

An investigation into molecular biomarkers of pulp inflammation and cell death and their potential use as a diagnostic tool

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Declaration

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Summary

Establishing an accurate clinical diagnosis of the level of pulp inflammation is currently unpredictable. Critical appraisal of the literature demonstrates a lack of standardisation in pulpal diagnostic accuracy, as well as biomarker identification and validation; which highlights a number of areas of particular focus. Diagnostic categorisation, substrate selection, collection techniques and testing methodologies all lack consistent standardisation, resulting in low quality studies with high risk of bias. With the growth in clinical techniques targeting pulp preservation techniques, the underutilisation of pulpal blood as an adjunct to clinical diagnosis is also apparent.

The overarching aim of the thesis was to present a robust method of collecting and analysing pulpal blood, to inform quality biomarker discovery with the potential to direct the ‘threshold’ between teeth amenable to vital pulp treatment and those that require root canal treatment. Using the information obtained from the literature review, a standardised methodology was formulated and presented in **Chapter 2**.

Practical aspects of pulpal blood collection and substrate analysis was investigated in **Chapter 3**. A robust method for whole blood collection and subsequent ELISA protein analysis for select candidate markers was presented. Microcapillary collection, although both feasible and reproducible, is technically sensitive and requires a degree of practical experience. The choice of substrate, serum or plasma, should be matched with the specific analyte under investigation. Volumetric methodology is preferred as a clinical translational approach as it can be used at the point of care.

In order to direct future therapeutic decisions relating to the maintenance of pulpal health, the visual appearance of the exposed pulp was correlated with traditional and novel pulp diagnostic categories, in **Chapter 4**. The potential to exploit pulpal blood both diagnostically and as a prognostic indicator was considered. A variety of reference standards were introduced including haemorrhage control, caries depth and symptoms, which were then related to treatment choice and outcome.

Focal necrosis is a definitive histologic feature of irreversible inflammation. In **Chapter 5** novel molecular diagnostic markers of end-stage pulpal inflammation, in the form of mediators of cell death, were analysed. Circulating nucleosomes and selective Danger Associated Molecular Patterns present novel diagnostic targets and prognostic indicators.

The complex regulation and interconnections of both inflammatory and cell death networks is apparent, such that the identification of a single mediator, appears unlikely. **Chapter 6** focused on the use of high-throughput protein analyses of pulpal serum. Validation assays were then conducted on a selection of candidate analytes that had demonstrated statistically significant differences between the carefully characterised samples, representing a range of health and progressive inflammation.

Chapter 7 summarises that within the limitations of the various models presented, an opportunity exists to direct larger, cross-sectional studies, to potentially direct future standards in biomarker identification of pulpal blood, that could be exploited to enhance clinical judgement and potential patient outcomes.

In conclusion, pulpal whole blood can be predictably collected and analysed. As a result, the potential does now exist to exploit biomarker identification as an alternative reference standard, to the current histological ‘gold-standard’, allowing for both diagnostic and prognostic assessments to be ameliorated.

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¹ Robert Burns; Epistle to The Rev. John M'math

² St. Brendan

³ Seamus Heaney; The Cure at Troy: A Version of Sophocles' Philoctetes

⁴ Seamus Heaney; in The Guardian

⁵ Samuel Beckett; Westward Ho!

⁶ Julius Henry “Groucho” Marx

⁷ William Butler Yeats; The Lake Isle of Innisfree

⁸ Patrick Kavanagh; Primrose

⁹ William Stanley Merwin; Separation

¹⁰ Marcus Aurelius; Meditations

¹¹ Samuel Beckett; adapted from Waiting for Godot

¹² Seán O’Casey; Red roses for me

¹³ Robert Frost; Devotion

¹⁴ Oscar Wilde

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Abbreviations

Abbreviation	Description
AAE	American Association of Endodontists
AAPD	American Academy of Pediatric Dentistry
abs	Absorbance
asymp	Asymptomatic
AU	Absorbance units
BSA	Bovine serum albumin
bw	Between
Calc	Calculation
Ca(OH)₂	Calcium hydroxide
cfDNA	Cell-free Deoxyribonucleic acid
CGRP	Calcitonin Gene Related Peptide
CH	Calcium hydroxide
citH3	Citrullinated Histone-3
Clin	Clinical findings
Co	Company
CO₂	Carbon dioxide (snow)
conc	Concentration
cw	Cotton wool pellet
D	Deep
DA	Diagnostic accuracy
DAMP	Damage associated molecular pattern
DDUH	Dublin Dental University Hospital
DNA	Deoxyribonucleic acid
doc	Document
dOR	Diagnostic odds ratio
DPC	Direct pulp capping
DPT	Direct pulp therapy
ECI	Ethyl chloride
EDTA	Ethylene diamine tetra-acetic acid
EF	ROEKO Endo Frost
ELISA	Enzyme-linked immunosorbent assay
EPT	Electric Pulp Test
excl	Exclusion
ExD	Extremely deep
EXT	Extraction
FC	Formocresol
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FP	Felypressin
g	Gravitational constant
GCF	Gingival crevicular fluid
GF	Growth factor
GmbH	Gesellschaft mit beschränkter Haftung
H₂O₂	Hydrogen peroxide
H3	Histone-3
H4	Histone-4
HA	Haemostasis achieved

H&E	Hematoxylin and eosin
Hep	Heparanised
HMGB1	High-mobility group box 1
HRP	Horseradish peroxidase
hrs	Hours
HS	High sensitivity
HSC	Hydraulic silicate cement
Hx	History
Ibu	Ibuprofen
ID	Identification
IFN-γ	Interferon gamma
IL	Interleukin
incl	Inclusion
incr	Increase
IP	Irreversible pulpitis
IPC	Indirect pulp cap
IPT	Indirect pulp therapy
K2EDTA	Ethylene diamine tetra-acetic acid dipotassium salt dehydrate
K3E	Ethylene diamine tetra-acetic acid tripotassium salt dihydrate
LoE	Level of evidence
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Ltd	Limited
Ly+	Elevated lymphocyte count
MAPK	Mitogen-activated protein kinase
MDD	Minimum detectable dose
Mfg	Manufacturing
mg	Milligram
min	Minutes
MiP	Mild pulpitis
ml	Millilitre
mL	Millilitre
mm	Millimetre
MMP	Matrix metalloproteinase
Mob	Mobility
MoP	Moderate pulpitis
MPLX	Multiplex assay
MPO	Myeloperoxidase
mRNA	Messenger RNA
MTA	Mineral trioxide aggregate
N	Normal
N+	Elevated neutrophil count
n	No
n =	Number
n*	Only exclusion listed
N/A	Not applicable
NaHep	Sodium heparin
NaOCl	Sodium hypochlorite
NE	Neutrophil elastase
NeP	Necrotic pulp
NET	Neutrophil extracellular trap

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NLR	NOD-like receptor
no	Number
NP	Normal pulp
NPV	Negative predictive value
Ns	Not stated
NS	Not significant (statistically)
OD	Optical density
op	Operative
PAI	Periapical index
Palp	Tender to palpation (Apical unless otherwise stated)
PAMP	Pathogen associated molecular pattern
PB	Pulpal blood
PBS	Phosphate buffered saline (pH 7.2)
PB(Se)	Pulpal blood supernatant (serum)
PCR	Polymerase chain reaction
pe	Pulpectomy
pg	Picogram
POCT	Point-of-care testing
pp	Pulpotomy
PPD	Periodontal pocket depth
ppt	Paper point
PPV	Positive predictive value
PrA	Protein assay
PRR	Pattern recognition receptors
PT	Pulp tissue
PTS	Pulp tissue supernatant
PVDF	Polyvinylidene fluoride
Q3	Third quartile
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
R²	Coefficient of determination
Rad	Radiographic
RAGE	Receptor for Advanced Glycation End-products
RBC	Red blood cells
r/c	Recall
Ref	Reference test
RIA	Radioimmunoassay
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
RoB	Risk of bias
ROS	Reactive oxygen species
RP	Reversible pulpitis
Rpm	Revolutions per minute
RT	Room temperature
s	Seconds
SeP	Severe pulpitis
sig	Significant
SIP	Symptomatic irreversible pulpitis
Sn	Sensitivity
SO	Single operator

Sp	Specificity
SP	Substance P
SSD	Statistically significant difference
StDev	Standard deviation
STROBE	STrengthening the Reporting of Observational Studies in Epidemiology
SWE	Stepwise excavation
symps	Symptoms
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TOC	Total Organic Carbons
ToPr	Total protein
TTP	Tender to percussion
U.K.	United Kingdom
U.S.A.	United States of America
V's	Versus
VI	Visual inspection
Vol	Volume/volumetric
VPT	Vital pulp therapy
v/v	Volume per volume
w	With
WB	Whole blood
WCC	White cell count
wrt	With respect to
w/v	Weight over volume
x	Times (multiple)
XLa	Extraction
y	Yes
Y*	Only inclusion listed
1°	Primary
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
μg	Microgram
μl	Microliter
%	Percentage
°C	Degrees Celsius
-	Minus
-	Negative response
+	Positive response
±	Plus, or minus
≤	Less than, or equal to
≥	Greater than, or equal to
#	Number
¶	Denoting haemolysed specimens
^	Denoting paper point collection, or signifying smoker cohort
┐	Denoting caries exposed samples
~	Denoting deep caries (primary molars)
*	Assumed cohort vital teeth without histologic verification

**	Assumed absence of disease without verification
@	at
?	Uncertain

Chapter 1

Literature Review

1.1 Introduction

Dental caries remains the most common cause of pulpal inflammation (Bjørndal et al. 2006, Hahn & Liewehr 2007a, Bernabé & Sheiham 2014). A relatively recently published analysis of the global burden of oral diseases, estimated the level of untreated caries in permanent teeth to be approximately 2.5 billion teeth (Kasebaum et al. 2017), a substantial increase on figures estimated to be in the region of 1.7 billion teeth in 1990. This rise was linked with population growth as well as ageing; speculatively could also have been influenced by increases in disadvantaged groups. Microbial contamination of the dentine-pulp complex from caries, or bacterial microleakage stimulates a cascade of immune and inflammatory-mediated events within the pulp (Izumi et al. 1995, Jontell et al. 1998, Stashenko et al. 1998). The carious process is a complex, dynamic process (Bjørndal et al. 2014); evidenced by variabilities in the individual's caries experience, restorative burden, specific virulence factors and the temporal elements of the disease process, all of which result in a range of reactions in the pulp (Reeves & Stanley 1966, Mjör 2002). The odontoblast cells form the first line of innate defence in the dental pulp, being involved in sensory stimulus transmission from the dentine, immunocompetence, as well as dentine matrix secretion (Couve et al. 2013). Dependent on the degree of insult incurred, a reactionary, or a reparative tertiary dentinogenic response will be induced, resulting in either upregulation of the secretory activity of the primary odontoblasts; or their replacement, subsequent to a sequence of recruitment, induction and differentiation into odontoblast-like cells (Smith et al. 1995). Once the extent of the ensuing inflammatory reactions persists, or exceeds the reparative

capacity of the dentinal-pulp complex, pulpal necrosis will ensue (Heyeraas et al. 2001).

Classically, pulpitis is defined clinically as being reversible, or irreversible based on a subjective history of symptoms and the findings of currently available special tests (Seltzer et al. 1963a; AAE 2013). These descriptors have been extrapolated from histological analyses of extracted teeth and are influenced by the perceived ability to preserve at least some, or all of the pulp (Bergenholtz & Kvist 2013, Frisk et al. 2013, Simon et al. 2013, Stangvaltaite et al. 2017).

1.2 Symptoms of pulpitis

The range of symptoms associated with pulpitis are variable and may not accurately reflect the 'true' extent of the inflammatory process, as demonstrated by classical histological studies (Seltzer et al. 1963a, 1963b, Garfunkel et al. 1973, Dummer et al. 1980). Histologically, irreversible pulpitis is denoted by the presence of discrete zones of necrosis within the coronal pulp, which are associated with bacterial colonization of the pulp tissue (Reeves & Stanley 1966, Anderson et al. 1981, Lin & Langeland 1981, Ricucci et al. 2014). Bacterial contamination has been demonstrated even in the absence of pulpal exposure; however, identification of this bacterial ingress into the pulp cannot yet be measured clinically (Lin & Langeland 1981). Ricucci et al. (2014) linked clinical pulpitis diagnosis to histological analysis and included teeth with severe symptoms associated with irreversible pulpitis, as well as teeth with descriptors related to normal, or reversibly inflamed pulp, which only portrayed the milder end of the spectrum. Case selection in this study did not account for the transitional phase between a clearly defined reversible and an acute

irreversible, or severe pulpitis; which is diagnostically more challenging to discriminate (Ricucci et al. 2014). Even in light of this, 5/32 cases diagnosed clinically as being irreversibly inflamed, were shown to exhibit reversible pulpitis histologically. A further two cases exhibiting clinically normal signs and symptoms were found to present with abscesses in the pulp chamber and would therefore be expected to fail if indirect pulp therapy had been provided. In 2010, the Swedish Council on Health Technology Assessment concluded that there is a lack of objective diagnostic methods available, to demonstrate whether inflamed pulp is saveable or not; consequently, pulpal disease diagnostics was highlighted as a priority area in endodontic research (Swedish Council on Health Technology Assessment; 2010).

Recently, new classifications of pulpal disease have been proposed, with Hashem et al. (2015) differentiating mild from severe pulpitis, based on spontaneous resolution of the reaction to a stimulus within 20 seconds; compared with pain which lingered for minutes, that required analgesia. Furthermore, Wolters et al. (2017) proposed a new categorisation of pulpitis linked to minimally-invasive management strategies (**Table 1.1**).

Wolters' 2017 classification categorised the inflamed pulp using recognised clinical findings, including subjective symptoms which rely on patient reported symptoms, in order to guide the clinician towards more minimally invasive treatment techniques. With the advent of hydraulic calcium silicate cements (HSCs), these minimally-invasive treatment concepts are generating more predictable treatment outcomes (Asgary et al. 2013, Simon et al. 2013, Asgary & Eghbal 2015, Quidemat et al. 2017, Taha & Abdelkhader 2018); however, the techniques remain limited by inaccurate prediction of pulp status and the materials by their non-specific mode of

action and inability to eliminate infection (Duncan et al. 2019a). The well-recognised limitations of current techniques in accurately diagnosing pulpal disease are frequently cited by

Table 1.1 Classification systems and proposed therapy according to pulp status [modified from Wolters et al. 2017; s: seconds]

Seltzer Classification	Reversible Pulpitis		Irreversible Pulpitis	
Proposed Therapy (Seltzer)	Conservative		Pulpectomy	
Wolters' Classification	Initial Pulpitis	Mild Pulpitis	Moderate Pulpitis	Severe Pulpitis
Clinical Features	Heightened response to cold, not lingering, no spontaneous pain.	Heightened & lingering response to cold/heat/sweet stimuli up to 20s, resolve. May be percussion sensitive.	Heightened prolonged reaction to cold, lingering minutes, may be percussion sensitive, spontaneous pain.	Severe spontaneous pain, warm and cold stimuli, range from sharp to dull throbbing, disturbed sleep often, percussion sensitive.
Proposed Therapy (Wolters')	Indirect Pulp Therapy	Indirect Pulp Therapy	Pulpotomy (partial, or complete)	Pulpotomy, or Pulpectomy

operators in questionnaire-based research, as one of the reasons for the failure to adopt more conservative treatment approaches (Stangvaltaite et al. 2013, Chin et al. 2016, Careddu et al. 2021).

1.3 Limitations of current diagnostic techniques

Currently, the most commonly used clinical tests to assess the status of the pulp, measure pulp sensibility (or neurogenic response). The integrity of the pulps' vascular supply is extrapolated indirectly from the ability of the sensory nerve components to

register a response to a stimulus. Absence of a response may be indicative of necrosis. The sensitivity to stimuli may be utilised to describe varying degrees of inflammatory injury (Olgart & Kerezoudis 1994).

Cold stimulation, followed by electric pulp testing are the most commonly utilised tools to determine the pulp status via sensibility (Jespersen et al. 2014). A combination of tests improves their validation in the context of pulpitis (Hyman & Cohen 1984, Fuss et al. 1986), however to date; no single, or combined measure is considered a perfect test, with false positive and negative responses common (Manikar & Kim 2018). The introduction of Laser Doppler flowmetry and pulse oximetry were welcomed as a breakthrough in the direct assessment of the pulp's vasculature and therefore a true measure of vitality with impressive results recorded in terms of diagnostic accuracy (Odor et al. 1986, Vongavssan & Mathews 1992, Evans et al. 1999); however, their associated limitations precluded their adoption into routine clinical practice.

The validity of a diagnostic test to accurately detect the presence, or absence of disease can be expressed in statistical terms as sensitivity, or specificity. A selection of studies relating to the diagnostic accuracy of cold testing to determine the state of the pulp is presented in **Table 1.2**. Verification of the index test is usually demonstrated by histological assessment of the pulp tissue, regarded as the 'gold standard'; or by use of a clinical reference test, usually the direct visual inspection once the pulp chamber has been accessed (Wieslander et al. 2009, Villa-Chavez et al. 2013, Jespersen et al. 2014). However, although direct visualization of pulp tissue can confirm vitality, it is more difficult to ascertain the level of inflammation of the tissue from visual inspection alone.

A lack of uniform correlation exists between the subjective and objective clinical findings and the histological condition of the pulp (Dummer et al. 1980). The histological description of the pulp states, as set out by Seltzer et al. (1963a) were used by Dummer et al. (1980). The patient's history and their responses to thermal and electric pulp testing were correlated with variable histologic presentations, which included intact uninfamed pulps, through progressively more severe inflammatory changes, up to the designation of total necrosis. Pain was reported in every category.

Table 1.2 Diagnostic accuracy of cold testing to determine pulp status [ECL: Ethyl Chloride; EF: Endo Frost; Ref: Reference test, Sn: Sensitivity, Sp: Specificity, PPV: Positive predictive value, NPV: Negative predictive value, DA: Diagnostic accuracy, dOR: Diagnostic odds ratio. * Assumed cohort vital teeth without histologic verification, ** Assumed absence of disease without verification]

Paper	Test	Ref	Sn (%)	Sp (%)	PPV (%)	NPV (%)	DA (%)	dOR (%)
Dummer 1980	Cold ECI	Histology	68	70				
Kamburoğlu 2005	Cold Coolan	Clinical*	93	98	97	94	95	
Pigg 2016	Cold	Clinical	89	80	81	88		31.4
Weislander 2009	Cold EF	Clinical	76	92	93	74		
Weislander 2009	Cold CO2	Clinical	76	89	90	73		
Jespersen 2014	Cold EF	Clinical	91.6	89.6	86.2	93.7	90.4	
Peterssen 1999	Cold ECI	Clinical*	83	93	89	90		
Peters 1994	Cold CO2	None**						
Villa-Chavez 2013	Cold EF	Clinical	88	100	100	90	94	

Variable sensitivities to thermal and electrical pulp testing were also recorded in each of the defined histological categories, underpinning the complexity of this aspect of pulp diagnosis. Factors known to influence the results of standard sensibility testing include patient anxiety, operator experience, metallic restorations, trauma and false positive or false negative responses. Interestingly, Dummer et al. (1980) calculated the prevalence of necrotic pulps to be 25%, in contrast to 67%, which he deemed to be non-saveable. The non-saveable pulp was defined as a pulp

exhibiting chronic partial pulpitis with necrosis-ranging to total necrosis. Anderson et al. (1981) afforded some uncertainty in their classification, which they described as “pulp disease of questionable reversibility”, which would allow for the possibility of providing more conservative treatment options.

In a relatively recent systematic review, Mejàre et al. (2012) concluded that the evidence relating to pulpal diagnosis remains low, due to poor study design, conduct and reporting. In particular, special circumstances (presence of an extra-coronal restoration, periodontal disease, an obliterated root canal system, cracked tooth, or a stressed pulp) are frequently omitted from these study populations. Alterations in disease presentation will significantly affect diagnostic accuracy, with reports suggesting that case control studies can overestimate both sensitivity and specificity (Whiting et al. 2013). This relates to studies that compare only the extremes of health and severe disease, which is often reported in studies relating to diagnostic accuracy of pulp testing (Mejàre et al. 2012). They further conclude that use of an inappropriate reference standard significantly affects estimates of accuracy, in particular sensitivity (Whiting et al. 2013).

1.4 Pulpal bleeding as a diagnostic aid

The presence, or absence of detectable bleeding within the pulp chamber has traditionally been relied upon to distinguish a vital from a necrotic pulp. The clinical reference standard most commonly cited is visible bleeding once the pulp chamber has been entered (Wieslander et al. 2009, Villa-Chavez et al. 2013, Jespersen et al. 2014). However, absence of detectable bleeding in the pulp chamber, or even the coronal aspect of the canals, but presence in the root canal system could lead to an

erroneous classification of necrosis (Petersson et al. 1999, Weislander et al. 2009) and an overestimation of disease prevalence, further diminishing the diagnostic accuracy (Rutjes et al. 2006). Therefore, it is highly questionable if total necrosis is a suitable reference standard in the measurement of pulpitis.

1.4.1 Diagnostic accuracy

The application of a dichotomous target condition (vital, or necrotic pulp; reversible, or irreversible pulpitis) to a reference standard when a wide spectrum exists is problematic, in diagnostic accuracy studies (Rutjes et al. 2007). Although histological analyses allow us to appreciate the very earliest minor changes, through to the most severe, advanced stages in the progression of the disease (albeit only as a snapshot); encompassing the entire spectrum of the disease state (Rutjes et al. 2007). The establishment of a clear link between diagnostic accuracy and biologically-driven treatment modalities remains unanswered in the current literature.

Disease prevalence will differ between target populations thereby precluding the ready transfer of data obtained from one study to another (Mejàre et al. 2012). Inappropriate selection of the target population may further bias the results obtained. Selection bias is an inherent difficulty in pulp diagnostic studies. The cohort under investigation may have been referred to a specialist unit, leading to alteration in the disease prevalence compared with that of the general population. The application of strict exclusion criteria further alters the disease spectrum. This may lead to difficulties for the clinician in cases where the diagnosis is complex. Operator experience was shown to have a significant effect on the accurate identification of diseased pulps when using cold testing in a large practice-based research network

(Pigg et al. 2016). Reported values can inflate estimates due to case selection, reference test, bias, disease prevalence, assumed knowledge of pulp status, blinding and inter/intra operator variation. As result; as dentists, we are likely over-treating and unnecessarily removing the injured pulp (Moynihan et al. 2013). In an era where the emphasis is clearly shifting in the direction of minimal intervention dentistry, there is a need to re-evaluate what the ideal reference standard ought to be. In order to achieve this, it is important that essential methodological standards are met (Bergenholtz & Kvist 2013).

1.4.2 Typical characteristics of pulpal bleeding

The condition of the bleeding vital pulp has not been clearly described in diagnostic studies to date, with just a few notable exceptions (Guthrie et al. 1965, Matsuo et al. 1996, Doğan et al. 2013). Guthrie et al. (1965), quantified the amount of pulpal haemorrhage observed by categorising it as slight, moderate, or profuse which was subsequently analysed histologically. This study examined 53 cariously-exposed pulps in the primary and permanent dentition. An additional 14 negative controls were included. The analysis focused on the location of leukocytes within, or beyond the pulp chamber, to distinguish good from poor risk groups, when considering vital pulp treatment (VPT) predominantly in primary molars. In order to conduct the histological assessment, the teeth were extracted and fixed in formalin, after the blood smear had been collected; thereby precluding an analysis of outcome of the proposed treatment. Notably, the character of the bleeding was not recorded for 8/53 cases, which could perhaps be because the bleeding did not fall clearly within the categories described;

1. Slight: No bleeding, or only 1-2 drops of blood apparent.
2. Moderate: Several drops of blood, ceased quickly.
3. Profuse: Blood filled cavity quickly and persisted.

A description of the quality of bleeding described by Guthrie et al. (1965) in the samples was aligned with their respective haemogram results, is summarised in **Table 1.3**. Inflammation confined to the pulp chamber, was defined as a good candidate for VPT, as opposed to poor; when the inflammation was identified in the root canals, was based entirely on their histological findings. Profuse bleeding was not demonstrated to correlate strongly enough with the histological finding classified as “poor risk” candidate, to conclude that excessive haemorrhage was a contraindication for VPT. Good VPT candidates were not demonstrated to have an elevated neutrophil count, despite moderate-profuse bleeding being noted in more than half of this category. Pain was noted in almost half the cases. Although the majority of the symptomatic cases were categorised as “poor risk” candidates, a small portion of painful pulpitis cases were identified as “good risk” candidates (Guthrie et al. 1965). However, in the absence of follow-up in the form of outcome of VPT procedures, this conclusion could be considered preliminary at best.

Table 1.3 Character of pulpal bleeding [adapted from Guthrie et al. 1965; N: normal; Ly+: Elevated lymphocyte count; N+: Elevated neutrophil count, no: number]

Poor Candidate				Good Candidate			
Bleed	Haemogram			Bleed	Haemogram		
	N	Ly+	N+		N	Ly+	N+
Slight (n=2)	1	1	0	Slight (n=12)	1	11	0
Moderate (n=9)	2	4	3	Moderate (n=12)	7	5	0
Profuse (n=7)	3	0	4	Profuse (n=3)	1	2	0

In another study, Matsuo et al. (1996) categorised the bleeding encountered upon accessing cariously-exposed pulps into four groups, into which time to achieve haemostasis was recorded.

1. Oozing.
2. Slight, but apparent from exposure site.
3. Overflowing, stopped within 30 seconds.
4. Overflowing, did not stop within 30 seconds.

The combined results from groups 3 and 4 demonstrated a successful outcome in 55.6% (5/9 cases treated by direct pulp capping) after 0-3 months, leading to the usefulness of bleeding as a clinical index being called into question (Matsuo et al. 1996). It should be noted however, that the number of cases analysed in categories 3 and 4 were very small.

Doğan et al. (2013) described the bleeding characteristics of the exposed pulps as normal, inflamed, or necrotic. Normal pulps exhibited little, or no bleeding and were red in colour. Inflamed pulps bled excessively and were deep red in colour. Both inflamed and necrotic cases were deemed not suitable for VPT perioperatively. They discovered, however, that 16 teeth, considered suitable for pulpotomy, were excessively inflamed even after completing a deeper amputation to the level of the root canal orifice. In a further seven cases deemed to be untreatable at the point of exposure, normal bleeding characteristics were discovered once the coronal pulp was completely amputated and these cases were then treated conservatively. Magnification is not mentioned in the methodology (Doğan et al. 2013) and their

classification of reduced vascularity, or absence of detectable bleeding could potentially have misclassified surface necrosis as a normal characteristic.

Collectively, these studies highlight the degree of subjectivity and complexity of aligning the visual inspection of cariously-exposed pulps, with recognised clinical diagnostic parameters. Indeed, this casts doubt as to what extent (if at all) visual inspection of pulpal bleeding assists with pulpal diagnosis. It is notable that the above studies make no reference to magnification or illumination and no images were provided for reference or to support the claims.

1.4.3 The colour of blood

A single paper has investigated the colour of pulpal blood in exposed pulps of primary molars undergoing pulpotomy, or pulpectomy (Aminabadi et al. 2017). Pulpal blood was collected via sodium heparinised microcapillary tubes and the colour assessed quantitatively using a spectrophotometer connected to a digital camera (SpectroShade™ Micro, MHT S.p.A, Verona, Italy). The blood colour was calculated using a colour difference formula based on reflectance and chromaticity. Treatment decisions were made according to the American Academy of Pediatric Dentistry Guidelines (AAPD 2015), or the inability to achieve haemostasis in under five minutes, using pressure applied with a saline-moistened cotton pellet. Pulpotomy was indicated in cases deemed to be reversibly inflamed upon mechanical, traumatic or carious exposure, whereas pulpectomy was preferred in irreversibly inflamed, or necrotic pulps (AAPD 2015). The collected blood was analysed post treatment. They concluded that the pulpectomy group presented with darker pulpal blood than the pulpotomy groups. The spectrophotometer was designed for tooth shade

assessment and the blood colour calculations, which were extrapolated indirectly, are not validated for this purpose. From a practical perspective the cost of the equipment could also be prohibitive and not a practical option for practitioners. Clinically, blood colour does not appear to be a useful marker of inflammation, particularly when the manner of magnification (if any) and the variety of light sources are clinician-dependent. The suggestion that darker coloured pulpal blood is indicative of a more advanced inflammatory state is not currently supported in the wider research.

1.5 The analysis of pulpal blood

There has been a focus in recent years to improve the quality (Bergenholtz & Kvist 2013, Duncan et al. 2016), conduct (Pigg et al. 2021) and reporting (Nagendrababu et al. 2020b) of research within the endodontic literature which aims to improve in the level of evidence and ultimately enhance patient outcomes (Collins & Varmus 2015, Giannobile 2015, Duncan et al. 2021). The volume of publications relating to VPT have increased substantially during the past 20 years (Kodonas et al. 2021). VPT studies have focused principally on treatment outcomes, efficacy of materials, or the incidence of pulp exposures. An opportunity now exists to review how studies in pulp biology are conducted to maximise their clinical translational diagnostic opportunities (Zehnder & Belibasakis 2021). There is a potential to harness a vastly underutilised substrate (pulpal blood), which could be handled chairside, to objectively and accurately assess pulpitis, and potentially link to a conservative treatment outcome.

To date, there are 13 published studies investigating pulpal blood as a diagnostic marker (**Table 1.4**), spanning more than 70 years. It is worth analysing various aspects of those studies specifically with regards to study design and reporting.

Table 1.4 Study design used in the analysis of pulpal blood [Power calc: power calculation; y: yes; n: no; Ibu: ibuprofen; STROBE: STrengthening the Reporting of OBservational studies in Epidemiology]

Author	Study Design	Power calc	Reporting Guideline	Ethical approval
Sharma 2021	Correlational, cohort? clinical trial	y	STROBE, clinical trial registered	y
Nguyen 2020	Clinical observational/interventional (Ibu)	y	-	y
Shmueli 2020	Clinical, observational	n	-	y
Lejri 2019	Prospective, cross-sectional, epidemiological	n	-	n
Mutluay 2018	Clinical, observational	n	-	y
Ghattas Ayoub 2017	Clinical, observational	n	-	y
Aaminabadi 2017	Clinical cohort observational	y	STROBE guideline report	y
Mente 2016	Prospective, 2-centre clinical study observational	n	-	y
ElSalhy 2013	Clinical, observational	n	-	y
Waterhouse 1999	Clinical cohort observational	n	-	n
Nakinishi 1995	Clinical cohort observational	n	-	n
Guthrie 1965	Clinical cohort observational	n	-	n
Prader 1949	Clinical cohort observational	n	-	n

1.5.1 Study design

Table 1.4 reveals that most studies in this area are observational in nature, investigating a specific clinical cohort. Primary research does play an important role in discovery and innovation; however, comparison of primary studies can be difficult if methodology, analysis and reporting are not standardised (Nagendrababu et al. 2020b). For contemporary research to feed into higher level evidence, the planning and implementation of each study must aim to consistently meet certain standardised criteria. Conforming to recognised reporting guidelines should ideally be universally adopted. Work is ongoing across various endodontic study designs to address these deficiencies (Nagendrababu et al. 2020b, Duncan et al. 2021, Pigg et al. 2021). Details of ethical approval and consent should routinely be registered and reported. The necessity to provide power calculations, should perhaps be reserved for studies that wish to demonstrate differences in outcome relating to specific interventions, or materials.

1.5.2 Inclusion/exclusion criteria

The level of detail revealed in the documentation of inclusion and exclusion criteria can assist the reader's interpretation of the quality of the study. Although regarded as common practice in contemporary literature, often relevant information can be overlooked. Three studies did not document inclusion, or exclusion criteria (**Table 1.5**) and perhaps unsurprisingly, these were the earliest published studies investigating pulpal blood as an analyte (Prader 1949, Guthrie et al. 1965, Nakinishi et al. 1995). Even from today's perspective, this finding should not necessarily be

regarded as a significant flaw, since the value of this research in terms of its historical context, cannot be overemphasised.

Four studies included a brief outline (**Table 1.5**) of inclusion criteria (Waterhouse et al. 1999, ElSalhy et al. 2013 and Aaminabadi et al. 2017, Mutluay et al. 2018). Of these, only three alluded to the patient's systemic health albeit in varying detail; from a simple statement of "non-contributory medical history" (ElSalhy et al. 2013, Mutluay et al. 2018), to a more detailed list of systemic conditions and medicaments that were noted to be absent (Aaminabadi et al. 2017). A variable amount of information was presented relating to the presence and degree of symptoms (which corresponded with the clinical categorisation of the study samples), as well as a brief mention of the absence of infection, or periapical pathologies.

Table 1.5 Details of the diagnostic tests recorded in the methodologies of studies analysing pulpal blood [Hx: Patient pain history recorded, Clinical: Standard clinical test findings recorded; Rad: Radiographic criteria defined; y: Yes; n: No, ns: Not stated, Consensus doc: Referenced consensus document; SO: Single operator study; incl/excl criteria: Inclusion & exclusion criteria recorded, n*: only exclusion listed, y*: only inclusion listed]

Paper	Hx	Clinical	Rad	Inc/Excl criteria	Consensus doc	Calibration examiners
Sharma 2021	y	y	y	y	AAE	ns
Nguyen 2020	ns	y	n	y	ns	ns
Shmueli 2020	y	n	y	y	ns	ns
Lejri 2019	n	y	n	n*	AAE	n
Mutluay 2018	y	No cold test	y	y*	ns	ns
Ghattas Ayoub 2017	n	y	y	y	AAE	ns
Aaminabadi 2017	y	No cold test	n	y*	AAPD	SO
Mente 2016	y	y	y	y	ns	y
ElSalhy 2013	y	y	y	y*	ns	ns
Waterhouse 1999	y	No cold test	ns	y*	ns	SO
Nakinishi 1995	y	y	ns	n	ns	ns
Guthrie 1965	y	y	ns	n	ns	ns
Prader 1949	n	n	n	n	n	n

Although Mutluay et al. (2018) presented a clear and detailed list of clinical, as well as radiographic inclusion criteria, they neglected to perform cold testing, a critical sensibility test necessary to determine the pulp status. Furthermore, the details of the number of cases that were ultimately excluded from this study was only presented in the flow chart demonstrating the designation of their study samples (Mutluay et al. 2018). The study groups were divided by those where haemostasis could be achieved at the exposure site, or at the canal orifice within a five-minute cut-off period. Eighty-four cases were recruited into the study; 29 cases were excluded based on the visual intra-operative identification of partial, or complete necrosis; a further 15 cases were excluded based on the inability to achieve haemostasis within the prescribed timeframe, resulting in a significant erosion of the study sample and potentially biased conclusions (Mutluay et al. 2018).

