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Identification and validation of novel biomarkers and therapeutics for pulpitis using connectivity mapping

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Abstract

Aim: To create an irreversible pulpitis gene signature from microarray data of healthy and inflamed dental pulps, followed by a bioinformatics approach using connectivity mapping to identify therapeutic compounds that could potentially treat pulpitis.

Methodology: The Gene Expression Omnibus (GEO) database, an international public repository of genomics data sets, was searched for human microarray datasets assessing pulpitis. An irreversible pulpitis gene expression signature was generated by differential expression analysis. The statistically significant connectivity map (ssCMap) method was used to identify compounds with a highly correlating gene expression pattern. qPCR was used to validate novel pulpitis genes. An *ex vivo* pulpitis model was used to test the effects of the compounds identified, and the level of inflammatory cytokines was measured with qPCR, ELISA and multiplex array. Means were compared using the t-test or ANOVA with the level of significance set at $p \leq .05$.

Results: Pulpitis gene signatures were created using differential gene expression analysis at cutoff points $p = .0001$ and .000018. Top upregulated genes were selected as potential pulpitis biomarkers. Among these, IL8, IL6 and MMP9 were previously identified as pulpitis biomarkers. Novel upregulated genes, chemokine (C-C motif) ligand 21 (CCL21), metallothionein 1H (MT1H) and aquaporin 9 (AQP9) were validated in the pulp tissue of teeth clinically diagnosed with irreversible pulpitis using qPCR. ssCMap analysis identified fluvastatin (Statin) and dequalinium chloride (Quaternary ammonium) as compounds with the strongest correlation to the gene signatures ($p = .0001$). Fluvastatin reduced IL8, IL6, CCL21, AQP9 ($p < .001$) and MMP9 ($p < .05$) in the *ex vivo* pulpitis model, while dequalinium chloride reduced AQP9 ($p < .001$) but had no significant effect on the other biomarkers.

Conclusions: AQP9, MT1H and CCL21 were identified and validated as novel biomarkers for pulpitis. Fluvastatin and dequalinium chloride identified by the ssCMap as potential therapeutics for pulpitis reduced selected pulpitis biomarkers in an *ex vivo* pulpitis model. *In vivo* testing of these licenced drugs is warranted.

KEYWORDS

dequalinium chloride, fluvastatin, irreversible pulpitis, pulp capping, ssCMap, vital pulp treatment

INTRODUCTION

The dental pulp responds to injury by mounting an inflammatory response characterized by recruitment of immune cells and release of inflammatory mediators and cytokines leading to pulpitis (Hahn & Liewehr, 2007). Clinically, pulpitis is diagnosed as either, reversible; indicating that the inflammation should resolve following appropriate management, or irreversible; indicating that the inflamed pulp is considered incapable of healing and for which root canal treatment is indicated (Levin et al., 2009). However, the clinical diagnosis for pulpitis is largely based on subjective symptoms and diagnostic methods that poorly correlate with the histological status of the dental pulp (Mejàre et al., 2012).

Attempts at improving the diagnosis of pulpitis using biomarkers have been suggested. Although limited by pulp accessibility for sampling, numerous studies have reported the use of biomarkers for pulpal diagnosis (Rechenberg et al., 2016; Zanini et al., 2017; Zehnder et al., 2011). Various inflammatory cytokines and mediators that are differentially expressed in healthy and inflamed pulps have been suggested as potential biomarkers. In these studies, however, candidate biomarkers were often limited in number and chosen *a priori* by investigators. An alternative molecular analysis using microarray gene expression data of pulpitis and healthy pulps would provide a more comprehensive method for the selection of biomarkers. Mining such data, a pulpitis specific gene signature could be created using differential expression analysis to identify novel biomarkers. Furthermore, bioinformatics platforms such as connectivity mapping (CMap) could be used to identify compounds that could potentially modify the pulpitis gene signature in favour of a healthy state.

