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

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Abstract

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 1st June 2018 which utilised 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 APPRASIAL & METHODS: In accordance with PRISMA guidelines, data was 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 CHARACTERSITCS: 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-immunosorbant-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 NSRCT. The most studied analytes were IL-1β and TNF-α.

IMPLICATIONS: Further research is required to develop an optimised PTF sampling and eluting protocol to overcome methodological heterogeneity and future studies are advised to follow a standardised approach to reporting data.

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 localised inflammatory response (Yamasaki *et al.* 1994, Stashenko *et al*. 1995). This reaction consists of various leukocytes (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 organised collection of leukocytes is a defensive response which acts to restrain endodontic pathogens inside the infected root canal (Metzger 2000, Márton & Kiss 2014). Additionally, its vascular nature results in production of an inflammatory exudate, which becomes enriched with key components of the immune response as the disease progresses (Nair 2004). In the literature this 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 supress 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 these 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 optimised, 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 warrants 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 is 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”.

Review

Methodology

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

- Pre-Registration

A protocol was pre-registered 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 utilising orthograde sampling techniques to retrieve PTF through the root canal during root canal treatment were included whilst those utilising 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. 1st 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 & 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 as per Liberati *et al.* (2009).

- 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 & 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 pre-piloted form to extract data items for evidence synthesis and quality assessment.

- Data items

Pre-determined information was extracted from published studies and organised 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 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 was unattainable it was coded as being not reported.

- Data synthesis & outcome measures

A qualitative synthesis was conducted on all studies that met the inclusion criteria. Briefly, key characteristics of each study were initially summarised 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. Target 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 & 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 randomised and non-randomised 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 S2). 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 the Appendix S1.

- Study characteristics & 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 6 evaluated the impact of clinical interventions on periradicular inflammatory mediator activity. Twenty-seven studies were observational in their design and 6 were interventional clinical trials, of which 4 were randomised. The objectives, design and results for individual studies can be found in Table 2.

Teeth sampled & 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/multi-rooted 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:

i) 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 ISO 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 (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, 2005b, Ehsani *et al.* 2012, Grga *et al.* 2012), eleven used 3 (Henriques *et al.* 2011, de Brito *et al.* 2012, Tavares *et al.* 2012, 2013, Rechenberg *et al.* 2014, Bambirra *et al.* 2015, de Brito *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 pre-coating 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 mms (Shahriair *el al.* 2011, de Brito *et al.* 2012, Tavares *et al.* 2012, 2013, Bambirra *et al.* 2015, de Brito *et al.* 2015, Ferreira *et al.* 2015, Martinho *et al.* 2015, 2016, Sette-Dias *et al.* 2016). 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).

ii) Fine needle aspiration (n=4): Two studies used a NeoDental 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 were not disclosed (Table 3).

iii) 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 3 times, two studies sampled 6 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 minutes 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, 2005b,

Ehsani *et al.* 2012). Seven studies excluded samples when "more than a small amount of blood" was retrieved (Kuo *et al.* 1998a, 1998b, Ataoğlu *et al.* 2002, Alptekin *et al.* 2005a, 2005b, 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, 2005b) 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

i) Proteomic/Metabolomic level (n=25): Enzyme-linked-immunosorbant 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 to 300 µls 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 minutes, five vortexed samples for 60 seconds, five centrifuged samples for 10–30 minutes at 4–15 000 x g, seven vortexed samples for 30 seconds and then centrifuged for 10 minutes at 5 000 x g and one incubated samples for 300 minutes, vortexed for 30 seconds and then centrifuged for 10 minutes at an unspecified gravity force. Further details on how samples were processed, including unspecified information, can be found in Table 3.