One study provided only minimal detail of the exclusion criteria (Lejri et al. 2019) (**Table 1.5**). Cases were excluded based on intraoperative discovery of necrosis, or an existing root filling and the inability to restore the tooth subsequently. Again, introducing bias into their findings. Critically, this paper is presented as a prospective study, but reads more like a retrospective case review.

Finally, five studies presented a varying level of detail in their inclusion and exclusion criteria (Mente et al. 2016, Ghattas Ayoub et al. 2017, Shmueli et al. 2020, Nguyen et al. 2020, Sharma et al. 2021). To improve the homogeneity of the data in this area and improve the applicability of the research findings, there is an urgent need to standardise the information collected and reduce the incidence of bias. Patient factors including cohort/recruitment, relevant systemic conditions, standardised age range, clinical/tooth factors symptoms, findings of special tests,

radiographic features, consent could be collated and presented in a standardised manner (Nagendrababu et al. 2020a), either as a check list, or in table format.

1.5.3 Diagnostic categorisation

When diagnostic controls are assessed in relation to pulpal blood analysis, the lack of standardisation becomes even more evident (**Table 1.6**). A single study (ElSalhy et al. 2013), presented what can be regarded as a “true” negative control, where health was specifically defined as a non-carious tooth, unaffected by periodontal disease, cracks, or restorations (**Table 1.6**). As an alternative, the proxy most often used to represent the ‘normal’ pulp, is an asymptomatic tooth, designated for root canal treatment for prosthodontic purposes (Guthrie et al. 1965, Nakinishi et al. 1995, Nguyen et al. 2020, Sharma et al. 2021). This does not account for the impact of the restorative burden (restoration, previous caries, accelerated ageing) leading up to the requirement for prosthodontic intervention, or its likely influence on the resultant inflammatory profile of the pulp.

Table 1.6 Diagnostic categories used in clinical studies analysing human pulpal blood [Ns: Not stated, "Normal" categorised as treatment indicated for prosthetic purposes; ~Caries exposed; ~ Deep caries in primary molars, Ibu: Ibuprofen, IP: Irreversible pulpitis, SIP: Symptomatic irreversible pulpitis, ?: Uncertain; ^: Smokers]

Paper	Healthy	'Normal'	Asymptomatic	Reversible	Irreversible	Other
Sharma 2021	0	10	0	0	40 SIP	0
Nguyen 2020	0	4	0	0	34 SIP	16/34 Ibu
Shmueli 2020	0	0	?	0	26 SIP	58 ~
Lejri 2019	0	0	73 IP	25	34 SIP	0
Mutluay 2018	0	0	0	0	0	44~
Ghattas Ayoub 2017	0	9	26	0	34	32^
Aaminabadi 2017	0	0	ns	ns	ns	211~
Mente 2016	0	0	8~	10~	26~	0
ElSalhy 2013	25	0	40~	0	43 SIP	0
Waterhouse 1999	0	0	0	0	0	39~
Nakinishi 1995	0	9	0	0	27	0
Guthrie 1965	0	14	0	0	0	53~
Prader 1949	Ns	Ns	Ns	Ns	Ns	21

There is further lack of standardisation between the various diagnostic categories when the spectrum of pulpitis from reversible to irreversible pulpitis is examined in more depth. Three studies (ElSalhy et al. 2013, Mente et al. 2016, Lejri et al. 2019) describe asymptomatic teeth, but do not provide enough clinical information regarding the potential level of pulpal insult, again highlighting the complexity of the presentation of pulpal inflammation. Two studies include asymptomatic, cariously-exposed teeth in an asymptomatic category (ElSalhy et al. 2013, Mente et al. 2016); while another describes asymptomatic irreversible pulpitis (Lejri et al. 2019); as distinct from reversible pulpitis and symptomatic irreversible pulpitis. Mente et al. (2016) categorised a range of teeth presenting with deep caries, which were diagnosed as asymptomatic, reversibly inflamed, or irreversibly inflamed, but only after pulp exposure occurred intra-operatively.

Most of the studies have focused on analysing pulpal blood in symptomatic irreversible pulpitis in permanent teeth (ElSalhy et al. 2013, Lejri et al. 2019, Nguyen et al. 2020, Shmueli et al. 2020, Sharma et al. 2021), with several studies introducing additional variables, including the use of systemic anti-inflammatories and primary molars and/or carious exposures. Furthermore, routinely accepted clinical descriptors, such as sensitivity to standard sensibility testing, or the presence of spontaneous, or provoked pain are often not reported (Prader 1949, Guthrie et al. 1965, Waterhouse et al. 1999, Aminabadi et al. 2017, Mutluay et al. 2018, Nguyen et al. 2020, Shmueli et al. 2020). Insufficient detail is reported by Prader (1949), relating to patient numbers, or their demographics, so it is not possible to determine if the cases were confined to the permanent dentition, or to which diagnostic category the cases were linked (**Tables 1.5, 1.6**). One study (Ghattas Ayoub et al. 2017) focused principally on the effect smoking had on the identifiable levels of selected mediators.

1.5.4 Methodological diagnostic detail

A lack of methodological detail in the available studies (**Tables 1.4, 1.5**), has generated data of low to moderate quality (**Tables 1.4, 1.7**), with the potential for a high risk of bias (Whiting et al. 2011, Herzog 2013). The quality assessment was conducted using a modified Newcastle-Ottawa-Scale (Gomes et al. 2013, Rechenberg et al. 2016a), is presented in **Table 1.7**. The three domains that were assessed were Selection, Comparability and Outcome. The decision rules are defined in **Appendix I** and scores were modified to range from 0 – 10, to conform with the literature being assessed. If the criterion was met (**Appendix I**), a score of one was awarded and zero,

if not. Selection criteria assessed details of the recruitment process, standardisation of the cohort, as well as details relating to inclusion and exclusion criteria and the population demographics. The maximum score that could be obtained in this domain was 2. In relation to the domain of comparability details relating to clinical and radiological assessment of both cases and controls was assessed. Gold standard, histologic assessment; or reference standard (direct inspection) relating to pulp inflammation was accepted as valid. The incorporation of a true negative control was considered essential. The maximum score in this domain was 4. The outcome domain assessed the level of detail provided to adequately define outcome measures, detailed information relating to methodology, identical tests performed on controls as test cases and an ability to compare with other published research. Scores rating from 0-4 were defined as low quality with a high risk of bias (Herzog et al. 2013). Scores of 5-6 were deemed moderate quality with a moderate risk of bias, whereas scores 7-8 were regarded as good with low risk of bias. Only one study scored as good, reaching an overall score of 7 (Mente et al. 2016). Two studies reached a score of 6 (Nguyen et al. 2020, Sharma et al. 2021), with a further three studies also qualifying as moderate quality with a moderate risk of bias (ElSalhy et al. 2013, Aminabadi et al. 2017, Mutluay et al. 2018). The remaining 7 studies were all deemed to be of low quality with high risk of bias, ranging in scores from 3-1.

Table 1.7 Assessment of the study quality using a Modified Newcastle-Ottawa-Scale [Dx: Diagnosis; tx: treatment]

Reference	Selection		Comparability				Outcome				Total quality score
	1. Selection of the cohort	2. Condition of the cohort	3. Dx of cases	4. Pulpal inflammation confirmation histologically	5. Quality of the controls	6. Calibration of examiners	7. Assessment of outcome	8. Same tx performed for cases & controls	9. Standardised method	10. Intra-study comparability	
Sharma 2021	1	1	1	1	0	0	1	0	1	0	6
Nguyen 2020	1	1	1	1	0	0	0	1	1	0	6
Shmueli 2020	1	0	1	0	0	0	0	0	0	0	2
Lejri 2019	0	0	0	1	0	0	0	0	0	0	1
Mutluay 2018	1	1	0	1	0	0	0	1	1	0	5
Aaminabadi 2017	1	1	0	1	0	0	0	1	1	0	5
Ghattas Ayoub 2017	0	0	1	0	0	0	0	1	1	0	3
Mente 2016	1	1	1	1	0	1	0	1	1	0	7
ElSalhy 2013	0	1	0	0	1	0	0	1	1	0	5
Waterhouse 1999	1	0	0	0	0	0	0	0	1	0	3
Nakanishi 1995	0	0	0	0	0	0	0	1	1	0	3
Guthrie 1965	0	0	0	1	0	0	0	1	1	0	3
Prader 1949	0	0	0	1	0	0	0	1	0	0	2

Most, but not all studies listed in **Table 1.5**, describe the patient history, with pain being the most common presentation. Symptomatic presentation however, represents a subcategory within a pulpitis population (Rechenberg et al. 2016b), as the condition is also known to progress in the absence of symptoms (Hasler & Mitchell 1970) and the mechanisms resulting in pain in some cases, but not others are poorly understood (Sessle 2011).

Radiographic examination findings have been consistently included in study methodology during the last two decades, but the associated radiographic criteria have been reported on with a variable level of detail. An itemised checklist of positive and negative radiographic findings and the consistent use of recognised indices, similar to those related to the appearance of apical periodontitis proposed by Brynolf 1967, would improve standardisation in reporting of studies using radiographs (Ørstavik et al. 1986). In relation to improving pulpal diagnostic research, universal adoption of recognised radiographic features and including measures of caries depth would also be beneficial moving forward (Bjørndal et al. 2019, Pigg et al. 2021).

The weakest aspect in this field, however, is the consistent absence of sufficient detail describing recognised diagnostic clinical findings; specifically, sensibility testing and its interpretation. Pulpal biomarker studies demonstrate broad heterogeneity both in the diagnostic parameters reported (**Table 1.6**) and the analytical methods used (**Tables 1.8 & 1.9**). Clinical diagnostic accuracy studies have relied upon histologic findings as the recognised “gold standard” in terms of validation, in the classic studies (Garfunkel et al. 1973, Anderson et al. 1981) and continue to be heavily cited in contemporary literature (Ricucci et al. 2021); however, in histological studies treatment outcome cannot be assessed as the tooth is

extracted for histological processing. The reference standard most often utilised in contemporary diagnostic accuracy research includes the direct visualisation of the exposed pulp and whether bleeding is identified (Wieslander et al. 2009, Villa-Chavez et al. 2013, Jespersen et al. 2014). However, the visualisation of bleeding pulp tissue may be sufficient for diagnostic accuracy studies involving sensibility testing, but does the mere presence of vital tissue represent a sufficient reference standard for biomarker studies analysing the intensity and location of pulpitis in the tooth. Furthermore, although the European Society of Endodontology position statement on the management of deep caries and the exposed pulp (Duncan et al. 2019a), recommended the use of an enhanced protocol using magnification and illumination, only one study in this review included these important factors (Mente et al. 2016). Indeed, the routine use of microscopes with their associated superior magnification and light source, are rarely included in the methodologies of VPT studies (Duncan 2022).

As few as four of the studies listed in **Tables 1.4** and **1.5** reference a diagnostic consensus, or guideline document detailing the criteria used (Sharma et al. 2021, Lejri et al. 2019, Aminabadi et al. 2017, Ghattas Ayoub et al. 2017); and of these, only two followed a recognised reporting protocol (Sharma et al. 2021, Aminabadi et al. 2017). Only one study documented the calibration of their examiners (Mente et al. 2016), three were conducted by single operators (Waterhouse et al. 1999, Aminabadi et al. 2017 and Sharma 2021); and in the remaining 9 studies, this detail was not included.

1.5.5 Collection methods and substrate handling

Table 1.8 outlines the various collection vehicles, substrates, dilutions and storage details reported in studies focused on the analysis of pulpal blood. No two studies are the same in terms of blood collection methodology. Guthrie et al. (1965), introduced the dental pulp haemogram to the English language literature, while before this Prader (1949) used Giemsa stain, originally designed to differentiate parasites in malaria diagnosis. Guthrie et al. (1965) used a more appropriate stain (Wright's), which was specifically designed to differentiate blood cell types. Both studies (Prader 1949, Guthrie et al. 1965) collected a single drop of pulpal blood directly, using a college tweezers and both investigators identified a consistent link between increased neutrophil count and more severe inflammatory degeneration.

Various absorbent materials have been used for pulpal blood collection including nylon fiber (Nakinishi et al. 1995), collagen membrane Periocol® (Waterhouse et al. 1999), paper points (Ghattas Ayoub et al. 2017) and cotton wool pellets (ElSalhy et al. 2013, Mutluay et al. 2018, Nguyen et al. 2020). Nakinishi et al. (1995) were the first group to analyse the effect of different absorbent materials on the recovery of specific analytes. They demonstrated that the nylon fiber had the best analyte recovery, followed by cotton wool pellets. Waterhouse et al. (1999) evaluated the extraction efficiency of the desired analyte, concluding that measurements required additional adjustment using an extraction efficiency formula to determine the correct concentrations. No subsequent studies have reported specifically on these aspects, rather favouring a post-hoc application of a total protein concentration to normalise any potential matrix effect exerted by the blood itself and standardise the template for comparison (ElSalhy et al. 2013, Nguyen et al.

2020). The use of a protein assay to normalise levels may not be well suited to pulpal blood research as the volume of available blood is unpredictable and very small.

Table 1.8 Collection data and substrate handling in the analysis of pulpal blood [Vol: Volume, s: sec; N/A: Not applicable; WB: Whole blood; Hep: Heparinised; PBS: phosphate buffered saline; RT: Room temperature; min: minutes; ns: Not stated; rpm: Revolutions per minute; g: gravitational constant; x: multiples; °C: Degrees Celsius; -: minus]

Paper	Vol (μL)	Collection	Substrate	Diluted	Centrifuge	Store
Sharma 2021	100	Micropipette	Serum	No	183.9 g 15 min RT 81.7 g 15 min RT	-80°C
Nguyen 2020	N/A	2 x Cotton pellet 60 s	WB	1000 μL Saline (Hep)	12,000 g 10 min 4°C	-20°C
Shmueli 2020	100	100 μL syringe	WB	No	N/A	N/A
Lejri 2019	N/A	N/A	N/A	N/A	N/A	N/A
Mutluay 2018	N/A	2 site Cotton pellet 45 s	WB	80 μL PBS	1,500 g 10 min 4°C	-80°C
Ghattas Ayoub 2017	N/A	4 x paper point 30 s	WB	400 μL PBS	10,000 g 15 min 4°C	-80°C
Aaminabadi 2017	ns	Microcapillary (Hep)	WB	N/A	N/A	N/A
Mente 2016	<10	Microcapillary (Hep)	Plasma	50 μL Saline	1000 rpm 1 min RT 1000 rpm 15 min RT	-70°C
ElSalhy 2013	N/A	1 x Cotton pellet 45-60 s	WB	1000 μL saline (Hep)	12,000 g 10 min 4°C	-20°C
Waterhouse 1999	N/A	PerioCol™	WB	N/A	ns	-80°C
Nakinishi 1995	N/A	Nylon Fiber	Serum	x100 PBS	1,700 g 5 min RT	-20°C
Guthrie 1965	N/A	College Tweezers	WB	N/A	N/A	N/A
Prader 1949	N/A	College Tweezers	WB	N/A	N/A	N/A

Table 1.9 Experimental analysis of pulpal blood [RBC: Red blood cells; WCC: White cell count; Ns: Not stated; N/A: Not applicable; SSD: Statistically significant difference; bw: between; NP: Normal pulp, SIP: Symptomatic irreversible pulpitis; wrt: with respect to; sig: significant; incr: increase; VPT: vital pulp treatment; asymp: asymptomatic; 1^o: primary; Ibu: ibuprofen; PE: pulpectomy; HA: haemostasis achieved]

Paper	Analyte	Assay	Sensitivity	Result
Sharma 2021	aMMP-9	Fluorometric R&D	0.005 ng/mL	SSD bw aMMP-9 NP v's SIP
Nguyen 2020	PGE ₂ , IL-1 β , IL-6, TNF- α , IFN- γ	ELISAs Life Technologies Corp	13.4, 0.05, 0.03, 0.13, 0.06 pg/mL	SSD less PGE ₂ , IL6, TNF α , IFN γ in Ibu group
Shmueli 2020	pH, pO ₂ , PCO ₂	Neonate Astrup	N/A	PE group sig higher PCO ₂ & pH
Lejri 2019	-	-	-	-
Mutluay 2018	IL-1 β , IL-2, IL-6, IL-8, IL-10, TNF, PGE ₂	ELISAs Invitrogen DIA Source ImmunoAssays	Ns	IL6 higher in group where HA <5 min
Ghattas Ayoub 2017	IL-1 β , TNF- α , hBD-2, hBD-3	Luminex Novex; Life technologies, ELISA R&D	3-10 pg/ml	Smokers sig lower TNF- α , hBD-2 than non-smokers
Aaminabadi 2017	Leukocytes, RBC, Eosinophils, Basophils, monocytes, lymphocytes	Haemogram	N/A	Ns diff bw groups WBC. Neutrophils sig higher PE
Mente 2016	MMP-9, TIMP-1	Fluorometric, ELISA R&D	<0.156 ng/mL, <0.08 ng/mL	SSD bw MMP-9 in asymp caries exposed vs SIP w NSAIDs
ElSalhy 2013	IL-2, IL-6, IL-8, IL-10, TNF- α , IFN- γ	ELISAs Bender Medsystems	0.4, 0.03, 0.05, 0.13, 0.06 pg/ml	Ratios IL6:IL10, IL8:IL10 sig in SIP
Waterhouse 1999	PGE ₂	ELISA Correlate-EIA	Ns	PGE ₂ detected & quantified in inflamed 1 ^o molars
Nakinishi 1995	IgA, IgG, IgM, Elastase, PGE ₂ , IL-1 α , IL-1 β , IL-6, TNF- α	ELISA, Cappel, Cayman Chemical Co., Gentzyme Research Products	Ns	SSD bw NP SIP wrt IgA, IgG, IgM, Elastase, PGE ₂
Guthrie 1965	Differential WCC	Haemogram	N/A	Elevated neutrophils not good for VPT
Prader 1949	Leukocytes, RBC, Eosinophils, Basophils, monocytes, lymphocytes, plasma cells	Haemogram	N/A	SSD incr Neutrophils & monocytes in Phase 3

Nguyen et al. (2020) followed the methodology described by ElSalhy et al. (2013), with some notable exceptions. They used two cotton wool pellets, whereas ElSalhy et al. (2013) used only one and their collection times varied. Both studies collected the pulpal blood in heparin coated tubes, whereas Mutluay et al. (2018), did not. The level of dilution (1000 μ L) applied to the collected samples, storage (-20°C) and centrifuge parameters (12,000g 10 min 4°C) was identical by ElSalhy et al. (2013) and Nguyen et al. (2020). In contrast, Mutluay et al. (2018) collected blood from two different sites (at initial exposure and from the canal orifices) with a single cotton wool pellet for each site, over for a shorter period of time. The collected samples were then diluted with only 80 μ L phosphate buffered saline in untreated collection vessels, stored at -80°C and centrifuged at a significantly lower gravitational constant (1,500g). What the three studies did have in common, was the substrate analysed was a diluted, centrifuged whole blood sample after removal of the cotton wool pellet. This differs from Nakinishi et al. (1995) who analysed the supernatant remaining after centrifuge, designated as serum. Again, sample storage, level of dilution and centrifuge parameters differ significantly.

Ghattas Ayoub et al. (2017) collected pulpal blood using four 25/04 taper root canal treatment paper points, in contact with the exposed pulp for 30 second intervals. It is not clear from their methodology if each paper point was stored in a single Eppendorf tube with the designated diluent (400 μ L of phosphate buffered saline [PBS]) for subsequent assay of the selected mediators.

One major drawback of the absorbent collection methods relates to the standardisation of the samples. The collected volume cannot be accurately measured or standardised, thereby introducing a matrix effect into the analysis (Chiu et al.

2010, Dupin et al. 2016) and relies on a subsequent total protein assay for standardisation and comparative analysis.

Finally, the remaining four (**Table 1.8**) studies (Mente et al. 2016, Aaminabadi et al. 2017, Sharma et al. 2020 and Shmueli et al. 2020) used aspiration methods to collect the pulpal blood. Mente et al. (2016) was the first group to use heparinised microcapillary tubes. Although an attempt was made to standardise the volume collected, it was not always possible to obtain the full volume of the microcapillary tube (10 μ L). Further corrective calculations were carried out using Excel software to standardise these findings; however, no further detail was described in their methodology. The designated substrate collected in this study was plasma, due to the pre-collection anticoagulant treatment of the microcapillary tubes, the only study to present data using plasma in the dental literature. Although the volume of diluent added was standardised at 50 μ L saline, the volume of whole blood collected was not reported to be 10 μ L in every sample, thereby introducing a variable diluent effect into the standardised volumetric method. Mente et al. (2016) used a double centrifuge method; centrifuging once before storage at -70°C, followed by a second, after thawing and removal of the supernatant. The samples were initially stored as centrifuged whole blood, followed by analysis of plasma upon thawing and repeat centrifuge. What effect, if any, this variation had on the detection of the analytes is unknown.

Heparinised microcapillary tubes were also used by Aaminabadi et al. (2017). No details were published of the volume capacity of the microcapillary tubes used. From the published images (Aaminabadi et al. 2017), it is evident that incomplete filling of the microcapillary tubes also occurred in this study. Their analysis focused

on digital blood colour assessment, haematologic and histologic findings. Although this study was one of the few studies to cite a diagnostic reference standard (AAPD) and a reporting guideline document (STROBE), they failed to align their findings to those clinical diagnostic standards; rather, the categories were divided by treatment (pulpectomy, accidental exposure and pulpotomy, carious-exposure and pulpotomy), or histologic findings (mild, moderate, or severe inflammation). They also elected not put the haematologic results in context with the only other literature based on the dental pulp haemogram (Prader 1949 and Guthrie et al. 1965).

Despite catalogued difficulties in collecting consistent volumes of pulpal blood, two research groups claim to have acquired 100 µL samples using sterile syringes, pre-treated with heparin in a paediatric cohort (Shmueli et al. 2020) and a micropipette (**Table 1.8**) in the posterior adult dentition (Sharma et al. 2020). The detail provided in these studies relating to this aspect is limited and no clinical images were included. Information was not provided regarding the syringe manufacturer, model, or needle gauge (Shmueli et al. 2020). Difficulties could reasonably be anticipated in relation to access, inadvertent introduction of air bubbles, clotting in the untreated micropipettes, as well as validity of obtaining the entire, expected volume on a predictable basis.

Table 1.9 shows very clearly the heterogeneity that exists in this field. None of the 13 listed studies have investigated the same analytes in the same experimental methodology. Manufacturer, kit sensitivity, study categorisations all varied, resulting in a paucity of evidence in this area. The specific discrepancies relating to each of the listed analytes will be explored in more detail throughout **Sections 1.6.3-1.7.2**.

1.6 Pulpal Inflammation

The pulp is equipped with native and recruited defence cells and inflammatory mediators that orchestrate the host response to microbial infection (Jontell et al. 1987, Hahn & Falkler 1992). Antigenic challenge triggers an intricate and complex cascade of immunological, inflammatory, cellular, vascular as well as neural events (Bergenholtz et al. 1977, Hahn & Liewehr 2007a, Hahn & Liewehr 2007b). Pathogen sensing is facilitated by the expression of pattern recognition receptors (PRRs) in the form of Toll-Like Receptors (TLR's). TLR-2 and TLR-4 have been most extensively studied of the PRRs, as they sense gram-positive bacterial cell wall components such as lipoteichoic acid (LTA) as well as lipopolysaccharide (LPS) associated with gram negative bacteria (Takeda & Akira 2005, Botero et al. 2006, Veerayutthwilai et al. 2007). Neutrophils are activated once the cell surface TLR's have ligated, leading to upregulation of their antimicrobial functions (Nicu & Loos 2016); which include phagocytosis and the production of cytokines and chemokines local to the site of injury. Histological analysis describes the cellular mechanisms and events occurring through progressive microbial insult. The transition from innate to adaptive immune response indicative of irreversible pulpitis, is particularly evident when the remaining dentine thickness is less than 2 mm (Izumi et al. 1995, McLachlan et al. 2004). Hasler & Mitchell (1970) demonstrated histologically the proportional increase in pulp inflammatory effects relative to diminishing dentine thickness over the pulp.

Increased expression and upregulation of chemical mediators also feature in acute inflammation and offer diagnostic and prognostic opportunities. Cytokines, produced by immune cells, regulate systemic and local inflammation through positive and negative feedback loops, via their designated pro- and anti-

inflammatory functions (Kawashima & Suda 2008). Together, cytokines and chemokines stimulate and perpetuate the immune/inflammatory response in triggering adaptive immune responses (Tanaka et al. 2014) and stimulation of leukocyte migration (Baggiolini et al. 1997). Consequently, other chemical mediators including arachidonic acid metabolites, nitric oxide, kinins and defensins trigger further inflammatory functions which regulate vascular permeability and pulpal homeostasis. Vasodilation of blood vessels and increased capillary permeability and precipitate oedema. Within the confines of the low compliance environment of the root canal system, vasodilation is succeeded by a secondary vasoconstriction which ultimately leads to necrosis (Kim & Dörscher-Kim 1989). Neurogenic inflammation, stimulated via activation of peripheral neurons will also lead to the release of neuropeptides such as Calcitonin Gene-Related Peptide (CGRP) and Substance P (SP), which further modulate the inflammatory response (Lundy & Linden 2004). Observed effects include an increase in blood flow, plasma extravasation and leukocyte accumulation, all of which function to promote tissue survival by facilitating the removal of the noxious stimulus. Increased levels of select neuropeptides have been linked with painful pulpitis (Byers et al. 1990, El Karim et al. 2006).

The adaptive immune response, predominantly comprising antigen-specific lymphocytes (T and B-cells); regarded as a learned, or targeted response following repeated exposure to the same antigen; functions to facilitate the defensive mechanisms in conjunction with the innate immune system. Cytokines, which form part of the innate immune response, are also produced by cellular components of the adaptive immune response. For example, IL-6 is produced locally by monocytes, macrophages, dendritic cells and fibroblasts. However, exclusive to adaptive

immunity, IL-6 is also produced by activated T-cells, which then lead to B-cell proliferation into plasma cells (Hahn & Liewehr 2007c) and is therefore referred to as a type-2 cytokine (**Appendix XV**). The proportion of innate, or adaptive response is determined by the severity of the infectious agent, as well as the host response and will show temporal expression in inflamed pulp tissue.

1.6.1 The need for next-generation diagnostic tests for pulpitis

A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001, Strimbu & Tavel 2010). The principle that blood is a relevant substrate to assist pulpal diagnosis, is that pulp exposure during operative intervention and subsequent treatment affords direct access to the pulp tissue and blood. Blood is rich in cellular components and as a result, will secrete several biomarkers including chemokines, cytokines, proteases, cell death markers (**Section 1.8**); the subsequent use of these biomarkers for diagnosis, or treatment has not been exploited to its full potential in the endodontic research to date. Blood has been effectively used elsewhere in medicine as a liquid biopsy for diagnostic evaluation (Swaminathan & Butt 2006, Kutcher et al. 2012), relating to treatment (Barnado et al. 2016) and outcome (Margraf et al. 2008, Hayase et al. 2018). The development of a valid objective molecular testing method within pulp diagnostics is of critical importance to potentially overcome many of the deficiencies in the current available methods used (Swedish Council on Health Technology Assessment 2010).

Next-generation diagnostic tests are required, as currently available techniques remain limited in their ability to reliably discriminate the threshold between reversible and irreversible pulpitis (Bjorndal et al. 2019). As a result, decisions regarding the possible retention of the pulp are based on crude techniques, rather than a rigorous biochemical assessment of pulpal biomarkers, using technology regarded as commonplace in medical disciplines. To date, the focus on molecular diagnostics in pulp biology has revealed significant methodological variations (**Sections 1.5.1-1.5.5**), such that no clear biomarker has been identified (**Table 1.10**).

Table 1.10 highlights the variability in methodology, substrates and vehicles utilised in this area, as well as the units of measurement by which the analytes under investigation are presented. Pulp tissue supernatant has been the most consistently examined substrate and enzyme-linked immunosorbent assay (ELISA) the most frequently method used. However, variation in the manufacturer of the ELISA as well as the sensitivity of the kits, does not allow for easy comparison between studies, even when the same target molecule is investigated. The quality of evidence in the research to date in this area is considered to be of a consistently low level (Rechenberg et al. 2016a, Hirsch et al. 2017). Although statistically significant differences have been demonstrated between ‘normal’ and inflamed pulps in certain selected studies, these findings are not well supported when traditionally ‘reversibly inflamed’ cases have been compared with more severely ‘irreversibly inflamed’ cases. This is problematic with regard to development of a potentially discriminatory test, which might provide for indirect, as opposed to direct pulp therapy. Molecularly speaking, could an asymptomatic cariously-exposed pulp exhibit vastly different

inflammatory markers compared with symptomatic irreversible pulpitis? The case selection and diagnostic criteria applied in many of the studies listed fail to provide adequate supporting evidence to date.

Table 1.10 (see over) Detection of chemical mediators in human pulpitis (selected studies) [ELISA: Enzyme-linked immunosorbent assay; RIA: Radioimmunoassay; PTS: Pulp tissue supernatant; PT: Pulp tissue; PB(Se): Pulpal blood supernatant (Serum); PB: Pulpal blood; MPLX: Multiplex assay; PrA: Protein assay; ppt: paper point; PP: pulpotomy; NS: not significant; SSD: statistically significant difference; Vol: volumetric method; N/A: not applicable, cw: cotton wool, ToPr: Total protein, Vol: volumetric, N: Normal, IP: Inflamed Pulp]

Target	Author	Method	Substrate	Collection	Mode	SSD	N:IP
PGE ₂	Cohen et al. 1985	RIA	PTS*	EXT	ToPr	y	5:140pg/mg
	Nakinishi et al. 1995	ELISA	PB	RCT	Vol	y	2:2-1120ng/ml
	Waterhouse et al. 1999	EIA	PB	RCT	Vol	N/A	N/A:1-2641ng/ml
	Waterhouse et al. 2002	EIA	PB	RCT	Vol	N/A	N/A:1-2641ng/ml
	Isett et al. 2003	ELISA	PT	EXT	Vol	y	286:3493pg/ml
	Petrini et al. 2012	RIA	PTS	EXT/RCT	ToPr	n	14:13pg/mg
IL-1 α	Nakinishi et al. 1995	ELISA	PB	Nylon	Vol	n	1:1-6ng/ml
	Zehnder et al. 2003	RT-PCR	PTS	EXT/RCT	mRNA	n	1:50% of β -actin
	Abd-Elmeguid et al. 2013	MPLX/IHC	PTS	EXT/RCT	ToPr	y	6:31pg/mg
	Ozdemir et al. 2015	ELISA	PTS	PP	ToPr	N/A	N/A:0.1pg/mg
IL-1 β	Nakinishi et al. 1995	ELISA	PB	Nylon	Vol	n	1:1-53ng/ml
	Zehnder et al. 2003	RT-PCR	PTS	EXT/RCT	mRNA	n	1:45% of β -actin
	Paris et al. 2009	RT-PCR	PTS	EXT/RCT	mRNA	y	1100:13000
	Silva et al. 2009	ELISA/IHC	PTS	EXT/RCT	ToPr	y	4:28pg/ml
	Abd-Elmeguid et al. 2013	MPLX/IHC	PTS	EXT/RCT	ToPr	y	2:12pg/mg
	Ghattas et al. 2017	Luminex	PB	ppt	ToPr	n	0.007:0.041pg/ μ g
IL-2	Rauschenberger et al. 1997	ELISA/H&E	PTS	EXT	ToPr	y	541:1945u/mg
	Anderson et al. 2002	ELISA	PTS	EXT	Vol	n	11:9pg/ μ l
	ElSalhy et al. 2013	ELISA	PB	cw	ToPr	n	0.05:0.06-12pg/ml
IL-6	Nakinishi et al. 1995	ELISA	PB	Nylon	Vol	-	0:low
	Barkhodar et al. 1999	ELISA	PTS	ns	ToPr	y	0.01:36pg/mg
	Zehnder et al. 2003	RT-PCR	PTS	EXT/RCT	Vol	y	0.2:60% of β -actin
	Abd-Elmeguid et al. 2013	MPLX/IHC	PTS	EXT/RCT	ToPr	y	0:8pg/mg
	ElSalhy et al. 2013	ELISA	PB	cw	ToPr	y	0:0.09-2.1pg/ml
	Ozdemir et al. 2015	ELISA	PTS	PP	ToPr	N/A	N/A:0.2pg/mg
IL-8	Huang et al. 1999	ELISA/IHC	PTS	EXT/RCT	ToPr	y	0.08:1.82pg/ml/ μ g
	Isett et al. 2003	ELISA	PT	EXT	Vol	y	120:977pg/ml
	Zehnder et al. 2003	RT-PCR	PTS	EXT/RCT	Vol	y	0.2:100% of β -actin
	Karapanou et al. 2008	ELISA	GCF	Periopaper	Vol	y	174pg/ml:302pg/ml
	Silva et al. 2009	ELISA/IHC	PTS	EXT/RCT	ToPr	y	2:46pg/ml
	Abd-Elmeguid et al. 2013	MPLX/IHC	PTS	EXT/RCT	ToPr	y	0.2:371pg/mg
	ElSalhy et al. 2013	ELISA	PB	cw	ToPr	y	0:4.3-594pg/ml
	Ozdemir et al. 2015	ELISA	PTS	PP	ToPr	N/A	N/A:1.2pg/mg
TNF- α	Nakinishi et al. 1995	ELISA	PB	Nylon	Vol	n	37:14-142ng/ml
	Pezelj-Ribaric et al. 2002	ELISA	PTS	EXT	ToPr	y	64:1962pg/g
	Karapanou et al. 2008	ELISA	GCF	Periopaper	Vol	-	-
	Paris et al. 2009	RT-PCT	PTS	EXT/RCT	mRNA	y	800:8000
	Abd-Elmeguid et al. 2013	MPLX/IHC	PTS	EXT/RCT	ToPr	y	0:3pg/mg
	ElSalhy et al. 2013	ELISA	PB	cw	ToPr	y	0.2:0.07-8.8pg/ml
	Ghattas et al. 2017	Luminex	PB	ppt	ToPr	n	0.002:0.002pg/ μ g
IFN- γ	ElSalhy et al. 2013	ELISA	PB	cw	ToPr	y	0.001:0.01-0.pg/ml

1.6.2 Identifying molecular targets of pulpitis

The first step towards employing molecular biomarkers for pulpal diagnosis is to identify key mediators (inflammatory, or other) and to increase our understanding of their role in both inflammation and regeneration (Cooper et al. 2011). Unfortunately, due to the complex modulation of pulpitis, it is perhaps unlikely that a single mediator will provide sufficient information to predictably and accurately diagnose irreversible pulpal disease. To date, 95 biomolecules have been analysed in the inflamed human pulp, in 850 separate studies (Rechenberg et al. 2016a). Notably, most of these studies have applied a candidate approach, in which the authors have pre-selected the biomolecule to be studied rather than analyzing via a high-throughput method the differential expression of a wide range of inflammatory, regenerative or other markers. Cytokines account for more than half the mediators studied, followed by proteases, enzymes, other inflammatory mediators (i.e. prostaglandins, Nitric oxide, neuropeptides) and growth factors (Rechenberg et al. 2016a, Hirsch et al. 2017, Zainini et al. 2017). Although statistically significant differences were detected between inflammation and health for two thirds of the biomarkers studied (Rechenberg et al. 2016a), no definitive candidate molecule has been identified. Data is clearly lacking in relation to optimizing the research methodologies to facilitate reference values between studies investigating similar analytes. Critically, as a result, it has not been possible, as yet, to identify a threshold that reflects the limits of reversible pulp tissue disease.

