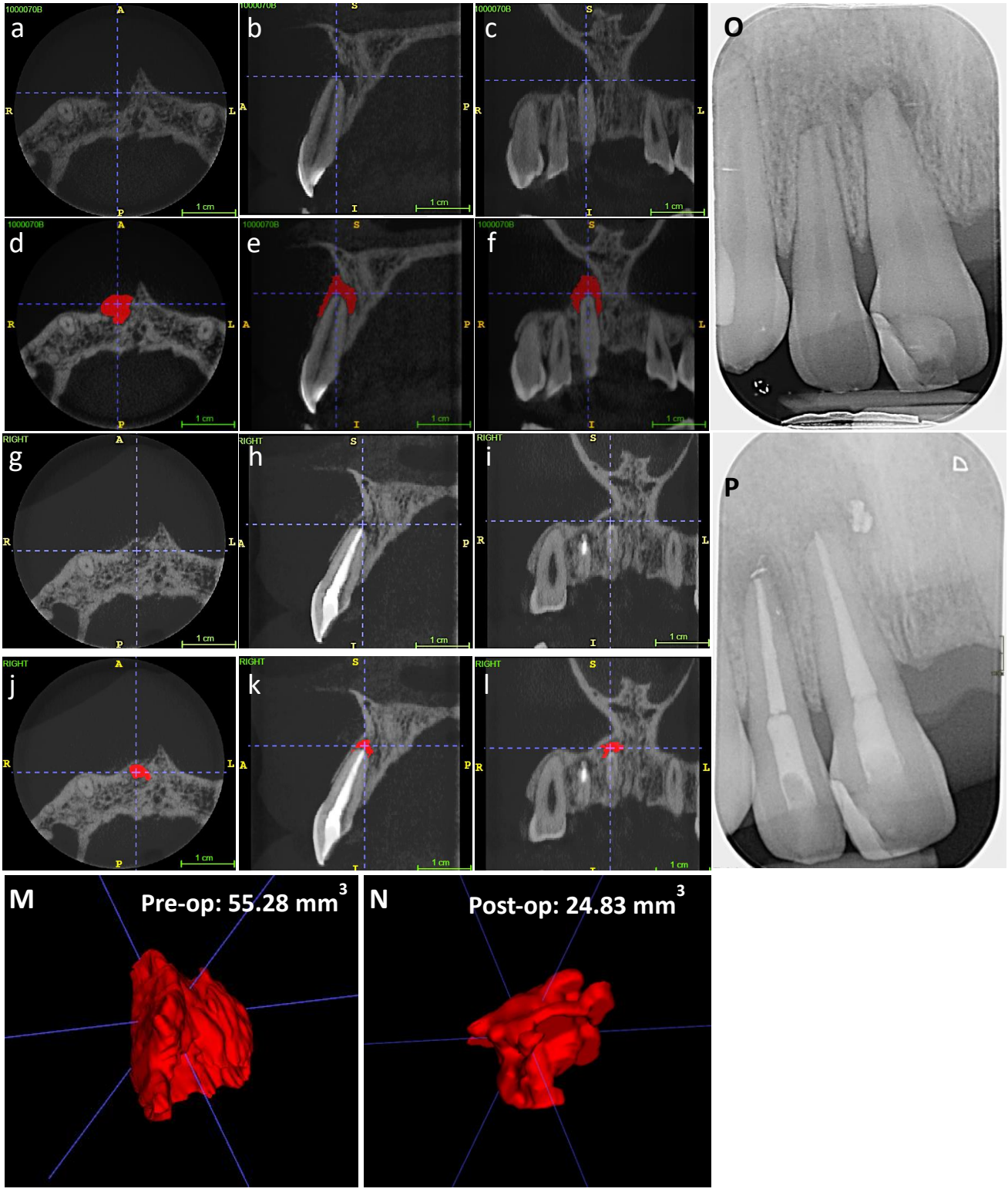

Figure 6: Longitudinal volumetric cone beam computer tomography analysis of periapical lesions following root canal treatment of the UR1. Example represents the axial [a, d, g, j], sagittal [b, e, h, k] and coronal [c, f, I, l] views alongside the pre- [M] and post-operative [N] volumetric analysis of a lesion that has reduced in size following 17% EDTA irrigation at the 12-month review interval [g-l]. The corresponding pre and post operative periapical radiographs are depicted in [O] and [P], respectively. Scale bars represent 1 cm.

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CHAPTER 7

GENERAL DISCUSSION

7.1 Rationale for the thesis

Apical periodontitis is a localised inflammatory condition of the periodontium that represents a dynamic equilibrium between putative endodontic microorganisms and the host's defence mechanisms (Nair 1997). The overall goal for treating this disease, is to regenerate damaged periradicular tissues. Current therapies aim to achieve this outcome indirectly by reducing microbial load from within root canals and thereby, initiating physiological periapical healing events. Despite improvements in the disinfection capabilities over recent decades, proportionate increases in success rates have not yet been witnessed with one in five teeth consistently failing to exhibit complete periapical bony healing (Ng et al. 2011). This suggests the effect of antimicrobial only approaches on treatment success may have exhausted. Thus, more predictable outcomes could be achieved by placing greater emphasis on directly enhancing periradicular regenerative wound healing processes.

Recently, *in vitro* studies have revealed a distinct niche of mesenchymal stem cells within the granulation tissues of apical lesions. These cells, formally termed PL-MSCs, have also been found to be possess tremendous immunosuppressive and regenerative potential (Liao et al. 2011). For these reasons, PL-MSCs have been implicated in regulating periradicular healing and make ideal candidates to target using novel tissue engineering therapies. The periapical wound healing process itself is co-ordinated by a broad range of growth factors, cytokines, chemokines and neuropeptides (Barrientos et al. 2008, Robson 1997). Notably, abundant reservoirs of these bioactive molecules are present locally in a sequestered state within the dentine's extracellular matrix and can be released by commonly used endodontic chelating agents, such as ethylenediaminetetraacetic acid (EDTA; Chae et al. 2018,

Galler et al. 2015, Smith et al. 2012, Zeng et al. 2016). The resulting extracts, formally termed dECMs, have been extensively studied for their therapeutic potential and have subsequently demonstrated their ability to upregulate regenerative events within other odontogenic MSC niches (Tomson et al. 2013 & 2017, Sadaghiani et al. 2016, Zeng et al. 2016, Widbiller et al. 2018, Jin et al. 2019) It was thus hypothesised that therapeutic irrigant regimes could be used to exploit dECMs during root canal treatment to upregulate PL-MSC regenerative activity within inflamed periradicular tissues and in turn, improve the predictability of treating apical periodontitis. The primary aim of the present thesis was to provide proof of concept for such an approach, with secondary aims being to overcome several of the challenges associated with the clinical translation of this treatment strategy.