Gene expression connectivity mapping (CMap) is an innovative bioinformatics platform, which uses a reference database of gene expression profiles obtained from microarray experiments in cell lines treated with various drugs. The basic concept is to use a reference database containing drug-specific gene expression profiles and compare it with a disease-specific gene signature. Theoretically, these genes may be important for the induction of the phenotype of interest, therefore, similar alteration of gene expression by any compound or drug identified would be predicted to induce the same phenotype. The technique is employed for drug repositioning approaches (Lamb, 2007) and further developed into the 'statistically significant connectivity map' (ssCMap) by using statistical means to control for false connections between gene signature and reference profiles (Musa et al., 2018; Zhang & Gant, 2009). The ssCMap has been successfully applied to phenotypic targeting and predicting effective drugs for several diseases (Malcomson et al., 2016; Ramsey et al., 2013) and recently for induction of osteo/odontogenic phenotypes of dental pulp cells (Rankin et al., 2020). It was therefore hypothesized that connectivity mapping could be used to identify compounds or drugs that could reverse the gene signature in pulpitis from inflammation to a closer to healthy phenotype. The study aims to use differential gene expression analysis of microarray data of dental pulps clinically diagnosed with irreversible pulpitis and healthy controls to create a pulpitis gene signature to identify potential biomarkers and use ssCMap to identify therapeutic agents to reverse this gene signature and experimentally validate their effects.

MATERIALS AND METHODS

Differential expression analysis

GEO databases were searched using the keywords: 'dental pulp', 'pulpitis', 'inflammation of dental pulp' and 'irreversible pulpitis'. The search identified two studies, GSE92681 and GSE77459. GSE92681 was excluded as it looked at long noncoding RNAs, which are not compatible with ssCMap. The Series Matrix Files (SMF) for GSE77459 data sets, which contain gene expression levels for all the probes used in the microarrays, were downloaded and opened in Microsoft Excel (Microsoft Corporation). The data sets (Galicia et al., 2016) were for six healthy dental pulps from sound teeth and six dental pulps from carious teeth with a diagnosis of irreversible pulpitis according to the American Association of Endodontists (AAE) classification (Levin et al., 2009). Two sample equal variance t-tests were performed on the gene expression levels measured in healthy and dental pulps diagnosed with irreversible pulpitis with Bonferroni post hoc test to control the expected number of false-positive differentially expressed genes (DEGs). Log2 fold changes were calculated by dividing the average expression level of the diseased samples by the average control for each gene, followed by taking the log2 of this value. If the log2 fold change was greater than 0, the genes were given a score of +1; if less than 0, they were given a score of −1.

ssCMap

The microarray was carried out on Affymetrix Human Gene 2.1 ST Array (GPL17692) and in order to be compatible with ssCMap, the significantly differentially expressed genes were converted to GPL96 probe IDs as previously described (Rankin et al., 2020). Following conversion, two queries using two gene signatures were created by differential expression analysis, the first query gene signature was generated using significance level cut-off $p = 1/N$ ($p = .000018$), where N is the total number of probes used in the array, and a second using a significance level cut-off $p = .0001$, This cut-off p-value was aimed to control the expected number of false-positive DEGs. The empirical False Discovery Rate

(eFDR) was readily calculated once the number of significant DEGs was obtained (Tsai et al., 2003; Zhang, 2011). Connectivity mapping was carried out with each signature gene query using Zhang and Gant's ssCMap (Zhang & Gant, 2009) with default settings, which included a false connection tolerance of 1.0. Compounds with a significant positive connection score were considered for further analysis. To identify potential biomarkers, top expressed genes with a log2 fold change greater than 0.5859 (1.5-fold change) were considered.

Quantitative real-time PCR

Pulp tissue from patients diagnosed with symptomatic irreversible pulpitis according to the AAE classification (Levin et al., 2009) and healthy pulps from sound third molars requiring extraction were obtained from patients attending the School of Dentistry, Queens University Belfast, Belfast, UK. Samples were collected in accordance with ethical approval from the Office for Research Ethics Committees (N. Ireland) (Ethical approval number 08/NIR03/15), and informed consent was obtained from all patients. RNA was extracted from pulp tissue ($n = 12$) and an *ex vivo* pulpitis model, using Maxwell RNA extraction kit (Promega Corporation). cDNA was prepared using the VILO Transcriptor First-Strand cDNA Synthesis Kit (Invitrogen). PCR reactions were performed using TaqMan specific primers and probes for CCL21 (Hs00171076_m1), MT1H (Hs00823168_g1) and AQP9 (Hs01033361_m1) (Thermo Fisher Scientific) as previously described (Al Natour et al., 2021). Changes in gene expression were analysed using the Mx3005P qPCR System (Agilent Technologies) and normalized to the housekeeping genes, glucuronidase-beta (GUSB) and β2-Microglobulin (B2M) (Thermo Fisher Scientific).