1.6.3 Experimental analysis and findings

As previously outlined (**Section 1.4.2, Table 1.9**), the very earliest published studies investigating pulpal blood were based on the visual appearance and cell counts of collected blood smears (Prader 1949, Guthrie et al. 1965). Although different staining methods were used; the findings were broadly in agreement, that the neutrophil count was elevated with increased clinically apparent inflammation (Prader 1949, Guthrie et al. 1965, Aminabadi et al. 2017).

The first group to use immunoassays for analysing pulpal blood was Nakinishi et al. (1995). The assay individually investigated IgA, IgG, IgM, and Elastase, the only group to analyse this particular group of immunoglobulins and a serine protease, in pulpal blood. Statistically significant differences were demonstrated between the normal and irreversibly inflamed pulp. From a total of nineteen inflamed cases, sixteen samples demonstrated detectable amounts of Elastase. The recovery of the other analytes became less consistent in the cytokine category; sometimes only recovered in half of the selected samples: PGE₂, IL-1 α , IL-1 β , IL-6, TNF- α . IL-6 was the least detectable of the analytes investigated, with only 2/18 inflamed samples registering a reading and only in very small quantities. It was not possible to detect IL-6 in normal samples.

1.7 Markers of inflammation in human pulp

1.7.1 Cytokines

Cytokines are intercellular mediators derived from numerous cell populations which function in the regulation of a range of cellular processes including immune cell networks, cell growth, tissue repair and inflammation (Degré 1996, Koj 1996,

Martinho et al. 2021). Some are constitutively present, whilst others are synthesised (Kupper et al. 1998). They are integral in the regulation of the duration and intensity of the inflammatory response (Menéndez et al. 2012). Their production is often by a cascade mechanism, which provides an effective signalling network (Singh et al. 2019). They may function synergistically, or antagonistically dependent on the input of various stimuli (Kushner 1993, Gabay & Kushner 1999). Cytokines have been extensively investigated as potential diagnostic biomarkers in pulpal research, due to their significance in the pathogenesis of infection and pronounced expression during inflammatory processes (Stashenko 1990, Rechenberg et al. 2016a, Hirsch et al. 2017).

Table 1.11 highlights that in almost all the studies presented, cytokines were detected in the normal pulp samples. Of the studies that failed to demonstrate the presence of cytokines in normal samples, questions could potentially be raised regarding the sensitivity of the assay employed as the cytokine may be expressed at least for some cytokines below the threshold of the assay (Nakinishi et al. 1995, Abdelmeguid et al. 2013, ElSalhy et al. 2013). Multiplex assays allow measurement of numerous mediators simultaneously, which is particularly desirable when only small volumes of substrate are available. However, the absence of results using this technology should be interpreted with caution as the sensitivities of the assays may be insufficient to detect certain mediators.

Focusing on the range of values detected from the various mediators investigated in irreversible inflammation (**Table 1.12**), the results demonstrate variations by several orders of magnitude between individual studies (perhaps due to dilution), which fail to offer a clear depiction of the cut-off between the normal

and acutely inflamed pulp. An observation is that samples normalised using the total protein do appear to demonstrate distinctly lower levels than those recorded using a volumetric method.

1.7.1.1 IL-1

IL-1 is a key mediator in immune and inflammatory networks (Dinarello 1996). Although IL-1 α was detectable in pulpal blood and pulp tissue (**Table 1.10**) using ELISA (Nakinishi et al. 1995, Ozdemir et al. 2015), a statistically significant difference was not demonstrated between normal and inflamed pulp (Nakinishi et al. 1995, Ozdemir et al. 2015). It is not clear which of the study parameters if any may have influenced these findings. The studies differed methodologically by volumetric and total protein methods, substrate (pulpal blood and pulp tissue supernatant) and collection vehicle (nylon fiber and spoon excavator), as well as employing alternative ELISA manufacturers with differing sensitivities. As previously highlighted these types of variances are seen throughout the results within this field (**Table 1.10**), and in the case of IL-1 further investigations have not been pursued; rather, a novel methodology, or an alternate mediator has been investigated.

Table 1.11 Reported levels of inflammatory biomarkers in uninflamed categories [*Some samples did not detect; Range, Mean ~]

Author Mediator	Nakinishi (ng/ml)	ElSalhy (pg/mg)	Ghattas Ayoub (pg/μg)	Nguyen (pg/ml)	Sharma (ng/ml)
IgA	0.06-0.3 mg/ml				
IgG	0.64-3 mg/ml				
IgM	0-0.11 mg/ml*				
Elastase	0-43.76 μg/ml				
PGE ₂	1.88,2.14			250-550	
IL-1α	1.3#				
IL-1β	0.9,1.9		0.0065~	0.2-0.4	
IL-2		0.005–1.37			
IL-6	0	0		0.25-0.45	
IL-8		0			
IL-10		0.048–0.61			
TNF-α	35.6	0.038–0.21	0.0021~	17.5-21	
IFN-γ		0.001–0.02		0-0.175	
hBD-2			0.2817~		
hBD-3			3.9720~		
aMMP-9					12-96*

Table 1.12 Reported levels of selected inflammatory biomarkers in inflamed categories [Range, Mean ~]

Author Mediator	Nakinishi (ng/ml)	Waterhouse (ng/ml)	ElSalhy (pg/mg)	Mente (ng/ml)	Mutluay (pg/ml)	Ghattas Ayoub (pg/μg)	Nguyen (pg/ml)	Sharma (ng/ml)
IgA	0.15-0.51 mg/ml							
IgG	1.71-5.91 mg/ml							
IgM	0.01-0.24 mg/ml							
Elastase	0-344.8 μg/ml							
PGE ₂	1.7-1120*	1-2641			0.03-4.76		410-590	
IL-1α	0.65-5.5							
IL-1β	0.6-52.8				0.03-1.7	0.0414~	0.2-3.4	
IL-2			0.06-18.9		0.05-0.56			
IL-6	Extremely low		0.02-2.09		0.03-1.16		0.65-1.75	
IL-8			2.3-593.6		0.2-2.8			
IL-10			0.16-17.92		0.05-4.19			
TNF-α	14.3-142.2		0.07-24.45		0.1-2.22	0.0021~	17.8-22.5	
IFN-γ			0.007-0.699		0.007- 0.7		0.02-0.2	
hBD-2						0.3954~		
hBD-3						3.060~		
aMMP-9								25.8–608
MMP-9				267-7991				
TIMP-1				11-2006				

1.7.1.1a IL-1 α

In relation to IL-1 α , Zehnder et al. (2003), investigated mRNA expression in healthy compared with inflamed human pulps by polymerase chain reaction (PCR). A statistically significant difference was not detected for IL-1 α . In 2013, Abd-Elmeguid and colleagues analysed a number of inflammatory cytokines including IL-1 α using a multiplex protein assay and immunohistochemistry. They established statistically significant differences between normal and inflamed human pulp for each of the five analytes investigated (**Table 1.10**). However, no significant differences could be demonstrated when the reversibly inflamed samples were compared with irreversibly inflamed cases (Abd-Elmeguid et al. 2013). In clinical practice, the necessity to differentiate a saveable versus a non-saveable pulp is becoming more important and the development of a discriminatory test to clearly delineate this threshold appropriately is key.

1.7.1.1b IL-1 β

IL-1 β a classic marker of inflammation, has produced valuable results relating to detection of advanced periodontal disease (Tsai et al. 1995), has demonstrated conflicting results in pulp research. Nakinishi et al. (1995) were unable to demonstrate significant differences between the normal and inflamed pulp, however Silva et al. (2009) was able to do so. Again, their methodologies differed in terms of volumetric (Nakinishi et al. 1995) and total protein extraction (Silva et al. 2009). It is interesting to note that Nakinishi et al. (1995) reported a range of 1-53 ng/ml of IL-1 β in their inflamed samples, compared with 28 pg/ml recorded by Silva and colleagues (2009). These results demonstrate a remarkable difference in the values

recorded by several orders of magnitude. The trend does appear to support the conclusion that equilibrating the sample using the total protein method demonstrated significantly lower values and this in turn may influence the level of detection in normal samples, which logically could be even lower, potentially beyond the sensitivity of the kit employed. In spite of this conclusion, one would expect that the pulp tissue would produce a larger mass of substrate than can be extracted from pulpal blood using an absorbent vehicle. Equally, mRNA expression reached a level of significance in Paris' et al. (2009) study; however, these findings were not supported by Zehnder et al. (2003). Luminex-multiplex assay presents another opportunity to investigate numerous analytes in various biological samples, in particular when limited volumes are available (Elshal & McCoy 2006). A significant limitation relating to this technology relates to the interpretation and referencing of analytes in particularly low concentrations. Ghattas et al. (2017) were unable to demonstrate significant differences for IL-1 β , or TNF- α in pulpal blood samples when normal and inflamed cases were compared. It is worth noting that their methodology applied a total protein, rather than a volumetric approach.

1.7.1.2 IL-2

Although IL-2 was detectable in pulp tissue supernatant (Rauschenberger et al. 1997, Anderson et al. 2002), as well as pulpal blood samples (ElSalhy et al. 2013); statistically significant differences were only demonstrated by Rauschenberger et al. (1997) when normal pulp tissues were compared with pulp tissues collected from irreversibly inflamed pulps. Methodologic accuracy reported by Rauschenberger et al. (1997) is questionable for a number of reasons (Hirsch et al. 2017). Principally, this

group devised their own assay plates and the units of measurement are not recognised by international standards (ISO <https://www.iso.org/foreword-supplementary-information.html>). The technical complexity of splitting the pulp longitudinally in order to provide samples for both assay and histology cannot be underestimated. Notably, Hirsch et al. (2017) therefore concluded that IL-2 was not a suitable biomarker for the purposes of pulp diagnostics.

Methodological questions could be raised concerning more recent studies (ElSalhy et al. 2013) regarding the collection vehicles chosen (cotton wool pellet soaked for 45-60 seconds) and even the subsequent level of dilution chosen (1 ml saline). Numerous studies have gone to significant lengths to identify superior collection vehicles and extraction methods, suggesting that nylon fiber (Nakinishi et al. 1995), or folded polyvinylidene difluoride (PVDF) membranes (Zehnder et al. 2011), were superior to cotton wool. Despite this finding ElSalhy et al. (2013), using paper points, was classified as being of moderate quality in a recent systematic review (Hirsch et al. 2017). A quality assessment score of five (out of a maximum score of 8) using a modified Newcastle-Ottawa Scale was applied to the same study (ElSalhy et al. 2013) in another systematic review (Rechenberg et al. 2016a).

1.7.1.3 IL-6

IL-6 retains a particular relevance during inflammation due to its role in the induction of the acute phase response (Gabay & Kushner 1999). Complement activation, induction of pro-inflammatory cytokines and neutrophils chemotaxis are just some of the functions of this pleomorphic cytokine (Degré 1996, Naka et al. 2002). In pulpitis research, IL-6 was the least detectable of all the molecules investigated by

Nakanishi et al. (1995). It was not detected in the healthy samples and only 2/18 inflamed samples detected IL-6 at very low levels, the exact details of which were not documented by the authors.

Three further research groups investigated IL-6 in pulpal blood (ElSalhy et al. 2013, Mutluay et al. 2018 and Nguyen et al. 2020). Methodological similarities and variances have already been discussed (**Sections 1.5.5, 1.6.3 & Tables 1.9, 1.11-1.12**). Unfortunately, direct comparison between the findings of ElSalhy et al. (2013) and Nguyen et al. (2020), is obscured by the expression of their data as picograms per milligram total protein and picograms per millilitre, respectively. In particular, these two groups had the most commonality in terms of their methodologies (cohort, collection vehicle, dilution, centrifuge parameters, heparinisation, storage and reported ELISA sensitivity). Both research groups utilised a Bio-Rad™ ‘Bradford’-style protein assay (Bio-Rad Laboratories, Hercules, CA USA) to determine the total protein concentration of the collected blood samples. One could speculate that this additional step was carried out in order to overcome a perceived limitation relating to inconsistent recovery of the substrate, which could reasonably be anticipated with an absorbent vehicle methodology. This is regarded as standard practice, particularly in studies investigating pulpal tissues, to which it is more readily applicable. Consideration should perhaps be given to standardising the volume of the substrate collected methodologically, as the availability of pulpal blood is so limited. Variability in the addition of diluents impacts the data in a manner that is not possible to standardise robustly.

The range of IL-6 (**Table 1.12**) recovered by Nguyen et al. (2020) in their inflamed samples [0.65-1.75 pg/ml] was narrower than that reported by both ElSalhy

et al. (2013) [0.02-2.09 pg/mg] and Mutluay et al. (2018) [0.03-1.16 pg/ml]. Notably, ElSalhy et al. (2013) did not recover any IL-6 in their “true” healthy control samples, compared with Nguyen et al. (2020), who used proxy (elective root canal treatment of asymptomatic restored teeth) control samples and registered levels similar to the lower end of the range of their inflamed samples [0.25-0.45 pg/ml]. Importantly, statistical significance was only demonstrated by ElSalhy et al. (2013). The incidence of recovery of IL-6 in caries exposed and symptomatic irreversible pulpitis was 52% and 69% respectively (ElSalhy et al. 2013). Notably, IL-6 produced the lowest range of any of the detected analytes of the inflamed samples, in this study.

The reported levels of IL-6 presented by Mutluay et al. (2018) were relatively similar to those shown by Nguyen et al. (2020), although their lowest levels differed by a factor of 10 (**Table 1.12**). The only significant differences that could be determined in this study were noted when the IL-6 levels at the initial exposure site were compared, based on the ability to achieve haemostasis (Mutluay et al. 2018). The applicability of these findings to an adult population is, of course, unknown.

Barkhordar et al. (1999) assessed pulp tissue supernatant obtained from inflamed and healthy control subjects and reported mean levels of 36 pg/mg and 0.01 pg/mg, respectively (**Table 1.10**). Conversely, AbdElmeguid et al. (2013) revealed an apparent down-regulation in the levels of IL-6 recovered in pulp tissue supernatant from teeth with signs and symptoms indicative of irreversible pulpitis [7.82 pg/mg] compared with reversible inflammation [15.95 pg/mg]. The difference was not statistically significant. Normal, healthy controls did not register any IL-6.

Zehnder et al. (2003) investigated the expression of mRNA of selected cytokines in healthy and inflamed pulp tissues by qRT-PCR (**Table 1.10**). The profile

of the samples designated in the inflamed category all had carious pulp-exposure. Significant differences were displayed between healthy and inflamed samples for IL-6, IL-8 and IL-18; but not IL-1 α , or IL-1 β . It is noteworthy that approximately 50% of the data was excluded from analysis due to either a). the standard deviation of a triplicate of one cytokine being greater than three times the average standard deviation, that sample was excluded and the observation designated as unobserved; or b). if more than one measurement in the triplicate analysis had to be disregarded, the result for that specific cytokine was classified as non-valid. This indicates a commendable level of honest, rigor and reproducibility in the conduct of their analyses.

The differential gene expression of selected cytokines may highlight the mechanistic, or temporal activation of these mediators and it is not known how the lack of altered protein expression can be accounted for.

1.7.1.4 IL-8

The expression of IL-8 is strongly associated with inflammatory and infective processes in many tissues, including human pulp (Huang et al. 1999). Its functions include chemotaxis and migration of neutrophils, induction of neutrophil degranulation, as well as influencing numerous other immunological cells, including B- & T-cells, leukocytes and natural killer cells (Baggiolini et al. 1994).

Only two investigators evaluated IL-8 in pulpal blood (**Table 1.12**) from inflamed cases (ElSalhy et al. 2013, Mutluay et al. 2018). IL-6 and IL-8 were the only cytokines that remained undetected using a high sensitivity ELISA, in the only study conducted on pulpal blood using ‘true’ negative controls (ElSalhy et al. 2013). The

incidence of recovery of IL-8 in caries exposed (45%) and symptomatic irreversible pulpitis (81%) varied widely across the analytes investigated by this group. How the sensitivity of the kit, or variations in the volume of substrate eluted during the assay influenced the detectability of the analytes cannot be deduced. IL-8 produced the widest range of cytokine recovered by ElSalhy et al. (2013) [2.3-593.6 pg/mg]. The range of IL-8 recovered by Mutluay et al. (2018) was consistent with the ranges of the other mediators detailed in their results (**Table 1.12**).

The levels of IL-8 have been widely studied in several other substrates (**Table 1.10**), including pulp tissue supernatants and gingival crevicular fluid, in cases with pulpitis (Huang et al. 1999, Isett et al. 2003, Zehnder et al 2003, Karapanou et al. 2008, Silva et al. 2009, Abd-Elmeguid et al. 2013, Ozdemir et al. 2015). Substrate sources have varied from extracted teeth, extirpated pulp, pulp fragments collected during pulpotomies, as well as various vehicles including cotton wool pellets, sterile paper points and Periopaper. Although ELISA has been the most consistent assessment method reported in the literature, other analyses have also been conducted including immunohistochemistry, multiplex assay and gene expression (RT-PCR). Despite substantial methodological heterogeneity between the studies of this mediator, IL-8 appears to be consistently expressed at a significantly higher level in inflamed compared with normal samples. Matched studies, employing similar methodologies did not produce comparable data (**Table 1.10**) however, with variations noted in the reported concentrations of IL-8 by Huang et al. (1999): 1.82 pg/ml/ μ g, Silva et al. (2009): 46 pg/mg and Ozdemir et al. (2015) 1.2 pg/mg. This further underpins the paucity of data that can be readily compared between research groups. The development of standardised reporting procedures that allow direct

comparisons in this field should ameliorate the quality and understanding of our diagnostic capabilities.

IL-8 appears to be consistently expressed at a significantly higher level in inflamed compared with normal samples (**Table 1.10**). This is reflected in numerous techniques ELISA, multiplex assay and gene expression analyses (Huang et al. 1999, Isett et al. 2003, Zehnder et al. 2003, Silva et al. 2009, Abd-Elmeguid et al. 2013). Statistical significance is supported regardless of volumetric, or total protein extraction; as well as substrate (pulp tissue supernatant, or pulpal blood). Lack of uniformity between studies, sampling similar substrates and using similar methodologies (i.e. pulp tissue supernatant using ELISA and total protein extraction) reveal consistently different results. Karapanou et al. (2008) measured levels of IL-8 and TNF- α in gingival crevicular fluid of patients with irreversibly inflamed pulps using Periopaper. IL-8 was shown to be significantly higher in the inflamed sites compared with the contralateral controls. Despite the fact that gingivitis and periodontitis were listed amongst their exclusion criteria, it is difficult however, to reliably exclude the influence of localised gingival inflammation around a painful tooth condition to the extent that the increased levels could be attributed to pulpal as opposed to periodontal origin. TNF- α was not detected in this study (Karapanou et al. 2008).

1.7.1.5 TNF- α

TNF- α has failed to reliably demonstrate significant differences between normal and inflamed human pulps (Nakinishi et al. 1995, Karapanou et al. 2002, Ghattas et al. 2017). The studies where statistical significance was established (Paris et al. 2009, Abd-Elmeguid et al. 2013, ElSalhy et al. 2013, Ghattas et al. 2017) have been

discussed above with the exception of Pezelj-Ribaric et al. (2002). The levels of TNF- α recorded by Pezelj-Ribaric et al. (2002) also reflect the inconsistencies noted in this particular field (**Table 1.10**). It is notable that five samples were lost in this study for technical reasons (Pezelj-Ribaric et al. 2002), which although representing transparency, may cast some uncertainty on the validity of the laboratory findings.

1.7.2 Proteases

Matrix metalloproteinases (MMP's) comprise a family of proteolytic enzymes which are involved in the degradation and/or remodeling of extracellular matrix proteins (Birkedal-Hansen et al. 1993). Their functions are broad ranging from physiological remodeling, to inflammatory and immune reactions in association with antigenic challenges (Visse & Nagase 2003). Twenty-five individual MMPs have been identified in humans and they are classified according to their substrate specificities. The full potential of their biological applications is yet to be fully understood. Their expression is tightly regulated and directly inhibited by specific tissue inhibitors: collectively known as Tissue Inhibitors of Matrix Metalloproteinases (TIMPS), of which, four groups have been identified. MMP's exist in latent (pro-) and active forms, requiring activation via a cysteine switch (Hadler-Olsen et al. 2011). It is becoming apparent that they demonstrate multiple specificities, not limited to immunity, inflammation, wound healing and vasodilation (Butler & Overall 2009), which means that they have a role both in inflammatory and reparative processes (Duncan et al. 2016). As a result, they have formed a particular research focus in terms of diagnosis and therapeutic targets within dental research (Tjäderhane et al. 1998, Sorsa et al. 1999, Duncan et al. 2016). The low pH environment caused by

bacterial acids and subsequent neutralisation phase during the carious process activates MMP's in dentin and saliva (Tjäderhane et al. 1998). In their active form, MMPs are capable of degrading extracellular matrix, a key feature in the progression of inflammation (Accorsi-Mendonca et al. 2013). Associations have been described in terms of up- and/or down-regulation of the enzymes corresponding with the progression of caries, or increasing levels of pulpal inflammation (Gusman et al. 2002, Tsai et al. 2005, Zheng et al. 2009, Accorsi-Mendonca et al. 2013).

MMP-2 and MMP-9 in particular, have been extensively studied in relation to pulpitis (Rechenberg et al. 2016a). In a rat pulp injury model Zheng et al. (2009) demonstrated upregulation of MMP-9 twelve hours after injury, with negligible change noted in MMP-2. MMP-9 appears to be consistently upregulated in inflamed human pulp (Gusman et al. 2002, Tsai et al. 2005, Zehnder et al. 2011, Accorsi-Mendonca et al. 2013). This finding was further supported by Tsai et al. (2005) when gene expression of MMP-9 mRNA was shown to be elevated, in comparison with healthy controls. The complex regulation of MMP's, however, leads to difficulties in demonstrating clear statistically significant differences across all test samples. This is particularly evident in the study by Zehnder et al. (2011), where they were able to demonstrate upregulation in just over half their test cases, presenting with clinical signs of symptomatic irreversible pulpitis. The remaining test samples and all of the healthy controls may have been recovered in such small quantities to be below the level of detection of the colourimetric kit used. The kit was selected specifically due to its sensitivity, reported to be two orders of magnitude greater than comparable ELISA on the market at that time. However, low yield may have been particularly relevant in this study as the substrate under investigation was dentinal fluid.

Although Zehnder et al. (2014) demonstrated that polyvinylidene fluoride (PVDF) provided a superior yield of quantifiable MMP-2 compared with cellulose with dentinal fluid, when utilising a different ELISA, they were only able to detect MMP-2 in 9/13 of the cases.

Mente et al. (2016) assessed levels of MMP-9 and TIMP-1 in pulpal blood by fluorometric and colourimetric protein assays respectively. They were able to demonstrate significant differences between selected categories of the cariously-exposed pulps. They were unable however, to present a threshold level above which irreversible inflammation could be determined. The predominant finding from the research conducted in human pulpitis models relies upon the comparison of 'irreversibly' inflamed with normal, or mildly inflamed samples. In order to present an effective chairside diagnostic test, it would be essential to demonstrate a unique biomarker which is exclusively expressed in the most severely inflamed models, or provide a valid threshold above which, the identified biomarker demonstrated severe inflammation and a resulting tissue damage in every case. To date, this has not been established in endodontic literature, due in part to the variability in the regulation of the immune, inflammatory and reparative mechanisms.

The assessment of active and latent forms of MMP-8 have proved discriminatory in periodontal research (Lee et al. 1995), such that the direct role of MMP-8 activity could be linked with connective tissue destruction. The usefulness of diagnostic tests should allow for evaluations to be performed over a wide spectrum of disease (McCulloch 1994) and critically, should benefit the patient, leading ultimately to improved patient outcomes. Although MMP-8 has been recovered from pulp tissues, periapical lesions and dentine (Shin et al. 2002, Wahlgren et al. 2002,

Aguirre-López et al. 2020), its application as a diagnostic adjunct has not yet been established in clinical endodontics.

1.8 Potential markers related to cell death

To date, most molecular-based diagnostic studies have investigated biomarkers related to inflammatory processes such as interleukins and proteases (Rechenberg et al. 2016a, Zanini et al. 2016, Hirsch et al. 2017). Indeed, acute pulpal inflammation is characterised by accumulation of phagocytes, principally neutrophils, macrophages and dendritic cells (Bergenholtz 1977, Warfvinge et al. 1985, El-Karim et al. 2021). Neutrophil granulocytes are the most abundant type of white blood cells and play a vital role in innate immunity by defending the host against invading pathogens. Neutrophil functions include phagocytosis, generation of reactive oxygen species (ROS), degranulation and the formation of Neutrophil Extracellular Traps (NETs) (Brinkmann et al. 2004, Urban et al. 2009). NETs are primarily composed of DNA (Brinkmann et al. 2004), which function to kill bacteria extracellularly. Cell death by netosis is a form of regulated cell death, characterised in neutrophils by the extrusion of histone containing fibers, known as NETs (Bordon et al. 2013). Neutrophils can release NETs as early as 10 minutes after activation and their release is dose dependent (Brinkmann et al. 2004). As well as NETs immobilizing and killing bacteria (Brinkmann et al. 2004), they also interact with the blood coagulation system (Soehnlein 2018). NETs have also been extensively studied in relation to sepsis, inflammatory disorders, oncology and autoimmune disorders (Berger-Achituv et al. 2013, Lee et al. 2017, Ebrahimi et al. 2018, Soehnlein 2018), most often utilizing fluorescence microscopy, or flow cytometric techniques (Gavillet et al. 2015).

When neutrophils undergo NETosis, nuclear and granular membranes disintegrate, the chromatin decondenses and diffuses into the cytoplasm, mixing with cytoplasmic proteins (Allam et al. 2014). The plasma membrane then ruptures leading to the release of chromatin (DNA and DNA-associated histone proteins), along with the granular proteins, into the extracellular space. This offers a potential opportunity for diagnostic assessment of discharged intracellular contents during cell death and necrosis (Fuchs et al. 2007).

Vitkov et al. (2009), were the first group to demonstrate NETs in gingival crevicular exudate suggesting their role in innate defence in periodontal disease. Cooper et al. (2013) further demonstrated NETs in gingival tissue biopsies in an attempt to correlate the role of NETs in the pathogenesis of periodontitis. NETs facilitate maximal targeted killing of microbes and although effective at clearing the pathogenic challenge, the associated collateral host tissue damage facilitates disease progression. White et al. (2018) and Magán-Fernández et al. (2019), have demonstrated applicability using an array of laboratory techniques including immunofluorescence and scanning electron microscopy, which have ameliorated our understanding of the processes. However, these type of laboratory techniques, do not readily translate to a clinical diagnostic test.

As a result, surrogate markers related to cell death processes have been investigated, with the 'gold standard' NET marker not yet identified (Masuda et al. 2016).

1.8.1 Extracellular histones

Extracellular histones mediate extensive cellular damage, haemostatic imbalance and amplification of the inflammatory response by inducing cytokine production (Xu et al. 2009, Chen et al. 2014). Histone-3 (H3) and Histone-4 (H4), are both reported to be biomarkers of disease progression as well as therapeutic targets in sepsis and other inflammatory diseases (Kutcher et al. 2012, Thålin et al. 2017). A new link has been identified between the cytokines IL-8 and IL-6, neutrophil activation, and the release of H3Cit in the blood stream (Thålin et al. 2018). Plasma histones have been shown to mediate cellular damage and severe inflammatory responses in various organs, which were linked with the TLR-4 receptor (Ekaney et al. 2014). In particular, their role in the development of sepsis has been widely studied (Clarke et al. 2007, Margraf et al. 2008). Neutrophil-derived circulating free DNA (cfDNA/NETs) are further proposed not just as diagnostic markers, but also indicators of therapeutic efficacy in the management of severe sepsis in humans (Margraf et al. 2008).

As IL-8 and IL-6 have been shown to induce NET formation *in vitro* (Thålin et al. 2018), this forms the rationale for considering a cell death detection ELISA targeting extracellular histones (Salgame et al. 1997, Holdenrieder et al. 2001a, Holdenrieder et al. 2001b, Kutcher et al. 2012, Elkon 2018) and comparing with an inflammatory cytokine IL-6, which has been shown to be discriminatively expressed in pulpitis. This could potentially be used to direct future therapeutic decisions in relation to the maintenance of pulpal health. IL-6 can induce acute phase response (Cannon et al. 1998) and plays a role in neutrophil activation (Calder et al. 2013). Cytokines are the most widely studied of the inflammatory mediators. Of these, IL-6 is one of the most frequently investigated (Nakinishi et al. 1995, Barkhordar et al.

1999, Zehnder et al. 2003, Abd-Elmeguid et al. 2013, Elsalhy et al. 2013). Experimentally induced pulpitis has revealed fluctuations in the levels of cytokines at different time points after induction of acute inflammation (He et al. 2017, Renard et al. 2016). The proposed choice of IL-6 as a candidate in this study relates to validation of a robust method for the collection of pulpal blood and an attempt to correlate the variances found within defined clinical diagnoses, in humans (Calder et al. 2013).

1.8.2 Opportunities for using extracellular histone as a marker of pulpal disease

Cell death detection assays have been validated in medical research and linked to the severity of a range of conditions (Salgame et al. 1997, Holdenrieder et al. 2001b, Kutcher et al. 2012, Ebrahimi et al. 2017, Thålin et al. 2017), but have not been applied to date as a potential diagnostic aid in pulpitis. As discussed in previous sections, the levels of inflammatory mediators *in-vivo* have proven to be highly variable in human studies, underpinning the complexity and dynamics of the underlying mechanisms and regulatory processes. It is necessary to identify a discriminatory mediator which should not be routinely expressed in health, or more importantly, even in mild inflammation. Specifically, the identification of the transition to the 'point-of-no-return'; namely necrosis (Ricucci et al. 2014), may provide the consistency lacking in the research to date.

1.8.3 High Mobility Group Box 1 (HMGB₁)

HMGB₁, first discovered 40 years ago, was initially considered to be a nuclear protein involved in maintenance of homeostasis (Goodwin et al. 1973), that interacted with histones and DNA, including transcription of chromatin (Rowell et al. 2012).

Extracellular HMGB₁, now recognised as an effective alarmin, acts in many ways dependent on the form of its release, leading to the recruitment of inflammatory cells, or amplification of the inflammatory response by stimulating cytokine and chemokine release, as well as recruitment of leukocytes (Wang et al. 2004, Kang et al. 2014, Huebener et al. 2015, Chen et al. 2022). Release of HMGB₁ is tightly conserved; either passively, indicating cell death through necrosis; or via active secretion, through a programmed cell death mechanism (Bustin 2002, Scaffidi et al. 2002, Raucci et al. 2007). Its activation has been shown to be strongly linked with TLR-2 and TLR-4 expression (Yu et al. 2020), mimicking effects usually associated with LPS exposure (Park et al. 2004) and contributing to organ damage (Allam et al. 2012), including pulpal responses to bacterial challenge (Mutoh et al. 2001).

HMGB₁ also has a high affinity with Receptor for Advanced Glycation End-products (RAGE), which functions to recruit neutrophils to the site of injury (Sims et al. 2010, Huebener et al. 2015, Yang et al. 2020). Significantly elevated levels of HMGB₁ have been demonstrated clinically as a late observation in sepsis, further suggesting that prolonged elevated serum levels were prognostic indicators of poor patient outcomes (Wang et al. 2004, Deng et al. 2019). The expression of RAGE together with HMGB₁ was investigated in pulp tissue and shown to be significantly higher in pulpitis compared with normal pulp tissues (Tancharoen et al. 2014). The group also demonstrated that HMGB₁ was expressed in a dose-dependent manner by LPS stimulated cells via RAGE pathway (Tancharoen et al. 2014).

1.9 Development of chairside diagnostic assays

Periodontal research has pioneered the use of diagnostic biomarker analysis to investigate disease severity and direct treatment (Lee et al. 1995, Sorsa et al. 1999). In, contrast, there is a paucity of endodontic research utilising molecular analyses to identify therapeutic or prognostic targets at least at the point-of-care, which is chairside (Mäntylä et al. 2003, Podzimek et al. 2015, Salminen et al. 2016, White et al. 2018). Notably, there are obvious constraints within endodontics, relating to the preoperative accessibility and invasiveness of testing the pulp directly; however, pulp exposure is a known intraoperative occurrence in Direct Pulp Therapy (DPT) that affords the clinician direct access to pulpal blood. Periodontal research has utilised gingival crevicular fluid (GCF), saliva and peripheral blood as substrates for clinical testing which can be carried out prior to treatment (Podzimek et al. 2015, McCrudden et al. 2016, Nicu et al. 2016, França et al. 2019, Gupta et al. 2022).

1.9.1 Blood collection and analysis

Pulpal blood forms an obvious clinical reference standard, which appears to have been neglected in the research to date (Rechenberg & Zehnder 2014). Serum is the liquid portion of the blood after it has been allowed to clot. It is free of clotting proteins but contains metabolites that result from the clotting process (Dupin et al. 2016). Due to platelets and cells being trapped within the fibrin mesh of the clot, serum is considered a 'cleaner' sample. Plasma, is the liquid portion of blood that has been prevented from clotting and therefore regarded as more representative of blood as it circulates in the body. Though it has an advantage over serum in that testing is not delayed by waiting 30 minutes for a clot to form, it is typically

contaminated by platelets and cellular elements that have the potential to alter and therefore bias analytical findings (Aziz et al. 1999, Dagur & McCoy 2015). At present, there is no consensus in the literature as to whether using serum or plasma is preferable (Tuck et al. 2009) when assessing the expression of pulpal biomarkers with some studies analysing one and some the other without obvious pattern (Nakinishi et al. 1995, Mente et al. 2016, Sharma et al. 2021).

1.9.2 Finding a suitable molecular test

Compared with other immunoassay methods, ELISA appears to be the most accurate, sensitive, and specific test for molecular evaluation of dental pulp inflammation. It is transferable to chairside and will further allow qualitative and quantitative analysis. Although time-consuming and expensive, the methods should be feasible to use in clinical practice and executable chairside to allow for their adoption into therapy. The results will be subsequently analyzed and correlated with the diagnostic and clinical findings. This type of testing has recently become commonplace during the COVID-19 pandemic, such that most people at least in the Western world are very familiar with this type of immunoassay technology.