7.2 Findings & Limitations

Overall, the hypothesis that endogenous dECMs can be exploited to treat apical periodontitis was supported by results presented in the previous chapters. Initial proof of concept for the clinical protocol introduced in Chapter 1 and Chapter 2 (Publication 1) was derived from the novel *in vitro* intact tooth model described in Chapter 3 (Publication 4). More specifically, and for the first time, periradicular bioavailability of dECMs solubilised into the root canals of mature permanent teeth was demonstrated by the careful apical placement of methylcellulose strips. Minimally pre-enlarging the apical foramen to 0.2 mm was found to be sufficient to facilitate this passage, which challenges guideline recommendations of pre-enlarging to 0.5 – 1.0 mm (Kim et al. 2018). This discrepancy could be explained by the fact the latter is based on the need for sufficient intraradicular influx of blood and cellular components for

revascularisation, which would invariably require a larger interface than the extraradicular efflux of smaller dECM molecules. Nevertheless, subsequent topical application of dECMs at the concentrations measured in the apically positioned methylcellulose strips were found to upregulate regenerative events in primary PL-MSC cultures that would otherwise be necessary for periradicular regeneration. This included cellular proliferation, migration, osteogenic differentiation and mineralisation without affecting cell viability, which has not been previously reported. Whilst these results need to be interpreted with caution, due to the *in vitro* nature of the experimental models, these findings were supported when this treatment approach was applied into the triple blinded randomised controlled pilot study, reported in Chapter 6. More specifically, irrigant regimes that promoted release of dECMs revealed similar clinical outcomes to those utilising conventional high strength NaOCl solutions during root canal treatment of single rooted mature permanent teeth diagnosed with apical periodontitis. Furthermore, there were no serious adverse events witnessed within the test group in which participants reported slightly lower, although statistically insignificant, pain scores compared with the conventional group where two acute exacerbations were witnessed. Once again, the present body of work is the first to report this finding, which has significant clinical implications. It is acknowledged however that these results are derived from a limited sample size and that additional fully powered trials are required to further support the conclusions of this study.

Considerable efforts were directed towards optimising the irrigant regime to promote release of dECMs into root canals. It is well established however that prior to any regenerative protocol, a pro-healing environment is a mandatory pre-requisite (Kim et al., 2018). This could be considered as one where the endodontic bacterial load is

below that necessary to initiate periradicular healing events, a threshold that is currently yet to be defined (Siqueira & Rôças, 2008). To date, NaOCl has long been the principal approach by which this is achieved owing to its organic solvent and bactericidal properties, however, it is recognised that its non-specific proteonacious properties negatively effect dentine matrix growth factor and MSC viability (Galler et al. 2015, Martin et al. 2014). To address this phenomenon, current regenerative endodontic protocols advocate limited contact times with low concentration of NaOCl solution prior to EDTA use however, these recommendations are based on MSC viability only and do not factor in the integrity of endogenous dECMs (Martin et al. 2014). The *in vitro* investigations reported in Chapter 3 nevertheless demonstrate that even these recommended regenerative irrigant protocols combined with even lower NaOCl concentration that otherwise have limited anti-microbial or organic solvent benefit still negatively impact dECM bioavailability. Whilst mechanically refurbishing dentine, a protocol that was informed by Virdee et al. (2020; Publication 4), improved detection of dECMs they were still not solubilised to the abundance measured when 17% EDTA was exclusively used. This was also reflected in the apically adapted absorbent methylcellulose paper strips that represented periradicular bioavailability.

Based on the above findings, it was determined that any use of recommended regeneration irrigation regimes would negatively impact clinical effectiveness of the proposed protocol and that the antimicrobial capability of the chemomechanical debridement regime would need to be derived from alternate mechanisms. For instance, EDTA only destabilises gram-negative bacteria by chelating cations from within their outer cell membranes (Finnegan & Percival et al. 2015). Whilst this effect alone may not always induce cell death, which would explain previous reports demonstrating its limited antimicrobial capabilities (Arias-Moliz et al. 2008 & 2009,

Fidalgo et al. 2010), it could potentially be sufficiently enhanced to do so when combined with mechanical instrumentation of root canals. This hypothesis was tested by Virdee et al. (2022; Publication 5), who found that chemomechanical preparation of root canals with 17% EDTA using a novel *in vitro* model eradicated the *E. faecalis* biofilm to the same level as 2% NaOCl treatment. It is acknowledged however there are limitations associated with this model, for instance the lack of tubular features, the use of a single species biofilm and the material used being different from dentine. Nevertheless, another alternate antimicrobial mechanism included the use of a multi-visit approach with calcium hydroxide intracanal medicament, which in itself has established antibacterial and organic solvent properties. Moreover, this medicament has demonstrated to not only have no negative impact on the integrity of dECMs, but it also promotes their release over a sustained period (Graham et al. 2006, Tomson et al. 2017).

It was anticipated that some of the inflammatory changes induced by topical dECM exposure would be subclinical and thus, undetectable using the clinical and radiographic diagnostics techniques currently available. Therefore, the sampling and analysis of PTF was optimised in Chapter 5 (Publication 6 & 7) to determine if there were biomarkers that could be utilised during root canal treatment to monitor disease activity. An initial systematic review highlighted considerable variability sampling and eluting protocols, which were for the first time subsequently refined and implemented clinically to identify a broad range of biomarkers in teeth diagnosed with asymptomatic apical periodontitis. In this cross-sectional study, the broad panel detected 18 of a potential 41 analytes (CCL-2, -3, -4; CSF-1; CXCL-8, -9; HGF; IL-1β, -6, -17A, -18; MMP-1, -12; OLR-1; OSM; TNFSF-10, -12; VEGF-A) in ≥75% of samples at statistically higher concentrations than those in normal apical tissues. Furthermore,

the CXCL-8, IL-1β, OLR-1, OSM and TNFSF-12 cluster were strongly correlated to apical periodontitis. 'Excellent' diagnostic performance was observed for TNFSF-12 and the PCA-derived cluster. When the same analysis was applied to the clinical study however, which sought to test the longitudinal differences in these analytes following completion of the test and control irrigant regimes, slightly different results were observed from the initial screen. Most notably, TNFSF-12 did not meet the minimum detection threshold to be included in the final analysis. As such, further correlational analyses were not conducted in recognition of the fact that a much larger sample size would be required to draw more robust conclusions of which analytes could reliably be correlated to treatment outcomes. Interestingly, the concentrations of IL-6 and IL-18 were found to be significantly higher in the test arm as opposed to the control arm immediately prior to obturation. Whilst the former is considered a pro-inflammatory cytokine, it has been associated with inhibiting IL-1β and TNF-α induced bone resorption, which results in larger periapical lesions, and potent regenerative activities in the liver and kidneys (Balto et al. 2001, Galun & Rose-John 2013, Kwan Tat et al. 2004).

7.3 Clinical Implications

There is now preliminary *in vivo* data, supported by *in vitro* mechanistic data, pertaining to the clinical safety and efficacy of irrigant regimes that optimise release of dECMs. As favourable outcomes were observed on par with 5.25% NaOCl, it can be inferred the bacterial load was reduced beyond the critical threshold necessary to facilitate periradicular healing (Siqueira & Rôças 2008). This raises the question of the heavy reliance on NaOCl for regenerative endodontic protocols, as its inhibitory effects

on dECM solubilisation and periradicular MSC-mediated events are well established, even at the lowest recommended concentrations (Galler at al. 2016). The proposed irrigant regime could thus enhance the clinician's ability to achieve the overall aims of regenerative endodontic procedures, particularly those that exploit signalling molecules from the dentine matrix (Kim et al. 2018). Further clinical applications outside of regenerative endodontic protocols could include high risk NaOCl extrusion scenarios to avoid severe swellings or damage to local neuroanatomy (Farook et al. 2014).

7.4 Future Direction

The overall aim of the newly developed proposed approach for treating apical periodontitis is to improve the relatively stagnant success rates of guideline standard root canal treatment by exploiting dECM bioavailability using biologically driven therapeutic irrigant regimes (Ng et al. 2007). A similar concept that has been clinically translated is the management of deep carious lesions where placement of calcium silicate materials solubilise dECMs that subsequently, upregulate DPSC-derived tertiary dentine deposition (Duncan et al. 2019). It is acknowledged however that the present body of work currently provides only proof of concept. Therefore, in order for such biologically driven and regenerative approaches to become fully realised in the clinical setting, further research is required to address some of the current limitations and strengthen knowledge.