ii) 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 minutes at 12 000 x g, precipitation centrifugation for 10 minutes at 12 000 x g and then incubation for 10 minutes at 55^{o}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-ɣ 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, 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, Osteoprotegrin [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 & 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, 2005b, 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, 2005b, 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 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 standardisation 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 optimised 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.* (1996) was the only article identified in this review to carry out a similar experiment however, this assayed only IL-1β and the influence of various eluting buffers and techniques on the recovery of 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 or 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 4). This may be for several reasons, firstly, 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 have 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 & CCTs) originated from the lack of clarity on the randomisation 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 pre-registered 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 standardisation 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 optimised 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) needs to be investigated to develop an optimised 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 optimised sampling and eluting protocol and a standardised 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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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

Table 1: PubMed search strategy

Study			Sample		Mediators Studied	Methodology		Results
Author	Objectives	Design	Diagnosis & Size (n)	Tooth Type		PTF Sampling	Assay Technique	
Safavi & Rossomando (1991)	Compare PTF TNF-α levels in teeth with & without apical periodontitis	Cross-sectional	CAP (n=6) NAT (n=5)	Single/multi rooted	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 & IL-1β levels & clinical findings in teeth with apical lesions	Cohort	"Apical lesion" (n=69)	Single rooted	IL-1α & IL-1β	Fine Needle Aspiration	ELISA	Pus containing PTF had higher IL-1α ($p<0.01$) & 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 & IgA levels & clinical findings in teeth with apical lesions	Cohort	"Apical lesion" (n=69)	Single rooted	IgG & IgA	Fine Needle Aspiration	ELISA	IgG levels higher than IgA ($p<0.01$). Larger lesions showed higher IgG & IgA levels ($p<0.01$). Throughout treatment, IgG & IgA decreased.
Shimauchi *et al.* (1996)	Develop a quantitative method for collecting & quantifying biomarkers in PTF	Cross-sectional	CAP/AAP (n=29)	-	IL-1β	Paper Points	ELISA	There is a curvilinear relationship between absorbed PTF & 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 & clinical findings of teeth with apical periodontitis	Cross-sectional	"Apical lesion" (n=77)	-	PGE_2	Paper Points	RIA	Higher PGE_2 levels in teeth with radiolucent areas ($p<0.05$) & 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 (n=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 &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" (n=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 & IL-1ra levels in teeth with apical lesions	Cross-sectional	"Apical lesion" (n=29)	-	IL-1β & IL-1ra	Paper Points	ELISA	High levels of IL-1ra, compared with IL-1β, and positive correlations between IL-1ra & IL-1β levels found ($P<0.05$). IL-1ra:IL-1β ratio from symptomatic lesions lower than asymptomatic lesions ($p<0.05$).