1.10 Aims and objectives

The overarching purpose of this study is to identify and develop predictable and accurate molecular diagnostic markers of pulpal inflammation; more specifically, the end-point of irreversible inflammation, namely necrosis.

In order to achieve a predictable and reproducible diagnostic testing methodology, several aims are proposed. Initial aims are to;

- Review the current literature in relation to molecular biomarker diagnostic testing for assessing pulpal disease to accurately predict the pulpal condition.
- Investigate the methodological aspects of pulpal blood collection, including the most appropriate vehicle in which to collect pulpal blood samples, the most effective blood analyte, as well as establishing the most appropriate laboratory testing technique by which to predictably analyze the pulpal condition.
- To correlate the visual appearance of the exposed pulp with traditional and novel pulp diagnostic categories based on symptoms. As a secondary aim, the visual appearance of the exposed pulps, symptoms and caries depth will be linked with VPT outcomes, after at least one year.
- The identification of the threshold of irreversible inflammation, namely necrosis, may present a novel target biomarker to differentiate pulpal states and indicate cases that may not, or are less likely to respond to therapy. An assay targeting circulating extracellular histones and capable of differentiating regulated from accidental cell death, with a view to developing a discriminatory chairside diagnostic test.
- To analyse the expression of an extensive panel of established inflammatory and regenerative mediators using a commercially available multiplex array in pulpal blood serum from healthy sound teeth, to teeth exhibiting signs and symptoms of severe pulpitis. A

secondary aim is to validate these findings on selective biomarkers using ELISA assays.

What follows is a suite of studies to develop and explore these stated aims. Using the information gathered from the literature review, a chapter outlining universal materials and methods is presented (**Chapter 2**). The purpose was to provide sufficient information on important aspects of the studies, deemed lacking in the bulk of the literature to date. This chapter provides detailed selection criteria, standardised examination and treatment protocols, as well as outcome measures used through the studies that follow.

After due consideration, pulpal blood was selected as the substrate of choice, due to its limited inclusion in pulpal biomarker research and its direct availability in the provision of direct pulp therapy. **Chapter 3** explores methodologic and practical aspects related to the collection, handling and analysis of pulpal blood, (since no standard protocol exists) and its relatability to recognised, clinical diagnostic parameters. It was necessary in this early stage of the studies to incorporate molecular analyses to interrogate the veracity of the methods adopted. To this end, one of the most widely reported analytes in pulpal biomarker research was selected (IL-6), coupled with a novel (Cell death) target for molecular evaluation.

Clinicians are required to evaluate the inflammatory status of the exposed pulp, visually, in order to determine the limit of vital pulp therapy, or the indication to proceed with root canal treatment. For such an important clinical determinant, limited data exists within the literature. **Chapter 4** proposes visual descriptors for the exposed pulp and examines their validity in the context of standard diagnostic

methods, the presence of associated symptoms and caries depth. The possibility of identifying the prognostic value of these factors as they relate to treatment outcome of pulpotomy was also considered.

Combining the data gleaned in the preceeding chapters, **Chapters 5 and 6** focus on the molecular evaluation of diagnostic targets within pulpal blood. **Chapter 5** concerns the investigation of two candidate cell death mediators by ELISA and their expression relative to the clinical diagnoses, treatments and presence of symptoms and caries depth. A profiling approach was adopted in **Chapter 6**, using a modified blood collection protocol and a high-throughput cytokine discovery assay. Pilot work revealed some promising trends, however several logistic issues precluded testing all samples and the study was modified to investigate a selection of candidate validation ELISA tests. **Chapter 7** explores the future potential of this body of work.

Chapter 2

General Materials and Methods

2.1 Introduction

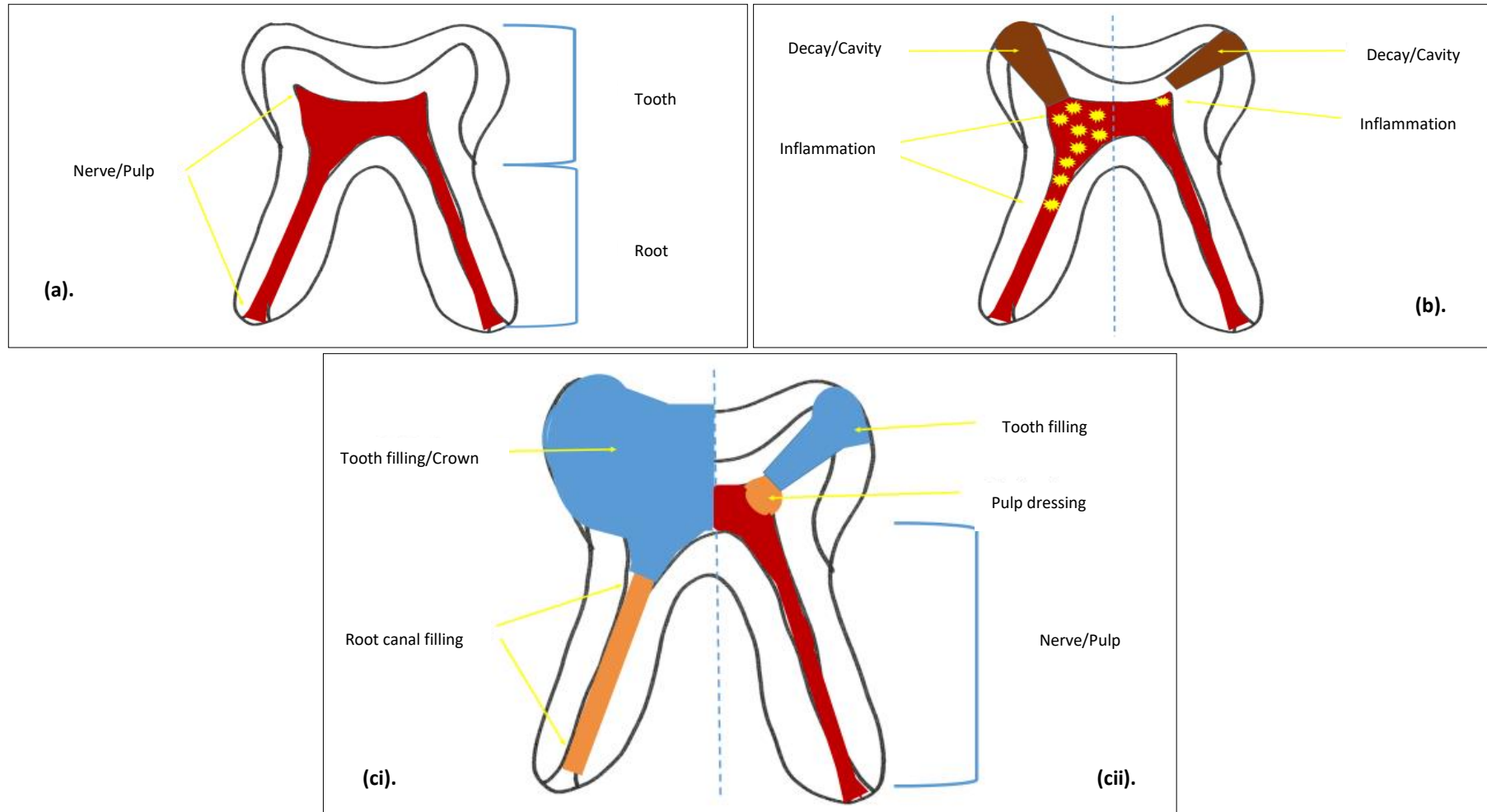
All clinical procedures were carried out after obtaining ethical approval and explicit, informed written consent. All consumables used in the transfer of samples from the clinic to laboratory and in subsequent analysis were certified as RNase- and DNase-free by the supplier as listed. Approved molecular biology grade reagents were used for all laboratory procedures.

2.2 Patient recruitment and consent

The protocol for the clinical and analytical aspects of this suite of studies was submitted to St. James' Hospital/Tallaght University Hospital Research Ethics committee on 26/06/2017 and approval was granted on 21/09/2017 (REC REF: 2017-07 Chairman's Action [16]; **Appendix II**). An amendment to the study protocol was sought on 19/10/2018 and approved on 05/11/2018 (2018-10 List 33[16]; **Appendix III**).

Patients were recruited in the Dublin Dental University Hospital (DDUH), through Restorative Dentistry, Orthodontic and Oral Surgery clinics from September 2017 until December 2021. Two distinct cohorts of patients were sourced; those requiring extraction of a 'sound' tooth for orthodontic, or prosthodontic purposes and those requiring root canal treatment, or vital pulp treatment (VPT) due to deep caries and pulpitis. Once identified, potential patients were canvassed by the lead investigator, informed verbally of the nature of the project and invited to join the study. Age-appropriate (under and over 18 years), pre-prepared patient information leaflets (**Appendix IVa** and **Appendix IVb**), were discussed at length with the patient and if applicable, their guardian; before illustrations (**Figure 2.1**) were shown and the

Figure 2.1 Schematic illustrations used for chairside patient education (a). A molar tooth indicating coronal, radicular and pulp tissues. **(b).** Molar tooth depicting mild and severe pulpal inflammation in response to carious challenge. **(c).** Same molar tooth highlighting likely treatment options: **(ci).** Conventional root canal treatment with extra-coronal restoration. **(cii).** Pulp preservation procedure.



clinical steps involved were carefully explained. If agreeable, their voluntary, informed consent was obtained (**Appendix V**). A copy of the written instructions was given to each patient to digest and refer to later. A 'cooling-off' period was only considered possible in patient presenting with 'sound' teeth, or certain asymptomatic cases. When these patients returned for the specified treatment, the standard pulp sensibility tests were repeated to ensure that the findings related contemporaneously to the samples acquired on the day of treatment.

Due to the symptomatic nature of pulpitis, a 'cooling-off' period was not possible in symptomatic, or deep carious teeth. Patients attending in pain, who needed operative treatment to relieve their presenting symptoms, or to ensure the vitality of the pulp was not further compromised, by delaying intervention. Patients were made aware verbally and in writing that they were entitled to withdraw from the study at any time of their choosing without the decision affecting their care in the Dublin Dental University Hospital (DDUH).

2.3 General patient history and examination

Clinically, pulp diagnoses were reached by assimilation of the patients' presenting complaint (often in the form a pain history), clinical examination, which includes the results of standard pulp sensibility tests (thermal testing and electric pulp testing) and the radiographic examination findings. These examination findings were detailed in a systematic manner and recorded in the clinical record template (**Figure 2.2**).

Figure 2.2 Clinical record template

Date:
Patient:
Tooth:

Pain History:

Sharp / Dull/ Throbbing
 Secs / Mins / Hrs/ Constant
 Duration since onset symps: Days / Weeks / Months / Years
 Onset: Sporadic / Spontaneous / Trigger / AM / PM
 Course: Static / Increasing / Subsiding
 Thermal: Hot / Cold / Exacerbate / Relieve
 Location: Specific / Generalised / Diffuse / Radiation
 Chewing
 Analgesia required: Y / N
 Analgesia details: Relief / No relief
 Details of most recent intake of analgesia: Drug: Dose: Time:
 Nocturnal
 Headaches / Migraines / Stress
 Trauma: RTA/ Cervical spine
 Sinusitis

NSAID's: Last dose & time

Sensibility/ Special tests:

Tooth	EF	EPT	TTP	Palp	PPD	Mob	Sinus	Colour

Key to symbols: + normal, ++ heightened, +++ heightened & lengthened –ve: negative
 N/A: not applicable, N: no, Y: yes +/-: present or absent; wnl: Within normal limits; Incr T: increased threshold;
 dec T: decreased threshold;

Radiographic findings:

Root development	Complete		Incomplete	
Apex	Closed		Open	
Periapical rarefaction	Present		Absent	
Depth of caries	No caries	Outer half	Inner half	Exposure

Diagnosis: Normal/vital
 Initial pulpitis/ Reversible pulpitis
 Mild pulpitis/ Irreversible pulpitis
 Moderate pulpitis/ Irreversible pulpitis
 Severe pulpitis/ Irreversible pulpitis

Clotting time:
Character of bleeding:
Hx:
Clinical findings:
Direct inspection:

2.3.1 Patient history

The presenting complaint was recorded for each patient, as well as a comprehensive medical history questionnaire and the details entered into the electronic dental record. A directed pain history enquiry was carried out and the information was entered into the clinical record template (**Figure 2.2**). The character, duration, nature, onset, location, as well as any triggers, exacerbators, or relivers were entered into the record. Details of any analgesia used, time since last dose and its effect on the nature, and/or severity of their pain were also recorded. Nocturnal pain and pain requiring analgesia were considered important (Tyldesley & Mumford 1970). Pain that was not alleviated by regular, over-the-counter analgesics was regarded as the most severe. Additional factors, suggestive of non-odontogenic sources, or complex pain conditions were also enquired of the patient; these included, but were not limited to sinusitis, headache disorders, cervical radiculopathy and/or emotional stressors. This information was then assimilated to categorise the patients' symptoms.

2.3.2 Clinical examination

The clinical examination included recording each tooth's response to cold testing. The procedure was explained to the patient beforehand. They were instructed to indicate when they felt a sharp, or cold sensation and the time for this unpleasant sensation to dissipate and/or completely resolve was also recorded. This allowed the operator to distinguish a normal from a heightened; or from a heightened, to a heightened and lengthened reaction to the stimulus (**Figure 2.2**).

The site was isolated with cotton wool rolls (ROEKO, Coltène/Whaledent® GmbH+Co. KG) and the teeth were air dried in preparation for cold and electric pulp testing. ROEKO Endo Frost (Coltène/Whaledent® GmbH+Co. KG) was applied to a size 0 ROEKO cotton wool pellet (Coltène/Whaledent® GmbH+Co. KG) and subsequently applied to each tooth (in the mid-labial aspect) individually, up to a maximum of 15 seconds. Electric pulp testing was carried out using a SybronEndo Vitality Scanner Model 2006 Electric Dental Pulp Tester (SybronEndo, California, U.S.A.). After the lip clip was secured, a conductive medium, Nupro Proply paste (Dentsply Sirona, Surrey, U.K.) was applied to the tip of the short probe, before testing the middle facial aspect of each tooth. The number at which the patient felt a sharp sensation, if any, was then recorded. Percussion tenderness was noted by tapping the occlusal aspect of the tooth with the 'butt' of the dental mirror. Palpation tenderness was examined by palpating the apical region with a gloved index finger. The presence of swelling, or sinus was also recorded. Periodontal probing was carried out using a Williams Periodontal Probe (Hu-Friedy Mfg. Co., Chicago, U.S.A.). Tooth mobility was assessed according to standard practice. Tooth colour was noted as either normal, or discoloured.

2.3.3 Radiographic examination

A long cone parallel periapical radiograph was taken using an appropriate size (size 1 or size 2) phosphor plate (Dürr Dental SE, Bietigheim-Bissingen, Germany), in a Rinn holder (Dentsply, Surrey, U.K.). The resultant images were then processed using Vista Scanner (Dürr Dental) and viewed using Planmecca Romexis Software (Panmecca OY, Helsinki, Finland). If a panoramic radiograph was already present, this was deemed

acceptable for orthodontic cases planned for extraction of disease-free premolars only. The radiographic report was detailed in the clinical template, with a particular emphasis on the following aspects: - Stage of root development, evidence of apical closure, presence of periapical rarefaction and depth of carious lesion (**Figure 2.2**).

2.3.3.1 Root development classification

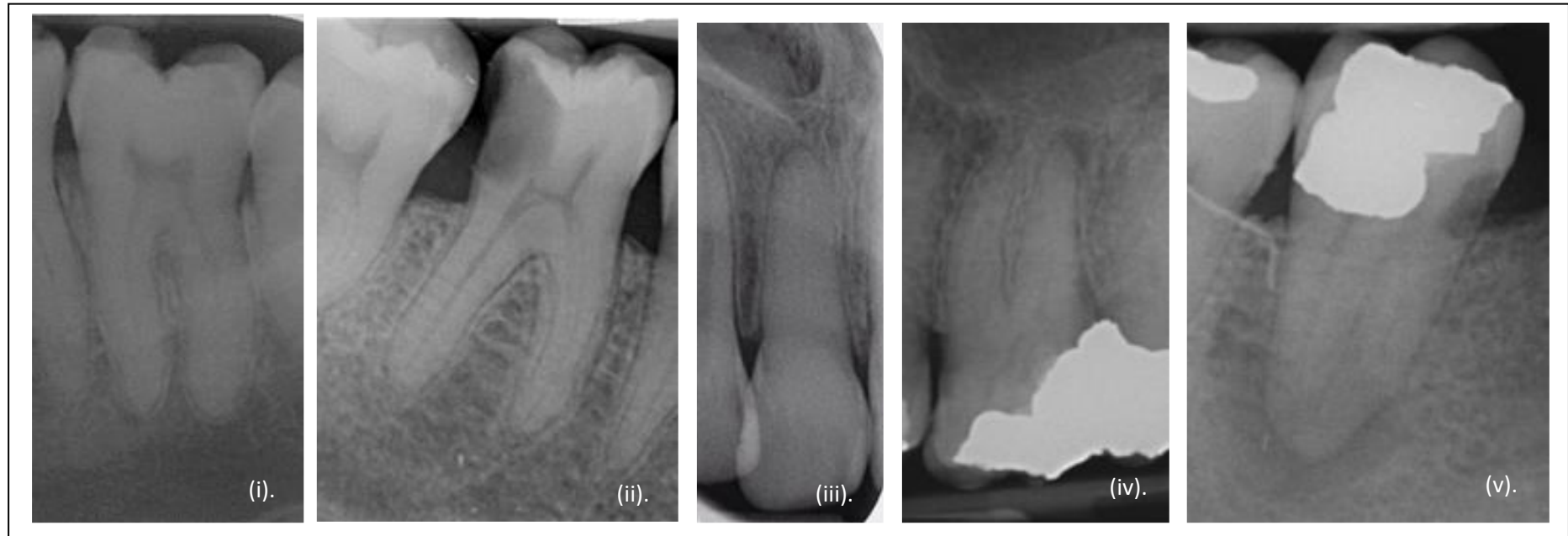
Root development and apical closure were assessed radiographically and classified according to Nolla 1960. Herein a scale is provided ranging from 1-10 describing the radiographic development of permanent tooth from the first radiographic evidence of the dental crypt (1), the initial calcification of the dental crown (2), to the completion of crown development (6). The final stages, 7-10 describe the development of the root, in thirds. One third of root development completed is scored as 7, two-thirds development as 8, root development almost complete (apex regarded as still open) as 9 and finally apical closure as 10.

2.3.3.2 The periapical index

The periapical index (PAI), a scoring system for the radiographic interpretation of periapical periodontitis introduced by Ørstavik et al. 1986 was used in this study. This index ranges from 1 (health), to 5 (severe apical periodontitis associated with an extensive periapical radiolucency). Scores of 1, or 2 are generally regarded as the cut-off point depicting probable health of the periapex. Scores of PAI 1 (A normal periodontal ligament space), or PAI 2 (A subtle widening of the periodontal ligament space, with no clear evidence of a developing radiolucency) were accepted in this study as health. Teeth exhibiting PAI scores of 3, or higher were excluded (at the

assessment stage), or regarded as failure in the subsequent outcome evaluation (Ørstavik et al. 2004). Examples of each are included in **Figure 2.3**. The PAI index was used both at the pre-operative assessment and at the outcome evaluations (described further in **Section 2.8.4**), routinely. Principally the index was devised using the combined radiographic and histologic evaluations limited to maxillary incisors only (Brynolf 1967). Its validity to accurately represent the radiographic interpretation of other regions of the jaws has been called into question (Wu et al. 2009). Despite this, and other known limitations of planar imaging, such as low specificity (de Paula-Silva et al. 2009) and superimposition of anatomic structures (Mallya & Lam 2019); the index continues to be heavily relied upon in the endodontic literature, both clinically and epidemiologically (Tibúrico et al. 2021, Kvist & Hofmann 2023).

Figure 2.3 Sample images denoting the PAI ranging from score 1-5 (i). Example of PAI score 1 (LL7) **(ii).** Example of PAI score 2 (LR6) **(iii).** Example of PAI score 3 (UR1) **(iv).** Example of PAI score 4 (UL6) **(v).** Example of PAI score 5 (LR7)



Condensing osteitis, if present, was also noted in the radiographic report, an example of which, is provided in **Figure 2.4**.

Figure 2.4 Example of condensing osteitis (LL6)



2.3.3.3 Caries depth

The depth of caries, or any existing restoration, relative to the pulp chamber was assessed pre-operatively, from the periapical radiograph, by the principal researcher.

It was then catalogued in the clinical template (**Figure 2.2**) as one of the following: -

1. Caries absent (no caries/restoration),
2. Outer three quarters of coronal dentine,
3. Inner quarters of coronal dentine (deep caries)
4. Certain exposure, according to Bjørndal et al. 2019 (extremely deep caries)

2.4 Inclusion criteria

1. Healthy control

- a. Normal pulp: Tooth unrestored, or minimally restored, with normal responses to standard sensibility testing. Under normal conditions, cold testing elicits a sharp, momentary pain in the tooth, at a threshold comparable with other normal, healthy teeth in the mouth. The pain does not have a heightened, or exaggerated character and does not linger beyond a few seconds. There is an absence of pain and no evidence of extensive periodontal disease, or abscess. Periapical health verified radiographically to be $PAI \leq 2$.

2. Inflamed pulp

- a. A range of caries depth from mild-moderate to deep/extremely deep, or restoration not extending beyond the pulpal quarter of dentine or close to the pulp. A range of responses from slightly exaggerated response to sensibility testing, indicating a heightened threshold relative to neighbouring 'normal' teeth to an exaggerated response to hot, or cold stimulus, mimicking their presenting discomfort and lingering (≥ 20 secs). The tooth may be percussion, or palpation tender. PAI scores of ≤ 2 were accepted.

In addition, only teeth that could be isolated with dental dam were included in the study. The use of non-steroidal anti-inflammatory drugs (NSAID's) by the patient was permitted; however, these categories were 'flagged' during data collection so that

they could be subsequently separated during the analysis, as patients who had taken NSAID's within the previous 8 hours and those who had not.

2.5 Exclusion Criteria

1. Patient factors: Uncooperative patients and those diagnosed with chronic inflammatory, or metabolic conditions (i.e. rheumatoid arthritis, diabetes, autoimmune disorders) and immunocompromised patients. If the patient refused to enrol in research study, or was pregnant. A patient history suggestive of a complex pain disorder, or any form of non-odontogenic pain.
2. Tooth factors: Presence of acute infection and moderate–severe marginal periodontitis. A necrotic pulp confirmed by negative response to thermal and electric pulp testing with clear evidence of a periapical radiolucency (PAI>2), or a condensing osteitis, or an internal or external resorption lesion. A previous history of dentoalveolar trauma, or a tooth unable to be isolated with dental dam.

2.6 Experimental diagnostic groups

From the evidence gathered during history, clinical examination, special tests and radiographic examination diagnostic categories were assigned. Two diagnostic systems were used; namely the Classic (Seltzer et al. 1963a) and novel (Wolters et al. 2017) descriptors of pulp status. These diagnoses ranged from normal pulps, through progressively more inflamed pulps, to pulpal necrosis (Introduction -**Table 1.1**) and were defined as follows:

2.6.1 Classic classification (Seltzer et al. 1963a)

- Normal pulp: Normal responses elicited to standard sensibility testing (cold and EPT). The tooth was not tender to percussion, or palpation.
- Reversible pulpitis: May elicit a normal, or heightened response to cold testing, which does not linger. No tenderness to percussion or palpation. The patient reports a history of thermal, or sweet sensitivity in response to a stimulus. The pain is sharp in character and does not linger.
- Irreversible pulpitis: A heightened response to a cold, hot, or electric stimulus. Tooth may be tender to percuss. The patient describes a range of severe pain symptoms, which may, or may not be triggered by a thermal stimulus, particularly heat. Spontaneous, lingering pain can be reported. The character can be initially sharp, which then develops into a dull throbbing long-lasting pain. Analgesia is often required and may, or may not, be effective in eliminating the pain. Sleep can be disturbed and chewing pain is also a common feature.

2.6.2 Wolters' classification (Wolters et al. 2017)

- Initial pulpitis: A heightened response elicited to cold stimulus, which does not linger. The patient describes an intermittent sensitivity to thermal, or sweet stimuli. The character is a low-grade discomfort which resolves instantly.
- Mild pulpitis: A heightened and lengthened response elicited by cold, or heat, or sweet stimuli; which lasts up to 20 seconds before resolving completely. May be percussion sensitive. The patient describes a pronounced, frequent

sensitivity to thermal, or sweet stimuli. The character is sharp and unpleasant, lasting up to 20 seconds.

- Moderate pulpitis: A heightened, prolonged reaction to cold stimulus, which lingers for minutes. May be percussion sensitive. The patient describes a severe pain, usually with a thermal trigger, although spontaneous pain can be a feature. Initially the character is sharp for up to 20 seconds, followed by a dull, ache lasting up to several minutes. Analgesia may be needed, but is usually effective for relief.
- Severe pulpitis: Severe, spontaneous pain, reactive to warm or cold stimuli, range from sharp to dull throbbing lingering and percussion sensitive. The patient presents with severe, persistent spontaneous pain, or triggered by warm and cold stimuli, which ranges from sharp and severe to a deep, dull throbbing ache, lasting for minutes to hours. Sleep is regularly disturbed and the tooth is usually percussion sensitive. Analgesia is often needed, but may not fully eliminate the pain.

2.7 Visual inspection of the exposed pulp

In order to add diagnostic information intraoperatively, the exposed pulp tissue was examined under dental dam, using an operating microscope (OPMI Pico, Carl Zeiss Ltd., Cambridge, U.K.) for all cases. The photographs were captured using a Nikon Coolpix 4500 (Nikon Corporation, Tokyo, Japan) through the microscope. The magnification ranged between 6x and 10x. Clinical photographs were captured at initial exposure of the pulp horn/chamber. A sequence of static images was recorded in each case, in order to demonstrate and illustrate the dynamic nature of the

bleeding; from surface necrosis, to reduced vascularity, to a microabscess, or profuse and overflowing. The final appearance of haemostasis, or subsequent refill (persistent bleeding) was also photographed. Bleeding time was not routinely recorded. The relative ease with which haemostasis was achieved was noted by the principal operator. This was evident as instantaneous haemostasis in some instances, or only minimal rinsing required to achieve haemostasis. For more detail, see **Chapter 4**.

2.8 Clinical procedures

2.8.1 Preoperative evaluation

When suitable candidates were identified, a dental examination was completed, before diagnosis and treatment plan options were discussed by the dentist and agreed with the patient; only at that stage was the purpose of the study then explained to them and their voluntary participation canvassed. If the patient expressed an interest in the study, the procedure itself was discussed, along with the likely drawbacks of any participation. The patient information leaflet was described in more detail and their informed consent was requested. The fact that their participation was voluntary, would not affect their treatment provision and that they were free to withdraw at any time was made clear to each patient. Afterwards, the patient was asked to confirm that they had understood what had been discussed and asked if they have any questions they wished to ask. Every interested patient was given a written patient information sheet (**Appendix IIIa, IIIb**) for them to study at home and discuss further with their family, or partners. It is accepted (by the very

nature of toothache) though that some patients who require emergency treatment would have to consent to treatment and participation on the same day.

The assessment, treatment and routine follow-up was provided by a single operator, the principal researcher. A standard endodontic examination was carried out. The patients' pain history, if any, was recorded as described in **Section 2.3.1**. Routine endodontic diagnostic tests were carried out and recorded in a template document (**Section 2.3** and **Figure 2.2**), of the tooth in question as well as its neighbouring units. Finally, the clinical diagnosis was noted in accordance with the classification systems outlined in **Sections 2.6.1** and **2.6.2**. The patients' pain history was approximated to these diagnostic parameters. Patients attending with 'sound' teeth were able to return on a subsequent appointment to proceed with the agreed treatment. Cases presenting with clinical signs of an inflamed pulp were treated on the same day.

2.8.2 Operative procedure and general aspects of sample collection

After explanation, consent, final clinical examination and diagnosis a tooth was included in the study. Thereafter, local anaesthetic without a vasoconstrictor (Scandonest® 3% w/v, Septodont Ltd., Kent, U.K.) was administered by infiltration, or regional block as necessary. All treatments were carried out under dental dam isolation, OraSeal™ caulking agent (Ultradent Products GmbH, Cologne, Germany) and with the use of dental operating microscope. The tooth surface was cleaned with 30% (w/v) hydrogen peroxide (Sigma-Aldrich, Arklow, Ireland) and rinsed with 2% (w/v) sodium hypochlorite (Milton® Sterilising Fluid, Milton International, Nantes, France), prior to accessing the tooth and removing the existing restoration, or caries

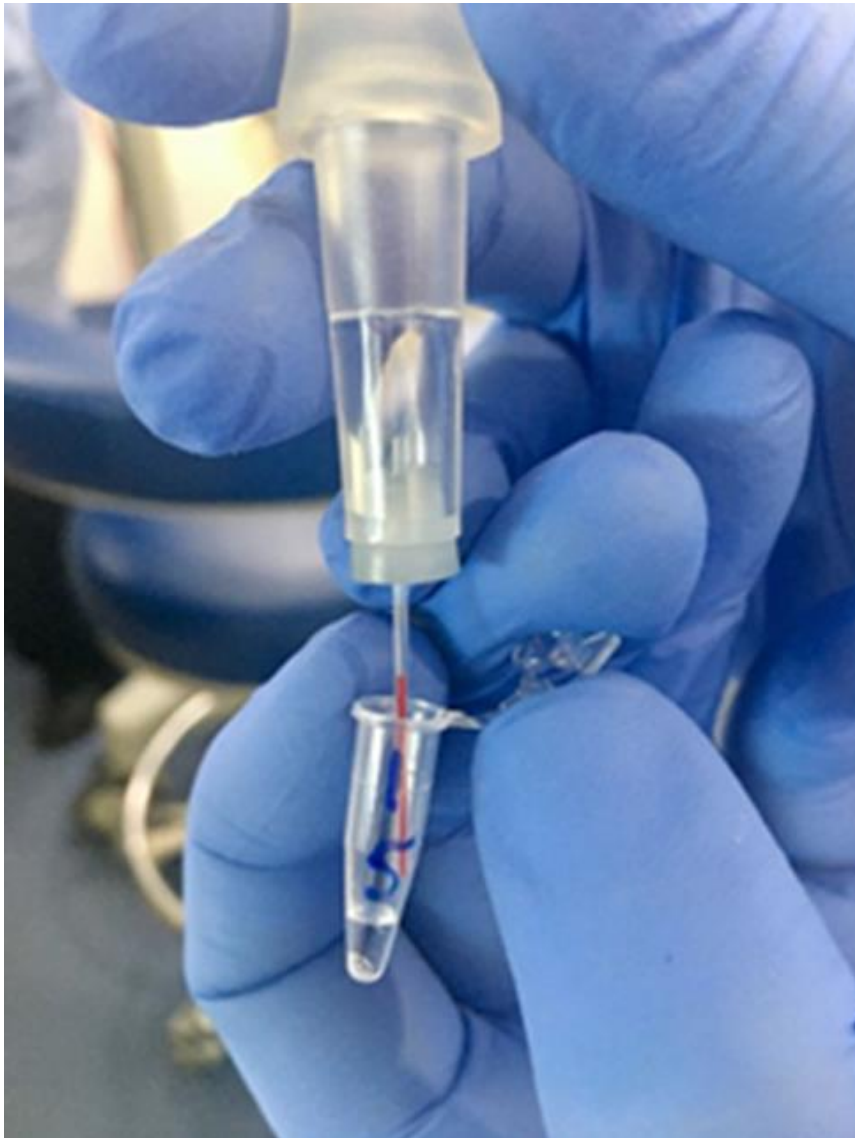
(if present). In line with standard operating procedures (Duncan et al. 2019a), the peripheral caries was removed prior to ‘unroofing’ the pulp chamber with a new, sterile diamond bur, under profuse water coolant. Excess water was carefully aspirated using Yankauer Suction Tubes (Romsons International, Enfield, London, U.K.), or paper points; being careful not to disturb the bleeding directly from the exposed pulp. The character of the initial bleeding encountered was noted and when possible, photographed (Nikon Coolpix 4500) as described in **Section 2.7**.

The pulpal blood samples were collected by microcapillary action (for more detail, see **Section 3.3.2, Appendix VIII**). Briefly, the collected blood was then expressed into a prepared polymerase chain reaction (PCR) tubes (Molecular BioProducts Inc., California, U.S.A.) (**Figure 2.5**) containing a predetermined amount of an anticoagulant (blood plasma samples only), a protease inhibitor (cOmplete ULTRA Tablets, Roche Diagnostics GmbH, Mannheim, Germany) and a known volume of PBS (Oxoid, Dublin, Ireland), prior to being stored on ice. The collected samples were designated for serum, or plasma extraction and stored upright, on ice until the procedure was completed and the patient discharged.

2.8.2.1 Pulpotomy procedure

Once the collection of pulpal blood had been completed and photographic images captured, the pulpotomy was then completed, using an enhanced protocol i.e. magnification, isolation and antiseptic lavage (Duncan et al. 2019a) by the principal researcher. All single rooted tooth had a full coronal pulpotomy carried out, with pulp amputation to the level of the root canal orifice. In multirrooted teeth, although partial pulpotomy was attempted in all cases, the majority of cases had full

Figure 2.5 Expression of collected blood into PCR from 5 μ L Minicap®, using a pipetting aid



coronal pulpotomy carried out. Amputation of the pulp was completed in each case with a new, sterile flat-ended, tapered diamond bur 012, or 014 (Frank Dental GmbH, Gmund, Germany), under water coolant. The exposed pulp was irrigated with 2% solution of sodium hypochlorite using a Leurlock Syringe 5ml (Monoject™, Covidien, Medtronic, Galway, Ireland) with a 27-gauge, side-vented needle (Appli-Vac™, Vista Dental, Wisconsin, U.S.A.). Gentle pressure was applied using a cotton wool pellet soaked in sodium hypochlorite before the tissues were inspected for haemostasis.

Irrigation, cavity wound cleaning and gentle reapplication of pressure was repeated in increments of 2 minutes, up to a maximum of three times, if necessary.

Once haemostasis was confirmed visually, and the cavity walls were cleaned of any blood residue, an HSC was prepared according to the manufacturer's instructions. Initially, White ProRoot MTA (Dentsply, Tulsa, OK, U.S.A.), was used and latterly, Biodentine™ (Septodont, Saint Maur des Fosses, France) was the HSC of choice. A glass ionomer cement (GC Fugii II LC®, GC Corporation, Tokyo, Japan) was placed over the HSC, once the initial set was evident. The tooth was then restored using dentine bonding agents and resin composite materials, once appropriate enamel and dentine etching with 37% phosphoric acid was completed and rinsed. This completed the pulpotomy procedure.

