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The influence of type 2 diabetes on ectopic calcification within the dentine-pulp complex

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ABSTRACT

Objective: To investigate the influence of Type 2 diabetes (T2D) on calcification within the dentine-pulp complex and examine the mRNA expression of mineralisation-associated genes in human dental pulp cells (hDPCs) cultured in a hyperglycaemic environment.
Design: Extracted non-carious molar teeth were collected from patients with well-controlled T2D (n=10) and non-T2D (controls) (n=10). The pulp was histologically examined using special stains. Primary hDPC lines (n = 3) were established from non-T2D tissue explants and grown in media containing 5.5mM- (control), 12.5mM- (prediabetes) and 25mM- D-glucose (T2D) for 7, 14 and 21 days. A PrestoBlue assay assessed the hDPC metabolic response to hyperglycaemia. The expression of mineralisation-associated genes *RUNX2, SPP1, SPARC, BGLAP, IBSP* and *DSPP* were analysed using quantitative real-time polymerase chain reaction. Data analyses were performed using GraphPad Prism and one-way ANOVA at $p < 0.05$.
Results: Diffuse amorphous calcifications and irregular predentine were consistently observed in T2D samples. Culturing hDPCs in 12.5 mM and 25 mM glucose significantly increased their metabolic activity. All genes were detected in hDPCs in the presence of hyperglycaemia over time. However, with the exception of *RUNX2* which was initially downregulated in response to hyperglycaemia, all genes were expressed independent of glucose levels.
Conclusion: T2D is associated with pulp calcifications similar to other body sites. Diffuse fibro-dentine foci of calcifications resembled the appearance of an 'aged' pulp and the gene expression for markers of mineralisation was independent of glucose levels. Calcifications may form due to the effects of chronic inflammation and prolonged glucose exposure.

1. Introduction

Type 2 diabetes (T2D) is a chronic metabolic disease characterised by hyperglycaemia, systemic inflammation and insulin resistance, and is primarily due to dysregulation or destruction of pancreatic β cells. As cells become resistant to insulin, blood glucose levels become elevated leading to prediabetes. Untreated, persistent hyperglycaemia frequently results in T2D (Echouffo-Tcheugui & Selvin, 2021). T2D is most commonly diagnosed using a glycated haemoglobin (A1c) test which measures an average plasma glucose concentration over the previous 2–3 months. Results are interpreted as (39–47 mmol/mol) 5.7–6.4 % as prediabetes and a level of (48 mmol/mol) 6.5 % or higher as T2D (World Health Organization, 2020). Patients with the disease have significant morbidity, a higher burden of risk factors and poorer health outcomes as the disease effects the function and inflammatory response associated with many organs and tissues (Daryabor et al., 2020; Gupta et al., 2020; Ong et al., 2023). Indeed, patients with T2D commonly present with complications throughout the body, including retinopathy, neuropathy, cardiovascular disease, tissue fibrosis, and impaired healing including within the pulp and periapical tissues (Alsamahi et al., 2022; Catrina & Zheng, 2021; Gregg et al., 2016; Patel et al., 2025; Segura-Egea et al., 2023).

In health, tissues respond to irritation and damage via inflammation and the physiological process of mineralisation through the organised deposition of minerals into a tissue matrix. In contrast, pathological responses include calcification which is associated with the ectopic deposition of mineral. This has been well illustrated in T2D where hyperglycaemia is associated with elevated inflammatory markers and vascular calcifications (Chen & Moe, 2003; Tsalamandris et al., 2019). Notably, animals studies have shown diabetic rats elicit greater pulp

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tissue inflammation and increased amounts of reactionary dentine in response to dental bleaching compared to normoglycemic controls (Cintra et al., 2017). In addition, histological analyses of the dental pulp obtained from patients with T2D have shown tissue resembling an 'aged pulp' with reduced vascularity and increased fibrosis and inflammation (Alsamahi et al., 2022). Furthermore, some samples contained calcified tissue within the pulp space in close association with thickened blood vessels (Patel et al., 2025) but as these were incidental findings it is uncertain if this is more typically associated with systemic disease.

In teeth with a vital pulp the physiological aging process, and secondary and tertiary dentinogenesis results in a reduction in the volume of the pulp space. Furthermore, radio-opacities within the pulp space are a common incidental finding on two-dimensional radiographs and three-dimensional scans (Turkal et al., 2013; Zhang et al., 2024). Clinicians often refer to these calcifications as pulp 'stones' and they are important to consider as part of treatment planning. In molars different types of stones have been described within the pulp based on their histological appearance, location and structure. Stones may be 'true' stones resembling dentine, or 'false' stones formed by degenerating cells, and they can be either 'free' within the pulp space, 'adherent' to the root canal walls or 'embedded' within canal walls (Goga et al., 2008). Clinically, calcifications are frequently found within the core of the pulp chamber during endodontic access cavity preparation and histologically these consist of irregular matrices of collagen fibres, surrounded by hydroxyapatite crystals (Moss-Salentijn & Hendricks-Klyvert, 1988). 'Denticles' describe calcifications composed of tubular dentine and are often surrounded by epithelial remnants whereas stones composed of non-tubular fibro-dentine are potentially produced by fibroblast-like cells. Notably, 'diffuse' pulp stones are amorphous and tend to increase with age (Goga et al., 2008). While the presence of calcifications within the pulp is not new, their significance, composition and morphology remain somewhat unexplained in patients with T2D.