Takeichi *et al.* (1998)	Quantify PTF NO levels in teeth with apical periodontitis	Cross-sectional	CAP/AAP (n=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 & IgM levels in teeth with apical lesions and explore correlations to clinical findings	Cohort	"Apical lesion" (n=32)	Single/multi rooted	βG, IL-1β, IgG, IgA & IgM	Methylcellulose filter paper strips	ELISA	Higher βG & IL-1β levels in pus containing PTF ($p<0.05$) & 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 & IgM levels when accessing the root canal of teeth with apical lesions	Cohort	"Apical lesion" (n=32)	Single/multi rooted	βG, IL-1β, IgG, IgA & IgM	Methylcellulose filter paper strips	ELISA	Mediator activity in less involved teeth did not change after treatment however, βG & IL-1β levels in teeth with pus containing PTF decreased after treatment whereas IgA & IgM increased ($p<0.05$).
Shimauchi *et al.* (2001)	Quantify PTF IL-8 & NO levels in teeth with apical lesions	Cross-sectional	"Apical lesion" (n=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 & TNF-α levels in teeth with apical lesions and explore correlation to clinical findings	Cross-sectional	"Apical lesion" (n=35)	Single rooted	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 & pulp MMPP-8 levels in teeth with necrotic pulps & apical lesions & monitor longitudinal changes during RCT	Cohort	CAP/AAP (n=11) NAT (n=10)	Single rooted	MMP-8	Paper Points	IFMA	MMP-8 levels decreased after treatment ($p<0.05$)
Liu *et al.* (2002)	Monitor longitudinal changes in PTF PGE_2 levels in teeth with AAP during RCT	Cohort	AAP (n=25)	Single/multi rooted	PGE_2	Paper Points	RIA	PGE_2 levels decreased after treatment ($p<0.0001$)
Alptekin *et al.* (2005a)	Explore correlations between PTF NE & PGE_2 levels & clinical findings in teeth with AAP & monitor longitudinal changes during RCT	Cohort	AAP (n=31)	Single rooted	PGE_2 & NE	Paper Points	ELISA	PGE2 & NE levels higher in teeth with clinical symptoms ($p<0.05$) however their levels did not change after treatment ($p>0.05$)
Alptekin *et al.* (2005b)	Explore correlations between PTF NE levels & clinical findings in teeth with AAP	Cross-sectional	AAP (n=31)	Single rooted	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 & explore correlations to clinical findings	Cross-sectional	AAP (n=20) CAP (n=40)	Single rooted	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 & without apical lesions refractory to endodontic treatment	Cross-sectional	CAP (n=20) NAT (n=20)	-	IL-1β, IL-17A, IFN-ɣ, IL-10, TNF-α & MCP-1	Paper Points	qPCR	Higher IFN-γ, TNF-α, IL-17A & MCP-1 mRNA expression in secondary lesions ($p<0.05$). No difference in IL-1β mRNA expression ($p>0.05$) between groups & IL-10 was insignificant in both groups ($p>0.05$).
Shahriari *et al.* (2011)	Evaluate impact of Ibuprofen on PTF IL-1β, TNF-α & PGE_2 levels in teeth with apical lesions undergoing RCT	Randomised controlled trial	CAP/AAP (n=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 & TNF-α reduction between groups before & after treatment ($p>0.05$).