2.8.2.2 Root canal treatment procedure

Root canal treatment was deemed necessary when haemostasis was not achieved at the deepest level of amputation at, or just below the root canal orifices; or if required for prosthodontic purposes. Failure to establish haemostasis was manifest when a persistent refill of blood into the pulp chamber continued after repeated applications of light pressure using a cotton wool pellet soaked in 2% sodium hypochlorite for 2-minute intervals, up to a maximum of 6 minutes, after the deepest point of amputation had been reached.

After completion of the access cavity, root canal treatment was carried out using a modified crown down technique with ReadySteel K-Flexofiles® (Dentsply Sirona, Ballaigues, Switzerland) and Maillefer ProTaper Gold® (Dentsply Sirona, Ballaigues, Switzerland) in an X-Smart™ Plus rotary motor (Dentsply Sirona

Baillaigues Switzerland). The working length was determined using an apex locator (Root Zx, J. Morita Corp., Osaka, Japan). Irrigation was carried out using 2% sodium hypochlorite throughout the procedure. The penultimate rinse used was 17% ethylene diamine tetra-acetic acid (EDTA) [Endo-Solution, PPH Cerkamed Stalowa Wola, Poland]. The final phase of intra-canal irrigation was completed with sonic activation using the EndoActivator® kit (Dentsply Sirona, Baillaigues Switzerland) for 60 seconds. Warm vertical obturation was completed using a zinc-oxide eugenol sealer (Pulp Canal Sealer™ EWT, Kerr™ Italia, S.r.l., Scafati, Italy), after an acceptable master cone (ProTaper Gold® Conform Fit™ Gutta Percha, Dentsply Sirona, Baillaigues Switzerland) demonstrated acceptable tug-back and clinical length, which was subsequently verified radiographically. Core filling was completed adhesively by the principal operator. This completed the root canal treatment procedure. The patient was returned to their primary dental provider to proceed with cuspal coverage, if indicated.

2.8.2.3 Extraction

The extraction of healthy control teeth was co-ordinated with the Oral Surgery Department within the DDUH. Patients requiring extraction of sound premolar teeth for orthodontic purposes were canvassed as described in **Section 2.2**. Once their informed consent was obtained, they were scheduled on an alternative date for the pulpal blood sampling followed immediately by the extraction. The patients were aware they could change their mind and withdraw from the study, if they wished and this decision would not adversely impact on their planned treatment. On their return for intervention, their explicit consent was again sought before administration of

local anaesthetic as described in **Section 2.8.2**. Pulpal blood collection was carried out in the same manner as for all cases, after the tooth had been cleansed and isolated under dental dam (**Section 2.8.2**).

On completion of the study aspects, the patient was then escorted directly to the Oral Surgery Clinic for completion of their planned extraction. Extraction were completed uneventfully by forceps and elevation, intact. Post-operative instructions and routine follow up were provided by the Oral Surgery Department. This concluded their involvement in the study. The patients were subsequently returned to their referring orthodontists for continuation of their treatments.

2.8.3 Patient follow-up

Patients were given appropriate post-operative instructions including the use, if needed, of analgesics. They were encouraged to return, or phone for advice, should any untoward event occur. This included severe post-operative pain, or swelling, or anything that caused the patient concern. On completion of the operative interventions the patient were returned to their primary dental caregiver for their continuing dental care.

2.8.4 Outcome evaluation

The outcome of operative interventions was followed up clinically and radiographically at regular intervals, according to standard clinical practice. Patients were invited to return for periodic review following pulpotomy and root canal treatment. A standard clinical examination was conducted including recording a pain history (if any), a routine endodontic examination including standard special tests

and a radiographic examination, as described in **Section 2.8.1**, with a few exceptions. Sensibility testing was not necessary for the endodontically treated teeth. Cold testing was conducted as a matter of routine, on teeth with pulpotomy, as well as their adjacent units. Percussion, palpation periodontal probing tooth mobility, presence of sinus /or swellings and tooth colour were all assessed as described earlier (**Section 2.3**). A periapical radiograph was recorded and evaluated by the principal researcher, using the PAI index (Ørstavik et al. 1986).

2.8.4.1 Pulpotomy outcome definitions

A pulpotomy was deemed successful when the patient reported no associated pain, or sensitivity; the clinical evaluation revealed no tenderness to percussion, or palpation, as well as absence of associated swellings, or sinus and the radiographic evaluation revealed a PAI score ≤ 2 . Although cold testing (Endo Frost, Coltène/Whaledent® GmbH+Co. KG) was carried out as a matter of routine, absence of a response to cold was not regarded as a negative finding in cases where a full pulpotomy had been carried out (Careddu & Duncan 2021, Duncan et al. 2021, Aravind et al. 2022).

The treatment was regarded as unsuccessful if the patient reported pain; the clinical evaluation revealed tenderness to percussion, or an apical radiolucency was noted (PAI ≥ 3). Other notable factors related to treatment failure included secondary caries and tooth fracture

2.8.4.2 Root canal treatment outcome definitions

Root canal treatment was deemed successful when the patient reported absence of pain; the clinical evaluation revealed no percussion, or palpation tenderness, absence of associated swellings, or sinus and radiographic evidence of a healthy periapical region, scoring PAI ≤ 2 (ESE, 2006).

The treatment was deemed unsuccessful if the patient described symptoms, or loss of function; the clinical evaluation revealed tenderness to percussion, or palpation, an associated sinus, or swelling had developed; or radiographic evaluation revealed a PAI score ≥ 3 (ESE, 2006).

2.9 Laboratory procedures

2.9.1 General

All laboratory procedures were carried out under aseptic conditions and in line with DDUH infection prevention protocols. Type I ultrapure water (Milli-Q Direct 8 Water Purification System, Millipore, Cork, Ireland), defined as ion-free and having low conductivity with Total Organic Carbons (TOC) was used for all relevant procedures. For accurate weighing of reagents, a calibrated analytical balance (ABS80-4N, Kern, Barcelona, Spain) was used. For liquid handling, Eppendorf single-channel pipettes were used, ranging in size from P-2.5 to P-5000 (Eppendorf, Hamburg, Germany). Sterile TipOne Filter Tips were used for all procedures (StarLab, Milton Keynes, U.K.). All centrifuge steps were carried out using a chilled centrifuge at 4°C (Eppendorf® Centrifuge 5425 R, Eppendorf AG, Hamburg, Germany), for 15 minutes at 13,552 x g.

2.9.2 Preparation of reagents

- Phosphate buffered saline: PBS with a pH of 7.5 was prepared by dissolving PBS tablets without calcium and magnesium in ultrapure water, which was then autoclaved at 121°C and cooled prior to use.
- Anticoagulant: A 1% stock solution of ethylene diamine tetra-acetic acid dipotassium salt dehydrate (K2EDTA) (Sigma-Aldrich, Arklow, Ireland) was made by dissolving one gram of K2EDTA in 100 ml ultrapure water, prior to storage at room temperature.
- Protease inhibitor: A 7% (v/v) stock solution of EDTA-free protease inhibitors (cOmplete ULTRA Tablets) was prepared according to the manufacturer's instruction. One tablet was dissolved in 1.5 ml ultrapure water immediately prior to use.

2.10 Statistical analysis

Descriptive statistics were produced using Microsoft Excel 2019 MSO. Statistical analyses were carried out on IBM SPSS Statistics 25 (IBM, Dublin, Ireland) and are described in each chapter in the appropriate Section.

Chapter 3

Methodological aspects of pulpal blood collection for diagnostic analysis

3.1 Introduction

3.1.1 The need to standardise pulpal blood collection in endodontics

A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001, Strimbu & Tavel 2010). Accurate identification of the threshold between reversible and ‘truly’ irreversible pulpitis is critical in endodontics and operative dentistry if minimally-invasive therapies are to be promoted, but this threshold has not yet been clearly established. Molecular tests hold significant promise in the area of pulp diagnosis, but rely on dentinal fluid, gingival crevicular fluid, pulpal blood, or a pulp tissue sample. Even with a predictable sample collection method, the interplay between chronic and acute inflammation, simultaneous reparative mechanisms and the complex regulation of the immune systems (Duncan et al. 2019b) means that the delineation of this threshold remains challenging. To date, the literature in this area has described the collection of pulpal blood using a range of poorly standardised methods, summarised in **Section 1.5** and in **Tables 1.4 - 1.9**.

3.1.2 Diagnostic limitations

Establishing a clear association between patient-reported symptoms and pulpal inflammation has traditionally been difficult (Hasler & Mitchell 1970, Michaelson & Holland 2002). Symptoms (when present) are often relied upon as indicators of the level of pulpal damage, with more pronounced symptoms being indicative of severe, or irreversible pulpitis. Patients, however, may present with no symptoms even when

advanced carious lesions are evident radiographically, can “silently” progress to necrosis in up to 40% of cases (Michaelson & Holland 2002). Diagnostically, in the absence of symptoms suggestive of pulpitis, it can be unclear whether indirect, or direct pulp treatment should be instigated (Bjørndal et al. 2019). Equally, the radiographic depiction of a pulp exposure, in the presence of extremely deep caries is regarded by some (Ricucci et al. 2014) as an insensitive tool. Despite its known drawbacks, the character of pulpal bleeding and/or the ability to achieve haemostasis within a reasonable timeframe, are often the intraoperative clinical parameters by which clinicians decide whether the pulp can be retained in part, or removed entirely (Matsuo et al. 1996; Careddu & Duncan 2021).

3.1.2 The need for molecular markers

Patients that present to an emergency or endodontic clinic, will be self-selecting, as their presenting pain will be their principal reason for attending (Rechenberg et al. 2016b). Diagnostic accuracy studies highlight some of the limitations in diagnosing the spectrum of presentations of pulpitis, including the strict exclusion criteria applied in such studies (Wieslander et al. 2009, Villa-Chavez et al. 2013, Jespersen et al. 2014, Pigg et al. 2016) that skew the distribution toward the extremes, making the subtler, or complex diagnoses even less certain. The value of the literature in the field of diagnostic accuracy is to confirm our ability to identify health and necrosis. This serves to potentially inflate our perception of our diagnostic accuracy. What is lacking is a test, or suite of tests to precisely measure where on the spectrum of pulpitis the patient presents (Donnermeyer et al. 2022, Richert et al. 2022), to

objectively inform clinicians when to intentionally provide direct, or indirect pulp therapy and when the pulp, or part of it, can be maintained.

3.1.4 Suitable substrate

The substrate, or analyte most commonly used for investigating pulpitis is pulp tissue biopsies collected during pulpectomy, or pulpotomy; or following extraction of the tooth, when the entire pulp has been removed with careful dissection (Cohen et al. 1985, Rauschenberger et al. 1997, Isett et al. 2003, Petrini et al. 2012). The pulp tissues are then processed for further investigation in a range of downstream assays including gene, or protein expression using candidate, or high-throughput profiling techniques (Rechenberg et al. 2016a, Zanini et al. 2017, Richert et al. 2022). These experimental models rely on extracted teeth, or extirpated pulps to provide a sufficient mass of tissue to analyse, thereby prohibiting conservative treatments and more importantly, analysis of the outcomes of those treatments (Duncan 2022). Furthermore, due to the nature of handling pulp tissue, they are not conducive to a chairside diagnostic test.

Dentinal fluid and gingival crevicular fluid present attractive, minimally-invasive alternatives to excising pulp tissue (Karapanou et al. 2008, Zehnder et al. 2011). Their principal advantage relates to the conservative nature of the test, hence applicability in indirect pulp therapy and the non-exposed pulp. The provision of indirect pulp therapy poses a particular diagnostic difficulty. According to Wolters' classification, the suggested treatment for initial and mild pulpitis is indirect pulp therapy (Wolters et al. 2017). The difficulty in adopting an indirect approach, relates to the presence of symptoms, or anticipated responses to sensibility testing (Duncan

et al. 2019a, Donnermeyer et al. 2022), or the fact that the reported symptoms may not accurately reflect the true inflammatory state of the pulp tissue (Dummer et al. 1980, El-Karim et al. 2006). Other disadvantages include the variability and minute volumes of dentinal fluid available for collection (Knutsson et al. 1994), as well as the practical difficulties in extracting proteins from the absorbent vehicles, to provide consistent downstream protein analysis (Zehnder et al. 2011). Furthermore, if the pulp is exposed collection of dentinal fluid is not possible.

Pulpal blood represents another relatively minimally invasive substrate which can only be studied if the pulp is exposed. The ability to detect carious pulp exposure radiographically is limited (Bjørndal et al. 2002) and the clinical decision to provide direct, or indirect pulp therapy is driven by other clinical parameters such as clinical symptoms, or residual dentine thickness (Dumsha & Hovland 1985, Bjørndal et al. 2014). The processes involved in pulpal repair of the unexposed pulp, compared with the exposed pulp, are regarded as simpler and more predictable, being reactionary rather than reparative in nature and do not involve cytodifferentiation of a progenitor cell, to form a new secretory cell (Smith et al. 2001, Bjørndal et al. 2010, Ricucci et al. 2014).

There is considerable potential to harness a vastly under-utilised substrate (pulpal blood), which can be collected chairside and objectively analysed. Pulpal blood biomarkers could be evaluated for diagnostic accuracy and, potentially linked to conservative treatment outcome; however, prior to that, there is a need to standardise collection methodology, including receptacle, blood handling and analysis (**Section 1.5**).

Serum is the liquid portion of the blood after it has been allowed to clot. It is free of clotting proteins but contains metabolites that result from the clotting process (Bowen & Remaley 2014). It is a cleaner sample typically free of cells and platelets because they are trapped in the fibrin meshwork of the clot (Alsaif et al. 2012). Plasma, on the other hand, is the liquid portion of blood that has been prevented from clotting and is more reflective of the blood as it circulates in the body. Though it has an advantage over serum in that testing is not delayed by waiting 30 minutes for a clot to form, it is typically contaminated by platelets and cellular elements that have the potential to alter analytical results (Gleissner et al. 2008, Yin et al. 2015). Neither substrate has been widely investigated in the endodontic literature.

3.2 Study aims and objectives

The overarching purpose of this study is to investigate, identify and develop predictable molecular diagnostic markers of pulpal inflammation in pulpal blood.

In order to achieve a predictable and reproducible diagnostic testing methodology, several other aims are proposed. Immediate aims are to; ascertain the most appropriate vehicle in which to collect the pulpal blood sample, investigate the most effective blood analyte (serum, or plasma), as well as establishing the most appropriate laboratory testing technique (total protein, or volumetric) to predictably analyze the pulpal condition.

3.3 Materials and methods

Suitable dental patients were recruited, informed, consented, diagnosed and treated as described in **Sections 2.2 - 2.8**. Briefly, 50 patients were identified as needing

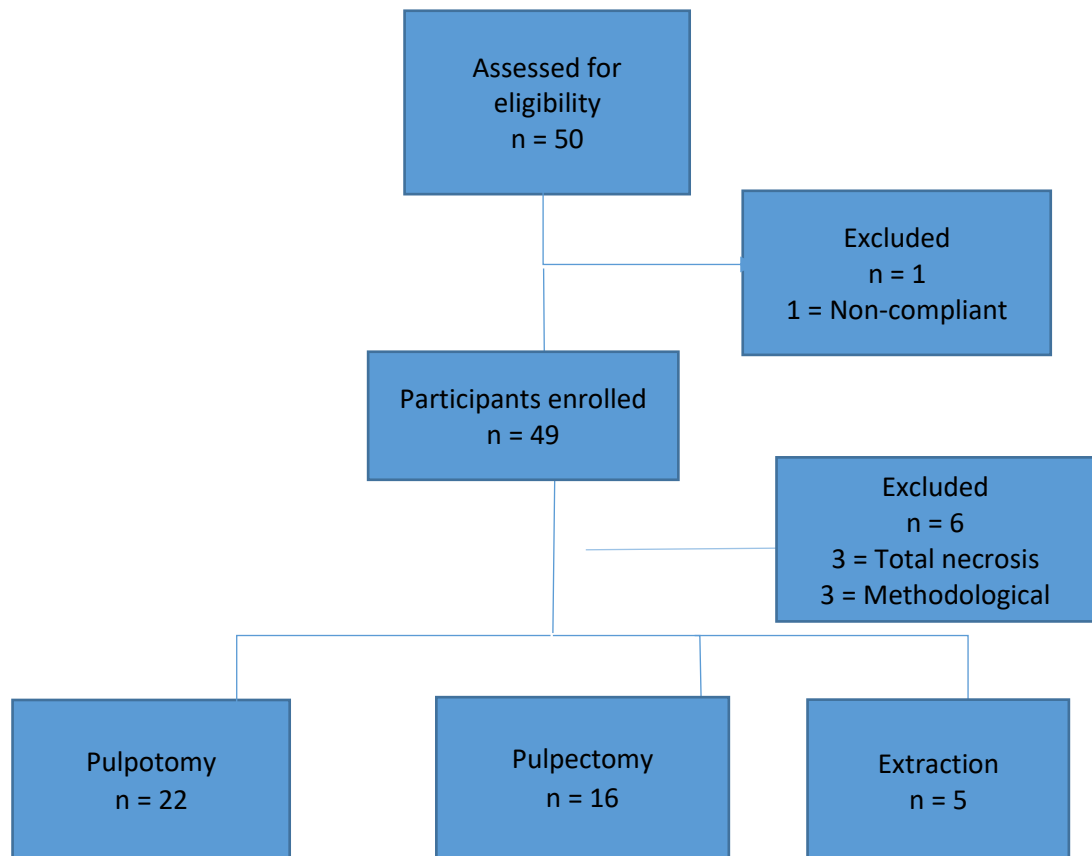
extraction, or restoration necessitating direct pulp therapy, or root canal treatment in the DDUH (**Figure 3.1**). The purpose of the research study was discussed with them and interested individuals were recruited to the study, once their informed consent was obtained. All patients were examined in a standardised manner (**Section 2.3**), inclusion (**Section 2.4**) and exclusion criteria (**Section 2.5**) were applied and a working clinical diagnosis (**Section 2.6**) and treatment plan (**Section 2.8**) were then formulated by a single operator.

In total, 50 patients were assessed for eligibility in the blood collection section of the study (**Figure 3.1**). One patient was excluded based on their inability to tolerate the dental dam. A further 6 patients were excluded from the analysis of pulpal blood after enrolment. Total necrosis was discovered on accessing three cases, clinically diagnosed as irreversible (severe) pulpitis. Three cases were excluded for methodological reasons, blood clotted in the microcapillary tube before it could be expressed into the collection tube; a second case was lost due to technical reasons as the blood spilled on transport to the laboratory and in the third case, it was not possible to collect pulpal blood immediately after extraction was completed.

The clinical procedure for all patients included the following common aspects (**Section 2.8.2**). Regional, or local infiltration anaesthesia was administered using local anaesthetic solution without a vasoconstrictor (Scanodnest 3% w/v). A dental dam was applied and secured with a suitable clamp. OraSeal™ (Ultradent Products GmbH) was applied before cleaning the tooth surface with 30% (w/v) hydrogen peroxide (Sigma-Aldrich) and rinsing with 2% (w/v) sodium hypochlorite solution. The pulp was ‘unroofed’ using a new, sterile diamond bur under water coolant, after

access preparation and/or non-selective caries removal had been completed. Careful suction was provided using a Yankauer (Romsons International) suction tube, being careful not to disturb the exposed pulpal fluid.

Figure 3.1 Flowchart depicting treatment designation in blood collection cohort [n=number]



3.3.1 Laboratory preparation in advance of blood collection

3.3.1.1 Introduction

In each phase of the studies presented, it was anticipated that the volume of blood collected could be standardised to a known level of dilution across all of the collected samples. Prior to sample collection, solutions had to be prepared in advance, based on a projected volume acquisition. The volume of available pulpal blood was expected to be limited to very small available volumes and the need for dilution of

the substrate was accepted, in line with similar published studies (ElSalhy et al. 2013, Mente et al. 2016). The decision to obtain a 1:6 solution of pulpal blood (**Figure 3.2**) was initially adopted based on the previous literature and initial testing.

3.3.1.2 Preparation of reagents

Reagents were prepared in advance, as described in **Section 2.9.2**. Flat Cap PCR tubes (0.2 ml) [Molecular BioProducts Inc.] were coded for planned serum, or plasma collection (based on the addition, or omission of an anticoagulant, respectively). Six separate solutions were prepared and stored on ice at chairside, on the basis of an anticipated 2 µl, 5 µl, or 10 µl whole blood collection (**Figure 3.2**) and each was identified alpha-numerically.

3.3.1.3 Preparation for blood plasma collection

In order to collect 10 µl of whole blood, 1 µl of the previously prepared 1% K2EDTA (Sigma-Aldrich) stock solution was added to each 0.2 ml PCR tube. Nine microliters of the 7% (v/v) stock solution of protease inhibitor (Roche Diagnostics) was added to the tube and 50 µl of PBS was added to all tubes. All tubes were then stored at -20°C until use, for up to three months. The same steps were carried out in respective proportions in order to collect 2 µl and 5 µl volumes (**Figure 3.2**).

3.3.1.4 Preparation for blood serum collection

To collect 10 µl of whole blood, 9 µl of the previously prepared 7% protease inhibitor stock solution (Roche Diagnostics) was added to 50 µl of PBS in the 0.2 PCR tube. All tubes were then stored at -20°C until required, or for a maximum of three months.

Figure 3.2 Dilution protocol to provide 1:6 solutions

a). 2 µL Collection

PLASMA SAMPLES ONLY K2 EDTA: {Sigma product 03659-100G, Ethylenediaminetetraacetic acid dipotassium salt dihydrate} 1% stock solution using distilled water Store at RT USE 0.2 µl for proposed 2 µl blood collection	ALL SAMPLES Protease inhibitor {cOmplete ULTRA Tablets, Mini, EDTA-free EASYpack 05 892 791 001} Stock solution dissolve one tablet in 1.5ml redistilled water (x7 Solution) Store 4 weeks -20°C USE 1.8 µl/2 µl	ALL SAMPLES Dilute all samples with 10 µl PBS

b). 5 µL Collection

PLASMA SAMPLES ONLY K2 EDTA: {Sigma product 03659-100G, Ethylenediaminetetraacetic acid dipotassium salt dihydrate} 1% stock solution using distilled Store at RT USE 0.5 µl for proposed 5 µl blood collection	ALL SAMPLES Protease inhibitor {cOmplete ULTRA Tablets, Mini, EDTA-free EASYpack 05 892 791 001} Stock solution dissolve one tablet in 1.5ml redistilled water (x7 Solution) Store 4 weeks -20°C USE 4.5 µl/ 5 µl	ALL SAMPLES Dilute all samples with 25 µl PBS

c). 10 µL Collection

PLASMA SAMPLES ONLY K2 EDTA: {Sigma product 03659-100G, Ethylenediaminetetraacetic acid dipotassium salt dihydrate} 1% stock solution using distilled water Store at RT USE 1 µl for proposed 10 µl blood collection	ALL SAMPLES Protease inhibitor {cOmplete ULTRA Tablets, Mini, EDTA-free EASYpack 05 892 791 001} Stock solution dissolve one tablet in 1.5ml redistilled water (x7 Solution) Store 4 weeks -20°C USE 9 µl/ 10 µl	ALL SAMPLES Dilute all samples with 50 µl PBS

Identical steps were carried out for the preparation of 5 µl and 2 µl volumes (**Figure 3.2**). The tubes were coded 'S' for serum, 'P' for plasma collection as well as 2, 5, or 10 according to the anticipated whole blood collection volume.

3.3.2 Blood collection

The pulpal blood samples were collected by microcapillary action (**Figure 3.3**), with initial pilot work necessary to establish how best to achieve this.

Figure 3.3 Blood collection by microcapillary action using a 10 µL Minicap



Table 3.1 outlines the details of the microcapillary tubes that were sourced from a variety of manufacturers (details provided in **Appendix VI**), to assess pulpal blood collection by way of a methodological pilot study and to determine the practicality of measuring protein expression of select candidate biomarkers by ELISA.

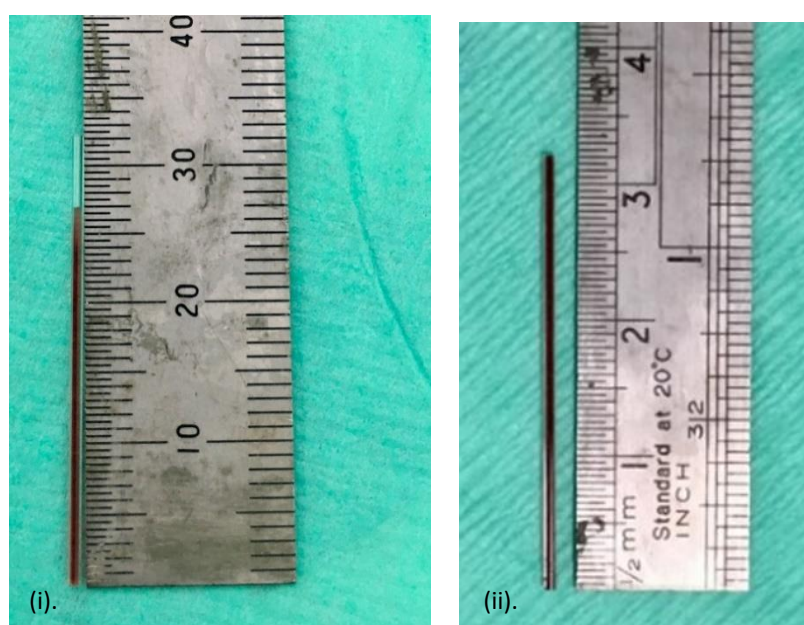
Three varieties of 20 µl microcapillary tubes were sourced: Minivette POCT (Sarstedt AG &Co. KG Nümbrecht, Germany), End-to-End Capillary 20 µl K3E (Sarstedt) and a

plain Minicap (Hirschmann, Eberstadt, Germany). The volume of the collected blood was precisely determined by measuring the penetration depth of the blood in the microcapillary tube, if incompletely filled (**Figure 3.4**) and then subsequently calculated relative to the volume capacity of the microcapillary tube.²⁴

Table 3.1 Details of the microcapillary tubes used in the pilot study for pulpal blood collection [A/C: Anticoagulant; Na-Hep: Sodium heparin; K3E: Ethylene diamine tetra-acetic acid tripotassium salt dehydrate]

Name	Supplier	A/C	Volume	Length	Internal diameter	External diameter
Minicaps	Hirschman	Plain	20 μ L	30 mm	1 mm	1.5 mm
Minicaps	Hirschman	Plain	10 μ L	32 mm	0.5	1 mm
Minicaps	Hirschman	Plain	5 μ L	32 mm	0.5 mm	1mm
Minicaps	Hirschman	Plain	2 μ L	32 mm	< 0.5mm	< 1mm
Minicaps	Hirschman	Na-Hep	10 μ L	32 mm	0.5 mm	1 mm
Ringcaps	Hirschman	Plain	10 μ L	125 mm	1.5 mm	2 mm
Minivette	Sarstedt	Plain	20 μ L	75 mm	1 mm	2 mm
End-to-end capillary	Sarstedt	K3E	20 μ L	29.5 mm	1 mm	2 mm

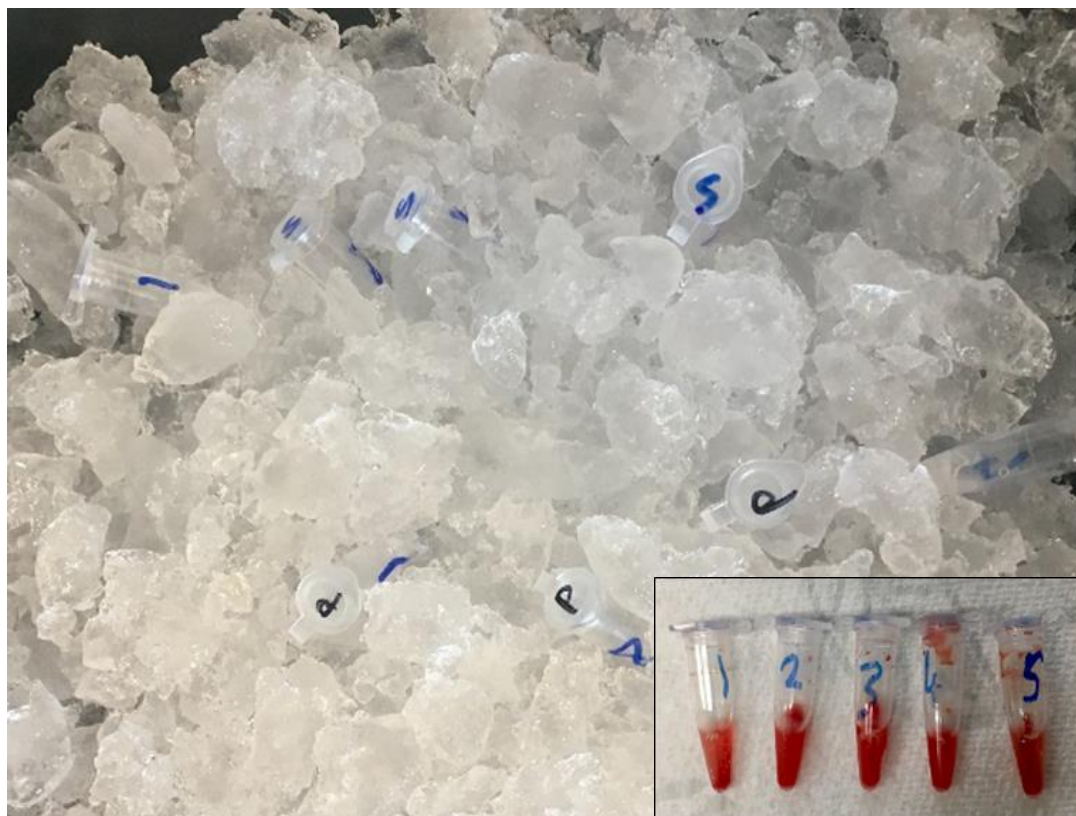
Figure 3.4 Measurement of collected blood volume from 10 μ L Minicap; (i). Incomplete fill; (ii). Complete fill



The collected blood was then expressed into the prepared PCR tubes (**Figure 2.5**) containing a predetermined amount of an anticoagulant (for the blood plasma

samples only), a protease inhibitor and a known volume of PBS, prior to being stored on ice. The collected samples were designated for serum, or plasma extraction and stored upright, on ice (**Figure 3.5**) until the procedure was completed and the patient discharged. Coding was alpha numeric sequence. A 'p' signified plasma collection, while an 's' signified serum collection. If multiple draws were possible, this was noted sequentially (p1, p2, p3 etc.).

Figure 3.5 PCR tubes coded and stored on ice prior to sample collection. Coded serum samples prior to centrifuge (inset)



3.3.3 Transport and storage of blood samples

The blood samples collected from the exposed pulp were transferred immediately to the prepared, coded 0.2 ml PCR tubes. The precise volume of blood collected in each

sample was calculated by manually measuring and recording the penetration depth of the blood in the microcapillary tube, corresponding to the maximum volume capacity of the microcapillary tube used (**Figure 3.4**). The samples were transported to the lab on ice (**Figure 3.5**), where they were 'spun' in a pre-chilled centrifuge (Eppendorf Centrifuge 5425 R), at 4°C (**Figure 3.6**) for 15 minutes t 13,552 x g.

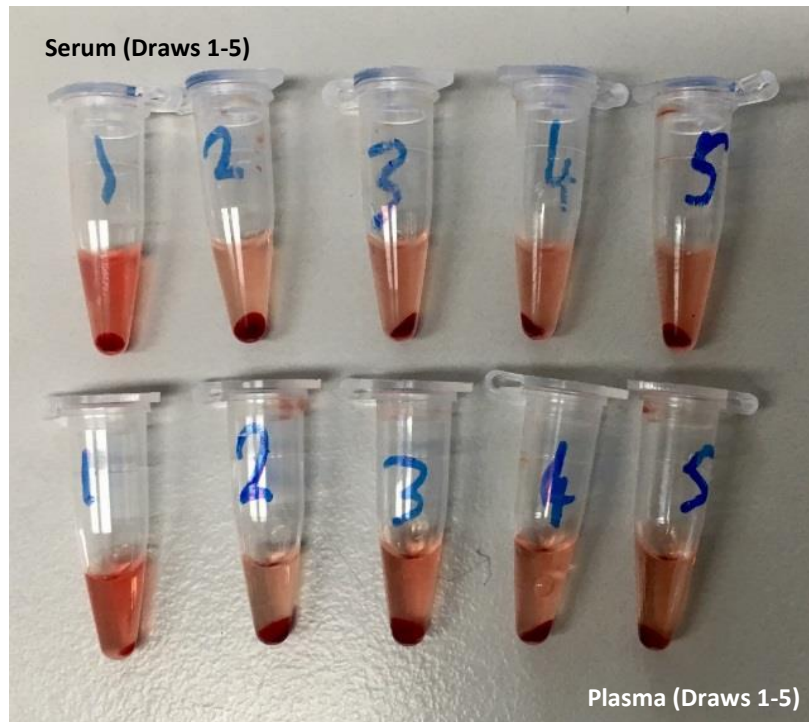
Figure 3.6 Samples in pre-chilled centrifuge at 4°C



The resultant supernatant (**Figure 3.7**) was extracted by pipette; from which, a known volume was then aliquoted and designated as either serum, or plasma (dependent on the addition, or omission of an anticoagulant during the preclinical handling [**Section 3.3.1.3 - 3.3.1.4**]). Each aliquot was pseudo-anonymously coded (**Figure 3.8**), prior to storage at -80°C, until ready for downstream molecular assay analysis.

The coded data were stored on a double encrypted computer, accessible by the principal researcher only, with the code held by a gatekeeper separate to all researchers in the study.

Figure 3.7 Coded serum and plasma samples after centrifuge



3.3.4 Laboratory testing of pulpal biomarkers-Introduction

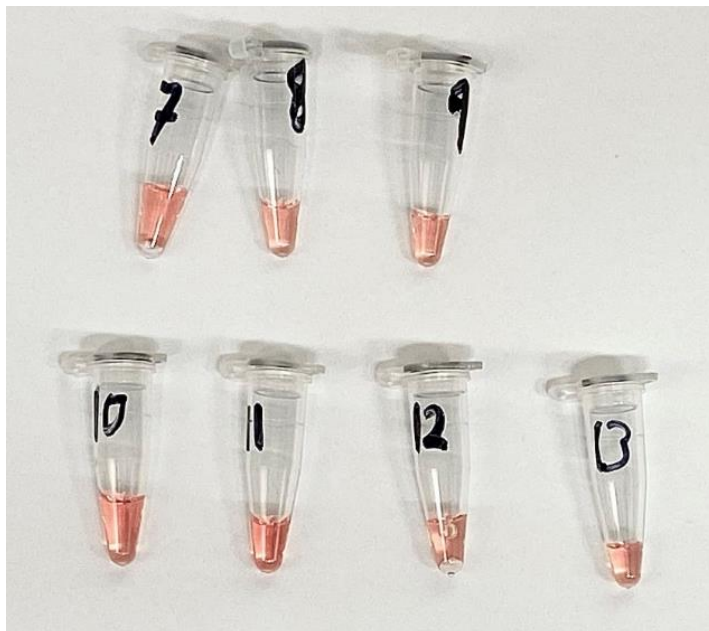
As one of the aims of the study was to ascertain the sensitivity and predictability of using molecular assays to analyse the protein levels of inflamed dental pulp blood samples; initially two commercially available ELISA's, a standard Human IL-6 ELISA (Invitrogen, ThermoFisher Scientific, Massachusetts, USA) and a Cell Death Detection (CDD) ELISA^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) were sourced and described in **Sections 3.3.4.2.1** and **3.3.4.3**.