A range of molecular markers are frequently used to evaluate tissue mineralisation activity with osteopontin (encoded by the *SPP1* gene), osteonectin (encoded by the *SPARC* gene), osteocalcin (encoded by the *BGLAP* gene), bone sialoprotein (encoded by the *IBSP* gene) and dentine sialophosphoprotein (encoded by *DSPP* gene) being commonly involved with calcification processes in bone and dentine. *RUNX2* is an important transcription factor in osteoblast and odontoblast differentiation. Thus far, however, the understanding as to how glucose influences these markers of mineralisation is limited and there appears no clear consensus on their expression in response to hyperglycaemia and how this may change with time and glucose levels. Indeed, the expression of markers for osteonectin, alkaline phosphatase and dentine sialoprotein are reported to be inhibited in human dental pulp cells (hDPCs) treated with 25 mmol/L glucose for 14 days (Horsophonphong et al., 2020). Conversely, the levels of osteopontin and alkaline phosphatase are elevated in rat dental pulp cells in response to high glucose (50 mmol/L) levels for an extended period of 28 days (Inagaki et al., 2010). Furthermore, osteopontin is detected in urinary stones and also plays a role in the development of atherosclerotic plaques (Ninomiya et al., 2001; Wolak, 2014) and in diabetic vascular complications (Li et al., 2023). Notably, our current understanding of calcification and mineralisation processes within the diabetic pulp has mostly been obtained from animal studies which often incur methodological differences, and from reviews which suggest pulp calcification may be associated with altered metabolic activity, oxidative stress, chronic inflammation and degeneration of odontoblasts (Inagaki et al., 2010; Pimenta et al., 2024). The varying types and aetiologies of pathologic calcifications illustrate the complexity of mineralisation in response to different environments and so the influence of hyperglycaemia on mineralisation regulators within the human pulp cells warrants further exploration.

There were two primary aims of this study which included: 1) determining the expression of mRNA levels in mineralisation-associated genes of interest (*RUNX2, SPP1, SPARC, BGLAP, IBSP* and *DSPP)* in human dental pulp cells (hDPCs) cultured in media containing a range of glucose concentrations over time, and 2) characterising the influence of T2D on the presence of calcifications within the coronal dentine-pulp complex. It was hypothesised that a hyperglycaemic environment would likely impair the metabolic activity of hDPCs in culture and affect the expression of mineralisation genes. Furthermore, it was predicted that ectopic calcifications would be more common in T2D samples.

2. Materials and method

2.1. Study design

Ethical approval was obtained from the University of Otago Human Ethics Committee (Health) (Ref. H21/080). The study included patients aged 30–65 years and mature permanent molar teeth from diabetic (T2D) (n = 10) and non-diabetic (non-T2D) (n = 10) patients were extracted for clinical reasons, for example third molars or molar teeth were extracted as part of a treatment plan for full denture treatment. Teeth were collected with written informed consent and understanding that anonymity would be preserved.

Inclusion criteria were healthy non-T2D participants (HbA1c <39 mmol/mol (<5.7 %)), or well-controlled T2D HbA1c tests (50–55 mmol/mol (6.7–7.2 %)). Diabetic patient inclusion criteria also ensured that individuals had a confirmed diabetes status > 12 months, were 'well controlled' based on medical records and lifestyle, and had no other systemic diseases. Study exclusions included: prediabetic HbA1c levels (39–47 mmol/mol (5.7–6.4)%), being a smoker, pregnancy, recent antibiotic use, regular anti-inflammatory medication use, pulpitis, periodontitis, and evidence of tooth wear.

All teeth collected were collected from age and sex matched individuals and met strict inclusion criteria including that they were: non-carious and diagnosed with a clinically normal pulp (American Association of Endodontists, 2009), asymptomatic, not sensitive to percussion or palpation, < 3 mm gingival pockets, and exhibiting a normal periapical appearance on radiographs.

2.2. Cell culture

Following extraction, non-T2D teeth (n = 3) were sectioned 2 mm below the cemento-enamel junction. Coronal pulp tissue was excavated to establish primary hDPC cultures using the explant method and DMEM supplemented with 10 % FBS, Antibiotic-Antimycotic (contains penicillin, streptomycin, amphotericin B) and gentamycin (Gibco™, ThermoFisher). Cells were cultured in 5.5 mM D-glucose (Gibco™, ThermoFisher) at 37℃ with 95 % air and 5 % CO_2. Cell outgrowth was expected within 30 days (Alsamahi et al., 2023). Cell cryopreservation was carried out at passage 3 and cells stored at −80℃ until required. For experimentation hDPC cells were seeded at a density of 5 × 104 cells/well in 24-well plates in supplemented DMEM containing 5.5 mM D-glucose. Following 24 hrs at 37℃ with 95 % air and 5 % CO_2 the medium was replaced with DMEM containing the 5.5 mM (control), 12.5 mM or 25 mM D-glucose to simulate the normoglycaemic, prediabetes and T2D environments (Fiorello et al., 2020; Milosavljević et al., 2018), and osteogenic supplement containing 0.2 mM 2-phospho L-ascorbic acid (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich) and 10 mM ß-glycerophosphate (Sigma-Aldrich). Three biological cell lines were utilised with experiments performed in duplicate.

2.2.1. HDPC cell viability and metabolic activity

A PrestoBlue™ assay (Invitrogen™, ThermoFisher) was used to measure the cellular metabolic activity. Assays were undertaken using the hDPC lines (n = 3) cultured in 5.5 mM, 12.5 mM or 25 mM D-glucose with osteogenic supplement and measurements taken at 7, 14, and 21 days. Two wells of duplicate 24-well plates provided quadruplicate data for each sample. Prestoblue™ Reagent (50 μl) was added directly to the well containing adherent cell cultures in 500 μl media. Following incubation for two hours at 37℃ in the absence of direct light.

Fluorescence (540/25 nm excitation and 620/40 nm emission) was measured using a Synergy™ 2 multi-mode microplate reader (BioTek Instruments).