In the first instance, participants from the clinical trial should be followed up for a longer review interval to more accurately assess for complete periradicular healing, a process that whilst occurs over two years in most cases can take up to four (Ørstavik, 1996;

Ng et al. 2011). Alongside this, an appropriately powered randomised control trial with a broader inclusion criterion and longer review intervals should be conducted. The inclusion of multirooted teeth, where irrigation protocols may play a greater role in treatment success (Laukkanen et al. 2021), or those that present with active clinical signs and symptoms of periradicular disease would be of particular interest to improve the generalisability of results. In the present trial, a secondary rationale of the multi-visit approach was to provide researchers with an opportunity to reversibly exit participants from the trial and onto conventional protocols in the event of any serious adverse effects. As these were not encountered the impact of a single visit approach, which by conventional standards has shown no significant differences to multi-visit protocols, could also be investigated (Mergoni et al. 2022).

Secondly, despite significant advancements in traditional pulp regeneration procedures, outcomes remain highly unpredictable with the formation of various types of repair tissue (Kim et al. 2018, Wei et al. 2022). One potential explanation for this could be the lack of dentine extracellular matrix-derived signalling molecules as a result of the NaOCl pre-conditioning regime (Galler et al. 2015). The aforementioned irrigation protocol could thus be considered and investigated for these procedures, which are also typically performed on single rooted albeit immature teeth, to potentially maximise dECM bioavailability for pulp regeneration.

It is a common theme that prior *in vitro* experimental conditions investigating dECM bioavailability do not reflect *in vivo* conditions well (Galler et al. 2015; Sadaghiani et al. 2016; Widbiller et al. 2018). More specifically, dentine powders and disks (Galler et al. 2015, Sadaghiani et al. 2016, Tomson et al. 2013, Widbiller et al. 2018) have often been used and analyses have been performed after many hours of immersing intact prepared teeth in media (Chae et al. 2018, Zeng et al. 2016). In contrast, the

experimental model utilised to harvest dECMs in the current investigation bridges the gap between laboratory-based protocols and *in vivo* application. For instance, teeth were left intact and root canals prepared to standardised dimensions leaving only a clinically representative surface area from which growth factors could be liberated. This model also facilitates the administration of proprietary solutions via conventional needle irrigation, which are capable of being activated by any means within a closed system. Irrigant exposure times, concentrations and volumes can also be tailored to those recommended by clinical guidelines (Galler et al. 2016). Finally, this was the first model to consider the interaction between dECMs solubilised into the canal and periradicular tissues. This model is thus well suited for future *in vitro* investigations that aim to investigate the efficacy of various chemomechanical debridement protocols on the intracanal and periradicular bioavailability of dECMs.

Another area of this project that is becoming increasingly prominent within the endodontic literature is the use of biomarkers to detect and diagnose disease, determine disease recurrence or progression; prognose compromised teeth; and predict response to treatment (Zehnder & Belibasakis 2022). The present diagnostic analysis reveals PTF as being a valid source of biomarkers for periradicular disease. Obtaining this objective information would be clinically useful for confirming the resolution of periapical inflammation immediately prior to obturation, particularly considering treatment outcomes are currently determined via long term temporal analysis of plain-film radiographs (ESE 2006). Specific clinical situations that would benefit include orthograde treatment of larger lesions where root end surgery may be anticipated or assisting diagnosis of nonspecific orofacial pain in patients with previously initiated root canal treatment. Additional investigations with considerably larger sample sizes that correlate these biomarkers to clinical signs and symptoms of

periradicular disease, treatment outcomes and biomarkers in more accessible tissue exudates of the same tooth, principally gingival crevicular fluid, are now required. Furthermore, cell culture experiments to determine the exact roles of some of the more prominently detected analytes, namely IL-6. IL-18, OLR-1, OSM, TNFSF-10 and -12, in periradicular pathophysiology are also warranted.

7.5 Conclusion

The prospect of regenerating damaged or diseased endodontic tissues has largely driven the current paradigm shift towards more biologically driven treatment strategies for managing endodontic diseases. Whilst these have typically been reserved for regenerating the pulp in immature permanent teeth, the discovery of PL-MSCs have ignited interests in applying similar principles to periradicular disease treatment in teeth with fully formed apices. The body of work presented here explores one such approach where endogenous dECMs are exploited using EDTA-based therapeutic irrigant protocols to directly upregulate intrinsic regenerative capacity of PL-MSCs, which in turn result in favourable clinical outcomes than conventional antimicrobial only approaches. Data from the various *in vitro* and *in vivo* experiments conducted have supported this hypothesis. More specifically, data has for the first time revealed the:

- deleterious effects of guideline standard regenerative protocols on dECM integrity
- confirmed the periradicular bioavailability of dECMs with EDTA based irrigation regimes
- the upregulation of regenerative events in PL-MSC cultures when dECMs were topically applied

- optimal methods for sampling and analysing PTF-derived biomarkers for detecting such subclinical changes
- one-year clinical outcomes of root canal treatment conducted with EDTA based therapeutic irrigant protocols being on par with conventional antimicrobial only approaches.

These findings have significant translation potential and now calls into question the need for the use of NaOCl in regenerative endodontic procedures. These paradigm shifting implications require further strengthening via appropriately powered multicentred phase III trials with longer review intervals and wider inclusion criteria.

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STATEMENT OF CANDIDATES CONTRIBUTION

Publication 1: Exploiting Dentine Matrix Proteins in Cell-Free Approaches for Periradicular Tissue Engineering.

SSV contributed to: Conceptualisation (lead); Data Curation (lead); Formal Analysis (lead); Funding Acquisition (supporting); Investigation (lead); Methodology (lead); Project Administration (lead); Resources (supporting); Visualisation (lead); Writing – original draft (lead); Writing – review & editing (equal).

Publication 2: Bioavailability of dentine extracellular matrix component with sodium hypochlorite irrigant regimes and their regenerative effects on periapical lesion-derived mesenchymal stem cells: an in vitro study

SSV contributed to: Conceptualisation (equal); Data Curation (lead); Formal Analysis (lead); Investigation (lead); Methodology (equal); Funding Acquisition (lead); Project Administration (equal); Resources (supporting); Validation (equal); Visualisation (lead); Writing – Original Draft Preparation (lead); Writing – Review & Editing (equal).

Publication 3: Current trends in endodontic irrigation amongst general dental practitioners and dental schools within the United Kingdom and Ireland: a cross-sectional survey

SSV contributed to: Conceptualisation (lead); Data Curation (lead); Formal Analysis (lead); Funding Acquisition (lead); Investigation (equal); Methodology (lead); Project

Administration (lead); Resources (supporting); Visualisation (lead); Writing – original draft (lead); Writing – review & editing (equal).

Publication 4: The influence of irrigant activation, concentration and contact time on sodium hypochlorite penetration into root dentine: an ex vivo experiment

SSV contributed to: Conceptualisation (lead); Data Curation (lead); Formal Analysis (supporting); Funding Acquisition (supporting); Investigation (lead); Methodology (lead); Project Administration (lead); Resources (supporting); Visualisation (lead); Writing – original draft (lead); Writing – review & editing (equal).

Publication 5: Antimicrobial Efficacy of Different Irrigant Solutions Using a Novel Biofilm Model: An In Vitro Confocal Laser Scanning Microscopy Experiment.

SSV contributed to: Conceptualisation (equal); Data Curation (lead); Formal Analysis (lead); Investigation (lead); Methodology (supporting); Funding Acquisition (supporting); Project Administration (lead); Resources (supporting); Validation (supporting); Visualisation (lead); Writing – Original Draft Preparation (lead); Writing – Review & Editing (equal).

Publication 6: A systematic review of methods used to sample and analyse periradicular tissue fluid during root canal treatment.

SSV Contributed to: Conceptualisation (lead); Data Curation (lead); Formal Analysis (lead); Funding Acquisition (supporting); Investigation (lead); Methodology (lead);

Project Administration (lead); Resources (lead); Visualisation (lead); Writing – original draft (lead); Writing – review & editing (equal).