Ex vivo pulpitis model

An *ex vivo* pulpitis co-culture model of primary human dental pulp cells (DPCs) and THP-1 macrophages was set up using a Transwell system (Corning Costar) as previously described (Rankin et al., 2020; Yonehiro et al., 2012). DPCs obtained from three donors were derived by explant culture from third molar teeth as previously described (About et al., 2000; El Karim et al., 2016) and grown in minimal essential medium with L-glutamine supplemented with 10% Foetal bovine serum (FBS), 100 UI/ml penicillin and 100 mg/ml streptomycin. THP-1 cells were grown in Roswell Park Memorial Institute medium (RMPI-1640) media supplemented with 10% FBS and differentiated into macrophages by incubation in 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) for 24 h. DPCs were seeded in the lower Transwell chamber at a density of 2×10^5 cells per well. THP-1 cells were seeded in the upper chamber at the density of 1×10^5 cells per well and differentiated into macrophages in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich). The upper and lower chambers were assembled as a co-culture, separated by 0.4 mm pore diameter membrane and co-cultured cells were then treated with 1 μg/ml ultrapure *E. coli* LPS (Sigma Aldrich) with or without the compound/drug of interest for 24 h.

MTT assay

DPCs and THP-1 were seeded in 96-well plates at a density of 3×10^4/ml and treated with control media or predicted compound at different concentrations for 24 h. MTT assay was performed by adding 10 μl MTT solution to each well for 2 h at 37°C, media was aspirated, wells were air-dried for 10 min and 200 μl DMSO was added and incubated at 37°C for a further 10 min. Sample optical density was measured at 510 nm using a Tecan GENios microplate reader (Tecan).

ELISA and Luminex Multiplex assays

The cytokines IL8, IL6, the chemokine (C-C motif) ligand 21(CCL21) and MMP9, were simultaneously measured from co-culture supernatant in the *ex vivo* pulpitis model using a magnetic Luminex assay kit and ELISA (R&D Systems). To establish a working concentration, the effect of each drug on DPCs treated with LPS was assessed by measurement of IL8 using Duoset ELISA (R&D Systems).

Statistical analysis

Data are presented and analysed as mean ± standard deviation (SD) using GraphPad Prism software, version 5.0 (GraphPad Software, Inc.). Means were compared using the t-test or ANOVA followed by a Bonferroni or Dunnet post-test with the level of significance set at $p \leq .05$.

RESULTS

Differential gene expression and ssCMap output

In total 53 619 probes were identified in the data sets and this number was used to calculate the cut-off point 1/*N*

($p = .000018$) for differential gene expression analysis. This cut-off p-value was aimed to control the expected number of false-positive DEGs as 1. Using this cut-off point a gene signature (154 genes, eFDR = 1/154 ≈ 0.6%) was created, which resulted in the identification of 76 compounds using ssCMap (Figure S1A). A longer gene signature (281 genes) was created using a cut-off point of $p = .0001$, for which the ssCMap revealed 232 compounds that could modulate these genes (Figure S1B). The longer gene signature was based on a slightly more relaxed p-value cut-off, which controlled the expected number of false-positive DEGs to about 5, resulting in an overall eFDR ≈ 5/281 ≈ 2% for this set of DEGs.

To identify potential biomarkers, a list of genes with log2 fold change greater than 0.5859 (1.5-fold change) was created (Table 1). The 1.5-fold change was chosen to prioritize genes with low but statistically significant fold change. These included cytokines that were previously suggested as potential biomarkers for pulpitis such as Interleukin 8 (IL8), Interleukin 6 (IL6) and Matrix Metalloproteinase 9 (MMP9) (Rechenberg et al., 2016). In addition, novel genes including chemokine (C-C motif) ligand 21 (CCL21), metallothionein 1H (MT1H), aquaporin 9 (AQP9), FBJ murine osteosarcoma viral oncogene homolog B (FOSB), peptidase inhibitor 3, skin-derived (PI3), metallothionein 1G (MT1G), C-type lectin domain family 4, member E (CLEC4E), serine dehydratase (SDS), selectin E (SELE), cholesterol 25-hydroxylase (CH25H) and a triggering receptor expressed on myeloid cells 1 (TREM1) were identified.