2.2.2. Gene expression analysis

Established primary hDPC lines (n = 3) were cultured in 5.5 mM, 12.5 mM or 25 mM D-glucose plus osteogenic supplements (as described above) with RNA collected for gene expression analysis using qPCR at 7-, 14-, and 21-days. Phosphate buffered saline was used to wash the attached cells which were detached with TRIzol® (Life Technologies) and the cell/TRIzol® homogenate stored at −80℃ until required. The Direct-zol™ RNA Miniprep kit (Ngaio Diagnostics Limited) was used according to the manufacturer's instructions for total RNA purification. A DNase 1 treatment was used to remove contaminating DNA from RNA samples.

Custom TaqMan® FAST qPCR arrays were utilised for gene analysis (Applied Biosystem™ Life Technologies). The genes of interest (GOI) included the mineralisation transcripts *RUNX2, SPP1, SPARC, BGLAP, IBSP* and *DSPP* and the reference gene *HPRT1*. Purified RNA for each of the 27 samples was diluted 1:10 with DNase and RNase-free water (5 μL), TaqMan® Virus 1-step FAST mastermix (ThermoFisher) (2.5 μL) and DNase and RNase-free water (2.5 μL) was dispensed into 16 wells of the 96-well custom Taqman qPCR array plate. Thermal cycling parameters were performed using the Quantstudio™ 6 Flex Real-time PCR system according to the manufacturer's instructions: polymerase activation (hot start) at 50°C for 5 min, 95°C for 20 s, 40 cycles at 95°C for 3 s and 60°C for 30 s. Following amplification and real-time data acquisition, the threshold and Cq value were calculated using the Quantstudio™ 6 Flex FAST Real-time PCR system software (ThermoFisher).

2.3. Cell culture data analysis and statistical analysis

Data are presented as means ± standard deviation and analysed using GraphPad Prism software V9 (GraphPad Software Inc., San Diego, USA). The normality of the data was first tested with a Shapiro-Wilk test. Metabolic activity data were analysed using one-way ANOVA followed by Bonferroni test for post-hoc comparison. Determination of mRNA expression differences between groups were based on the transformed $2^{-\Delta Cq}$ values and the one-way ANOVA followed by Bonferroni's test and Tukey's test for post-hoc comparison. Statistical significance was set at $p < 0.05$.

2.4. Histology and special stains

Twenty teeth (T2D (n = 10) and non-T2D (n = 10)) were used for histological examination. Following extraction, teeth were sectioned at mid-root level and the coronal portion was placed in a tissue fixative of 10 % neutral buffered formalin for 48 h prior to decalcification and tissue processing. The specimens were decalcified in 10 % ethylenediaminetetraacetic acid (EDTA) solution agitated at 4℃ for 8–11 weeks until ready for analyses. Samples were placed in phosphate-buffered saline (ThermoFisher) for 24 h to remove residual EDTA prior to tissue processing and embedding in paraffin. Tissue sections of 4 μm thick, were obtained and the initial section was stained with haematoxylin and eosin (H&E) for histological examination by a pathologist blinded to the sample selection to confirm the plane of section and the relative absence of pulp inflammation, as previously described (Alsamahi et al., 2022). Pulp tissue samples were viewed at 100x, 200x and 400x objective magnification and histologically diagnosed as uninflamed when there was an evident intact odontoblast layer, no evidence of tissue necrosis and congested blood vessels, and appearance of normal distribution of fibroblasts. Specifically, immune cells also appeared sparsely distributed with no localised collections of neutrophils or lymphocytes indicating inflammation. Further sections were stained with toluidine blue (Sigma-Aldrich) to provide distinction between mineralised and unmineralized tissue (Bain et al., 1990) and a Modified Gallego's stain was used to identify and differentiate hard tissues: dentine (stains light green-yellow) and cementum/bone (stains red), (Dhouskar et al., 2019).

Sections were examined using a light microscope (Leica CTR5000, Leica Microsystems, Wetzlar, Germany) at 100x, 200x and 400x objective magnification. Photomicrographs were obtained in the correct orientation of the representative fields using the Olympus© DP-23 camera (EVIDENT Technology Center Europe GmbH, Münster, Germany) with the cellSens™ software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) for image capture. All tissues sections were evaluated for the presence, type and location of hard tissue staining and findings were described qualitatively.

3. Results

3.1. Patient data

The mean age of non-T2D participants was 46 years ± 4.3 standard deviation (SD) and 48 years ± 4.8 SD for T2D individuals. There was no statistical difference in age between the groups (p = 0.06). There were 6 males and 4 females in each group and the mean time since diagnosis was 2.6 years. The mean HbA1c of the T2D patients was 52.5 mmol/mol ± 1.5 SD or 7 % ± 0.2 SD.

3.2. Cell culture

3.2.1. HDPC metabolic activity

Cells were more metabolically active in response to hyperglycaemia conditions (F = 6.9; p = <0.001) (Fig. 1). At 7 days the metabolic rate of the cells exposed to glucose was greater for 12.5 mM (+49 %; p = -0.004) and 25 mM glucose (+42 %; p = 0.017) compared to 5.5 mM glucose. At 14 days metabolic activity remained high compared to 5.5 mM controls with exposure to 12.5 mM (+29 %; p = 0.049) and 25 mM glucose (+35 %; p = 0.010). By day 21 only a significant difference in metabolic activity could be detected when the cells were cultured in 25 mM glucose (+33 %; p = 0.031).