Publication 7: Periradicular tissue fluid-derived biomarkers for apical periodontitis: An in vitro methodological and in vivo cross-sectional study

SSV Contributed to: Conceptualisation (lead); Data Curation (lead); Formal Analysis (supporting); Funding Acquisition (lead); Investigation (lead); Methodology (lead); Project Administration (lead); Resources (lead); Visualisation (lead); Writing – original draft (lead); Writing – review & editing (equal)

Publication 8: Therapeutic Irrigant Procedures for Treating Apical Periodontitis (TIPTAP): A triple blinded parallel group randomised controlled phase I/II trial.

SSV Contributed to: Conceptualisation (equal); Data Curation (lead); Formal Analysis (lead); Funding Acquisition (supporting); Investigation (lead); Methodology (lead); Project Administration (equal); Resources (equal); Visualisation (lead); Writing – original draft (lead); Writing – review & editing (equal)

APPENDICES

APPENDIX 1

CHAPTER 2

PUBLICATION 1

IN VIVO STUDIES EVALUATING THE THERAPEUTIC POTENTIALS OF DENTAL MESENCHYMAL STEM CELLS

Supplementary Table S1: *In vivo* studies evaluating the therapeutic potentials of of dental mesenchymal stem cells. Studies have been grouped based on primary disease treated. The references associated with this table are listed below.

Study	Target Disease	Niche	Animal	Key finding
Yamaza *et al.* (2010)[1]	Autoimmune	SHED	Mouse	SHED transplantation reversed systemic lupus erythematous associated disorders
Ishikawa *et al.* (2016)[2]	Autoimmune	SHED	Mouse	SHED-conditioned media infusion prevented exacerbation of rheumatoid arthritis symptoms
Shimojima *et al.* (2016)[3]	Autoimmune	SHED	Mouse	SHED-conditioned media infusion reduced neuronal injury associated with autoimmune encephalomyelitis
Gandia *et al.* (2008)[4]	Cardiovascular	DPSC	Rat	DPSC injection improved cardiac function after myocardial infarction
Yamaguchi *et al.* (2015)[5]	Cardiovascular	SHED	Rat	SHED-conditioned media infusion reduced infarct size after myocardial infarction
Chiu *et al.* (2016)[6]	Cardiovascular	DPSC	Rat	DPSC implantation promoted neurogenesis and angiogenesis in the presence of hypoxia-ischemia
Lee *et al.* (2016)[7]	Cardiovascular	DPSC	Rat	DPSC transplantation induced angiogenesis and improved neurological function after stroke
Miura *et al.* (2003)[8]	Dento-alveolar	SHED	Mouse	SHED transplantation increased dentine tissue-like deposition
Seo *et al.* (2004)[9]	Dento-alveolar	PDLSC	Mouse	PDLSC transplantation increased periodontal tissue regeneration in surgically created periodontal defects
Sonoyama *et al.* (2006)[10]	Dento-alveolar	PDLSC / SCAP	Mouse	PDLSC / SCAP transplantation increased root/periodontal complex regeneration capable of supporting porcelain crowns
Liu *et al.* (2008)[11]	Dento-alveolar	PDLSC	Swine	PDLSC transplantation increased periodontal tissue regeneration in surgically created periodontal defects
Cordeiro *et al.* (2008)[12]	Dento-alveolar	SHED	Mouse	SHED transplantation improved regeneration of pulp-like tissue in tooth slices
Ding *et al.* (2010)[13]	Dento-alveolar	PDLSC	Swine	PDLSC transplantation increased periodontal tissue regeneration in surgically created periodontal defects
Huang *et al.* (2010)[14]	Dento-alveolar	DPSC / SCAP	Mouse	DPSC and SCAP seeding increased regeneration of vascularised pulp-like tissue in emptied root canals

Alsanea *et al.* (2011)[15]	Dento-alveolar	DPSC	Mouse	DPSC transplantation increased dentine regeneration in endodontic perforation defects
Park *et al.* (2011)[16]	Dento-alveolar	DPSC / PDLSC	Canine	PDLSC transplantation increased periodontal tissue regeneration in surgically created periodontal defects
Khorsand *et al.* (2013)[17]	Dento-alveolar	DPSC	Canine	PDLSC transplantation increased periodontal tissue regeneration in surgically created periodontal defects
Rosa *et al.* (2013)[18]	Dento-alveolar	SHED	Mouse	SHED injection increased regeneration of vascularised pulp-like tissue in emptied root canals
Yu *et al.* (2013)[19]	Dento-alveolar	GMSC	Canine	GMSC transplantation increased periodontal tissue regeneration in surgically created periodontal defects
Nakashima & Iohara (2014)[20]	Dento-alveolar	DPSC	Canine	DPSC seeding increased regeneration of vascularised pulp-like tissue in emptied root canals
Kanafi *et al.* (2013)[21]	Endocrine	SHED	Mouse	SHED-derived islet cell transplantation reversed streptozotocin-induced diabetes
Cho *et al.* (2015)[22]	Hepatic	DPSC	Mouse	DPSC transplantation suppressed liver fibrosis and improved liver function
Yamaza *et al.* (2015)[23]	Hepatic	SHED	Mouse	SHED transplantation improved liver dysfunction and reduced fibrosis
de Mendonça Costa *et al.* (2008)[24]	Musculoskeletal	DPSC	Rat	DPSC transplantation increased bone regeneration in cranial defects
d'Aquino *et al.* (2009)[25]	Musculoskeletal	DPSC	Human	DPSC transplantation increased bone and periodontal ligament regeneration in mandibular bony defects
Zheng *et al.* (2009)[26]	Musculoskeletal	DPSC	Swine	DPSC transplantation increased bone regeneration in mandibular bony defects
Yang *et al.* (2010)[27]	Musculoskeletal	DPSC	Mouse	DPSC transplantation increased muscle regeneration in muscle defects
Honda *et al.* (2011)[28]	Musculoskeletal	DFSC	Rat	DFSC transplantation increased bone regeneration in calvaric defects
Pisciotta *et al.* (2012)[29]	Musculoskeletal	DPSC	Rat	DPSC transplantation increased bone regeneration in calvaric defects
Riccio *et al.* (2012)[30]	Musculoskeletal	DPSC	Rat	DPSC transplantation increased bone regeneration in calvaric defects
Giuliani *et al.* (2013)[31]	Musculoskeletal	DPSC	Human	DPSC transplantation increased bone regeneration in mandibular bony defects
Maraldi *et al.* (2013)[32]	Musculoskeletal	DPSC	Rat	DPSC transplantation increased bone regeneration in calvaric defects
Annibali *et al.* (2014)[33]	Musculoskeletal	DPSC	Rat	DPSC transplantation increased bone regeneration in calvaric defects

Fujii *et al.* (2018)[34]	Musculoskeletal	DPSC	Mouse	DPSC transplantation increased bone regeneration in calvaric defects
Liu *et al.* (2020)[35]	Musculoskeletal	ABMSC	Rabbit	DPSC transplantation increased bone regeneration in calvaric defects
Arthur *et al.* (2009)[36]	Neurodegenerative	DPSC	Avian	DPSC transplantation guided axon generation and neuroplasticity
Wang *et al.* (2010)[37]	Neurodegenerative	SHED	Mouse	SHED transplantation improved parkinsonian behaviour disorders
Matsubara *et al.* (2015)[38]	Neurodegenerative	SHED	Rat	SHED-conditioned media infusion increased functional recovery after spinal cord injury
Mita *et al.* (2015)[39]	Neurodegenerative	SHED	Mouse	SHED-conditioned media infusion increased cognitive function in Alzheimer's model
Yamamoto *et al.* (2016)[40]	Neurodegenerative	DPSC	Rat	DPSC transplantation induced myelinated fibre regeneration
Nicola *et al.* (2017)[41]	Neurodegenerative	SHED	Rat	SHED transplantation increased functional recovery after spinal cord injury
Zhang *et al.* (2018)[42]	Neurodegenerative	SHED	Rat	SHED transplantation improved parkinsonian motor defects
Gomes *et al.* (2010)[43]	Ophthalmic	DPSC	Mouse	DPSC transplantation induces cornea regeneration in corneal defects
Syed-Picard *et al.* (2015)[44]	Ophthalmic	DPSC	Mouse	DPSC injection induces cornea regeneration in corneal defects
Ueda *et al.* (2010)[45]	Dermatological	SHED	Mouse	SHED-conditioned media infusion improved wound healing and reduced UV damage
Wakayama *et al.* (2015)[46]	Respiratory	SHED	Mouse	SHED-conditioned media infusion attenuated lung injury and improved survival rates