de Brito *et al.* (2012)	Quantify PTF CD4+CD28+ & CD8+ derived inflammatory cytokine mRNA expression in teeth with CAP & monitor longitudinal changes during RCT	Cohort	CAP (n=20)	Single/multi rooted	IL-1β, IL-10, IL-17A, TNF-α, IFN-ɣ, MCP-1, MIP-1β, RANTES, RANKL, CXCR4, & CCR5	Paper Points	qPCR	IFN-γ, IL-1β, RANKL & RANTES mRNA expression reduced ($p<0.05$) & increase in IL-10 & CxCR4 mRNA expression after treatment ($p<0.05$). No difference in TNF-α, IL-17A, MCP-1, MIP-1β & CCR5 levels after treatment.
Ehsani *et al.* (2012)	Evaluate impact of Ibuprofen & N-acetylcysteine on PTF TNF- α, IL-6 and IL-17A levels in teeth with CAP & explore correlations to clinical findings	Randomised controlled trial	CAP (n=80)	Single/multi rooted	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-randomised controlled trial	CAP (n=20)	Single/multi rooted	IL-1β, IL-10 IL-17A, TNF-α, IFN-ɣ & MCP-1	Paper Points	qPCR	IL-1β, IL-10, IFN-ɣ mRNA expression in teeth receiving a calcium hydroxide dressing lower than in those that did not ($p<0.05$).
Grga *et al.* (2013)	Monitor longitudinal changes in PTF PGE2 levels during RCT in vital teeth with or without a large restorations	Cohort	NAT (n=47)	Single rooted	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-randomised controlled trial	CAP (n=20)	Single/multi rooted	IL-1β, IL-17A, IL-10, TNF-α, IFN-ɣ & MCP-1	Paper Points	qPCR	IL-1β, IL-10, MCP-1 & 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 & IL-8 levels in teeth with irreversible pulpitis to those with AAP	Cross-sectional	AAP (n=27) NAT (n=21)	Single/multi rooted	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 (n=20)	Single/multi rooted	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+ & CD8+ derived cytokine mRNA expression in patients with & without HIV	Cohort	CAP (n=53)	Single/multi rooted	IL-1β, IL-10, IL-17A, TNF-α, IFN-γ, MCP-1, MIP-1β, RANKL, RANTES, CXCR4 & CCR5	Paper Points	qPCR	Increased IL-10 & CXCR4 mRNA expression & reduced RANKL, IFN-γ, IL-1β & RANTES after treatment in HIV negative patients ($p<0.05$). Increased RANKL, IFN-γ, IL-1β, TNF-α, IL-17A, RANTES & 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 & without SCA	Cross-sectional	CAP (n=36)	-	IL-1β, IL-10, IL-17A, TNF-α, IFN-γ, MCP-1, MIP-1β & RANKL	Paper Points	qPCR	No significant difference observed in inflammatory mRNA expression in SCA positive & 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	Randomised controlled trial	CAP (n=30)	Single rooted	TNF-α, IFN-γ, IL-2, IL-4, IL-5 & IL-13	Paper Points	ELISA	Lower IL-2, TNF-α, IFN-γ levels ang higher IL-4, IL-5 & IL-13 levels after use of dressing ($p<0.05$). No difference observed between types of dressings ($p>0.05$).
Martinho *et al.* (2016)	Quantify PTF levels of MMPs, TIMPs and MMP/TIMP complexes in teeth with CAP and explore their correlation to clincial findings	Cross-sectional	CAP (n=20)	Single rooted	MMP1, MMP2, MMP9, TIMP1, TIMP2, MMP1,2,9/TIMP1,2	Paper Points	ELISA	Higher MMP1, -2 & -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 (n=12) NAT (n=12)	Single/multi rooted	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.* (2016)	Evaluate impact of minocycline interappointment dressing on PTF levels of IL-7A in teeth with AAP	Randomised controlled trial	AAP (n=16) NAT (n=16)	-	IL-17A	Paper Points	CBA	Lower IL-17A levels in calcium hydroxide and minocycline groups after treatment ($p<0.05$)