Upon thawing, the samples were homogenised by vortexing at 1800 g in a Heidolph REAX top (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for

20 seconds. The samples were assayed either by using a volumetric method, in which the samples were diluted prior to assay, in a standard volume of ELISA diluent; or, the concentration of total protein was calculated first, using a Bradford assay (Bio-Rad Laboratories GmbH, München, Germany) [Section 3.3.4.1] in order to standardise the protein level between samples.

All reagents were prepared according to the specific manufacturer's instructions, in a laminar flow hood in the Tissue Culture Room, Dental Material Science Laboratory, DDUH. A plate shaker, Titramax 100 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) was used when indicated, for selected ELISA steps.

Figure 3.8 Known volumes of supernatant aliquoted and coded prior to storage at -80°C



The sample concentrations were determined (when possible) using a standard curve constructed in Microsoft Excel 2016 from seven serial dilutions of the

standard supplied with the kit and the concentrations reported in pg/ml. All biological samples were assayed in duplicate.

3.3.4.1 Total protein determination

The total protein concentration of the collected blood samples was determined using a Bradford assay (Bio-Rad Laboratories GmbH). Dye reagent (Bio-Rad Laboratories GmbH, München, Germany) was prepared by mixing 10 ml reagent with 40 ml distilled water and filtered through Whatman™ no. 1 filter paper (GE Healthcare Ltd., Buckinghamshire, UK). Bovine serum albumin (BSA) (Sigma-Aldrich, Arklow, Ireland) was used as a standard solution and serially diluted 7 times to establish a standard curve. The concentrations ranged from 0-1 mg/ml (0, 0.05, 0.1, 0.4, 0.8 and 1.0 mg/ml). One millilitre of dye reagent was added to 10 µl of the standard and its colour intensity read through a NanoDrop™ 2000/2000c Spectrophotometer (ThermoFisher Scientific, Massachusetts, USA) at 595 nm. The concentrations were calculated using the information obtained from the standard curve (**Appendix VII**). These samples were then further analysed by the candidate ELISA's (**Section 3.3.4.2.2, Section 3.3.4.3**). Due to limited volumes of serum and plasma available, in order to provide a constant sample volume of 110 µl it was necessary to add substrate diluent to provide sufficient sample solution (50 µl) to assay in duplicate.

3.3.4.2 Inflammatory marker interleukin-6 (IL-6) ELISA

The principle of the test was based on a sandwich design with anti-human IL-6 monoclonal antibody adsorbed onto microwells, which binds to the human IL-6 in the sample, or standard. Following a series of steps including antibody capture,

incubation and washing to remove the biotin conjugate anti-human IL-6 antibody and subsequent removal of streptavidin-HRP, a coloured product was formed proportional for the amount of IL-6 present in the sample or standard. The reaction was terminated and the absorbance measured at 450 nm using Tecan GENios™ Spectrophotometer (Unitech, Dublin, Ireland). The human IL-6 sample concentration was determined using a standard curve constructed from seven serial dilutions of the IL-6 standard supplied with the kit and concentrations reported in pg/ml.

3.3.4.2.1 Standard human IL-6 ELISA - Volumetric method

In the standard IL-6 ELISA (Invitrogen), 10 µl of the collected sample was further diluted with 90 µl assay buffer, allowing samples of 50 µl to be assayed in duplicate (1:9 dilution). A subsequent assay applied a 1:4 dilution (20 µl sample solution with 80 µl assay buffer). The limit of detection described by the manufacturer was 0.92 pg/ml.

3.3.4.2.2 High Sensitivity human IL-6 ELISA – Total protein methodology

The high-sensitivity (HS) human IL-6 ELISA (Bender MedSystems, Vienna, Austria) was carried out according to manufacturer's instructions in a similar manner to described above (**Section 3.3.4.2.1**) with the addition of an amplification reaction and has been successfully reported in the endodontic literature (ElSalhy et al. 2013). Concentrations were extrapolated from the standard curves, which were corrected for the dilutions and varying initial sample volumes (Excel, version 2016; Microsoft, Redmond, WA) and are reported in pg/ml of collected blood. The limit of detection as determined by the manufacturer was 0.03 pg/ml.

3.3.4.3 Cell Death Detection ELISA^{PLUS}

Cytoplasmic histone-associated-DNA-fragments were assayed using a sandwich ELISA Cell Death Detection (CDD) ELISA^{PLUS} kit (Roche Diagnostics). In brief, 10 µl of the aliquoted plasma, or serum sample was added to 40 µl incubation buffer, giving a total of 50 µl of test solution. This allowed for the samples to be assayed in duplicate. Thereafter, 20 µl of the test solution was diluted 1:4 in 1% BSA, 0.5% Tween, 1 mM EDTA in PBS, and added to streptavidin-coated microtiter plates, containing biotinylated mouse-anti-histone antibody and peroxidase-conjugated anti-DNA antibodies. After the completion of multiple standard washing steps, peroxidase activity of the retained immune-complexes was developed by incubation with ABTS (2, 2'-azino-di[3-ethylbenzthiazoline-sulfonate]) and read in a Tecan GENios™ Spectrophotometer (Unitech) at 405 nm. Subsequent results were reported as absorbance units (AU) as recommended by the manufacturer.

3.3.5 Statistical analysis

Descriptive statistics are presented including minimum, maximum, mean, median and standard deviations. The number and cumulative relative frequencies of the collected pulpal blood volumes were produced using Microsoft Excel 2019 MSO. Correlation graphs and data charts were constructed using Microsoft Excel 2019 MSO.

3.4 Results

Pulpal blood was collected by microcapillary action using a variety of microcapillary tubes, detailed in **Table 3.1** and **Appendix VI**. The decision as to which volume

capacity to select was made empirically, chairside. Multiple draws were attempted in every case. There was a great deal of variation in the rate of collection of pulpal blood between cases, which did not predictably relate to the apparent, visible haemorrhage observed by the principal operator. Even in the presence of profuse haemorrhage and blood pooling, the microcapillary action exhibited considerable variability. Speculatively, this may relate to intrapulpal pressure, or other factors (Chien 1985), which are beyond the remit of this study. Notably, it was possible to fill some tubes in a matter of seconds, whereas others would fill very slowly taking up to 2 minutes. The number of draws collected related to the ease of blood sampling. When the tubes filled quickly, multiple draws could readily be acquired. However, in cases where the rate of collection was especially slow, further draws were generally not possible. The raw data obtained from the blood collection arm of the study is catalogued in **Appendix VIII**, which lists the numbers of draws obtained from each case and the designation by substrate of the draws that were collected (for downstream serum, or plasma analysis). The vehicle by which the whole blood was collected and the measured volumes for serum, or plasma analysis.

3.4.1 Pilot study findings

Details of the dimensions of each of the microcapillary tubes used in the pilot study are summarised in **Table 3.1**. Tubes ranged in length from 29.5 mm to 125 mm. Internal and external diameters ranged from < 0.5 mm - 1.5 mm and from < 1 mm – 2 mm, respectively. The shorter length of the ‘End-to-End’ capillary (Sarstedt) was advantageous in terms of facilitating access (29.5 mm), however it became apparent that both the diameter (2 mm) and the volume capacity (20 µl) were not suited to

the collection of pulpal blood from teeth *in situ* and were designed for blood collection from other parts of the body. This became particularly obvious when the Minicap (Hirschmann) 2 µl and 5 µl were introduced to the study (**Section 3.3.2, Table 3.1**). In fact, no pulpal blood could be effectively collected using the 'End-to-End' capillary 20 µl K2E (Sarstedt), or the plain 20 µl Minicap (Hirschmann).

The 20 µl Minivette POCT (Sarstedt) was initially expected to be an ideal alternative collection vehicle, because the tube was tapered, it was coated with K3E (ethylene diamine tetra-acetic acid (EDTA) tripotassium salt dihydrate potassium salt), the preferred anticoagulant and the plunger design was expected to exert a negative pressure ensuring a rapid draw. The difficulties encountered with its use; however, ruled it out as the ideal collection vessel. These included, the wide tip diameter (2 mm), which precluded use in the confined space available during a pulpotomy procedure. A bulky plunger was impractical intraoperatively and its length > 75 mm, prevented access to certain anatomical areas of the mouth. The included plunger only facilitated the expression of fluid after collection and the tube diameter/volume capacity lead to an unacceptably slow microcapillary action. It was not possible to obtain a 20 µl volume of pulpal blood. The largest volume collected was approximately 5.3 µl (Minivette 20 µl). In addition, its cylindrical, variable taper did not lend itself to a precise measurement of exact volume.

Due to the limitations of the 20 µl Minivette POCT, the sodium heparinised 10 µl Minicap (Hirschmann) were investigated as a possible alternative. Plain microcapillary tubes in 2 µl, 5 µl and 10 µl Minicap (Hirschmann) were also sourced. Paper point collection was employed in selected samples, when the microcapillary

action failed, however, this introduced an additional variable into methodology as it was not possible to measure volume.

3.4.2 Pulpal blood collection

3.4.2.1 The effect of the collection vehicle

The internal diameter of the collection vessel appeared to have the most significant effect on the microcapillary action. The 2 µl Minicap (Hirschmann) was consistently the fastest to fill; followed, in order by the 5 µl Minicap, 10 µl Minicap and the 20 µl Minivette POCT (Sarstedt). The 20 µl Minicap and the 'End-to-End' Capillary 20 µl K2E (Sarstedt) were too slow to be useful clinically and were not used beyond the pilot for further analyses. The microcapillary action was noticeably more efficient when 5 µl Minicap vessels were used, which were more consistent in collecting the full capacity of the tube than the 10 µl Minicap. As a result, the 5 µl Minicap was selected for the bulk of collections and the 2 µl Minicap only introduced into the study for selected cases in which pulpal blood collection was particularly challenging.

The external diameter of the microcapillary tubes (**Table 3.1**) ranged from a maximum of 2 mm (20 µl), through 1 mm (5 µl, 10 µl) to 0.5 mm (2 µl). From a purely practical perspective, the larger diameters were more difficult to seat into the pulp chamber and did not lend themselves to a minimally-invasive pulpotomy technique. The 1 mm diameter vessels were easier to use, but did pose difficulties in certain premolar and incisor cases that were narrow in the mesio-distal and labio-palatal dimensions, respectively. The 0.5 mm diameter tubes allowed rapid microcapillary fill, but were more fragile and prone to fracture *in situ* during collection. Neither the

2 mm, nor the 1 mm diameter vessels lent themselves readily to a conservative approach as the pulp exposure needed to be relatively large in order to collect blood.

It was therefore concluded that the Hirschmann Minicaps plain microcapillary tubes were best suited for the collection of small volumes of pupal blood (**Figure 3.3, 3.4**). As a result of the variability in the efficiency of microcapillary collections observed in the pilot work, a selection of the following sizes was routinely prepared and brought to the clinic for samples collection: 2 μ l, 5 μ l and 10 μ l, using the dilution protocols presented in **Figure 3.2**.

3.4.2.2 Vehicle

The practical implication of wider diameter microcapillary tubes relates to the time taken to fill even a small portion of the tube. During the first collection incomplete filling exceeded 4 minutes, as a result the blood clotted inside the tube and it was no longer possible to express the blood into the pre-prepared 0.2 ml PCR tube. Subsequent 'draws' of pupal blood from this first case were also unsuccessful. As a result of this experience, extensive methodological pilot work was then conducted on a variety of collection vessels (**Section 3.3.2, Table 3.1**).

The maximum volume collected in a single case was 114 μ L, while the minimum volume was 3.75 μ L. The average volume collected over 29 cases was 44 μ L, with a median volume of 34 μ L. The numbers of draws ranged widely from 2-21 (**Table 3.2**).

3.4.2.3 Hirschmann Minicaps

Table 3.2 lists the number of complete and incomplete fills obtained using 2 μl , 5 μl and 10 μl Minicaps. The highest number of complete fills (92) were acquired using the 5 μl Minicaps. An equal number of incomplete fills ($n = 41$) were acquired using the 5 μl and 10 μl Minicaps. The lowest number of incomplete fills were obtained using the 2 μl Minicaps.

Table 3.2 Details of pulpal blood collection related to the various volume capacities and acquisition of the Minicap vessels used

Details of vehicle	Frequency
Incomplete 2 μl fills	10
Complete 2 μl fills	23
Incomplete 5 μl fills	41
Complete 5 μl fills	92
Incomplete 10 μl fills	41
Complete 10 μl fills	35

Figure 3.9 presents the cumulative relative frequencies of complete and incomplete fills according to their volume capacity (2 μl , 5 μl , or 10 μl), in each case.

Figure 3.9 (i). shows that the 2 μl microcapillary tube was completely filled once only per case 38% of the time, twice per case 13% of the time and with an incidence of 25% each, of four and five fills in each individual case. The incidence of a single incomplete fill (2 μl , **Figure 3.9 (ii).**) per case was 33.33%. Three incomplete 2 μl fills (**Figure 3.9 (ii).**) were recorded with a frequency of 66.67% in individual cases.

3.4.2.4 Volume

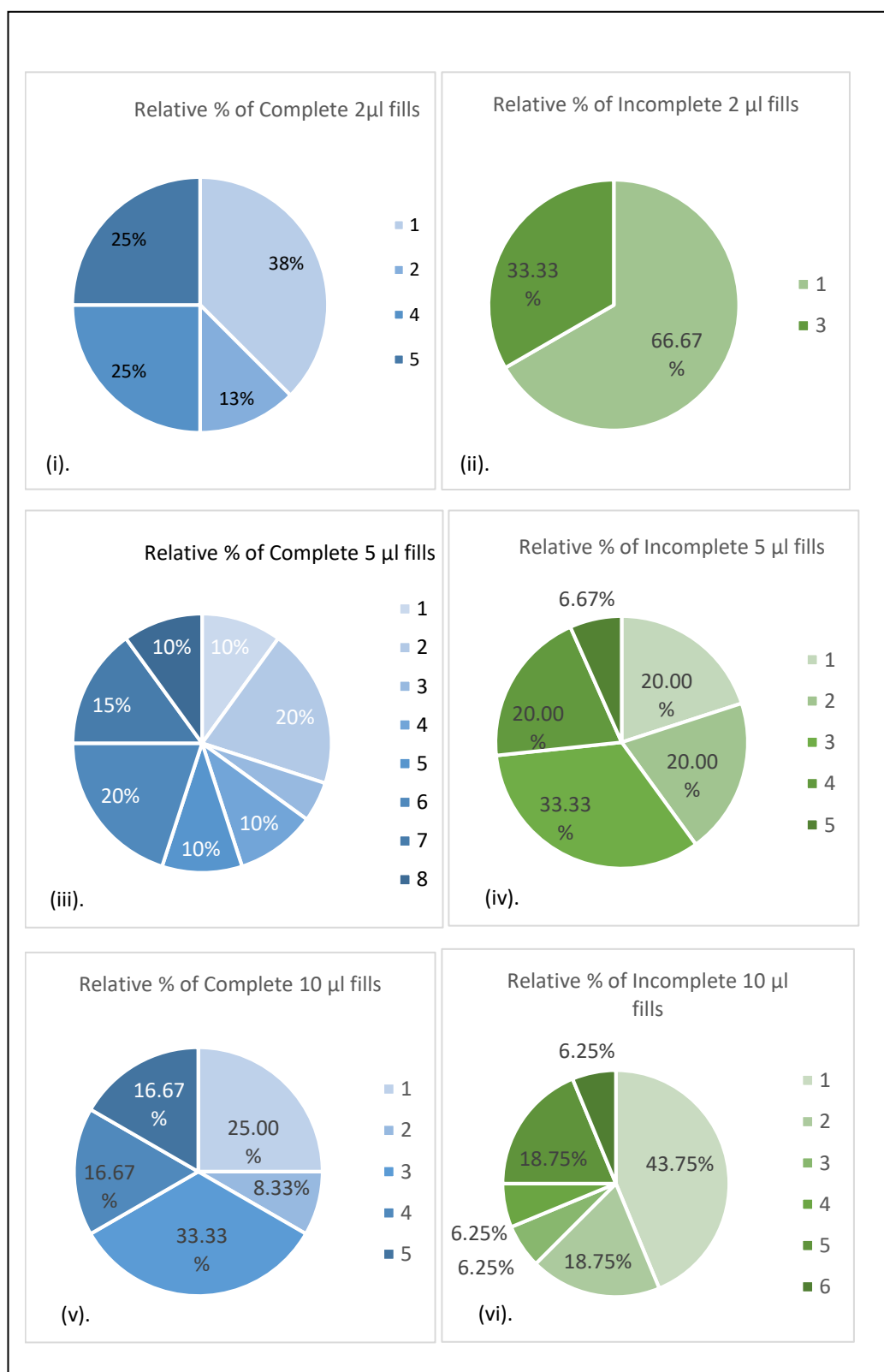
Pulpal blood was collected by capillary action using a variety of microcapillary tubes (detailed in **Section 3.3.2** and **Table 3.1**). Volumes of blood were collected via numerous draws in order to maximise the substrate available for analysis (**Appendix VIII**). **Table 3.3** provides a brief summary of overall blood volume collections. The maximum volume collected in a single case was 114 μL , the minimum was 3.75 μL . The average volume collected was 44 μL and the median was 34 μL . The number of blood draws per case ranged from 2 up to 21.

Table 3.3 Summary data of whole pulpal blood collections

Blood Collection	
Maximum volume (μL)	114
Minimum volume (μL)	3.75
Average volume (μL)	44
Median volume (μL)	34
Number of draws (Range)	2-21

Interrogation of the raw data documented in relation to blood volume collection (**Appendix VIII**) revealed a number of cases (Tooth numbers 2, 3, 4, 5, 10, 15, 28) where the collected volumes were at, or below 10 μL . Qualitative review of the associated clinical and radiographic data (**Appendix XI**), revealed a consistent observation of pulp chamber sclerosis and reduced vascularity. A representative case is depicted in **Figure 3.10**.

Figure 3.9 Cumulative relative frequencies of complete and incomplete Minicap vessel collections;
(i). Relative % of complete 2 μ l fills; (ii). Relative % of incomplete 2 μ l fills; (iii). Relative % of
complete 5 μ l fills; (iv). Relative % of incomplete 5 μ l fills; (v). Relative % of complete 10 μ l fills; (vi).
Relative % of incomplete 10 μ l fills [%: percentage]



3.4.3 Which substrate? Serum, or plasma?

The combined serum and plasma results of the HS human IL-6 ELISA (total protein method) and CDD ELISA^{PLUS} (volumetric method) are presented in **Figures 3.11** and **3.12**, respectively. The individual readings from multiple draws were averaged and presented as a single value, with error bars indicating their standard deviations. The correlation between serum and plasma using the HS IL-6 ELISA was low ($R^2 = 0.2591$, data not shown), as demonstrated by the total protein results (**Figure 3.11**), possibly due to the low number of samples for comparison, as well as the necessary dilutions applied to conduct the assays (**Section 3.4.4.1**). The correlation demonstrated by the CDD ELISA^{PLUS} volumetric data was high [$R^2 = 0.8648$] (**Figure 3.13**) indicating, at least for this assay, that serum, or plasma could be used as a substrate for analysis of pulpal blood.

Figure 3.10 Case representing (i). the radiographic appearance of pulp chamber sclerosis, with images depicting (ii). initial appearance upon unroofing the pulp chamber and (iii). reduced vascularity associated with pulp chamber sclerosis.

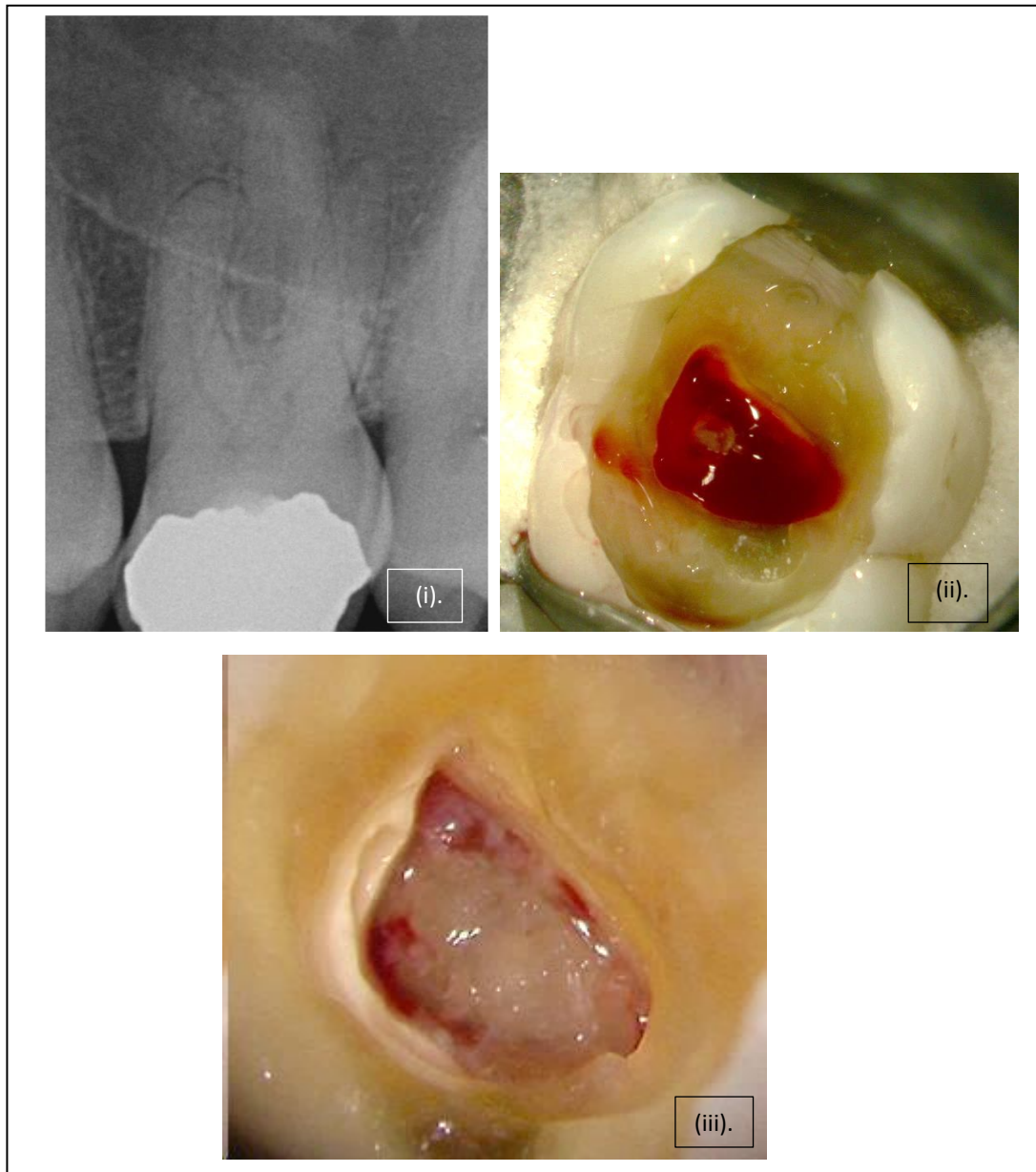


Figure 3.11 Summary findings comparing serum and plasma by HS Human IL-6 ELISA using a total protein method [Tooth 12 is the negative control]

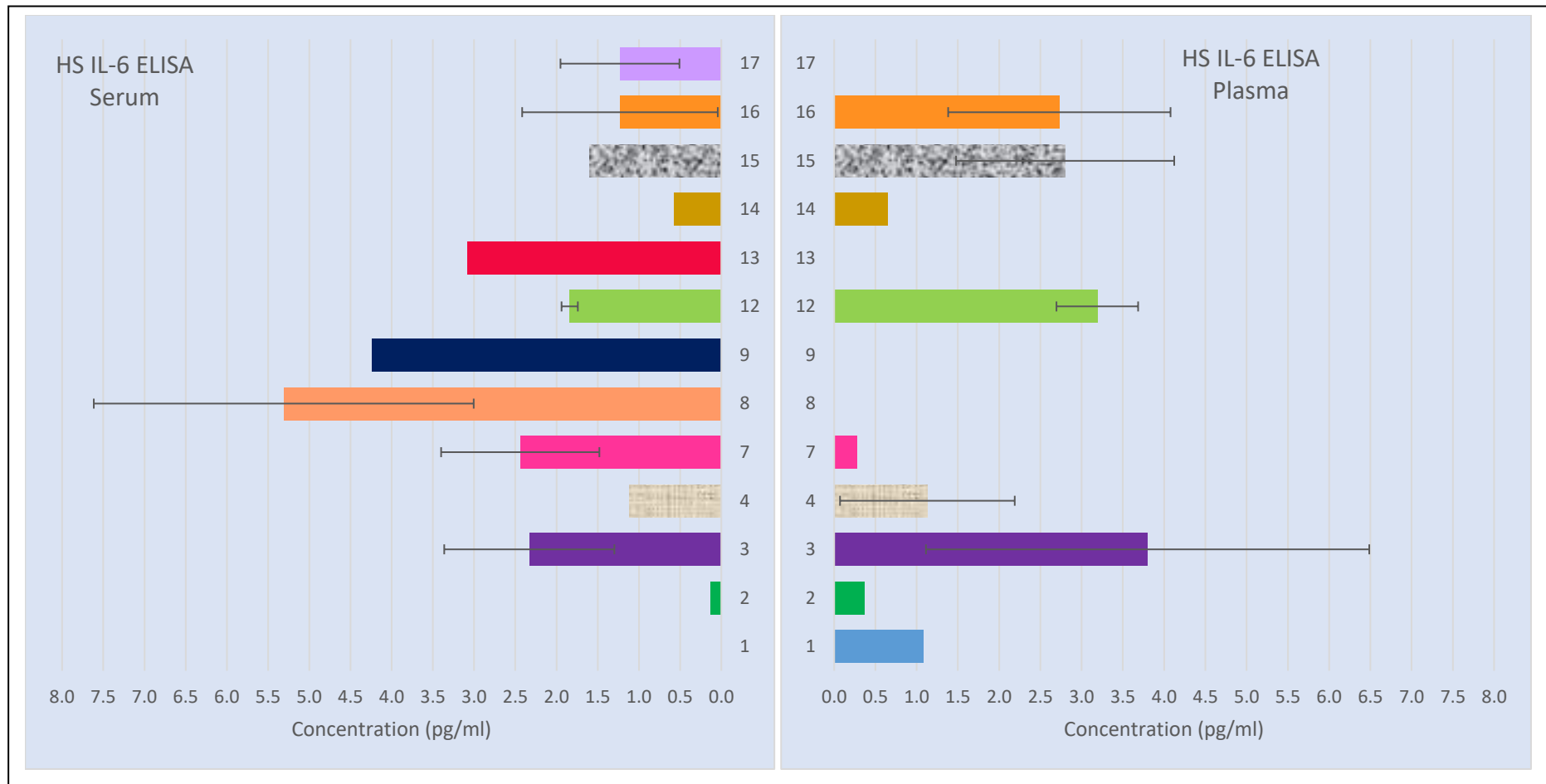


Figure 3.12 Summary findings comparing serum and plasma by CDD ELISA^{PLUS} using a volumetric method [Tooth 12 is the negative control]

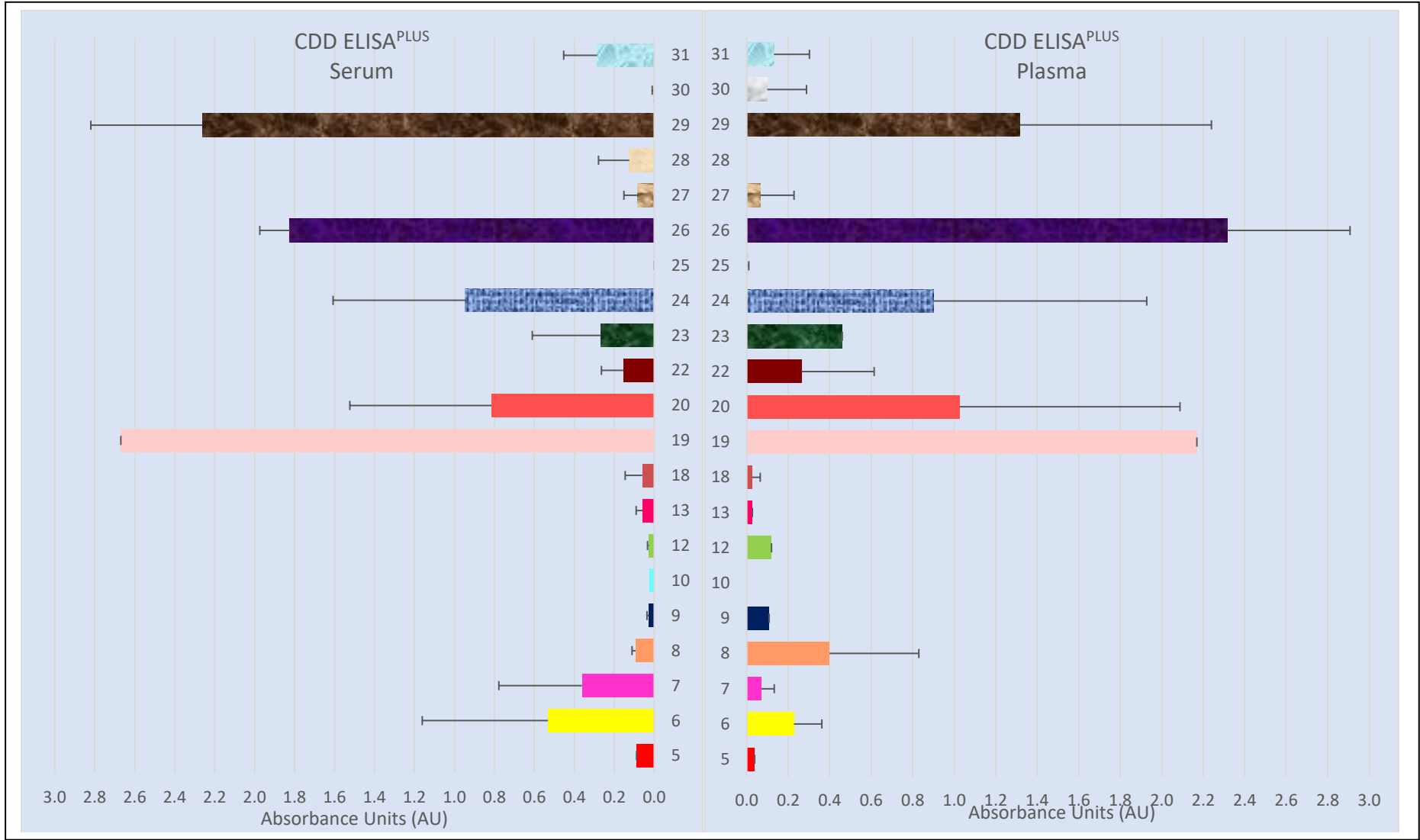
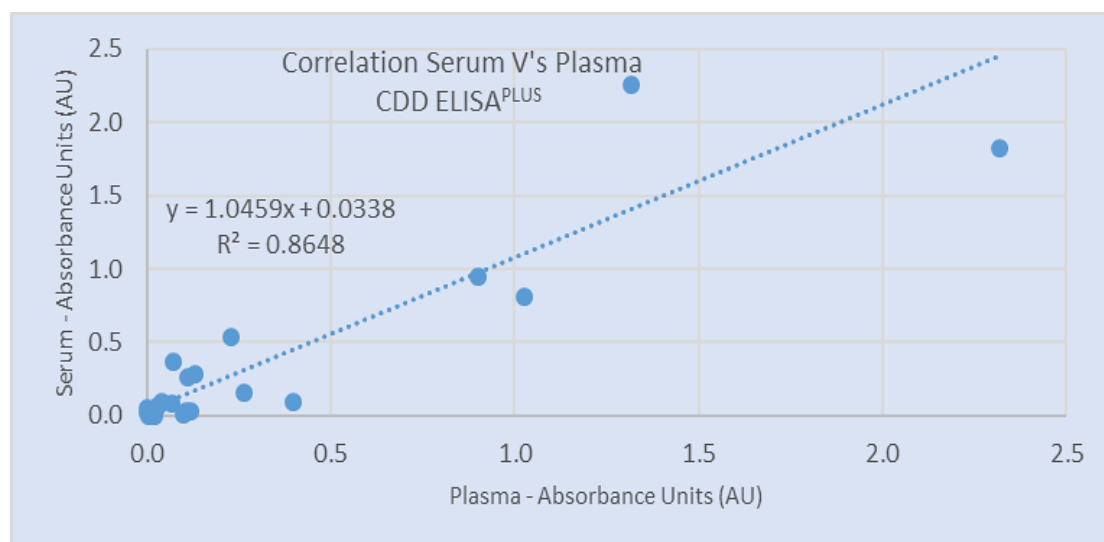


Figure 3.13 Correlation graph comparing CDD ELISA^{PLUS} serum and plasma results by volumetric method [R^2 : coefficient of determination; Each dot on the graph represents individual readings]



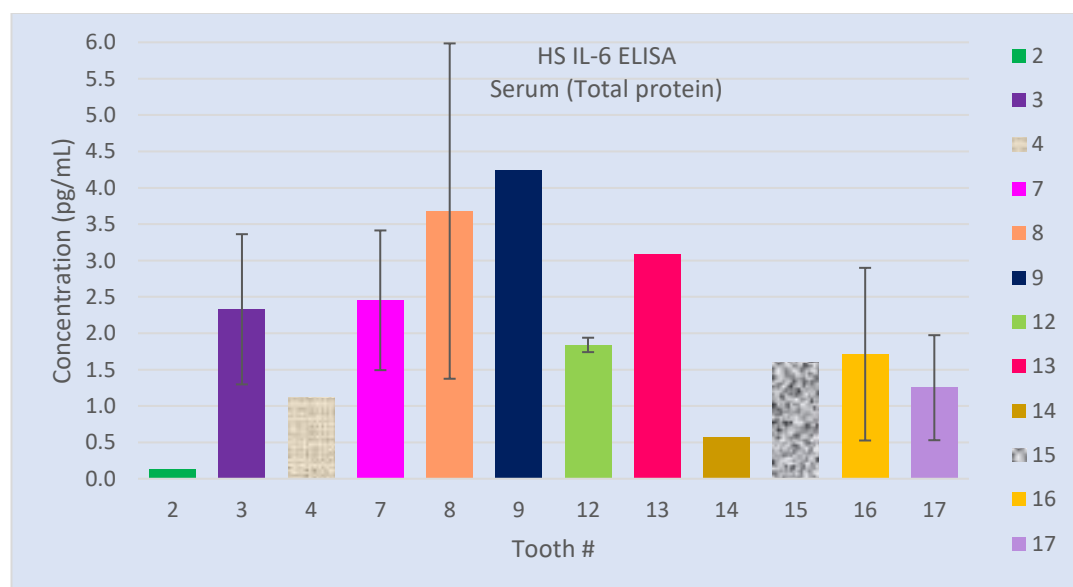
3.4.4 Method of analysis

3.4.4.1 Total protein methodology

The raw data acquired from the Bradford assays is presented in **Appendix VII**. In view of the finite volumes of collected pulpal blood available for analysis, the most suitable concentration was determined to be 30 µg/ml. In a small number of cases ($n = 9$), there was an insufficient volume of substrate available to provide a protein concentration of 30 µg/ml, it was therefore necessary to use a smaller concentration of 20 µg/ml. These cases are identified in **Appendix VII**. Using this kit, the results were below the detection limit of the kit for all samples, stated by the manufacturer to be 0.92 pg/ml. As a result, a more sensitive ELISA needed to be sourced (**Section 3.3.4.2.2**) and is reported on below. The results of the total protein methodology achieved using the HS Human IL-6 ELISA (Bender MedSystems) and Cell Death Detection ELISA^{PLUS} (Roche Diagnostics) are shown in **Figures 3.14 - 3.16**.