ABMSC: alveolar bone mesenchymal stem cell; DPSC: dental pulp stem cell; DFSC: dental follicle stem cell; GMSC: gingival mesenchymal stem cells; SCAP: stem cells of the apical papilla; SHED: stem cells from human exfoliated deciduous teeth

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APPENDIX 2

CHAPTER 4

PUBLICATION 3

SAMPLE QUESTIOAIRRES TO DENTAL SCHOOLS

AND GENERAL DENTAL PRACTITIONERS

Supplementary 1: Sample questionnaire distributed to endodontic course leads in UK & Ireland dental schools

Q1 Which of the following irrigants do you teach undergraduate students to use during primary root canal treatment? (Please note you may choose more than one option)

- Chlorhexidine
- Citric Acid
- Dual Rinse HEDP
- Ethylenediaminetetraacetic acid (EDTA)
- Hydrogen Peroxide
- Iodine
- Local Anaesthetic
- Saline
- Sodium Hypochlorite (NaOCl)
- Other (please specify)

Q2 If NaOCl was selected, please could you specify the concentration that you teach undergraduate students to use during primary root canal treatment?

- N/A (do not use NaOCl)
- 0.5 – 1.0%
- 1.1 – 2.0%
- 2.1 – 3.0%
- 3.1 – 4.0%
- 4.1 – 5.0%
- 5.1 – 6.0%
- Other (please specify)

Q3 If NaOCl was selected, please could you specify the average time period in minutes that you teach undergraduate students to use this irrigant for during primary root canal treatment:

- N/A (do not use NaOCl)
- > 0 – 5
- > 5 – 10
- > 10 – 15
- > 15 – 20
- > 20 – 25
- > 25 – 30
- > 35 – 40
- Other (please specify)

Q4 If EDTA was selected, please could you specify the average time period that you teach undergraduate students to use this irrigant for during primary root canal treatment?

- N/A (do not use EDTA)
- > 0 – 5
- > 5 – 10
- > 10 – 15
- > 15 – 20
- > 20 – 25
- > 25 – 30
- > 35 – 40
- Other (please specify)

Q5 If both NaOCl and EDTA were selected, what penultimate & final rinse sequence do you teach undergraduate students to use during primary root canal treatment?

- N/A (do not use NaOCl and/or EDTA)
- NaOCl penultimate and EDTA final
- EDTA penultimate and NaOCl final
- Other (please specify)

Q6 Do you teach undergraduate students to routinely heat the irrigants during primary root canal treatment?

- Yes
- No
- Other (please specify)

Q7 Which of the following irrigant activation techniques do you teach undergraduate students to use during primary root canal treatment?

(Please note you may choose more than one option)

- N/A (do not agitate irrigants)
- Apical Negative Pressure (e.g. EndoVac)
- Manual Dynamic Activation (GP Pumping)
- Passive Ultrasonic Irrigation
- Sonic Irrigation (e.g. EndoActivaor)
- Other (please specify)

Q8 If you teach undergraduate students to routinely use irrigant activation techniques during primary root canal treatment, please could you specify the recommended duration in seconds per canal?

- N/A (do not agitate irrigants)
- > 0 – 30
- > 30 – 60
- > 60 – 90
- > 90 – 120
- > 120 – 150
- > 150 – 180
- Other (please specify)

Q9 Please could you specify a reason for selecting your irrigant choice in Q1?

Q10 Do you have any further comments or information you would like to add?

Supplementary 2: Sample questionnaire distributed to GDPs in the UK

Q1 Did you attain your primary qualification from a dental institute within the UK or Ireland?

- Yes
- No

Q2 If selecting yes to Q1, please can you state the dental school you attained your primary qualification from?

Q3 How many years have you practiced as a general dentist within the UK?

Q4 Which country do you predominately practice in?

- England
- Northern Ireland
- Scotland
- Wales

Q5 Approximately what proportion of your practice would you deem private?

- 0 – 50 %
- 51 – 100%

Q6 Which of the following irrigants do you use during primary root canal treatment? (Please note you may choose more than one option)

- Chlorhexidine
- Citric Acid
- Dual Rinse HEDP
- Ethylenediaminetetraacetic acid (EDTA)
- Hydrogen Peroxide
- Iodine
- Local Anaesthetic
- Saline
- Sodium Hypochlorite (NaOCl)
- Other (please specify)

Q7 If NaOCl was selected, please could you specify the concentration that you routinely use during root canal treatment?

- N/A (do not use NaOCl)
- 0.5 – 1.0%
- 1.1 – 2.0%
- 2.1 – 3.0%
- 3.1 – 4.0%
- 4.1 – 5.0%
- 5.1 – 6.0%
- Other (please specify)

Q8 If NaOCl was selected, please could you specify the average time period in minutes that you use this irrigant for during primary root canal treatment:

- N/A (do not use NaOCl)
- > 0 – 5
- > 5 – 10
- > 10 – 15
- > 15 – 20
- > 20 – 25
- > 25 – 30
- > 35 – 40

Q9 If EDTA was selected, please could you specify the average time period that you use this irrigant for during primary root canal treatment?

- N/A (do not use EDTA)
- > 0 – 5
- > 5 – 10
- > 10 – 15
- > 15 – 20
- > 20 – 25
- > 25 – 30
- > 35 – 40
- Other (please specify)

Q10 If both NaOCl and EDTA were selected, what penultimate & final rinse sequence do use during primary root canal treatment?

- N/A (do not use NaOCl and/or EDTA)
- NaOCl penultimate and EDTA final
- EDTA penultimate and NaOCl final
- Other (please specify)

Q11 Do you routinely heat the irrigants during primary root canal treatment?

- Yes
- No
- Other (please specify)

Q12 Which of the following irrigant activation techniques do you to use during primary root canal treatment? (Please note you may choose more than one option)

- N/A (do not agitate irrigants)
- Apical Negative Pressure (e.g. EndoVac)
- Manual Dynamic Activation (GP Pumping)
- Passive Ultrasonic Irrigation
- Sonic Irrigation (e.g. EndoActivaor)
- Other (please specify

Q13 If you routinely use irrigant activation techniques during primary root canal treatment, please could you specify the duration in seconds per canal?

- N/A (do not agitate irrigants)
- > 0 – 30
- > 30 – 60
- > 60 – 90
- > 90 – 120
- > 120 – 150
- > 150 – 180
- Other (please specify)

Q14 Please could you specify a reason for selecting your irrigant choice in Q5?

Q15 Do you have any further comments or information you would like to add?