Table 2: Characteristics of all studies that met the inclusion criteria (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 immunosorbant assay, FAK: focal adhesion kinase, HSP: heat shock protein, IFMA: immunofluorometric assay, IFN: interferon, Ig: immunoglobulin, IL: interleukin, Il-1ra: interluekin-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: osteoprotegrin, OPN: osteopontin, PGE_2: prostaglandin-E2, PMN: polymorphonucleurcyte, 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: tranforming growth factor, TIMP: tissue inhibitor of metalloproteinase, TNF: tumour necrosis factor, βG: β-glucuronidase & -: not reported)

Technique	Study	Parameters used to sample PTF via root canals					Timing of PTF samples		Parameters used to elute & prepare PTF samples				
		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 & FCS (100 µl)	60	-	-
	Shimauchi *et al.* (1996)	Kerr	40	WL	30	1	Before Instrumenting	-	ELISA	PBS + Tween 20 (?? µl)	-	-	10 @ 10 000 g
	Takayama *et al.* (1996)	Kerr	40	WL	30	1	After Instrumenting	-	RAI	PBS (150 µl)	-	30	10 @ 5 000 g
	Shimauchi *et al.* (1997)	Kerr	40	WL	30	2	After Instrumenting	7-10 days	RAI	PBS (150 µl)	-	30	10 @ 5 000 g
	Shimauchi *et al.* (1998)	Kerr	40	WL	30	1	After Instrumenting	-	ELISA	PBS (150 µl)	-	30	10 @ 5 000 g
	Shimauchi *et al.* (2001)	Kerr	40	WL	30	1	After Instrumenting	-	CA	PBS (150 µl)	-	30	10 @ 5 000 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 @ 4 000 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 @ 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 @ 12 000 g
	Grga *et al.* (2013)	Kerr	40	WL	30	2	After Instrumenting	3 days	RIA	PBS (150 µl)	-	30	10 @ 5 000 g
	Tavares *et al.* (2013)	-	-	2 mm past WL	60	2	After Instrumenting	15 days	qPCR	TRIzol (?? µl)	10	-	25 @ 12 000 g
	Rechenberg *et al.* (2014)	Orbis	-	2 mm past WL	30	1	After Instrumenting	-	ELISA	PBS (300 µl)	300	30	10 @ ?? g
	Bambirra *et al.* (2015)	-	-	2 mm past WL	60	2	After Instrumenting	7 days	qPCR	TRIzol (?? µl)	10	-	25 @ 12 000 g
	de Brito *et al.* (2015)	-	-	2 mm past WL	60	2	After Instrumenting	7 days	qPCR	TRIzol (?? µl)	10	-	25 @ 12 000 g
	Ferreira *et al.* (2015)	-	-	2 mm past WL	60	1	After Instrumenting	-	qPCR	TRIzol (?? µl)	10	-	25 @ 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 @ 12 000 g
	Zhi *et al.* (2016)	-	30	WL	30	2	After Instrumenting	7 days	CBA	-	-	-	30 @ 4 000 g
		Brand	**Size**	**Insertion Depth**	**Time (s)**				**Assay Tech.**	**Elution Buffer**	**Incubated (mins)**	**Vortexed (secs)**	**Centrifuged (mins)**
Filter Paper Strip	Kuo *et al.* (1998a)	Interstate	-	-	"few sec."	7-14	Before Instrumenting	3 mins	ELISA	PBS + Tween 20 (50 µl)	-	-	-
	Kuo *et al.* (1998b)	Interstate	-	-	"few sec."	7-14	Before Instrumenting	3 mins	ELISA	PBS + Tween 20 (50 µl)	-	-	-
		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 @ 15 000 g
	Matsuo *et al.* (1995)	Neo Dental	-	-	-	6	-	14 days	ELISA	PBS + Tween 20 (?? µl)	-	-	10 @ 15 000 g
	Takeichi *et al.* (1996)	Hamilton	-	-	-	1	Before Instrumenting	-	ELISA	-	-	-	10 @ 10 000 g
	Takeichi *et al.* (1998)	Drumond Sci.	-	-	-	1	-	-	CA	-	-	-	-

Table 3: Basic parameters of the sampling methods used to retrieve periradicular tissue fluid via the root canal. (-: not reported, WL: working length, BSA: bovine serum albumin, FCS: fetal calf

serum, HCl: hydrochloric acid, PBS: phosphate buffered solution, PTF: periradicular tissue fluid, Tris: trisaminomethane, g: gravity force, ELISA: enzyme-linked-immunosorbant assay, RIA: radioimmunoassay, CA: colorimetric assay, IMFA: immunofluorometric assay, CBA: cytometric-bead-array, qPCR: quantitative polymerise chain reaction)