Plasma samples were assayed from 10 test teeth, and serum from 12 teeth. The samples were matched using a total of nine test teeth, due to insufficient availability of substrate to allow multiple assays to be conducted on every case. The samples were coded consecutively as either plasma, or serum, followed by the draw number in brackets. The first draw; p1, or s1, the second draw p2, or s2 etc. Notable fluctuations in protein concentrations occurred as the draw number increased. Technical replicates produced consistent protein concentrations in some [Appendix VII, Tooth 1: P5 (p3, pe3); Tooth 4: P20, P21 (p2, p2); Tooth 7: P45, P47 (p2, p2)], but not all cases [Appendix VII, Tooth 3: S7 (s1, s1); Tooth 15: P93, P94, P95 (p3, p3, p3)]. Haemolysis and paper point sampling did not reveal a clear pattern, due to low numbers available for comparison, no further conclusion can be drawn, from this observation.

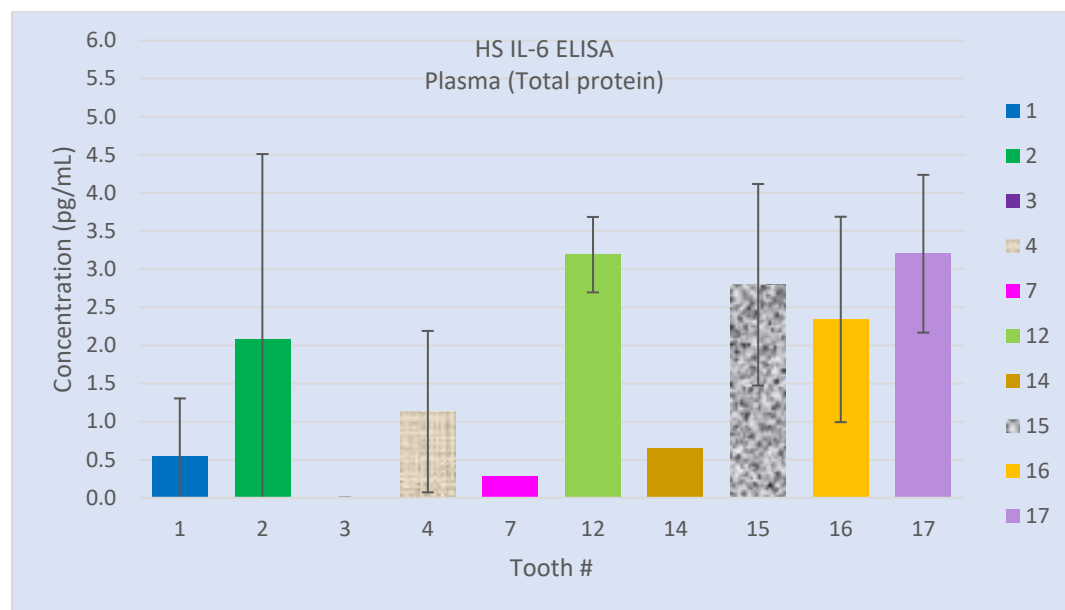
Figure 3.14 HS Human IL-6 ELISA results depicting serum collections using a total protein method [#: Number; Tooth 12 is the negative control]



3.4.4.1.1 High sensitivity Human IL-6 ELISA

IL-6 was reliably expressed within the threshold of the kit in all samples shown (**Figure 3.14** and **3.15**), with the exception of two plasma samples (Tooth 3, p1 and Tooth 4, p1). The combined results of the HS IL-6 ELISA are depicted in **Figure 3.11**.

Figure 3.15 HS Human IL-6 ELISA results depicting plasma collections using a total protein method
[#: Number; Tooth 12 is the negative control]



All draws relating to a single tooth were averaged, with error bars demonstrating their standard deviations. There were insufficient samples to provide simultaneous assays for all cases, for both serum and plasma. The range of expression detected was 0.13 pg/ml - 5.31 pg/ml in the serum samples and 0.0 pg/ml – 4.46 pg/ml in the plasma samples. Mean values of 1.93 pg/ml (serum) and 2.08 pg/ml (plasma), were recorded. Standard deviations ranged from 0.09 - 2.3 pg/ml and 0.49 - 2.6 pg/ml, in serum and plasma samples, respectively.

3.4.4.1.2 CDD ELISA^{PLUS}

The CDD ELISA^{PLUS} failed to produce readings in more than half of the samples assayed using the total protein technique (**Figure 3.16 (i, ii).**). This may reflect the level of sensitivity of the kit, or the limited volumes of available substrate to produce sufficient protein concentrations across all samples. The concentration of total protein selected for the assays was 30 µg/ml, in most instances. However, some exceptions were unavoidable due an insufficient volume of available substrate to maintain 30 µg/ml in all samples (**Appendix VII, Table 3.4**). There are insufficient samples to determine how this alteration in protein concentration could have exerted on the readings obtained.

The range of expression of circulating histones found in plasma was 0.00 – 0.631 AU and in serum 0.00 – 0.124 AU, using a total protein methodology. The corresponding mean values were 0.06 and 0.02, respectively. Their standard deviations ranged from 0.01 - 0.24 AU in plasma and 0.00 – 0.08 AU for the serum samples.

The pattern of results obtained from the total protein method raises some concern that minute sample volumes could render the analytes below the detectable threshold for certain ELISA assays. This can be seen in **Figure 3.16 (i, ii).**, where many of the samples assayed using the total protein method did not register any reading, or readings very close to zero (**Table 3.4**), despite their corresponding matched volumetric samples producing consistent readings (**Figure 3.16 (iii, iv).**). This may be due to the fact that some of the total protein corrections, meant that increased sample dilution meant that the sample was below the ELISA assay threshold.

It is worth noting that Tooth 12 (the true negative control) was expressed in a range between 2 - 3.5 pg/ml by total protein method using the HS IL-6 ELISA (Figures 3.11, 3.12). However, with the exception of a single plasma sample (p4); Tooth 12 failed to produce readings using the CDD ELISA^{PLUS} (Figure 3.16 (i, ii)., circled red) in the total protein methodology.

A number of plasma samples failed to produce readings using the total protein methodology with CDD ELISA^{PLUS} (Tooth 4, Tooth 12, Tooth 14, Tooth 16 and Tooth 17), were all highly expressed by HS IL-6 ELISA ranging from 1 - 3.5 pg/ml (Summarised in Table 3.4). Similarly, a number of serum samples (Tooth 3, Tooth 7, Tooth 8, Tooth 12, Tooth 16 and Tooth 17) also failed to produce readings when their corresponding samples were highly expressed using the HS IL-6 ELISA, ranging from 1 - 5.25 pg/ml (summarised in Table 3.4).

Figure 3.16 (See over): CDD ELISA^{PLUS} results arranged according to method (Total protein (i)., (ii). and Volumetric (iii)., (iv).) and analyte (Serum (i)., (iii). and Plasma (ii)., (iv).) [Tooth 12 is true negative control, circled red]

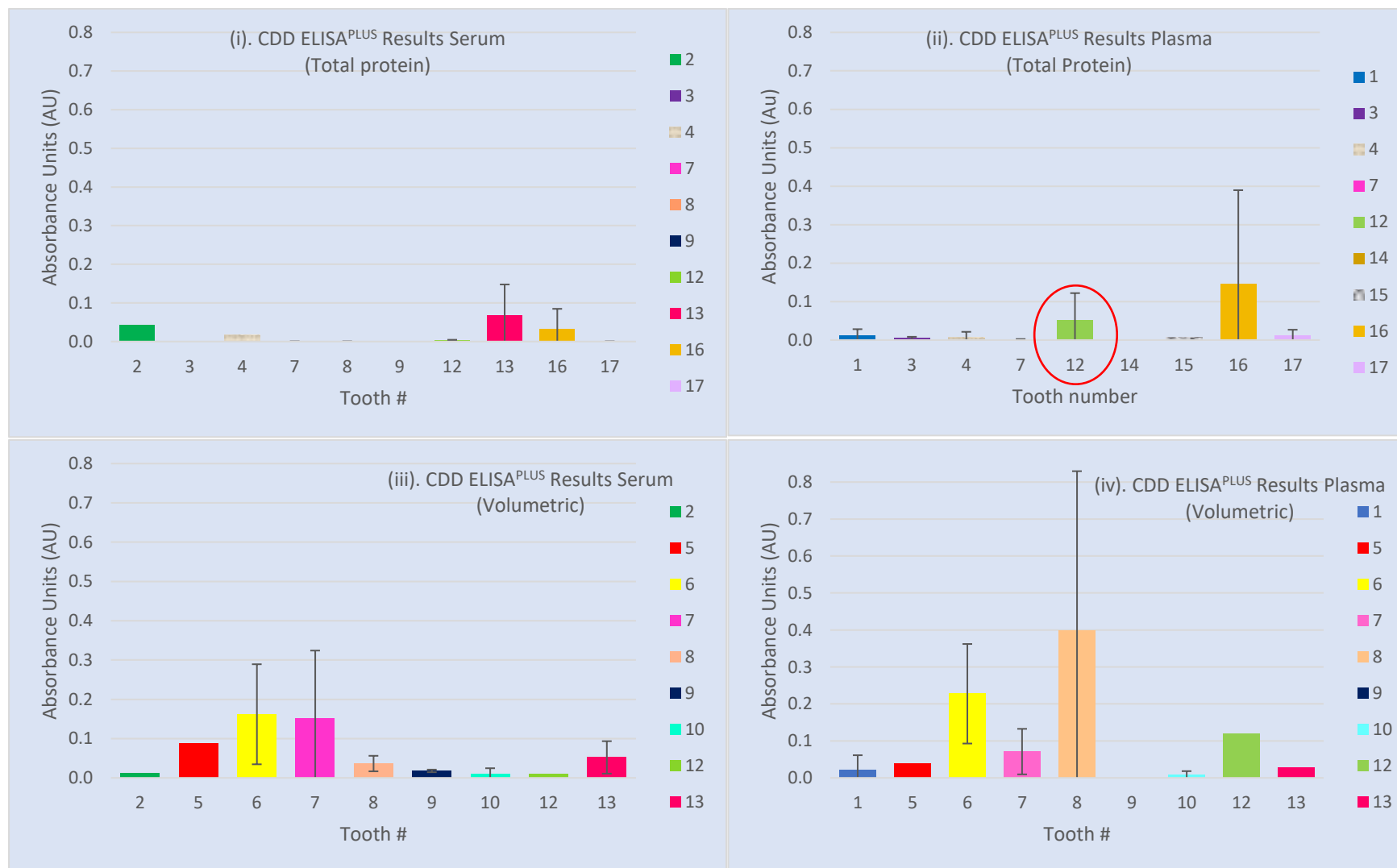


Table 3.4 Snapshot of the CDD ELISA^{PLUS} samples which failed to produce a reading by total protein methodology, aligned with their corresponding HS human IL-6 ELISA samples [Chart is divided by substrate (plasma/serum)]

Code	Draw	Tooth	CDD (AU)	IL-6 (pg/mL)	Protein (µg)
					30
P18	p1	4	0.0000	0.00	20.66
P22	p3	4	0.0000		14.33
P76	p3	12	0.0000	2.84	4.21
P107	p3	16	0.0000	4.46	1.21
P118	p1	17	0.0000	3.93	3.88
S7	s1	3	0.0000	1.60	25.01
S40	s2	7	0.0000	3.40	5.91
S40	s2	7	0.0000	2.48	5.91
S56	s1	8	0.0000	2.05	5.29
S56	s1	8	0.0000	5.31	7.67
S119	s2	16	0.0000	1.32	7.84
S121	s2	16	0.0000	0.03	3.75
S133	s2	17	0.0000	0.71	8.33
S139	s4	17	0.0000	2.20	9.24

3.4.3.2 Volumetric methodology

A volumetric method was investigated initially using a standard human IL-6 ELISA (Invitrogen) and the CDD ELISA^{PLUS} (Roche Diagnostics). The threshold of the IL-6 ELISA kit was not conducive to the study of pulpal inflammation because no readings could be obtained from the samples assayed. Once it was established that the initial volumetric samples used were below the limit of detection of the standard human IL-6 ELISA (Invitrogen), a more sensitive kit (HS human IL-6 ELISA, Bender) was sourced. There were insufficient remaining samples to repeat the volumetric ELISA with the new kit and to allow comparisons to be drawn with the subsequent total protein results.

The CDD ELISA^{PLUS} demonstrated consistently more positive responses using the volumetric compared with the total protein method [Figure 3.16 (iii, iv) and Figure 3.16 (i, ii).]. The mean absorbance (AU) calculated in the volumetric CDD ELISA^{PLUS} was 0.48 AU (plasma) compared with 0.55 AU (serum); whereas the values

in the total protein method were 0.017 AU and 0.055 AU, respectively for serum and plasma. The ranges were 0.0 AU- 2.7 AU (plasma) and 0.0 AU- 2.95 AU (serum) in the volumetric method, and 0.0 AU-0.63 AU (plasma) and 0.0 AU-0.124 AU (serum) using the total protein method. The standard deviations for the volumetric samples ranged as follows: 0.00 – 0.55 AU for serum samples and 0.004 – 0.767 AU for the plasma samples.

3.4.4.2.1 Influence of the sample volume collected

The standardisation of pulpal whole blood collection proved challenging throughout the clinical aspects of the study, the details of which, have been discussed in **Sections 3.4.1 and 3.4.2.**

Inevitable variations in the dilution ratios. For example, in Tooth 6 (p1), 6.25 µl of whole pulpal blood was collected and expressed into a PCR tube prepared for a 10 µl collection, 9.69 µl was obtained for p3 and expressed into a PCR tube prepared for 10 µl collection, 7.5 µl (s3) was collected as 10 µl protocol etc. This led to unintentional, small fluctuations in the dilutions obtained.

This information serves to underpin the importance of establishing and maintaining consistent whole blood volume acquisition (**Appendix IXa**). Despite the noted variations in whole blood volumes acquired, the pattern of absorbances did not alter in a corresponding linear manner and requires further analysis.

Consideration of utilisation of centrifuge rather than brief period of vortex to achieve consistent homogenisation of samples prior to assays may also be warranted.

3.4.5 Influence of sample handling

Inadvertent aspiration of red blood cells during the aliquoting procedure within some samples was observed (**Figure 3.17**), and haemolysis noted in a small number of specimens (**Figure 3.18**). This occurred after the chilled centrifuge stage and prior to extraction of the supernatant. The associated ELISA^{PLUS} data is presented in **Figure 3.19** to appreciate the potential influence on the resultant absorbances measured.

In total, 9 samples demonstrated a visual appearance of haemolysis and are presented in **Figure 3.19**, alongside their matched, unaffected samples, for comparison. In all but one sample (Tooth 31, p5¶) the presence of either haemolysis, or aspiration of blood cells led to a significant increase in the CDD ELISA^{PLUS} readings (**Figure 3.19**). It should be noted, that the haemolysed, or red-cell-aspirated samples were assayed on the same day, under the same experimental conditions, as their corresponding unaffected samples.

Figure 3.17 Aspirated red blood cells



The substrate extracted from samples collected by paper points was visibly not as translucent as 'normal' (non-haemolysed) samples. The effect of paper point sampling on the absorbance recorded is displayed in **Figure 3.20**. With the exception of the serum samples obtained from Tooth 20 and Tooth 22, the expression of samples collected by paper point was lower than the preceding plasma, or serum sample. It should be noted that paper point extraction was limited to cases where blood collection was hampered significantly (limited volume, or poor microcapillary action), or as the final collected sample.

Figure 3.18 Haemolysed specimen [Noted after chilled centrifuge, prior to extraction of the supernatant, disruption of the clot and release of red blood cells is apparent]

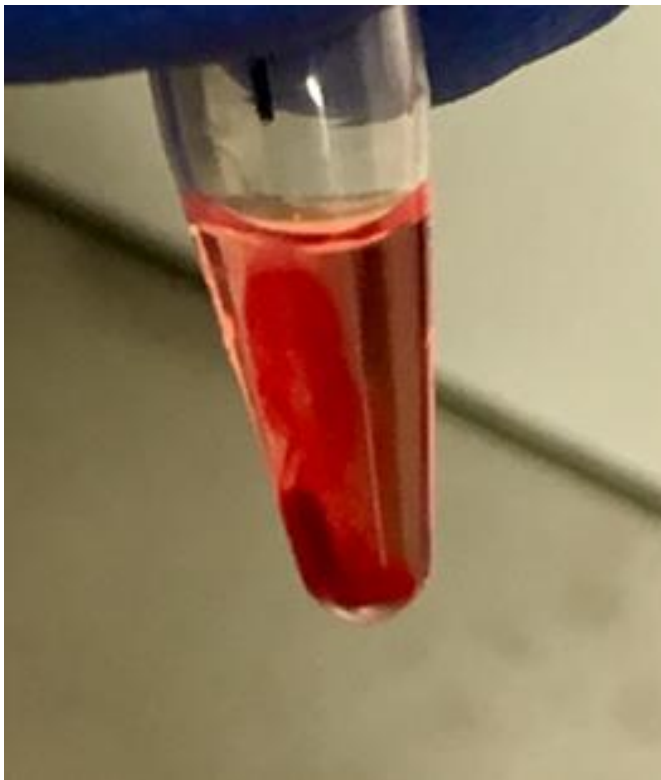


Figure 3.19 Chart depicting the possible influence of aspiration of blood cells, or haemolysis on the absorbance recorded by CDD ELISA^{PLUS} [#: Number; Draw number indicated as p1, p2, p3 etc.; Aspirated blood cells/haemolysed samples denoted by ¶ after draw number (p1¶). All haemolysed samples included in graph]

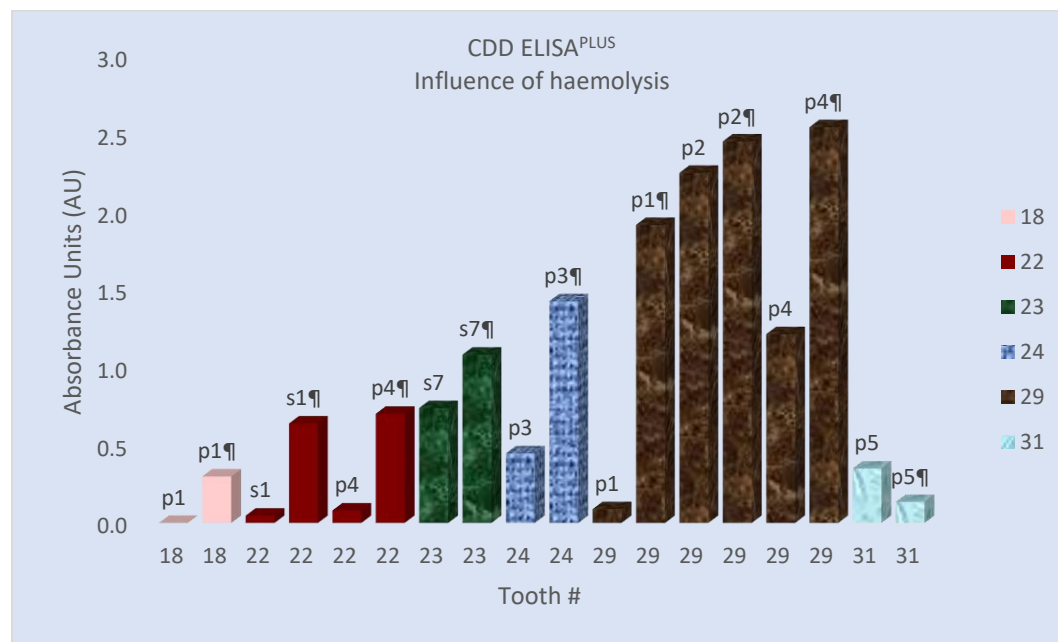
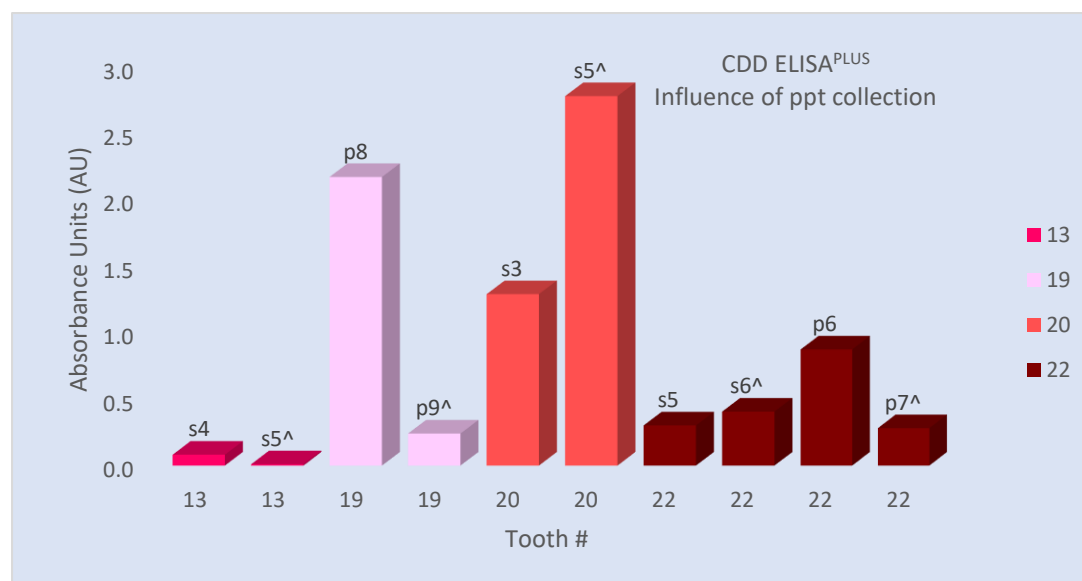


Figure 3.20 Chart depicting the possible influence of paper point collection on the absorbance recorded by CDD ELISA^{PLUS} in consecutive samples [#: Number; Draw number indicated as p1, p2, p3 etc.; Paper point samples denoted by ^ after draw number (s5^). All samples collected with paper point charted in graph]



3.5 Discussion

3.5.1 Blood collection

3.5.1.1 Collection vehicles - Microcapillary action

The inability to consistently fill the capacity of the microcapillary tube became apparent immediately during the first case. In this instance, the rate of collection was particularly slow, to the extent that the first few microliters of blood clotted within the tube and it was not possible to transfer to the PCR tube for downstream analysis. The total time which elapsed, prior to the sample clotting was less than 4 minutes. Therefore, practicality and efficiency influenced the clinical decision to accept incompletely filled vessels, rather than risking blood clotting by waiting to obtain a completely filled vessel. For this reason, two minutes was empirically chosen as the maximum practical collection time, ensuring clotting would not impede sample transfer to the designated PCR tube. Additionally, if the microcapillary action noticeably ceased for 30 seconds an incomplete fill was also accepted. Incomplete collections were accepted and the exact volume was calculated by measuring penetration depth relative to the maximum calibrated penetration depth of the tube (Figure 3.4).

3.5.1.2 Efficiency of collection

The proficiency in the collection of pulpal blood improved with each case (Appendix VIII). It would appear through this arm of the study, that there was a learning curve and development of experience is required in the collection of pulpal blood. The smaller volume capacities (2 µl, 5 µl) proved more consistent, in the ability to obtain complete fills (Figure 3.9, Table 3.2). Operator experience and calibration should be

considered in the development of future studies. The decision as to which collection vessel to use was made empirically by the principal operator. This aspect was necessary from a practical perspective, but could have introduced inconsistency in the methodology, which could be regarded as a limitation of the study.

The observation of sclerosis within the pulp chamber had a marked effect on the ability to collect pulpal blood (**Section 3.4.3.4, Appendix VIII, Appendix XI**) which was not anticipated in the development of the study. This aspect should be considered and standardised as a potential exclusion factor in the design of future studies.

3.5.1.3 Volume of analyte

With experience, the ability to consistently achieve complete fills of the collection vessels allowed for improved standardisation of the collected volumes. In view of developing future study protocols, eliminating factors which could impact on the interpretation of results is of great importance. Consistency in the volume collected as well as the level of dilutions applied are critical for standardisation purposes. This aspect has not been considered by previous studies in the area (Mente et al. 2016, Aminabadi et al. 2017), or in studies using absorbent paper point, or another carrier methodology (Ghattas Ayoub et al. 2017, Mutluay et al. 2018, Nguyen et al. 2020) where standardisation of the analyte volume is impossible.

Variation in the draws was likely to have been influenced by variations in whole blood collection and the dilutions required to facilitate the assays. Some variation was observed in the absorbances obtained from technical replicates (**Appendix IXa**). As a result, alteration to the homogenisation protocol of samples

after thawing, may require refinement (as noted in the protein concentrations of technical replicates **Appendix VII**), to ensure consistent distribution of the analytes.

3.5.1.4 Whole blood versus paper point

Paper point collection was not initially considered in the original study protocol, as the principal aim was to assess the serum and plasma extraction from a given volume of whole pulpal blood. The effects of blood handling on protein recovery have been widely studied (Skogstrand et al. 2008, Keustermans et al. 2013). Although a dried blood spot can offer greater long-term stability, efficient extraction of disease-associated cytokines can be significantly compromised (Pfleger et al. 2008). In spite of this, paper point collection of pulpal blood was included in some cases in this project, at least in part, because methodological comparison was an aim of this study. It may offer some information on the differences between the collection methods and allow for analysis of the available pulpal blood that was impossible to collect by microcapillary action. This aspect was not initially considered in the study design with paper point reserved for cases where collection was impossible and as a result of very small numbers for analysis, no clear pattern could be observed. Future studies could focus on the comparison between paper point sampling and standardised volumes collected by microcapillary action.

3.5.1.5 Problems unique to the collection of pulpal blood

In order to access pulpal blood, invasive, operative intervention is necessary. The availability of true healthy control subjects presents a unique challenge in the study of pulpal blood sampling. 'True healthy' controls are teeth designated for extractions,

usually for orthodontic purposes. These (often young) patients are asked to submit to an additional intervention immediately prior to providing the prescribed extraction (**Section 2.8.2, 2.8.2.3**). As a compromise, proxy healthy controls are often sourced (Nakinishi et al. 1995, Ghattas Ayoub et al. 2017, Nguyen et al. 2020, Sharma et al. 2021). These can include patient requiring elective endodontic treatment for prosthodontic purposes, which may, or may not have a completely unaffected normal, or uninflamed pulp and may influence the information obtained (**Section 1.5.3**).

The time taken to collect completely fill the collection vessel will be influenced by the speed, or relative ease of the microcapillary action. In view of the inadvertent variations incorporated into this study (accepting incomplete fills), it would be reasonable to consider collecting multiple draws to reach a predefined volume; for example, 25 µl via multiples of 5 µl (**Section 6.3**).

Collection time does require consideration from a patient comfort and co-operation point of view. Pulpal haemodynamics is known to be influenced neural networks both locally (sympathetic vasomotor functions, via intradental sensory nerve fibers) and via remote mechanisms (autonomic stimulation) [Olgart 1996]. Studies investigating the functional physiology of pulpal haemodynamics further demonstrated the influence exerted by capillary resistance (Meyer 1980) and capillary permeability (Kim 1985).

In addition to the collection of the analyte, the completion of the clinical procedure needs to be carried out in an efficient manner. It is of particular relevance if this is to be considered as a chairside diagnostic technique in the future. In relation to VPT, it will necessitate competent tissue handling, management of haemorrhage,

disinfection, placement of the HSC and adhesive restoration of the tooth (**Section 2.8.2.1**). In endodontic treatment, ideally the root canal filling should be completed and the tooth restored (**Section 2.8.2.2**). These aspects could influence the time available for blood collection and the resultant volumes that could be obtained (**Appendix VIII**).

3.5.2 Substrate handling

3.5.2.1 Pre-analytical considerations

The specific pre-analytical blood preparation method employed (whole blood, serum, or plasma) has been reported to affect the detection of various proteins, or antibodies (Haab et al. 2005). It is not yet clear in the dental literature, which is the most appropriate vehicle, or analyte, or anticoagulant to use when collecting pulpal blood samples.

In terms of anticoagulant, EDTA was chosen for plasma collection in this study as it was deemed the most suited to cytokine evaluation (Thavseu et al. 1992, de Jongh et al. 1997). EDTA plasma and serum samples have demonstrated similar levels of IL-6, compared to lithium heparin and sodium citrate; both of which, resulted in lower levels of demonstrable cytokines (Flower et al. 2000). Haab et al. (2005) suggest that EDTA may have a protective effect on the stability of cytokines, potentially due to its role as a protease inhibitor. The use of pre-treated collection vessels (Sodium heparin and K3EDTA) in this study, resulted in lower values obtained when compared with similar samples from the same cases using plain microcapillary tubes (**Section 3.4.2.1, Appendix IXa**); however, this may not have been only due to the anti-coagulant used but also the vessel itself. Cannon et al. (1988), suggest that

EDTA-plasma represents a truer reflection of free circulating cytokines, compared with serum; on the basis that additional cytokines could be secreted from leukocytes during the clotting process. Others (Zhou et al. 2010), suggest that the effect on serum, or plasma collection is dependent on the specific marker under investigation. As a result, one methodological aim of this study was to investigate the most appropriate substrate (plasma, or serum) for the detection of cell death markers.

The relationship between the inflammatory and coagulation systems has also been widely studied (Esmon 2005). IL-6 increases platelet production and in this study the majority of plasma samples produced higher values than the corresponding serum values with the HS IL-6 ELISA (**Figure 3.11**). The correlation between serum and plasma (**Section 3.4.3**) was found to be low ($R^2 = 0.2591$), using the HS IL-6 ELISA, but higher using the CDD ELISA^{PLUS} ($R^2 = 0.8648$), suggesting that the substrate of choice may be kit dependent.

3.5.2.2 Half-life of analytes

The half-life of IL-6 is reported to range from 2-15 hours (Wirtz et al. 2000). It has been previously demonstrated that IL-6 levels decreased in unseparated samples left at room temperature after 4 hours (Flower et al. 2000). Furthermore, *in vitro* synthesis of IL-6 was noted after 3 hours storage at body temperature (Riches et al. 1992), so ideally collection should be completed within this relatively short time frame to best reflect the *in vivo* status of circulating cytokines.

A single study has reported the half-life of histones in plasma to be 4.6 minutes (Ekaney et al. 2014). Plasma from healthy volunteers was spiked with calf thymus histones at a concentration of 100µg/ml and using western blotting it was

noted that protein degradation, reached a plateau at 30 minutes after which, no further change could be detected for up to 6 hours. Caution should be exercised in the interpretation of spiked analysis, as it may not reflect the temporal dynamics *in vivo*. However, the level of stability reported by Ekaney et al. (2014) after reaching a plateau at 30 minutes, saw no further degradation of the protein for a period of up to 6 hours. In this current study, all samples were collected with a protease inhibitor (**Figure 3.2**) and were processed within 2 hours of collection. The time elapsed from the sample collection to centrifuge and separation did not exceed two hours, which is well inside the 4-hour window suggested by Skogstrand et al. (2008). Delays longer than 4 hours at room temperature have been shown to increase the detectable levels of inflammatory cytokines, including IL-6.

3.5.2.3 Sample storage

The ideal temperature for storage of whole blood samples was found to be 4°C (Skogstrand et al. 2008). This was not possible to achieve during the clinical steps and as a compromise, in line with laboratory practice, samples were stored during the clinic on ice, in an upright position until the dental procedure was complete and the patient discharged. In order to avoid repeated freeze-thaw cycles, the samples were centrifuged in a chilled centrifuge (4°C) prior to aliquoting into defined volumes, before finally storing at -80°C. Previous research has indicated that freeze-thaw cycles did not affect the levels of IL-6, but was previously identified as problematic with TNF- α after three cycles (Flower et al. 2000). After 4 hours at room temperature, levels of IL-6 decreased significantly (Aziz et al. 1999). However, TNF- α increases were identified between 4-6 hours at room temperature. This highlights the specific

requirements should be tailored to the specific analyte under investigation. Graham et al. (2017), however, suggested that freeze-thaw cycles do not affect IL-6 recovery for up to 5 freeze-thaw cycles.

3.5.2.4 Blood handling

Care must be exercised in the handling of blood samples to avoid haemolysis *in vitro* (Carraro et al. 2000). This can potentially occur as a result of blood being forced under excessive pressure through a fine needle, or centrifuging the sample before the clotting process is complete. Haemolysis occurs when the cell membrane is damaged and haemoglobin, or other cellular components are released into serum, or plasma. The light absorption of haemoglobin ranges between 340-440 nm and 540-580 nm, which has been reported to affect the results of assays within these wavelengths (Katzman & Baumann 2016). Although blood is used throughout medical diagnostics (Gabay & Kushner 1999, Berger-Achituv et al. 2013, Hayase et al. 2018), Rechenberg & Zehnder (2014) suggested that blood could be an unsuitable substrate for protein analysis in the pulp, for a number of reasons which we will discuss. The presence of interfering substances, such as endogenous antibodies, are known to affect ELISA readouts (Brown et al. 1989). Other groups have reported that modifications focused on the purification of proteins, have been successful in eliminating such interferences from the assay (Grebentchikov et al. 2002). As the level of technical proficiency relating to blood collection and sample processing improved throughout the study, it became apparent that certain samples could have potentially been haemolysed (**Figure 3.18**), or evidence of red blood cells becoming aspirated into certain aliquots (**Figure 3.17**). Details of these samples were specifically noted and identified, in order

that their results could be correlated/explained as ‘pure’, or potentially ‘spoiled’ samples (**Figure 3.19**). *In vitro* haemolysis is a leading cause of pre-analytical laboratory error, which impacts significantly on medical diagnostics (McCaughey et al. 2016), leading to the requirement to provide repeat testing and delays in the processing of results. This form of haemolysis consistently alters analytical results, including ELISA, by delivering significantly different values than reference standards (Lippi et al. 2008, Heireman et al. 2017). This is particularly important in the development of new diagnostic testing when no reference standard exists.