Validation of selected novel pulpitis genes

For validation, the top three novel genes CCL21, MT1H and AQP9 were selected for qPCR quantification in the dental pulps of sound and carious teeth with a clinical diagnosis of symptomatic irreversible pulpitis. AQP9, MT1H and CCL21 were all expressed in the dental pulp tissue and their expression is upregulated in samples of irreversible pulpitis compared to the healthy pulp. Expression of AQP9 is significantly higher compared to MT1H ($p < .05$) and CCL21 ($p < .001$) in dental pulps with irreversible pulpitis (Figure 1).

Compounds selection and their effects on cell viability

The ssCMap identified 232 compounds for the long query and 79 for the short query that could potentially modulate gene signature. Compounds were selected based on their highest connection scores. Fluvastatin and dequalinium chloride were the two compounds identified by both queries with the highest connection scores and therefore selected for experimental validation. Fluvastatin is a Hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor (statin) class of drugs, while dequalinium chloride is Quaternary ammonium cation (Table 2). MTT assay showed that fluvastatin, had no effect on DPCs or THP-1 macrophages viability at any of the concentrations tested (Figure 2). However, dequalinium chloride significantly reduced cell viability at a higher concentration ($p < .05$) (Figure 2).

TABLE 1 List of genes with a log2 fold change greater than 0.5859 (1.5-fold change) selected as potential biomarkers

GPL96_ID	Gene symbol	Gene name	Log2 fold change	p-value	Regulation
202859_x_at	IL8	Interleukin 8	1.241889632	6.74E-05	1
204606_at	CCL21	Chemokine (C-C motif) ligand 21	1.053933825	6.89E-10	1
206461_x_at	MT1H	Metallothionein 1H	1.032697802	1.36E-06	1
205568_at	AQP9	Aquaporin 9	0.935287545	8.06E-05	1
203936_s_at	MMP9	Matrix metallopeptidase 9	0.89000635	5.57E-08	1
202768_at	FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.84370843	5.03E-07	1
203691_at	PI3	Peptidase inhibitor 3, skin-derived	0.783932029	6.44E-13	1
204745_x_at	MT1G	Metallothionein 1G	0.778623636	2.34E-10	1
219859_at	CLEC4E	C-type lectin domain family 4, member E	0.748959137	3.9E-05	1
205695_at	SDS	Serine dehydratase	0.747035464	6.59E-06	1
206211_at	SELE	Selectin E	0.736467219	3.43E-09	1
206932_at	CH25H	Cholesterol 25-hydroxylase	0.733972379	1.35E-08	1
219434_at	TREM1	Triggering receptor expressed on myeloid cells 1	0.706776916	3.61E-06	1
205207_at	IL6	Interleukin 6 (interferon, beta 2)	0.671590863	4.42E-07	1