3.2.2. Gene expression analysis

All mineralisation-associated GOI transcripts were detected following hDPC culture exposure to 5.5 mM, 12.5 mM or 25 mM glucose

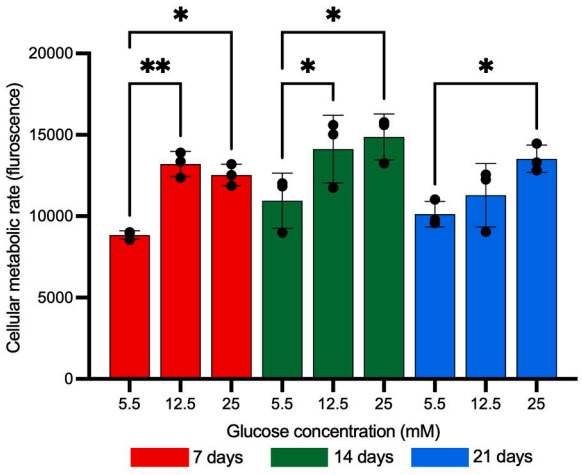

Fig. 1. – hDPC metabolic activity following exposure to glucose. Cells were cultured in DMEM media containing 5.5 mM (control), 12.5 mM or 25 mM for 7 days (red), 14 days (Green) and 21 days (blue). relative levels of cellular metabolic activity were determined by PrestoBlue assays. Data are presented as mean ± Standard deviation, $n = 3$. unpaired one-way ANOVA followed by Bonferroni's test for post-hoc comparison.

for 7, 14 and 21 days.

The quantity of mRNA expression for *SPP1, SPARC, BGLAP, IBSP* and *DSPP* were unaffected (at a level of significance) when hDPCs were cultured in the presence of 12.5 mM or 25 mM glucose at three time points compared to those cultured in 5.5 mM glucose. hDPC *RUNX2* mRNA was the exception, with expression significantly affected *(*$F = 2.8$*;* $p = 0.034$*)* transiently decreasing at day 14 (-4.2-fold; $p = 0.042$) when cultured with 12.5 mM glucose (Fig. 2).

3.3. Histological qualitative analysis

When examined microscopically following H&E stain, all processed specimens in the T2D and non-T2D groups met the histological assessment criteria for a normal pulp, and the tissue was diagnosed as

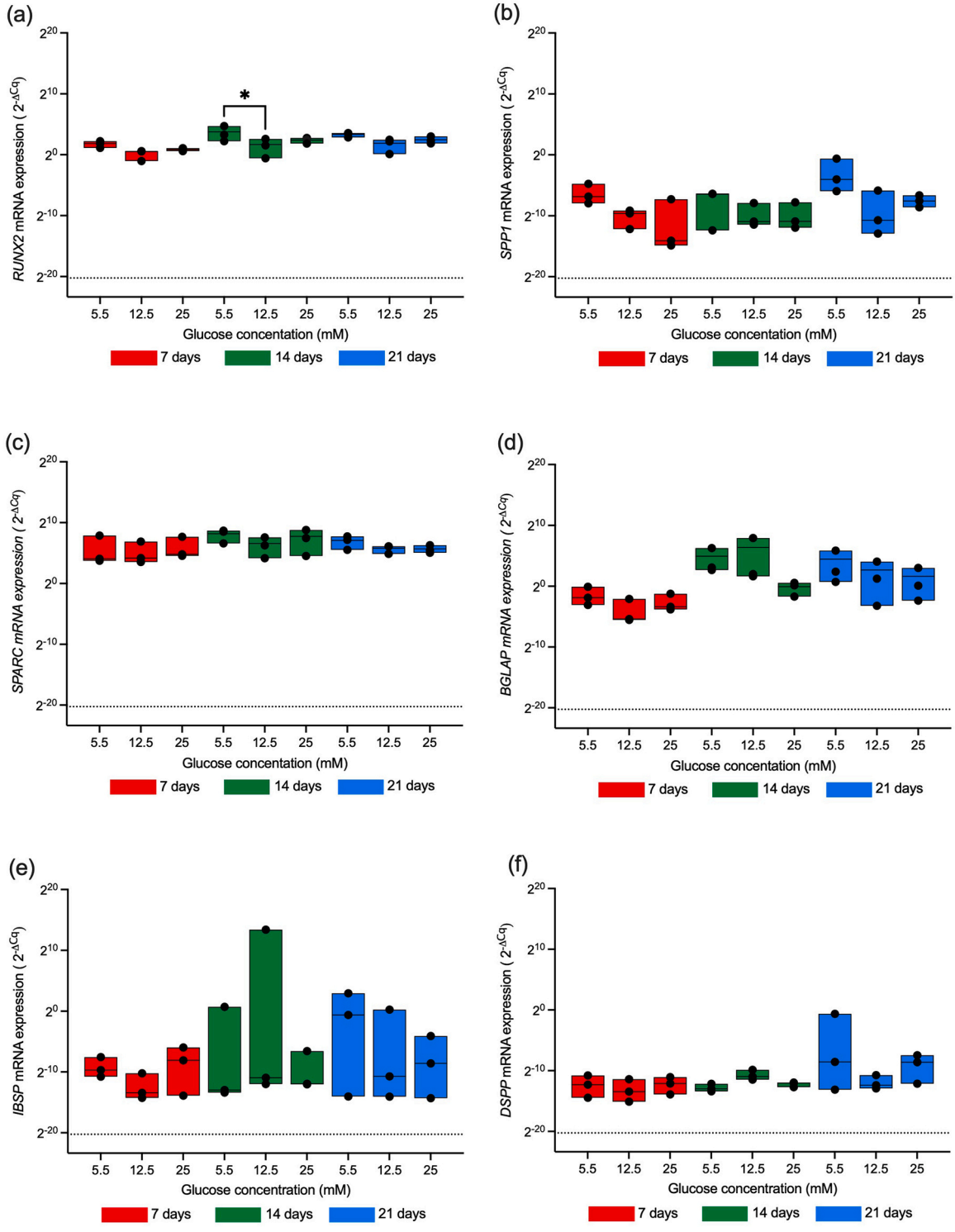

Fig. 2. – hDPC mineralisation-associated gene expression following exposure to glucose. (a) *runx2* (b) *spp1* (c) *sparc* (d) *bglap* (e) *ibsp* (f) *dspp*. Cells were cultured in DMEM media containing 5.5-, 12.5- or 25-mM D-glucose for 7 days (red), 14 days (Green) and 21 days (blue). data are expressed as a log transformation of cq ($2^{-\Delta Cq}$). the box-whisker plot shows the minimum and maximum range of mRNA detected, $n = 3$. dotted line indicates limit of detection. Unpaired one-way ANOVA followed by Tukey's test for post-hoc comparison.