APPENDIX 3

CHAPTER 5

PUBLICATION 6

DESIGN SPECIFIC CRITERIA USED FOR ASSESSING THE BIAS IN STUDIES WHICH MET THE INCLUSION CRITERIA IN THE SYSTEMATIC REVIEW

Bias Domain	Criterion	RCTs	CCTs	Cohort	Cross-section
Selection	Was the allocation sequence generated adequately (e.g., random number table, computer-generated randomization)?	x			
	Was the allocation of treatment adequately concealed (e.g., pharmacy-controlled randomization or use of sequentially numbered sealed envelopes)?	x			
	Were participants analysed within the groups they were originally assigned to?	x	x		
	Did the study apply inclusion/exclusion criteria uniformly to all groups?	x	x	x	x
	Did the strategy for recruiting participants into the study differ across study groups?	x	x	x	x
	Does the design or analysis control account for important confounding and modifying variables through matching, stratification, multivariable analysis, or other approaches?	x	x	x	x
Performance	Did researchers rule out any impact from a concurrent intervention or an unintended exposure that might bias results?	x	x		
	Did the study maintain fidelity to the intervention protocol?	x	x	x	x
Attrition	If attrition (overall or differential nonresponse, dropout, loss to follow-up, or exclusion of participants) was a concern, were missing data handled appropriately (e.g., intention-to-treat analysis and imputation)?	x	x	x	x
Detection	Were interventions implemented consistently across all study participants?	x	x		
	In prospective studies, was the length of follow-up different between the groups?	x	x	x	
	Were the outcome assessors blinded to the intervention or exposure status of participants?	x	x	x	x
	Were outcomes assessed using valid measures and implemented consistently across all study participants?	x	x	x	x
Reporting	Were the potential outcomes pre-specified by the researchers? Are all pre-specified outcomes reported?	x	x	x	x

APPENDIX 4

CHAPTER 5

PUBLICATION 6

EXCLUDED ARTICLES AT FULL TEXT EVALUATION

	Study	Reason for Exclusion
1	Rocca *et al.* (1987)	Did not sample periradicular tissue fluid
2	Barkhordar *et al.* (1999)	Did not use orthograde sampling technique
3	Noda *et al.* (2000)	Did not sample periradicular tissue fluid
4	Yu *et al.* (2002)	Unable to attain access to full text
5	Siqueira & Rôças (2003)	Did not use orthograde sampling technique
6	Siqueira & Rôças (2004)	Did not use orthograde sampling technique
7	Silva *et al.* (2005)	Did not use orthograde sampling technique
8	Vernal *et al.* (2006)	Did not use orthograde sampling technique
9	Machado de Oliveira *et al.* (2007)	Did not use orthograde sampling technique
10	Yan *et al.* (2007)	Unable to attain access to full text
11	Nonaka *et al*. (2008)	Did not use orthograde sampling technique
12	Soares *et al.* (2008)	Did not sample periradicular tissue fluid
13	Gazivoda *et al.* (2009)	Did not use orthograde sampling technique
14	Dezerega *et al.* (2010)	Did not use orthograde sampling technique
15	Wang *et al.* (2010)	Did not sample periradicular tissue fluid
16	Ferreira *et al.* (2011)	Did not use orthograde sampling technique
17	Martinho *et al.* (2012)	Did not sample periradicular tissue fluid
18	Amaya *et al.* (2013)	Did not sample periradicular tissue fluid
19	Hernádi *et al.* (2013)	Did not use orthograde sampling technique
20	Provenzano *et al.* (2013)	Did not use orthograde sampling technique
21	Araujo-Pires *et al.* (2014)	Did not use orthograde sampling technique
22	Sousa *et al.* (2014)	Did not use orthograde sampling technique
23	Keleş & Alçin (2015)	Case report
24	Baeza *et al.* (2016)	Did not sample periradicular tissue fluid
25	Carvalho *et al.* (2016)	Did not sample periradicular tissue fluid
26	Alfenas *et al.* (2017)	Did not use orthograde sampling technique
27	Pourhajibagher *et al.* (2017)	Did not sample periradicular tissue fluid

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APPENDIX 5

CHAPTER 5

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RESULTS TABLE FOR RISK OF BIAS ASSESSMENT

Study	Selection	Performance	Attrition	Detection	Reporting	Overall Bias
Safavi & Rossomando (1991)	+	+	+	-	?	Medium
Matsuo *et al.* (1994)	+	+	+	?	?	Medium
Matsuo *et al.* (1995)	+	+	+	?	?	Medium
Shimauchi *et al.* (1996)	?	?	?	-	?	High
Takayama *et al.* (1996)	+	+	+	-	?	Medium
Takeichi *et al.* (1996)	+	+	+	?	?	Medium
Shimauchi *et al.* (1997)	-	-	+	-	?	High
Shimauchi *et al.* (1998)	-	+	+	-	?	Medium
Takeichi *et al.* (1998)	+	?	+	?	?	Medium
Kuo *et al.* (1998a)	+	-	+	-	?	Medium
Kuo *et al.* (1998b)	+	-	+	-	?	Medium
Shimauchi *et al.* (2001)	-	+	+	-	?	Medium
Ataoğlu *et al.* (2002)	+	-	?	-	?	High
Wahlgren *et al.* (2002)	+	+	?	+	?	Medium
Liu *et al.* (2002)	+	+	+	-	?	Medium
Alptekin *et al.* (2005a)	+	-	+	-	?	Medium
Alptekin *et al.* (2005b)	+	-	+	-	?	Medium
Pezelj-Ribarić *et al.* (2007)	?	+	+	-	?	Medium
Henriques *et al.* (2011)	+	+	+	+	?	Low
Shariar *et al.* (2011)	?	-	+	+	?	Medium
de Brito *et al.* (2012)	+	+	+	?	?	Medium
Ehsani *et al.* (2012)	?	-	+	+	+	Medium
Tavares *et al.* (2012)	+	+	+	+	?	Low
Grga *et al.* (2012)	+	+	+	+	?	Low
Tavares *et al.* (2013)	+	+	+	-	?	Medium
Rechenberg *et al.* (2014)	+	?	+	-	?	Medium
Bambirra *et al.* (2015)	+	?	+	-	?	Medium
de Brito *et al.* (2015)	-	?	+	-	?	Low
Ferreira *et al.* (2015)	-	?	+	-	?	Low
Martinho *et al.* (2015)	?	+	+	+	?	Medium
Martinho *et al.* (2016)	+	+	+	+	?	Low
Sette-Dias *et al.* (2016)	-	-	+	-	?	High
Zhi *et al.* (2017)	?	+	+	+	?	Medium

APPENDIX 6

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COMPLETE TARGET 48 PANEL WITH FREQUENCY OF MISSING DATA

Target-48 cytokine panel	Detection Threshold Met	Missing Data Frequency
Chemokine Ligand [CCL]-2	Yes	2%
Chemokine Ligand [CCL]-3	Yes	0%
Chemokine Ligand [CCL]-4	Yes	3%
Chemokine Ligand [CCL]-7	No	46%
Chemokine Ligand [CCL]-8	No	30%
Chemokine Ligand [CCL]-11	No	68%
Chemokine Ligand [CCL]-13	No	48%
Chemokine Ligand [CCL]-19	No	66%
Chemokine Ligand [CXCL]-9	Yes	22%
Chemokine Ligand [CXCL]-10	No	29%
Chemokine Ligand [CXCL]-11	No	60%
Chemokine Ligand [CXCL]-12	No	68%
Colony Stimulating Factor [CSF]-1	Yes	7%
Colony Stimulating Factor [CSF]-2	No	63%
Colony Stimulating Factor [CSF]-3	No	30%
Epidermal Growth Factor [EGF]	No	61%
Fms-related Tyrosine Kinase 3 Ligand [FLT3LG]	No	70%
Human Growth Factor [HGF]	Yes	0%
Interferon Gamma [IFNG]	No	54%
Interleukin [IL]-1β	Yes	0%
Interleukin [IL]-2	No	70%
Interleukin [IL]-4	No	68%
Interleukin [IL]-6	Yes	0%
Interleukin [IL]-7	No	70%
Interleukin [CXCL]-8	Yes	0%
Interleukin [IL]-10	No	69%
Interleukin [IL]-13	No	57%
Interleukin [IL]-15	No	66%
Interleukin [IL]-17A	Yes	12%
Interleukin [IL]-17C	No	68%
Interleukin [IL]-17F	No	66%
Interleukin [IL]-18	Yes	0%
Interleukin [IL]-27	No	70%
Interleukin [IL]-33	No	68%
Lymphotoxin [LT]-α	No	42%
Matrix Metallopeptidase [MMP]-1	Yes	7%
Matrix Metallopeptidase [MMP]-12	Yes	9%
Oncostatin M [OSM]	Yes	0%
Oxidised Low Density Lipoprotein Receptor [OLR]-1	Yes	0%
Thymic Stromal Lymphopoietin [TSLP]	No	66%
Transforming growth factor [TGF]-α	No	41%
Tumour Necrosis Factor [TNF]	No	57%
Tumour Necrosis Factor Superfamily [TNFSF]-10	Yes	5%
Tumour Necrosis Factor Superfamily [TNFSF]-12	Yes	12%
Vascular Endothelial Growth Factor [VEGF]-A	Yes	0%