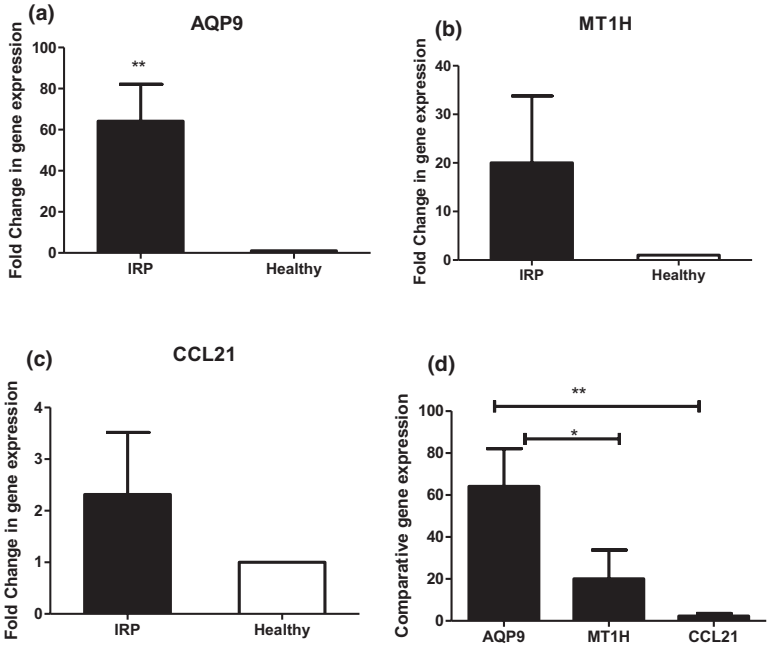

FIGURE 1 Expression of the novel genes AQP9, MT1H and CCL21, validated in teeth clinically diagnosed with irreversible pulpitis (IRP). The expression of the genes was determined using qPCR and fold change was calculated in relation to healthy pulps. AQP9 is significantly upregulated in dental pulp with IRP compared to MT1H and CCL21. Unpaired t-test and one way ANOVA, *$p < .05$, **$p < .001$

TABLE 2 Top candidate drugs identified by ssCMap using two gene queries. The connection sores show the correlation of the drugs with the input gene signature. Negative scores indicate a negative correlation, that is the input genes are downregulated when treated with the particular drug. Drugs with the highest connection scores were selected for laboratory validation. Fluvastatin and dequalinium chloride were among the top candidates for both queries

	Connection scores	Set scores	*p* value	Drug class and effect
Fluvastatin	−5.28287[a]	−0.186540827	.0001	HMG-CoA reductase inhibitor, anti-lipemic, anti-inflammatory
	−3.838490829[b]	−0.183546829	.0001	
Dequalinium chloride	−5.12676[a]	−0.18403886	.0001	Quaternary ammonium, antiseptic, bacteriostatic, antifungal
	−4.460235046[b]	−0.21327705	.0001	

[a]Long query.

[b]Short query.

Effect of selected compounds on pulpitis biomarkers

In preliminary work to determine the working concentration of the compounds, their ability to reduce IL8 levels in LPS treated DPCs was measured by ELISA. Of the concentrations tested, 10 μM fluvastatin and 100 nM dequalinium chloride reduced IL8 levels (Figure S2). Next, the effect of fluvastatin and dequalinium chloride on pulpitis biomarkers protein and gene expression was examined using the *ex vivo* pulpitis model. As shown in Figure 3, fluvastatin treatment resulted in a significant reduction in the levels of IL8, IL6, CCL21 ($p < .001$), MMP9 ($p < .05$) and gene expression of AQP9 ($p < .001$). Dequalinium chloride resulted in a significant reduction in AQP9 gene expression ($p < .001$) and reduced IL8, IL6, MMP9 and MT1H, but the reduction was not significant (Figure 4).

DISCUSSION

The current description of pulpitis as reversible/irreversible has become questionable, given advances in minimally invasive vital pulp treatments (VPT) and the evidence that maintenance of pulp vitality is possible in teeth with cariously exposed pulp and those with signs and symptoms of irreversible pulpitis (Cushley et al., 2019, 2020; Li et al., 2019). The success of minimally invasive VPT requires parallel development in diagnosis, with novel treatments for pulpitis (Duncan et al., 2019). In line with this, the present study employed comprehensive gene expression analysis and bioinformatics approaches to identify biomarkers as well as compounds that could treat pulpitis. The results of this study showed that using the above methods, a disease-specific gene signature for irreversible pulpitis was created, and this gene signature could be modified indicating the possibility of reversing the disease, at the level of *in silico* modelling and in an *ex vivo* pulpitis model.