uninflamed. In the H&E stained T2D samples, the predentine appeared more irregular and prominent ectopic amorphous calcifications were observed throughout the pulp space (Fig. 3 A & 3 C) compared with non-T2D samples (Fig. 3B & 3D). This finding was confirmed in the toluidine blue staining which distinguished the mineralized and unmineralised tissue. The predentine appeared thicker in the T2D samples and calcifications had a marbled appearance with reversal lines (Fig. 4 A & 4 C). The Modified Gallegos stain indicated that the location of the calcifications in T2D samples was non-specific, and they commonly exhibited a diffuse amorphous appearance. Calcifications were of variable size and located free within the pulp space, attached to the pulp chamber walls or embedded within the walls within the pulp chamber and in the coronal third of the radicular pulp (Fig. 5 A & 5 C). Furthermore, this stain differentiated the hard tissues and suggested that calcifications were of a fibro-dentine content. The dark pink staining indicated that some calcifications were more cementum-like while other calcifications resembled the appearance of tertiary dentine which exhibited as a mixture of pink and yellow-green staining (Fig. 5 A & 5 C). Those calcifications that were free within the pulp space were often observed adjacent to blood vessels and neurovascular bundles (Fig. 5 C).

4. Discussion

This study was conducted to investigate the potential influence of T2D and hyperglycaemia on the presence of calcifications within the coronal dentine-pulp complex. Notably, findings suggest that elevated glucose may exert similar pathological calcification effects as is observed at other body sites. Histological analysis including the use of special stains indicated that T2D was associated with irregular and thickened predentine and discrete ectopic calcifications which were widespread within the pulp tissue either free within the chamber and attached to or embedded within dentine walls and resembled stones associated with an aged pulp (Goga et al., 2008). Notably and consistent with Palatyńska-Ulatowska et al. (2021) the calcifications identified within the pulp chamber were mostly nodular and of fibro-dentine-like content resembling mixed cemental and dentine/bone-like tissues. The addition of 12.5 mM and 25 mM D-glucose to hDPC cultures simulated prediabetes and T2D and their metabolic rate in these hyperglycaemic environments was relatively stable over time. Interestingly, while mineralisation-associated genes were expressed at all time points, *RUNX2* was the only transcript which was affected when hDPCs were cultured in elevated glucose media and this was most evident at the initial time point. All other GOIs were expressed at levels that were independent of the influence of glucose concentration and time. The original hypothesis that calcifications would be more common in the presence of systemic disease was subsequently supported. However, the hypothesis was rejected in relation to cell metabolism which was predicted to be reduced in the hyperglycaemic environment along with the expression of mineralisation markers which did not alter appreciably.

The co-morbidities associated with diabetes creates challenges in the collection of relatively large numbers of human teeth from participants who also meet strict inclusion criteria. While a sufficiently large sample is preferred, the more limited sample size used for tooth specimens was nevertheless comparable with studies using similar sample numbers which showed differences between T2D and non-T2D patients (Ferreira et al., 2018; Nakajima et al., 2015), and findings were consistent with published data from our laboratory (Alsamahi et al., 2023). Cell culture models are commonly used to standardise conditions and explore the effects of diabetes (Fiorello et al., 2020; Kopp et al., 2023), however this comes with limitations and provides only a 'snap-shot' of data over a relatively short timeframe. T2D and low-grade chronic inflammation in response to hyperglycaemia occur over a prolonged period and often patients present with co-morbidities, consequently these models cannot fully recapitulate the complex *in vivo* environment. The histological findings and reversal lines identified allude to this and acknowledge that *in vivo* complications may develop due to chronic hyperglycaemia, in response to abrupt changes in glucose levels. In addition, other lifestyle factors and calcifications may undergo periods of calcific activity and inactivity. Furthermore, while the laboratory environment enabled standardisation, and the quantitative analysis of gene expression in