PRISMA 2009 Flow Diagram

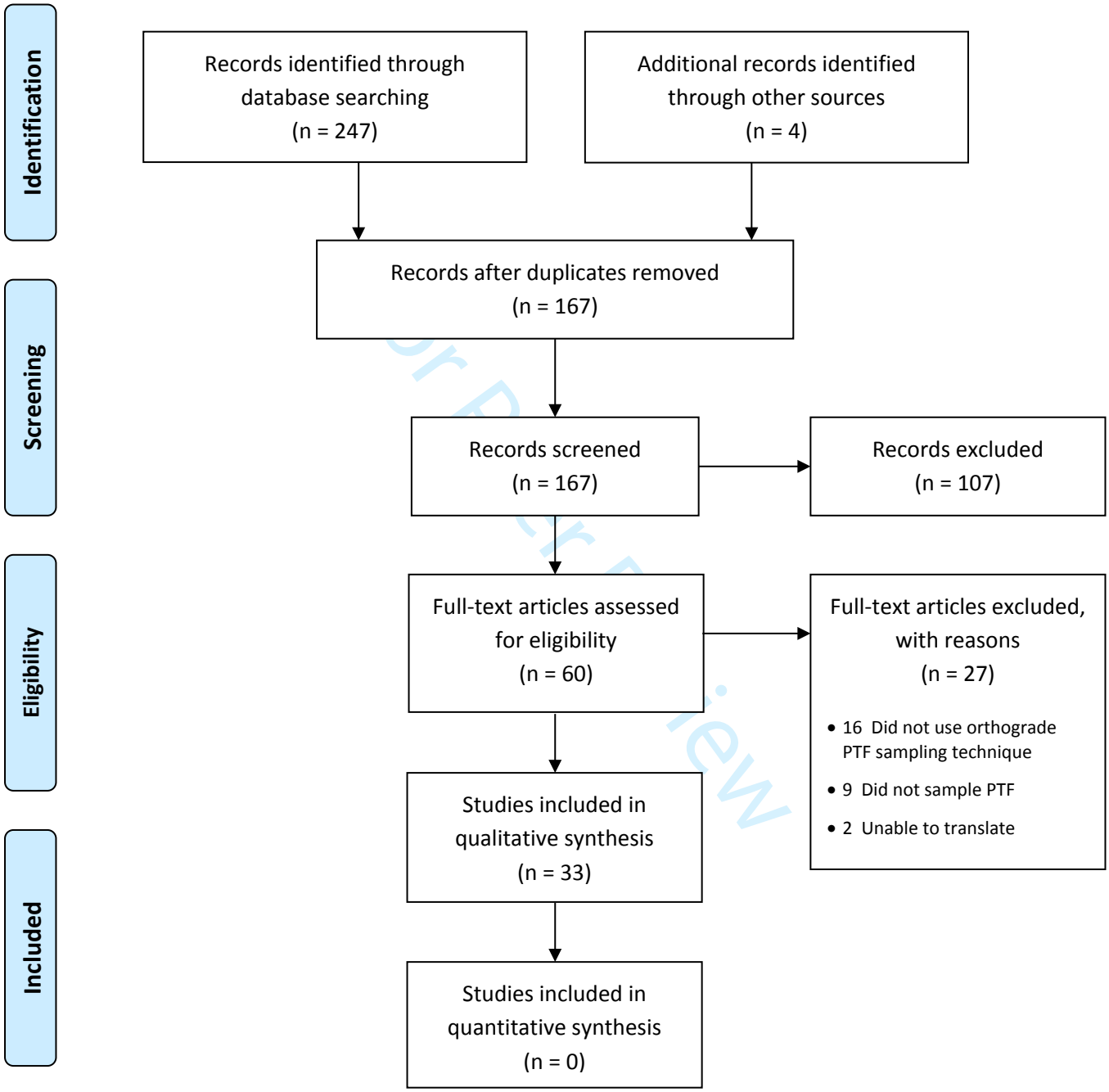

Figure 1: PRISMA flow diagram depicting the flow of information through the phases of the systematic review