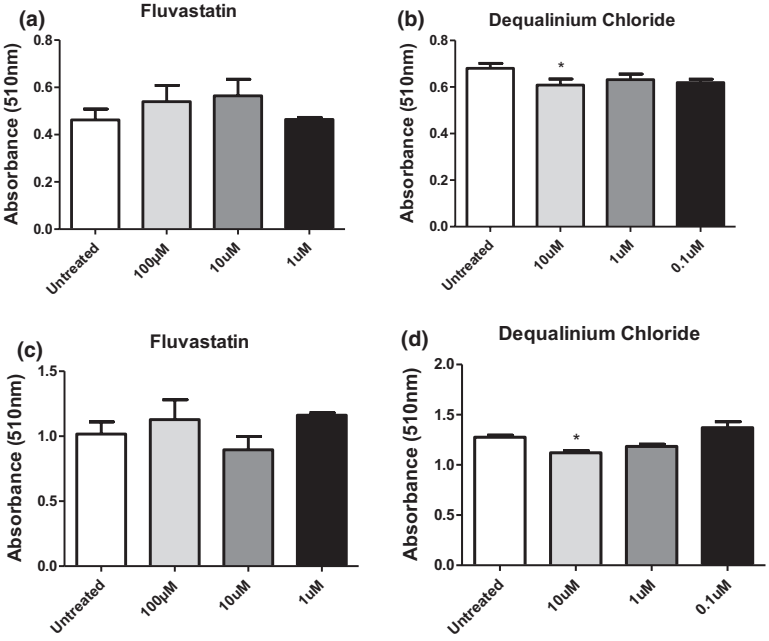

FIGURE 2 Effect of the selected compounds fluvastatin and dequalinium chloride on the viability of dental pulp cells (DPCs) and THP-1 macrophages. Fluvastatin had no significant effects on the viability of DPCs (a), or THP-1 macrophages (c) at any of the concentrations tested. Dequalinium chloride significantly reduced both DPCs (b) and THP-1 macrophage (d) viability at 10 μM concentration. *$p < .05$. One way ANOVA

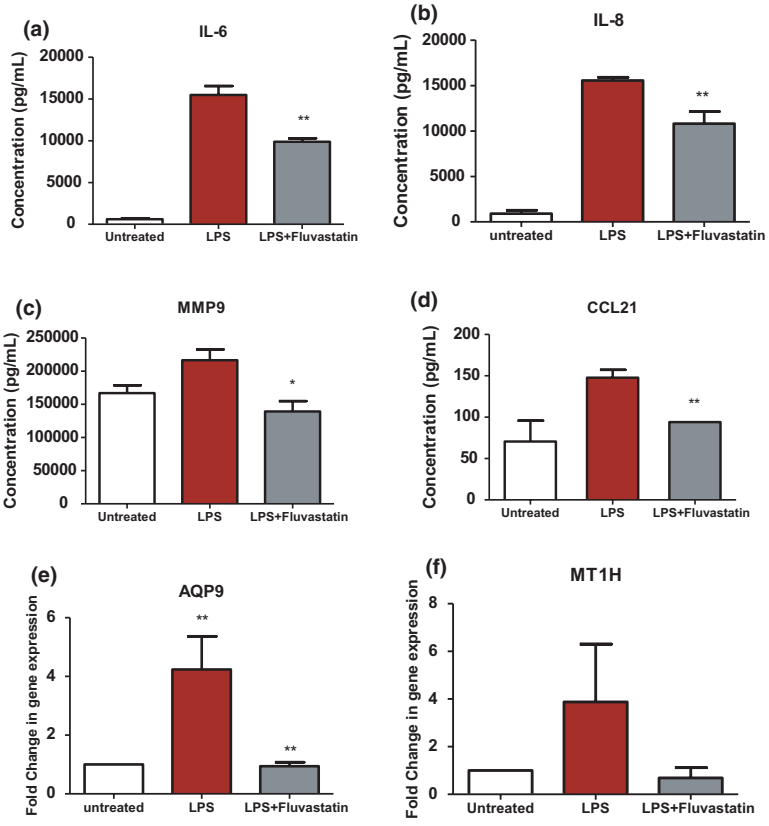

FIGURE 3 Effects of Fluvastatin on pulpitis biomarkers measured using a multiplex assay for IL8, IL6, MMP9, CCL21 and qPCR for AQP9 and MT1H. Biomarkers were significantly upregulated with LPS treatment. Fluvastatin significantly reduced, IL8, IL6, CCL21, MMP9, AQP9 but not MT1H. *$p < .05$, **$p < .001$. One way ANOVA

The gene signature revealed genes previously shown to be upregulated in irreversible pulpitis such as IL8, IL6 and MPP9 (Rechenberg et al., 2016), which confirms the validity of this approach and provides further evidence for these genes as pulpitis biomarkers. In addition, three novel genes including CCL21, M1H and AQP9 were also identified and validated using qPCR of dental pulps with the clinical diagnosis of irreversible pulpitis.