Appendix S1: Complete Target-48 Panel with frequency of missing data. Rows highlighted in green indicate analytes that met the detection threshold and were used for subsequent analyses.

APPENDIX 7

CHAPTER 5

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DIAGNOSTIC PERFORMANCE DATA OF PTF-DERIVED ANALYTES

Analyte	TP	TN	FP	FN	Accuracy	Sens.	Spec.	PPV	NPV	Youden
Chemokine Ligand [CCL]-2	27	9	4	4	0.82 [0.70-0.93]	0.87 [0.77-0.97]	0.69 [0.56-0.83]	0.87 [0.77-0.97]	0.69 [0.56-0.83]	0.67 [0.67-0.67]
Chemokine Ligand [CCL]-3	26	11	5	2	0.84 [0.73-0.95]	0.93 [0.85-1.00]	0.69 [0.55-0.82]	0.84 [0.73-0.95]	0.85 [0.74-0.95)	0.79 [0.79-0.80]
Chemokine Ligand [CCL]-4	23	11	8	2	0.77 [0.65-0.90]	0.92 [0.84-1.00]	0.58 [0.43-0.72]	0.74 [0.61-0.87]	0.58 [0.43-0.72]	0.76 [0.76-0.76]
Colony Stimulating Factor [CSF]-1	26	11	5	2	0.84 [0.73-0.95]	0.93 [0.85-1.00]	0.69 [0.55-0.82]	0.84 [0.73-0.95)]	0.85 [0.74-0.95]	0.80 [0.79-0.80]
Chemokine Ligand [CXCL]-9	22	11	9	2	0.75 [0.62-0.88]	0.92 [0.84-1.00]	0.55 [0.40-0.70]	0.71 [0.58-0.84]	0.85 [0.74-0.95]	0.59 [0.59-0.59]
Hepatocyte Growth Factor [HGF]	27	12	4	1	0.89 [0.79-0.98]	0.96 [0.91-1.00]	0.75 [0.62-0.88]	0.87 [0.77-0.97]	0.92 [0.84-1.00]	0.89 [0.89-0.89]
Interleukin [IL]-1β	26	11	5	2	0.84 [0.73-0.95]	0.93 [0.85-1.00]	0.69 [0.55-0.82]	0.84 [0.73-0.95]	0.85 [0.74-0.95]	0.80 [0.79-0.80]
Interleukin [IL]-6	21	12	10	1	0.75 [0.62-0.88]	0.95 [0.89-1.00]	0.55 [0.40-0.69]	0.68 [0.54-0.82]	0.92 [0.84-1.00]	0.71 [0.71-0.71]
Interleukin [CXCL]-8	20	11	11	2	0.70 [0.57-0.84]	0.91 [0.82-0.99]	0.50 [0.35-0.65]	0.65 [0.50-0.79]	0.85 [0.74-0.95]	0.68 [0.68-0.68]
Interleukin [IL]-17A	25	13	6	0	0.86 [0.76-0.97]	1.00 [1.00-1.00]	0.68 [0.55-0.82]	0.81 [0.69-0.92]	1.00 [1.00-1.00]	0.84 [0.84-0.84]
Interleukin [IL]-18	28	11	3	2	0.89 [0.79-0.98]	0.93 [0.86-1.00]	0.79 [0.66-0.91]	0.90 [0.82-0.99]	0.85 [0.74-0.95]	0.85 [0.74-0.95]
Matrix Metallopeptidase [MMP]-1	27	13	4	0	0.91 [0.82-0.99]	1.00 [1.00-1.00]	0.76 [0.64-0.89]	0.87 [0.77-0.97]	1.00 [1.00-1.00]	0.90 [0.90-0.90]
Matrix Metallopeptidase [MMP]-12	23	10	8	3	0.75 [0.62-0.88]	0.88 [0.79-0.98]	0.56 [0.41-0.70]	0.74 [0.61-0.87]	0.77 [0.64-0.89]	0.76 [0.76-0.76]
Oxidised Low Density Lipoprotein Receptor [OLR]-1	28	12	3	1	0.91 [0.81-0.99]	0.97 [0.91-1.00]	0.80 [0.68-0.92]	0.90 [0.82-0.99]	0.92 [0.84-1.00]	0.86 [0.86-0.86]
Oncostatin M [OSM]	23	13	8	0	0.82 [0.70-0.93]	1.00 [1.00-1.00]	0.62 [0.48-0.76]	0.74 [0.61-0.87]	1.00 [1.00-1.00]	0.77 [0.77-0.77]
Tumour Necrosis Factor Superfamily [TNFSF]-10	26	11	5	2	0.84 [0.73-0.95]	0.93 [0.85-1.00]	0.69 [0.55-0.82]	0.84 [0.73-0.95]	0.85 [0.74-0.95]	0.87 [0.87-0.87]
Tumour Necrosis Factor Superfamily [TNFSF]-12	29	13	2	0	0.95 [0.89-1.00]	1.00 [1.00-1.00]	0.87 [0.77-0.97]	0.94 [0.77-0.97]	1.00 [1.00-1.00]	0.97 [0.97-0.97]
Vascular Endothelial Growth Factor [VEGF]-A	28	12	3	1	0.91 [0.82-0.99]	0.97 [0.91-1.00]	0.80 [0.68-0.92]	0.90 [0.82-0.99]	0.92 [0.84-1.00]	0.86 [0.86-0.86]
PCA Cluster	29	13	2	0	0.95 [0.89-1.00]	1.00 [1.00-1.00]	0.87 [0.77-0.97]	0.94 [0.86-1.00]	1.00 [1.00-1.00]	–

Appendix S2: Diagnostic performance data presented with [95% confidence intervals]. [TP] true positive; [TN] true negative; [FP] false positive; [FN] false negative; [Sens.] sensitivity; [Spec.] specificity; [PCA] principal component analysis; [PPV] positive predictive value; [NPV] negative predictive value.