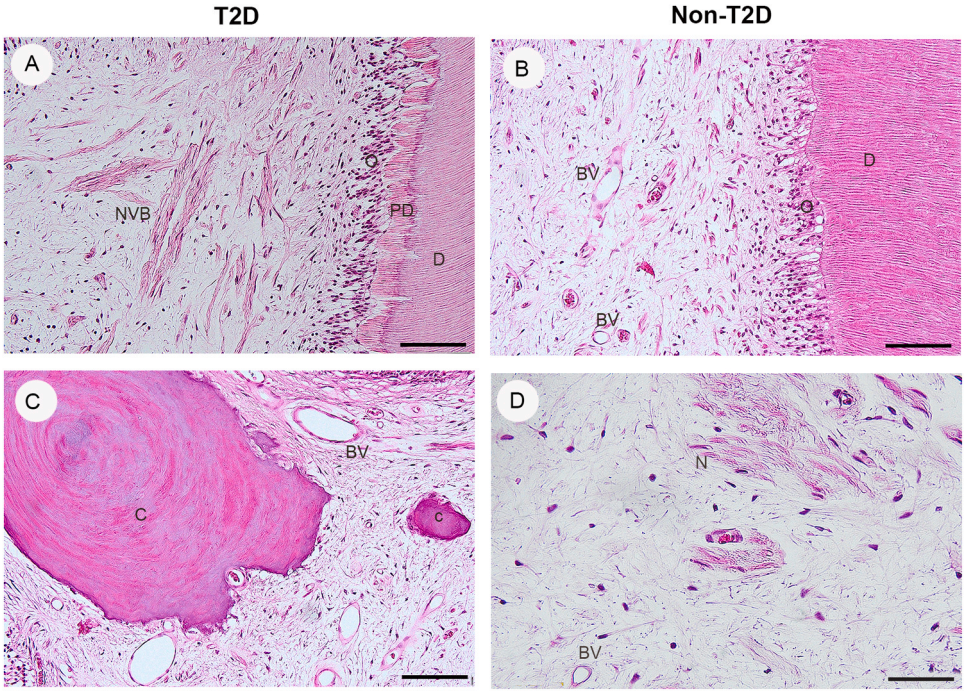

Fig. 3. – haematoxylin and eosin stained images showing the histomorphology of the clinically normal dental pulp-dentine complex from age-matched T2D and non-T2D samples. (A) And (C) representative T2D dental pulp and (B) and (D) representative non-T2D dental pulp. Images show T2D samples associated with an irregular predentine layer adjacent to odontoblasts and calcifications of various sizes within the pulp space. (A-D; 40x scale bar 50 μm). Nvb-neurovascular bundle, N-nerve tissue, PD-predentine, D-dentine, BV-blood vessels, O-odontoblasts.

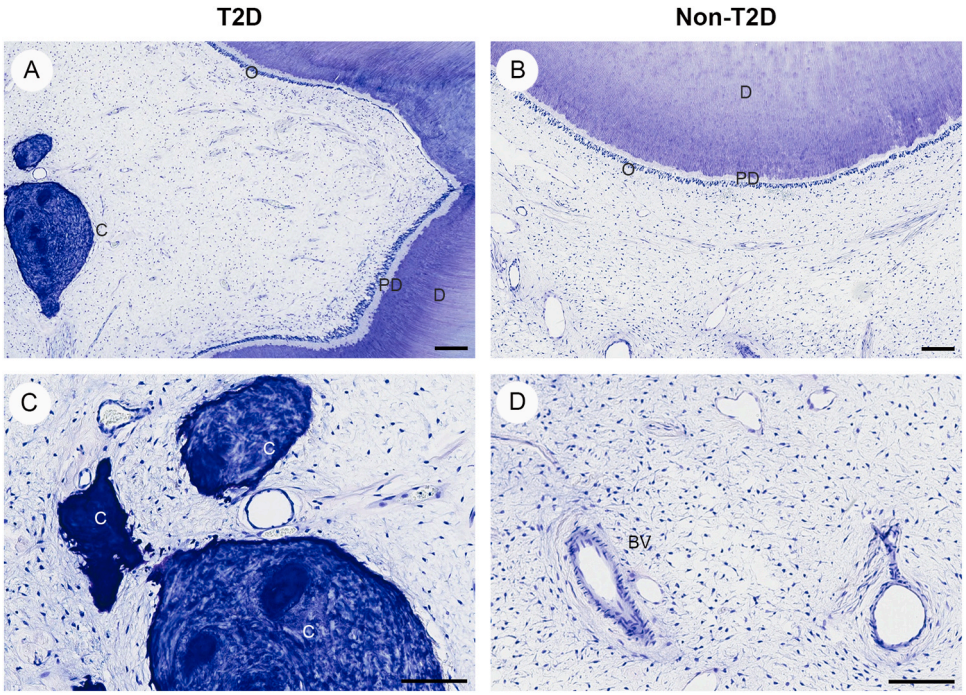

Fig. 4. – toluidine blue stained images of the pulp-dentine complex from age-matched T2D and non-T2D samples. (A) And (C) representative T2D dental pulp and (B) and (D) representative non-T2D dental pulp. Images show T2D samples associated with a pale blue unmineralised irregular predentine layer and dark blue calcifications of various sizes a marbled appearance and reversal lines. (A-B; 20x scale bar 50 μm, C-D; 40x scale bar 50 μm). Pd-predentine, D-dentine, BV-blood vessels, O-odontoblasts, C-calcification.