The dental pulp is enclosed within rigid dentinal walls creating low interstitial compliance, which was traditionally considered to limit the ability of the pulp to recover from severe inflammation (Yu & Abbott, 2007). During pulpitis the pulpal fluid volume increases, causing the tissue pressure to increase and leading to compression of blood vessels and subsequent ischaemia and necrosis (Heyeraas & Berggreen, 1999). Therefore, tight regulation of pulpal fluid

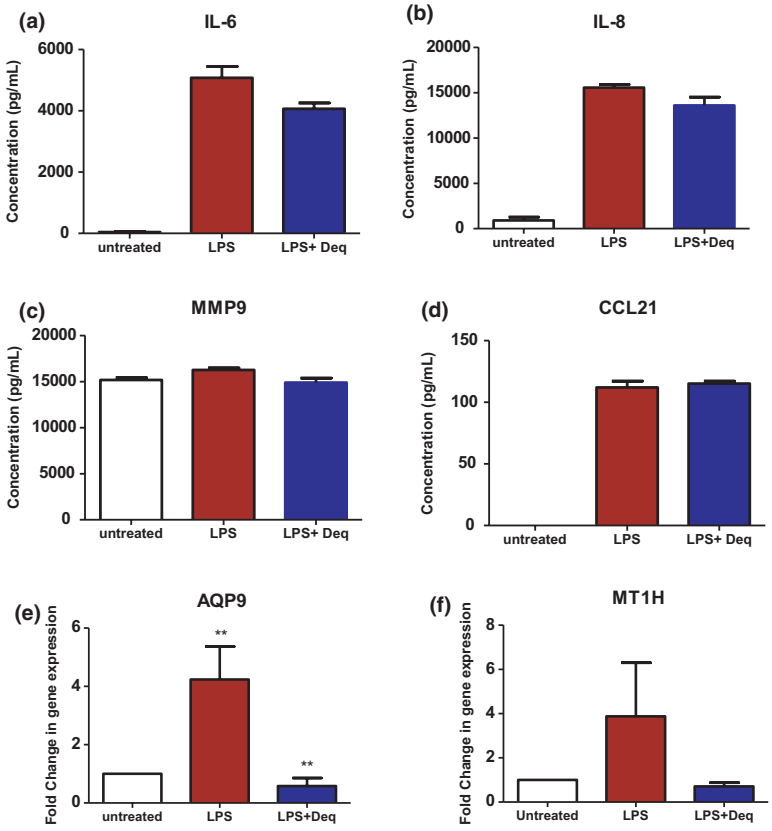

FIGURE 4 Effects of Dequalinium chloride on pulpitis biomarkers measured from co-culture using a multiplex assay and ELISA for IL8, IL6, MMP9, CCL21 and qPCR for AQP9 and MT1H. Biomarkers were upregulated with LPS treated. Dequalinium chloride (Deq) significantly reduced AQP9 but the reduction was not significant for IL8, lL6 MMP9, CCL21 and MT1H. *$p < .05$, **$p < .001$, One way ANOVA

dynamics is important in the resolution of pulpitis. Cellular fluid movement is regulated by aquaporins (AQP), the cell membrane-embedded proteins that facilitate water movements by increasing membrane water permeability and water flux in response to osmotic gradients (Agre & Kozono, 2003). Of these, AQP9 has previously been found to be associated with brain oedema (Badaut et al., 2014). In the current study, AQP9 was highly expressed in irreversible pulpitis, in which oedema is also often reported (Trowbridge, 1981). It is likely that increased expression of AQP9 in irreversible pulpitis contributes to the development of oedema and increase pulpal fluid volume by facilitating water movement. AQP9 also plays a critical role in leukocyte and dendritic cells function during systemic inflammation (De Santis et al., 2018; Matsushima et al., 2014) and its level is upregulated in patients with infectious endocarditis (Thuny et al., 2012). These suggested roles in oedema and response to infection make AQP9 an important novel biomarker for pulpitis. Indeed the results of this study, which demonstrated higher expression of AQP9 compared to other novel biomarkers for pulpitis and its consistent reduction by both fluvastatin and dequalinium chloride in the *ex vivo* pulpitis models further support this notion.