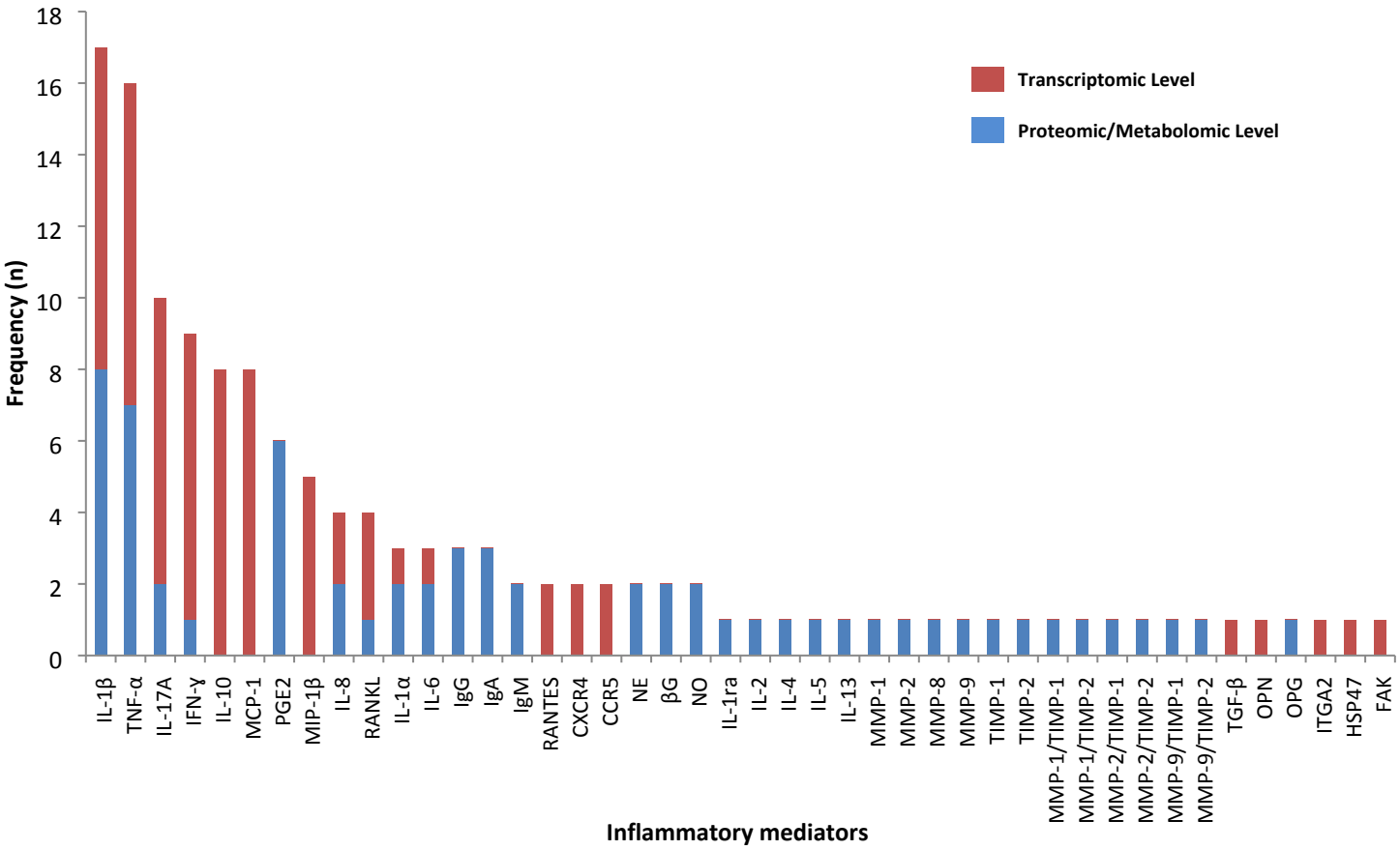

Figure 2: The types of inflammatory mediators targeted and the frequency at which they were studied at a proteomic/metabolomic and transcriptomic level (CCR5: chemokine receptor type 5, CXCR4: chemokine receptor type 4, FAK: focal adhesion kinase, HSP: heat shock protein, IFN: interferon, Ig: immunoglobulin, IL: interleukin, IL-1ra: interluekin-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: osteoprotegrin, 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 & -: not reported)

	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

Appendix S1: Excluded articles at full-text evaluation with reason

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 S2 – Design specific criteria used for assessing the bias in studies which met the inclusion criteria in this systematic review. Table has been adapted and modified from Viswanathan *et al*. 2012. (RCT = randomised controlled trial, CCT = controlled clinical trial) overall

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