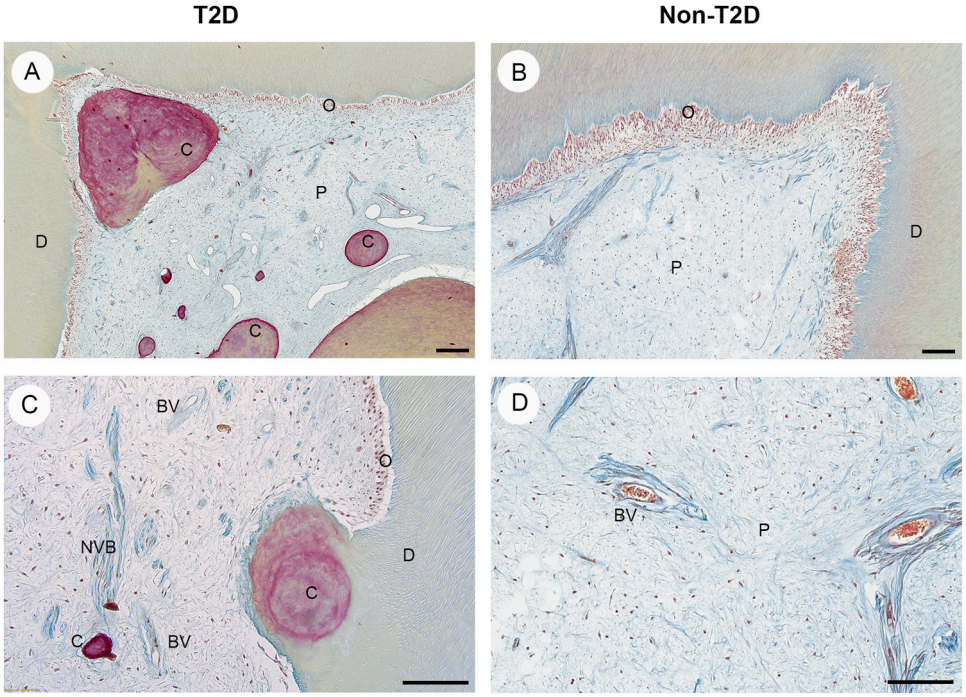

Fig. 5. – modified gallegos stain images of the pulp-dentine complex from age-matched T2D and non-T2D samples which differentiates hard tissues: dentine (stains light Green-yellow) and cementum/bone-like tissue (stains red). (A) and (C) representative T2D dental pulp and (B) and (D) representative non-T2D dental pulp. Staining shows calcifications were of mixed composition but predominantly cementum/bone-like and were seen free within the pulp space or attached to dentine walls. Calcifications were not seen in the non-T2D samples (B) and (D). (A-B; 20x scale bar 50 μm, C-D; 40x scale bar 50 μm). NVB-neurovascular bundle, BV-blood vessels, O-odontoblasts, PD-predentine, D-dentine, P-pulp, C-calcification.

culture, variability between samples was still observed. Such variability is expected in laboratory studies and is likely due to genetic and environmental differences, along with different host responses (Arora et al., 2022; Balasankar et al., 2022; Pimenta et al., 2024).

Determining differences in the deposition of hard tissue in histological samples can provide a better understanding of pulp changes due

to T2D and non-T2D. The descriptive and qualitative analysis performed here showed clear differences and diffuse and variable presentation of the calcifications observed within the coronal radicular pulp-dentine complex (embedded within dentine, attached to dentine walls, or free within the pulp space). Future studies including the use of micro-CT analysis could provide more comprehensive three-dimensional data which would enable enhanced characterisation of differences in mineralisation associated with T2D progression. Despite these limitations, it is important to emphasise that the findings reflect the effects of T2D elsewhere in the body and extends existing understanding of the different types of pulp calcifications observed clinically (Goga et al., 2008). Furthermore, the consistent histological pattern in pulp tissue from all T2D samples explains the hard friable nature of the pulp which has been previously described and reconciles with the difficulty establishing T2D hDPC lines using the explant technique (Patel et al., 2025). Indeed it is likely that the metaflammation, which resembles aging-induced inflammation and characterises T2D in other tissues, is a contributing factor to the fibrotic texture of pulp tissue in patients with the systemic disease (Prattichizzo et al., 2018).

Given the pulp and dentine are intimately related and have a common embryology, the appearance of an irregular and thickened layer of predentine in T2D is interesting and seems inconsistent with the increased presence of calcifications in the adjacent pulp tissue. However, this finding confirms observations in animal studies (Inagaki et al., 2010) and most recently a systematic review of the influence of T2D on the physical and mechanical properties of dentine has highlighted that T2D may negatively affect dentinogenesis and mineral apposition (Hwang et al., 2025).

There are several recognised types of ectopic calcifications found in dental pulp tissue and while the aetiology remains unclear these findings suggest that T2D may lead to the formation of ectopic aggregates of hard tissue and often in the proximity of blood vessels. Notably, this finding bears some resemblance to the 'aged' pulp where collagen bundles, blood vessels and nerve sheaths often form loci for calcifications (Bernick & Nedelman, 1975). The current findings support most recent histological evidence showing a positive correlation between metabolic diseases, tissue aging and the prevalence of pulp stones (Khan et al., 2024).

Calcifications related to T2D have been attributed to the increased concentration of advanced glycation end-products (AGEs). AGEs are produced physiologically via a non-enzymatic Maillard reaction when reducing sugars react with lipids and proteins. The process is slow and leads to damage of proteins and cross linking of collagen (Khalid et al., 2022). While AGES are a normal part of aging, in T2D this process is accelerated, and the accumulation of AGEs can upregulate inflammation and oxidative stress. This leads to an increased glycation activity which has been reported within the pulp of patients with T2D (Alsamahi et al., 2023; Khalid et al., 2022). Moreover, animal studies have demonstrated that AGEs enhance the early calcification of rat dental pulp cells (Sugiyama et al., 2022). The fibro-dentine appearance of the calcifications suggests that non-odontoblastic cells may be involved. Hyperglycaemia provides an environment where concentric layers of glycated collagen may aggregate and mineralise. Furthermore, the complexity of AGEs and calcification processes are complex and take time to exert their effects which may offer an explanation as why the GOIs showed limited change over 21 days. Future studies may consider observation over an extended time-period.

Ectopic calcifications elsewhere in the body are increased in patients with T2D, particularly in the vasculature where they represent a risk factor for cardiovascular disease. Notably, vascular cells associated with T2D potentially undergo trans-differentiation to osteoblast-like cells and show higher expression and upregulation of bone related genes including *RUNX2* and *OPN* (Avogaro & Fadini, 2015; Poetsch et al., 2020). However, this phenomenon was not observed in the hDPCs where markers of biomineralization were not significantly affected by hyperglycaemia. Notably, *RUNX2* was down-regulated initially, however this effect has been emphasised by others who have highlighted the role of the RUNX2 transcription factor in determining the maturity of osteoblasts and odontoblasts. In particular, *RUNX2* levels need to be down regulated for the terminal differentiation of odontoblasts and dentinogenesis, and RUNX2 is able to induce the trans-differentiation of odontoblasts into osteoblasts (Komori, 2010).