APPENDIX 8

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COMPLETE TARGET 48 PANEL WITH FREQUENCY OF MISSING DATA

Target 48 Analyte	Missing Data (%)	Raw Analyte Concentration (pg / mL)			
		Baseline (S1)		Pre-obturation (S2)	
		5% NaOCl	17% EDTA	5% NaOCl	17% EDTA
Chemokine Ligand [CCL]-2	3	8.5 [2.30 – 16.34]	10.3 [5.36 – 66.88]	1.02 [0.16 – 7.47]	2.1 [0.59 – 10.83]
Chemokine Ligand [CCL]-3	5	7.6 [0.97 – 17.38]	7.4 [2.23 – 18.67]	0.5 [0.16 – 2.01]	0.5 [0.13 – 2.51]
Chemokine Ligand [CCL]-4	8	19.7 [4.34 – 52.53]	27.0 [7.25 – 50.46]	1.1 [0.40 – 5.43]	1.78 [0.13 – 10.07]
Chemokine Ligand [CCL]-7	70	-	-	-	-
Chemokine Ligand [CCL]-8	50	-	-	-	-
Chemokine Ligand [CCL]-11	93	-	-	-	-
Chemokine Ligand [CCL]-13	73	-	-	-	-
Chemokine Ligand [CCL]-19	95	-	-	-	-
Chemokine Ligand [CXCL]-9	35	-	-	-	-
Chemokine Ligand [CXCL]-10	70	-	-	-	-
Chemokine Ligand [CXCL]-11	93	-	-	-	-
Chemokine Ligand [CXCL]-12	100	-	-	-	-
Colony Stimulating Factor [CSF]-1	10	1.6 [0.42 – 2.51]	1.5 [0.70 – 3.41]	0.1 [0 – 0.97]	0.3 [0.14 – 0.87]
Colony Stimulating Factor [CSF]-2	93	-	-	-	-
Colony Stimulating Factor [CSF]-3	55	-	-	-	-
Epidermal Growth Factor [EGF]	90	-	-	-	-
Fms-related Tyrosine Kinase 3 Ligand [FLT3LG]	90	-	-	-	-
Hepatocyte Growth Factor [HGF]	3	41.7 [11.94 – 109.66]	62.7 [31.10 – 102.17]	2.7 [0.48 – 7.44]	8.2 [0.50 – 33.24]
Interferon Gamma [IFNG]	78	-	-	-	-
Interleukin [IL]-1β	3	70.0 [23.93 – 185.92]	146.28 [47.27 – 249.04]	0.5 [0.11 – 1.59]	0.8 [0.40 – 4.27]
Interleukin [IL]-2	100	-	-	-	-
Interleukin [IL]-4	100	-	-	-	-
Interleukin [IL]-6	5	2.8 [0.77 – 6.35]	1.7 [0.69 – 4.84]	0.1 [0.02 – 0.44]	0.7 [0.10 – 6.07]
Interleukin [IL]-7	100	-	-	-	-
Interleukin [CXCL]-8	3	271.6 [46.02 – 1254.32]	709.0 [53.60 – 1953.12]	16.5 [3.83 – 225.94]	23.9 [3.97 – 312.31]

Target 48 Analyte	Missing Data (%)	Raw Analyte Concentration (pg / mL)			
		Baseline (S1)		Pre-obturation (S2)	
		5% NaOCl	17% EDTA	5% NaOCl	17% EDTA
Interleukin [IL]-10	78	-	-	-	-
Interleukin [IL]-13	93	-	-	-	-
Interleukin [IL]-15	100	-	-	-	-
Interleukin [IL]-17A	35	-	-	-	-
Interleukin [IL]-17C	100	-	-	-	-
Interleukin [IL]-17F	90	-	-	-	-
Interleukin [IL]-18	0	41.0 [19.66 – 91.86]	54.9 [34.18 – 81.15]	0.2 [0.19 – 4.52]	13.78 [1.46 – 41.45]
Interleukin [IL]-27	100	-	-	-	-
Interleukin [IL]-33	90	-	-	-	-
Lymphotoxin [LT]-α	93	-	-	-	-
Matrix Metallopeptidase [MMP]-1	20	18.7 [1.53 – 67.80]	10.80 [2.03 – 60.40]	0.5 [0 – 14.76]	1.8 [0.02 – 21.06]
Matrix Metallopeptidase [MMP]-12	20	265.9 [12.94 – 2222.72]	143.32 [22.44 – 547.28]	41.4 [4.38 – 125.25]	26.1 [1.54 – 94.9]
Oncostatin M [OSM]	8	9.7 [2.05 – 29.18]	15.3 [5.83 – 39.55]	0.19 [0.06 – 3.06]	0.23 [0.04 – 3.63]
Oxidised Low Density Lipoprotein Receptor [OLR]-1	5	694.0 [155.40 – 1020.85]	804.6 [414.41 – 931.45]	11.2 [2.05 – 115.58]	11.16 [0.73 – 208.13]
Thymic Stromal Lymphopoietin [TSLP]	95	-	-	-	-
Transforming growth factor [TGF]-α	63	-	-	-	-
Tumour Necrosis Factor [TNF]	80	-	-	-	-
Tumour Necrosis Factor Superfamily [TNFSF]-10	8	15.1 [4.41 – 39.22]	27.2 [14.06 – 36.85]	0 [0- 0.57]	0 [0 – 2.86]
Tumour Necrosis Factor Superfamily [TNFSF]-12	33	-	-	-	-
Vascular Endothelial Growth Factor [VEGF]-A	3	40.0 [15.36 – 79.90]	63.7 [32.51 – 90.28]	4.4 [1.07 – 25.02]	6.9 [2.27 – 20.84]

Appendix 1: Complete Target-48 Panel with missing data frequency (%) and raw concentration values (pg/mL) for analytes that met the detection threshold.

APPENDIX 9

PUBLICATIONS & PRESENTATIONS ARISING FROM THIS DOCTORAL RESEARCH

PAPERS ACCEPTED FOR PUBLICATION

Virdee, S. S., Bashir, N. Z., Krstic, M., Camilleri, J., Grant, M. M., Cooper, P. R., & Tomson, P. L. (2023). Periradicular tissue fluid-derived biomarkers for apical periodontitis: An in vitro methodological and in vivo cross-sectional study. *International Endodontic Journal*, *56*(10), 1222–1240.

Virdee, S. S., Albaaj, F. S., Grant, M. M., Walmsley, D., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2023). Antimicrobial Efficacy of Different Irrigant Solutions Using a Novel Biofilm Model: An In Vitro Confocal Laser Scanning Microscopy Experiment. *The European Journal of Prosthodontics and Restorative Dentistry*, 31(1), 50–58.

Virdee, S. S., Bashir, N., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2022). Exploiting Dentine Matrix Proteins in Cell-Free Approaches for Periradicular Tissue Engineering. *Tissue Engineering. Part B, Reviews*, *28*(4), 707–732.

Virdee, S. S., Ravaghi, V., Camilleri, J., Cooper, P., & Tomson, P. (2020). Current trends in endodontic irrigation amongst general dental practitioners and dental schools within the United Kingdom and Ireland: a cross-sectional survey. *British Dental Journal*, [*Online: ahead of print]*.

Virdee, S. S., Farnell, D. J. J., Silva, M. A., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2020). The influence of irrigant activation, concentration and contact time on sodium hypochlorite penetration into root dentine: an ex vivo experiment. *International Endodontic Journal*, *53*(7), 986–997.

Virdee, S. S., Butt, K., Grant, M., Camilleri, J., Cooper, P. R., & Tomson, P. L. (2019). A systematic review of methods used to sample and analyse periradicular tissue fluid during root canal treatment. *International Endodontic Journal*, 52(8), 1108–1127.

CONFERENCE PRESENTATIONS

2023 British Endodontic Society Spring Scientific Meeting - London

2021 British Endodontic Society Annual General Meeting – Birmingham

2019 European Society of Endodontic Biennial Congress – Vienna

2019 British Society of Oral & Dental Research Annual Meeting - Leeds

2018 British Endodontic Society Spring Scientific Meeting - London

AWARDS

2023 British Endodontic Society Poster Prize

2021 British Endodontic Society Aspiring Researcher Award

GRANTS

2022 Oral & Dental Research Trust Glaxo-Smith-Kline Grant (£7 500)

2021 Royal College of Surgeons Edinburgh Pump Priming Grant (£10 000)

2020 British Endodontic Society Annual Research Grant (£15 000)

2020 European Society of Endodontics Young Investigator Research Grant (£5 000)

2019 British Endodontic Society Overseas Travel Grant (£250)