Another novel gene identified in this study was MT1H, which belongs to a family of small metal-binding cellular proteins that bind to heavy metals such as zinc (Kalinowski & Richardson, 2010). MT1H plays a role in regulating cellular zinc homeostasis as well as other functions, including neuroprotection, anti-inflammatory and defence against oxidative damage (Chung et al., 2009; Coyle et al., 2002). CCL21 is another novel gene identified in the study. It is a potent pro-inflammatory chemokine that regulates dendritic cell migration and has been implicated in various chronic inflammatory pain conditions, including rheumatoid arthritis and neuropathic pain (Biber et al., 2011; Pickens et al., 2012). In the central nervous system, CCL21 expression is increased in damaged neurons, leading to increased microglial P2X4 expression (Biber et al., 2011), a finding that is likely to be replicated in the dental pulp as highly innervated tissue. However, the expression of both MT1H and CCl21 in irreversible pulpitis in this study was variable and the difference between healthy and diseased samples did not reach significance, suggesting the inherited variability in pulpitis diagnosis that rely on subjective patients symptoms (Mejàre et al., 2012).

Another interesting finding of this study was the identification of compounds that could modify the gene signature associated with irreversible pulpitis and thereby potentially reverse the disease. Among the compounds identified, fluvastatin is a member of the 3-hydroxy-3-m ethyl-glutaryl–coenzyme-A (HMG-CoA) reductase inhibitors (statins) drug class that function by slowing the production of cholesterol. In addition to their classic function, statins have well-documented anti-inflammatory effects (Björkhem-Bergman et al., 2010; Leung et al., 2003). Among the stains, simvastatin has been shown to reduce

the production of cytokines in the dental pulp (Jung et al., 2017) and to enhance odontogenic differentiation of dental pulp stem cells (Miyazawa et al., 2015; Okamoto et al., 2009). A biphasic calcium phosphate cement containing simvastatin is recently developed as novel pulp capping material (Chang et al., 2020). The findings of the current *ex vivo* study, that fluvastatin reduced inflammatory cytokines associated with irreversible pulpitis, provide further support for the anti-inflammatory properties of statins and their repurposing for treatment of pulpitis.

Dequalinium chloride was also identified by ssCMap as a compound highly correlated with the irreversible pulpitis gene signature. It is a bis-quaternary ammonium compound with a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts and protozoa (Tischer et al., 2012) and currently used as a topical treatment for vaginal infections (Mendling et al., 2016). Although dequalinium chloride was not as effective as fluvastatin in reducing all the cytokines tested in this study, its broad antimicrobial effect is very advantageous in the context of caries-induced pulp inflammation, as control of infection is key to a successful vital pulp treatment (Bjørndal et al., 2019; ESE, 2019). In fact, antimicrobial activity for fluvastatin has also been demonstrated (Jerwood & Cohen, 2008). Therefore, the identification of compounds with antimicrobial activity to treat pulpitis gives confidence to the ssCMap approach, as pulpitis is primarily a bacterial-induced disease. Although reduction of cytokines is a desirable treatment outcome, control of bacterial infection is essential and further studies to evaluate antimicrobial effects against endodontic pathogens are warranted. It is essential also that the anti-inflammatory and antimicrobial effect of these drugs be investigated *in vivo* to validate these *ex vivo* findings.

The ssCMap is a very useful tool for repurposing drugs already licenced for human use and therefore reduces the need for the lengthy drug discovery process, however, the method is not without limitations. For instance, the gene signature created is not inclusive of all differentially expressed genes as many have not been cloned or their function identified yet, therefore, a future analysis may reveal further biomarkers and more compounds as potential therapeutics for pulpitis. The limited number of GEO datasets available to be included in this study is also another limitation as likely a larger number of data sets may result in different gene signatures and consequently additional compounds. Nevertheless, the genes and the compounds identified and validated in this study are relevant to pulpitis disease pathophysiology and potential treatment.

CONCLUSION

ssCMap and gene expression analysis can be successfully used to identify biomarkers and compounds for the treatment of irreversible pulpitis. AQP9, MT1H and CCL21 were identified and validated as novel biomarkers for pulpitis. Fluvastatin and dequalinium chloride identified by the ssCMap as potential therapeutics for pulpitis, reduced selected pulpitis biomarkers in an *ex vivo* pulpitis model. Reduction of biomarkers with fluvastatin and dequalinium chloride suggest a potential use as pulp capping/dressing materials in treating pulpitis, and their *in vivo* validation could reveal novel therapeutic applications.

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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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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