To better and more efficiently understand the mineralisation response in hDPCs exposed to a range of glucose conditions, a panel of candidate genes were analysed in a time-dependent manner to identify differentially regulated transcripts which encode molecules with a range of functionalities. Notably, differential mRNA expression is also frequently used as a starting point for investigating functional changes in cells or tissues, as it not only provides information on individual molecule involvement but can also indicate cellular signalling and pathway activation (Koussounadis et al., 2015). There is an assumption that changes in mRNA levels lead to changes in protein levels and, consequently, changes in cellular response. Furthermore, mRNA is generally easier to isolate, quantify, and analyse than proteins, and due to its relative ease of use in high-throughput analysis and cost efficiency, it can also enable interrogation of a range of molecular functions. Given the identification of the differential expression of *RUNX2,* the use of ELISA in future studies may assist in providing additional information regarding the levels of RUNX2 protein expressed temporally by cultured cells and the associated molecular mechanisms activated by this transcription factor.

Animal studies have also shown that hyperglycaemia leads to increased ectopic calcification in the dental pulp of rats and in agreement with the current study, expression of genes associated with calcification, were not consistently affected by 25 mM/L glucose exposure (Inagaki et al., 2010; Sugiyama et al., 2022). Notably, Sugiyama and colleagues found that common mineralisation-related genes (*OPN, OCN, DMP1* and *DSPP)* were not involved with the early calcification of rat pulp cells (Sugiyama et al., 2022). Taken together, while dysregulated calcium metabolism is recognised in T2D (Pittas et al., 2007) and the cellular metabolic rate increases over time with increased concentration of glucose, it is possible that pulp calcification induced within a hyperglycaemic environment of the pulp is less related to mineral imbalances and biomineralization events and may be more related to low-grade inflammation and AGEs (Alsamahi et al., 2023; Sugiyama et al., 2022).

Low grade inflammation is associated with the development of T2D, and evidence suggests it is an important cofactor in the calcification response (Bessueille & Magne, 2015). In particular, C-reactive protein (CRP), IL-6 and tumour necrosis factor (TNF-α) have been implicated in low grade inflammation, glucose control as well as in the pathogenesis of T2D and its complications (Mirza et al., 2012). Furthermore, lipopolysaccharide (LPS) has been implicated in T2D and atherosclerosis pathogenesis. LPS is also present on the cell surface of most Gram-negative bacteria and is consequently implicated in pulpitis via its stimulation of the release of pro-inflammatory cytokines (Chung et al., 2011; Creely et al., 2007). Potentially, cells in a diabetes model may develop some tolerance to high glucose, and so future studies which further develop the *in vitro* T2D model by including the additional chronic stress of proinflammatory molecules, such as cytokines and bacterial components, are warranted to further evaluate and characterise the calcific response.

Ectopic calcifications frequently form in response to chronic inflammation and irritation as well as the ongoing attempt of the pulp to instigate repair mechanisms. Irrespective of the aetiology, the increased likelihood of encountering calcifications in patients with T2D holds clinical relevance particularly related to pulp healing, identifying root canals during endodontic access, encountering and managing canal obstructions and they pose potential risks for procedural errors. The presence of pulp calcifications may block access to root canal orifices, entrap infected pulp tissue and impair adequate chemo-mechanical preparation of root canals leading to poorer healing outcomes.

Removal of pulp stones can be challenging, complex, and result in additional dentin destruction. If calcifications are visible on conventional radiographs, a limited field of view CBCT scan preoperatively may provide additional three-dimensional information on the size and position of the stone. Furthermore, the use of more advanced endodontic techniques including the use of elongated long neck round burs, ultrasonic instrumentation with copious sodium hypochlorite irrigation, and high magnification with the aid of a dental operating microscope can assist clinicians in their removal. More recently, micro-guided endodontics using CBCT scans and printed templates have been used to effectively guide burs within calcified root canals although there is a learning curve with this technique and long-term clinical studies are required (Peña-Bengoa et al., 2023). Overall, this higher likelihood of encountering pulp calcifications in patients with diabetes highlights the significance of considering the patients systemic health alongside their endodontic needs. This is important for establishing case difficulty and long-term prognosis, treatment planning and in conversations with patients as part of the informed consent processes.

In summary, this study has provided new data to better understand the association of systemic disease with pulp and periapical disease and tissue healing. The physiological processes of mineralisation can enhance the mechanical and protective properties of the pulp-dentine complex, however ectopic calcifications associated with T2D are common and may pose an increased risk of complications for clinicians. Potentially, the calcifications seen in association with systemic disease resemble an 'aged' pulp and it is possible this may be more related to chronic low-grade inflammation and glycation than biomineralization processes. Additionally, it will be important to ascertain whether calcifications associated with systemic disease are apparent radiographically to assist clinicians in treatment planning. Furthermore, well controlled studies comparing high resolution advanced three-dimensional imaging and conventional radiographs may advance this understanding and inform future clinical decision-making.

5. Conclusion

Hyperglycaemia and T2D may alter the mineralisation dynamics of predentine and lead to calcifications within the dental pulp, similar to that observed at other bodily sites. Interestingly, the diffuse nature, fibro-dentine appearance and proximity to blood vessels and nerve bundles resembles age-related calcifications. Notably, the hDPC expression of mineralisation genes independent of the hyperglycaemic environment may support a role for prolonged hyperglycaemia, metaflammation and glycation in the formation of calcific foci. These findings offer a new insight which recognises the systemic status of the patient and how this can inform case difficulty, treatment planning and informed consent processes.

CRediT authorship contribution statement

Paul Cooper: Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Friedlander Lara T:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Howard Chao:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Haizal Mohd Hussaini:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Trudy Milne:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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