Pulpal blood was collected in this study, principally using a range of glass microcapillary tubes, a recognised route of obtaining pulpal blood for analysis (Mente et al. 2016, Aaminabadi et al. 2017). The process of blood collection using this method, meant that the operator had to be more aggressive when handling the pulp than would be standard practice during VPT. Generally, inflamed pulp tissue should be removed as atraumatically as possible, in order to limit tissue damage and encourage healing and repair (Fouillen et al. 2023). However, even in the presence of copious bleeding, variation was noted in the rate and ease of microcapillary blood collection. Therefore, in order to stimulate additional bleeding, small pumping actions into the pulp tissue were employed in order to accomplish better filling of the microcapillary tubes. This may have inadvertently led to dental pulp cell damage and potentially some of the *in vitro* haemolysis observed in selected samples.

As cytokine detection can be influenced by the length of time that serum and plasma are in contact with erythrocytes, resulting in a false estimation of circulating mediators. As a result, it is recommended that the extracted supernatant (serum/plasma) should be visibly free of erythrocytes (Esmon 2005). The level of

haemolysis can range from slight to gross breakdown (Hawkins 2002). The effect of the degree of haemolysis contamination exerted on serum biochemistry was investigated and levels of erythrocytes between 0.1% - 1% did not significantly alter results obtained (Laessig et al. 1976). Nakinishi et al. (1995) demonstrated that nylon fibre was superior in the recovery of serum IgG and their method was not affected by haemolysis, when compared with cotton wool, glass wool, Teflon fibre, or pipetting as the standard. The extensive pilot work demonstrated that the 5 µl and 2 µl Minicaps were the most efficient collection vessels. As the study progressed, the consistent collection of full volumes was readily and predictably achieved. This was considered the method of choice for all future blood collections within the study.

3.5.3 Analytical considerations

3.5.3.1 Level of detection

Zhou et al. (2010) reported problems achieving reliable readings when very small volumes of analyte were available, noting that after dilution, the level of the biomarker could be rendered lower than, or exceed the narrow dynamic range of the kit employed. For example, after Nakinishi et al. (1995) applied a 100-fold dilution to supernatant extracted from nylon fibre, IL-6 was detected at such a low level, that it was not reported in their paper (Nakinishi et al. 1995). This could have been due to the level of dilution, or alternatively that the level may be below the sensitivity of assay used. This finding is tentatively supported in the current study, where the initial IL-6 ELISA (Invitrogen, ThermoFisher Scientific), was unable to demonstrate the presence of IL-6 using a volumetric method (**Section 3.4.4.1**); however, when a more sensitive kit was employed (Bender MedSystems), IL-6 was detected in all samples

using a total protein methodology which incorporated additional substrate diluents (**Section 3.4.4.1.1**). Furthermore, the relative inability to detect circulating histones (CDD ELISA^{PLUS} [Roche Diagnostics]) in specific samples when further dilutions were performed to standardise their protein content; in spite of their corresponding volumetric samples producing readouts, highlights the importance of the specific kit threshold in identifying the molecule of interest. (**Figure 3.16**).

Due to the very small volumes of pulpal blood available for collection from the exposed pulp, standardisation of the diluents added requires pre-analytical consideration.

3.5.3.2 Volumetric, or total protein methodology

The vast bulk of pulp biology literature examining inflammatory mediators (e.g. cytokines), have focused on the analysis of pulp tissue (Rechenberg et al. 2016a); however, this does not have the potential to extend to a chairside method. The application of a Bradford Assay to standardise the total protein concentration lends itself well to investigation of pulp tissue due to a greater volume and concentration of fluid to analyse. After pulp tissue homogenisation the protein level in the resulting cell lysate can be quantified and standardised between samples. In the original experimentation a total protein method was employed, in which the protein level is equilibrated prior to analysis. This method is routinely used in other laboratory techniques such as Western Blot. Although, the HS Human IL-6 ELISA (Bender MedSystems) data presented shows consistent readings using the total protein methodology, its limit of detection was defined by the manufacturer as 0.03 pg/ml, which meant very small volumes of analyte were possible. The findings in this part of

the study are consistent with the existing literature on inflammatory cytokines (Nakinishi et al. 1995, Waterhouse et al. 1999, 2002, ElSalhy et al. 2013, Mutluay et al. 2018), where values are most often reported in a range rather than a defined threshold. Discriminatory thresholds differentiating health from disease have not been defined in the literature. ElSalhy et al. (2013) reported the range of IL-6 in pulpal blood of normal and inflamed cases as 0.0-2.09 pg/mg total protein. In the current study this range was demonstrated to be 0.0-5.31 pg/mg total protein, using the same HS Human IL-6 ELISA kit (Bender MedSystems).

When the total protein methodology was applied to the Cell Death Detection ELISA^{PLUS} (Roche Diagnostics), the sensitivity of the assay appeared to be insufficient to produce meaningful results. Due to the limited substrate volume available to standardise the protein concentration at a sufficiently high level to yield an absorbance within the threshold of the kit (**Appendix VII**). These findings are consistent with results in other studies (Zehnder et al. 2011). This particular kit does not produce a standard curve to allow calculation of concentration of the extracellular H3, nor does it state a defined sensitivity. What published studies have done to overcome this drawback, is to identify the highest quartile Absorbance Units (AU) from the assay to signify elevated levels of circulating histones (Kutcher et al. 2012). This rationale has been applied to the analysis of the Cell Death Detection ELISA^{PLUS} (Roche Diagnostics) results, which will be discussed further in **Chapter 5**. The Cell Death Detection ELISA^{PLUS} (Roche Diagnostics), did however, produce consistent readings using the volumetric method. Since the Cell Death Detection ELISA^{PLUS} correlation between serum and plasma ($R^2 = 0.8648$) was shown to be comparable, the conclusion that either serum, or plasma are suitable substrates for

investigation at least with the Cell Death Detection ELISA^{PLUS} (**Figure 3.13**) was reached.

3.6 Conclusion

At present, there is a lack of methodological consideration and standardisation within this field. As a result, an effective, simple and reproducible method of collecting pulpal blood was investigated and analysed. The data has highlighted some practical, technical and logistic difficulties with pulpal blood collection and has highlighted pulp sampling methods that work, the value of plasma over serum and the analysis methods that could be introduced into routine practice and research activities.

Total protein methodology, although beneficial in basic scientific research, was not as applicable to a chairside technique. At this stage, a volumetric technique, at least for point-of care analysis is preferred. The volumetric method is more translational, in that a dentist collecting blood chairside to assay biomarker levels is not going to use a total protein method, but rather a practical, volumetric one. The value of this information relates to devising standard operating procedures and identifying, as well as minimising, potential pitfalls relating to the collection of pulpal blood for the purpose of volumetric analysis of protein expression.

Chapter 4

A correlation of the visual appearance of exposed pulps with symptoms, caries depth and vital pulp treatment outcome

4.1 Introduction

4.1.1 The visual appearance of the exposed pulp

Achieving haemostasis after pulp exposure is implicit in the provision and success of VPT (Matsuo et al. 1996, Asgary & Parhizkar 2021, Duncan et al. 2023). Unusually, for such an important determinant, there is limited descriptive material relating to the appearance of the exposed pulp in the existing literature (Ricucci et al. 2019). Qualitative evaluations often fail to sufficiently describe, or categorise the condition of the exposed pulp. For example, the differentiation between an initial and a mild pulpitis is challenging to accurately distinguish clinically (Donnermeyer et al. 2022), or describe visually (Richert et al. 2022). The translation and uptake of digital dentistry is now a widely accepted norm in clinical practice (Schwendicke 2020, Spagnuolo & Sorrentino 2020) and an invaluable adjunct to the clinician's armamentarium. Digital images can be used to educate the patient, to document clinical information, to demonstrate outcome, as well as to communicate with colleagues. Although the routine use of an operating microscope is considered the standard of care within the discipline of endodontics (AAE Special Committee to Develop a Microscope Position Paper 2012), it is considered beyond the scope of most general dental practitioners (Forgie et al. 1999) and therefore qualitative standards based on the appearance of the pulp may not be a pragmatic solution. Notably, in a recent position statement by the American Association of Endodontists (AAE) it was concluded that direct observation of pulp tissue during and after achieving haemostasis offers additional diagnostic information about the condition of the pulpal tissue (AAE, 2021). To that end, a range of descriptors have been relied upon in the literature to direct the clinician towards the most appropriate treatment.

Characteristics of pulpal bleeding have been reviewed in detail in **Section 1.4**, as well as highlighting certain flaws in the exiting literature surrounding the analysis of pulpal blood (**Section 1.5 - 1.6**).

4.1.2 Haemorrhage control in diagnosis

It is essential during VPT, that pulpal haemorrhage is controlled, as arrest of bleeding is regarded as the ultimate intra-operative diagnostic criterion (Shoaf et al. 1979, Stanley 1989, Matsuo et al. 1996). The ability to achieve haemostasis is considered a positive prognostic indicator (Duncan et al. 2023), while bleeding and clotting control are also important from a practical perspective for successful material placement (Schröder 1973). Contemporary research on the cariously-exposed pulp has not adequately addressed the limitations in the operative determination of the condition of the inflamed pulp (Bjørndal et al. 2019). The descriptor most frequently cited is time to achieve haemostasis (**Table 4.1**). As yet, no consensus has been reached regarding the optimal time point, within which, haemostasis should be achieved or indeed how to standardise the method of achieving haemostasis (**Table 4.1**). Likewise, descriptions of the character of pulpal bleeding demonstrate low specificity and cannot be readily validated by objective measures. Furthermore, the level of tissue amputation is influenced by the operator's assessment of the bleeding encountered during the procedure (Schröder 1987, Raslan & Wetzel 2006).

The heterogeneity in treatment protocols outlined in a selection of studies presented in **Table 4.1**, is considerable and this precludes ready comparison between studies. The manner in which haemostasis is achieved is shown to range from gentle irrigation with saline, or sodium hypochlorite at various concentrations; to the

application of a variety of agents or astringents, aldehydes, calcium hydroxide powder, cytotoxic irrigants and/or the application of pressure (Heilig et al. 1984, Matsuo et al. 1996, Waterhouse et al. 2000, Quidemat et al. 2017, Taha et al. 2022).

Table 4.1 Selected papers relating to control of pulpal haemorrhage [cw: cotton wool pellet; FC: Formocresol; CH: Calcium hydroxide; min: minutes; s: seconds, Ns: not stated; Y: yes; N: no; FP: Felypressin; A: Adrenalin]

Author	Dentition	Time	Method	Agent	Vasoconstrictor
Heilig 1984	Primary	6 min 1 – 5 min	Moist cw pellet 1min, 2 nd moist pellet 5 min	Saline	Ns
Matsuo 1996	Permanent	30 s	Irrigate 10% NaOCl & 3% H ₂ O ₂	10% NaOCl 3% H ₂ O ₂	Ns
Nosrat & Nosrat 1998	Permanent	1-2 min	Tap water/CH	Water CH	Y FP
Waterhouse 2000	Primary	3-10 min	Dry cw, FC cw 5min or CH	FC CH	Y A-1:80,000
Aeinehchi 2007	Immature permanent	3 min	Moist cw saline/FC	Saline FC	Y A-1:100,000
Doğan 2013	Primary	5 min	Irrigate saline	Saline	Ns
Nosrat 2013	Immature permanent	5-10 min	Moist cw	Water	Y A-1:80,000
Aminabadi 2017	Primary	5 min	Moist cw	Saline	Ns
Linsuwanont 2017	Permanent	5-10 min	Irrigate 2.5% NaOCl	2.5% NaOCl	Y A-1:100,000
Quidemat 2017	Permanent	5-25 min	Irrigate 5% NaOCl	5% NaOCl	Ns
Taha & Khazali 2017	Permanent	2-6 min	Flush 2.5% NaOCl soaked cw	2.5% NaOCl	Y A-1:80,000
Mutluay 2018	Primary	6 + 6min	Dry cw, 2 nd moist cw, repeated at orifice	Water	N
Taha 2022	Permanent	2-8 min	2.5% NaOCl cw gentle pressure	2.5% NaOCl	Y A-1:100,000

Furthermore, the addition of a vasoconstrictor in the local anaesthetic which could reasonably impact the detectability of the bleeding encountered (Pitt Ford et al. 1993), is not routinely reported in the methodologies, or considered in the results. Furthermore, primary teeth will differ physiologically from the permanent dentition (Fuks et al. 2012) as recognised resorptive processes within the primary dentition

(Harokopakis-Hajishengallis 2007) will have large impact on the vasculature and the appearance of pulpal bleeding (Caliskan 1993).

4.1.3 Time as an objective variable in haemorrhage control

The variation in time to achieve haemostasis in vital pulp studies has been reported to range from 1-25 minutes (**Table 4.1**). Of the one hundred and seventeen studies published since 2000, only half of the twelve studies which met the inclusion criteria in a recent review of VPT in mature molars presenting with irreversible pulpitis (Santos et al. 2021), reported the time taken to achieve haemostasis. According to Asgary & Parhizkar (2021) the observation of high blood flow, or profuse bleeding upon accessing the pulp, should not be considered a contraindication to providing successful VPT. The necessity to establish immediate haemostasis is not considered essential (Asgary et al. 2022) and does not appear to significantly influence the outcome of pulpotomy procedures (Bogen et al. 2008, Alquaderi et al. 2014, Linsuwanot et al. 2017); however, there is a lack of quality data addressing this issue (Zanini et al. 2019). Moving forward it is important that this information is better standardised in order to disseminate a clear message to dentist working in a range of environments.

4.1.4 Treatment outcome in vital pulp treatment

The outcome of VPT can be difficult to determine clinically, unless clear signs of failure become evident. Sensibility testing is not expected to elicit predictable responses after coronal pulpotomy (Careddu & Duncan 2021, Aravind et al. 2022) and success focuses on the absence of indicators of failure (Tan et al. 2020); such as

persistent, or developing pain; or signs of resorption, or an emerging periapical rarefaction which may develop at a later stage (Asgary & Eghbal 2013, Kunert et al. 2015). The longevity of treatment in the primary dentition could be expected to be finite, with follow-up ranging from 1-4 years. The permanent dentition, is expected to function throughout the patient's lifetime. Difficulties cited by clinicians relate to the perceived complexity of the potential future endodontic treatment (Careddu et al. 2021, Edwards et al. 2021, Chevalier et al. 2022, Hatipoğlu et al. 2023); or the removal of a HSC, or inaccessible root canal systems due to the presence of dystrophic calcification (McCabe & Dummer 2011). Unlike root filled teeth (ESE, 2021), there is no consensus on the indication to provide cuspal coverage for teeth following VPT procedures (Mertz-Fairhurst et al. 1998, Downer et al. 1999, Abu-Awwad 2019, Tan et al. 2020).

The volume of publications per year relating to VPT procedures has grown dramatically during the past 20 years (Kodonas et al. 2020). Despite sustained interest in this area being reflected not only by primary research but also by secondary research including; systematic reviews, meta analyses, scoping reviews, umbrella reviews and scientometric analysis (Kodonas et al. 2020, Leong & Yap 2021, Sabeti et al. 2021, Santos et al. 2021), the value of data highlighted in reviews should be interpreted with an element of caution. This is due to the erosion of studies that failed to meet accepted inclusion criteria. For example, in a recent systematic review and meta-analysis where 1100 articles were screened, only 11 articles were ultimately deemed suitable for inclusion (Ather et al. 2022). If the quality of the evidence is to improve, the importance of incorporating universally agreed-upon

reporting standards in primary clinical research cannot be overemphasised (Cushley et al. 2022, El- Karim et al. 2023).

4.1.5 Symptomology and its role in diagnosis

Clinical pulp diagnosis is centred around three pillars:

1. Symptoms (if any) described by the patient,
2. Clinical findings determined by the results of standard special tests including; responsiveness to sensibility tests and radiologic interpretation,
3. Intraoperative findings in the form of the visual appearance once the pulp chamber has been accessed.

Certain aspects have already been reviewed in **Sections 1.1 - 1.3**.

4.2 Study aims and objectives

The principal aim of this study was to correlate the visual appearance of the exposed pulp with a traditional and novel pulp diagnostic classification that are based on symptoms. As a secondary aim, the visual appearance of the exposed pulps, symptoms and caries depth were linked with VPT outcomes, after at least one year.

4.3 Materials and methods

Ethical approval, patient recruitment, examination, consent, diagnostic categorisation and treatment was carried out in a standardised manner as described previously (**Sections 2.1 - 2.8, Appendices II - V**). Briefly, 50 adult patients attending the Department of Restorative Dentistry and Periodontology in the DDUH, requiring

either VPT, or root canal treatment were evaluated, informed of the purpose of the study and enrolled once they had considered the information provided.

4.3.1 Experimental diagnostic categorisation

The clinical diagnostic categorisation used both Classic (Seltzer et al. 1963a) and Novel (Wolters et al. 2017) descriptors (**Section 2.6**). Additionally, the patient's symptoms were aligned to descriptors commonly used in the Classic, or novel nomenclatures (**Section 2.6**). This information was then collated, as described in **Sections 2.3** and **2.8.1**. Inclusion and exclusion criteria were adhered to, detailed in **Sections 2.4** and **2.5**.

4.3.2 Visual appearance of the exposed pulp

Images depicting the various bleeding characteristics of the exposed pulp are presented in **Figures 4.1 - 4.6**. The photographs were captured at pre-determined stages of the treatment. Immediately upon penetrating the pulp horn or roof of the pulp chamber, initial access, refined access and finally once and/if haemostasis was achieved. Pulpal blood was collected from the exposed pulp using microcapillary tubes, detailed in **Sections 3.3.1** and **3.3.2**.

4.3.3 Categorisation of the exposed pulp

A descriptive classification was created and proposed as part of this project according to the images obtained (**Figures 4.1 - 4.6**) and presented in **Table 4.2**:

- Normal pulp: Minimal bleeding on exposure, haemostasis achieved spontaneously, or quickly without additional measures (**Figure 4.1**).

- Reduced vascularity: No visible bleeding on exposure, or minimal bleeding, revealing active bleeding on deeper amputation of the pulp tissue (**Figure 4.2**).
- Profuse: The volume of bleeding clearly extends beyond the pulp chamber, is overflowing, but bleeding can be arrested (**Figure 4.3**).
- Profuse - Overflowing: - Persistent bleeding (**Figure 4.4**).
- Micro abscess: Purulent discharge visible on exposure of the pulp, profuse bleeding evident upon deeper amputation, bleeding persists (**Figure 4.5**).
- Total necrosis: No visible bleeding within the pulp chamber of root canal system. May contain serous fluid (**Figure 4.6**).

Figure 4.1 Normal Pulp - minimal bleeding on exposure

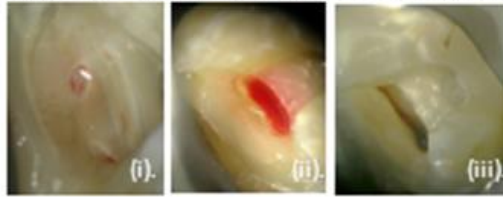


Figure 4.2 Reduced vascularity - subsequent active bleeding, arrested with deeper amputation

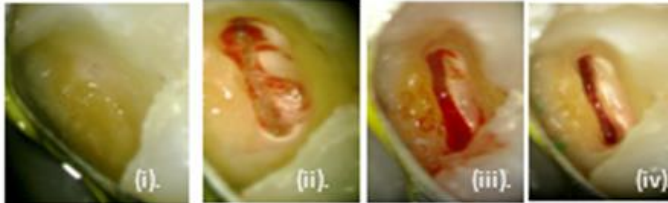


Figure 4.3 Profuse, overflowing, but bleeding can be arrested

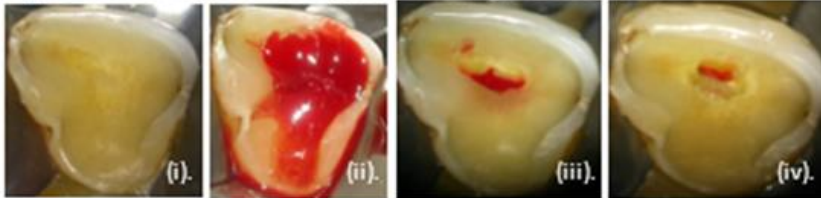


Figure 4.4 Profuse, overflowing, persistent bleeding

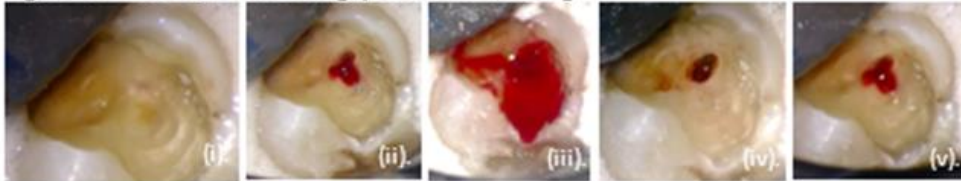


Figure 4.5 Micro-abscess – purulent discharge, profuse bleeding

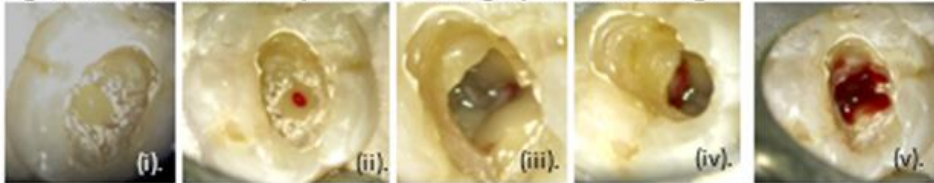
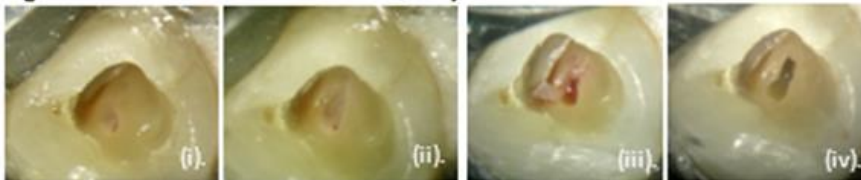


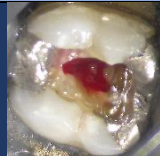
Figure 4.6 Total necrosis – avascular, may contain serous fluid

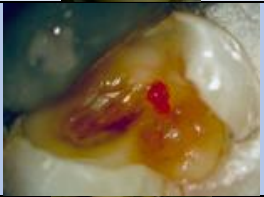
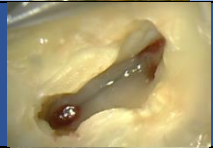


4.3.4 Narrative description of the exposed pulp

Figures 4.1 - 4.6 highlight an attempt to categorise the visual appearance of the exposed pulp. **Figure 4.1 (i).** depicts minimal bleeding on initial penetration of the buccal and palatal pulp horns in an upper premolar, followed by a more active bleeding (**Figure 4.1 (ii).**) once unroofing of the pulp chamber was completed and initial haemostasis (**Figure 4.1 (iii).**) after blood collection was complete. **Figure 4.2 (i).** illustrates a distinctly reduced vascularity on initial penetration of the roof of the pulp chamber. With further unroofing of the pulp chamber (**Figure 4.2 (ii).**) a damaged pulp, exhibiting reduced vascularity is evident. Copious bleeding is revealed during tissue removal (**Figure 4.2 (iii).**). Initial haemostasis is pictured in **Figure 4.2 (iv).** Initial entry into the pulp reveals an avascular pulp (**Figure 4.3 (i).**), after which, profuse bleeding is encountered (**Figure 4.3 (ii).**) overflowing the pulp chamber. Arrest of the resultant haemorrhage is controlled with minimal effort **Figure 4.3 (iii).** and **Figure 4.3 (iv).** **Figure 4.4 (i).** reveals a pink hue just prior to piercing the pulp horn, to reveal a profuse (**Figure 4.4 (ii).**), overflowing (**Figure 4.4 (iii).**) haemorrhage. Despite deeper amputation (**Figure 4.4 (iv).**), continuous refill of the pulp chamber (**Figure 4.4 (v).**) is noted. **Figure 4.5 (i).** illustrates a micro abscess upon penetration of the roof of the pulp chamber (**Figure 4.5 (ii).**). Purulent discharge is evident (**Figure 4.5 (ii).** and **Figure 4.5 (iii).**), under which, profuse bleeding is revealed (**Figure 4.5 (iv).**). Continuous refill of the pulp chamber is not possible to control (**Figure 4.5 (v).**), despite deeper amputation, gentle irrigation and repeated application of pressure. **Figures 4.6 (i).** reveals a distinctly avascular pulp space. A pink hue is evident just prior to unroofing **Figure 4.6 (ii).**, revealing a blood-stained, serous fluid within the pulp chamber (**Figure 4.6 (iii).**). The root canal space is devoid of any connective

Table 4.2 Proposed theoretical categorisation of the visual inspection of the exposed pulp aligned with (i). Classic and (See over) (ii). Novel classifications of pulp status

Diagnostic category		Visual inspection	Representative image	
(i). Classic Classification	Normal pulp	Minimal bleeding on exposure, haemostasis achieved spontaneously, without additional measures.		
	Reversible pulpitis	Bleeding evident on exposure, haemostasis readily achieved, sometimes application of light pressure may be needed. No deeper amputation required. Blood flow may be apparent, but does not flood pulp chamber.		
	Irreversible pulpitis	Profuse bleed, overflowing pulp chamber, may persist after deeper amputations. Micro abscess may be visible. Pressure and antimicrobial irrigation not always effective to achieve haemostasis.		

Diagnostic category		Visual inspection	Representative image		
(ii). Novel Classification	Normal pulp	Minimal bleeding on exposure, haemostasis achieved spontaneously, without additional measures.			
	Initial pulpitis	Bleeding evident on exposure, small volume, does not flow beyond site of exposure. Haemostasis achieved spontaneously, no additional measures.			
	Mild pulpitis	Bleeding evident on exposure, flow extends beyond site of exposure and may overflow pulp chamber. Haemostasis readily achieved with gentle irrigation and/or light pressure.			
	Moderate pulpitis	Reduced vascularity may be a feature at the site of exposure. Profuse bleed will be evident, overflowing pulp chamber, deeper amputation required to achieve haemostasis. Application of pressure, or antimicrobial disinfection effective to achieve haemostasis.			
	Severe pulpitis	Profuse bleed, overflowing pulp chamber persists after deepest amputation. Micro abscess may be visible. Pressure and antimicrobial disinfection does not stem the flow.			

tissue (**Figure 4.6 (iv).**).

4.3.5 Operative procedures

After exposure, the operative procedures were completed in a standardised manner, described in **Section 2.8.2**. Pulpotomy, pulpectomy with the subsequent root filling and restoration, or extraction was carried out, as previously described in **Sections 2.8.2, 2.8.2.1, 2.8.2.2 and 2.8.2.3**. All patients were followed up in a process described in more detail in **Section 2.8.3**. Patients were advised that they could return for unscheduled review, if required, if their symptoms did not improve after intervention, or if any new symptoms developed prior to their planned clinical review to evaluate treatment outcome.

4.3.6 Outcome evaluation

Patients were invited to return for a scheduled review at regular intervals to evaluate their response to treatment. Details of the outcome evaluation, as well as the definition of success for pulpotomy and root canal treatment are detailed in **Sections 2.8.4, 2.8.4.1 and 2.8.4.2** respectively. Patients who failed to return for scheduled review were contacted again, if necessary up to a maximum of three times, by phone and/or by post.

4.3.7 Statistical analysis

Descriptive statistics were prepared using Microsoft Excel 2019 MSO. Maximum and minimum values, means, medians, ranges and standard deviations are reported in relevant sections. Further statistical analyses were carried out using SPSS Statistics

25 (IBM). Measures of agreement were produced using Cohen's and Fleiss Kappa statistics. Diagnostic accuracy was reported in percentages relating to sensitivity, specificity, false positive and false negative rates, positive-, and negative-predictive values and disease prevalence. A Chi-squared analysis was conducted to compare qualitative data between the various diagnostic parameters, as well as between the two treatment groups (pulpotomy and pulpectomy).

4.4 Results

4.4.1 Study population, demographics and outcome

Fifty patients were recruited to this prospective, observational study (**Figure 4.7**). Two patients were excluded; one due to the inability to collect pulpal blood immediately after extraction (methodological) and one due to an inability to tolerate the dental dam (uncooperative). The mean age of patients enrolled in the study was 39.1 years (**Table 4.3**), which encompassed an age range of 14-78 years. The ratio of female: male participants was nearly 2:1

In total, 35 molars, 10 premolars, two canines and three incisors received an intervention; either pulpotomy, pulpectomy (and subsequent root canal treatment), or extraction (**Table 4.3**). The distribution by tooth type, age and gender is summarised in **Table 4.3**. Twenty-four pulpotomies were carried out in twenty-three patients, nineteen pulpectomies/root canal treatments were provided for eighteen patients and five patients required extraction (summarised in **Figure 4.7**).

Figure 4.7 Flowchart of recruited and analysed cases [n=: number, @: at; r/c: recall]

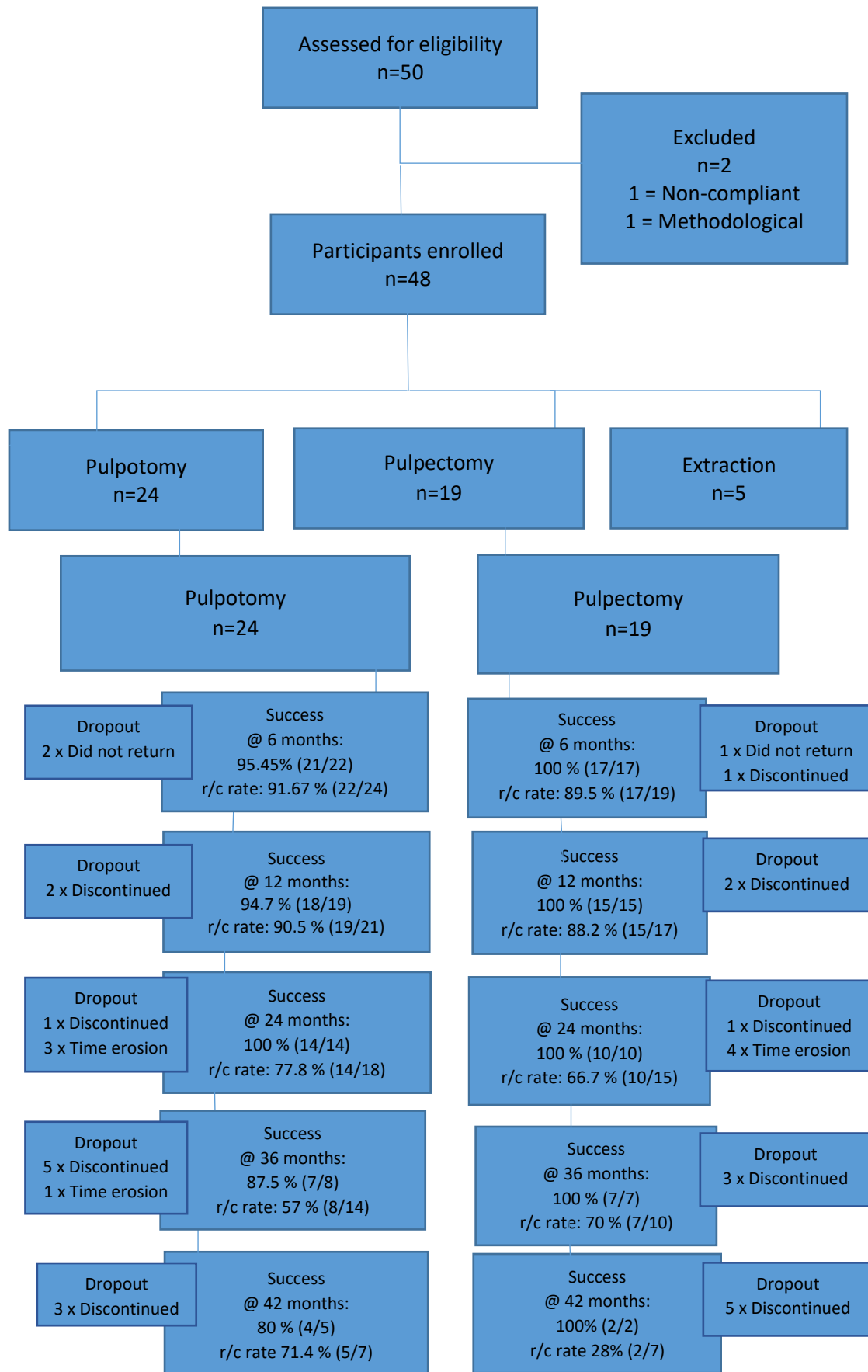


Table 4.3 Summary of study demographics by treatment, symptoms and caries depth
[pp=pulpotomy; pe=pulpectomy; xLa: Extraction; n=number; Ex Deep: Extremely deep, F: female, M: male]

	Age (years) Mean [Range]	Gender	Molar	Premolar	Canine	Incisor
All	39.1 [14-78]	F: n = 32; M: n = 17	35	10	2	3
pp	40.5 [17-76]	F: n = 16; M: n = 8	17	4	2	1
pe	41.8 [14-78]	F: n = 11; M: n = 8	15	2	0	2
xLa	29.0 [15-57]	F: n = 5	2	3		
Pain	40.1 [14-76]	F: n = 25; M: n = 13	n = 38			
Deep caries	44.1 [15-78]	F: n = 12; M: n = 3	n = 15			
Ex Deep caries	38.5 [14-69]	F: n = 17; M: n = 11	n = 28			

Thirty-eight patients presented with pain, 15 teeth were diagnosed with deep caries and 28 teeth were diagnosed with extremely deep caries (**Table 4.3**). Age and gender difference were not statistically significant between the treatment (pulpotomy and pulpectomy) groups ($p < .05$) [data adjusted to exclude teeth that were extracted].

4.4.2 Diagnostic distribution of cases

Tables 4.4 - 4.5 show the distribution of the diagnostic parameters for both classifications and clearly highlight disparity between the patient complaint (history), the information that is ascertained clinically and what is discovered after entering and visualising the inflamed pulp tissue. Wolters' classification highlights the spectrum of findings associated with a diagnosis of "irreversible" pulpitis. Of the 37 cases clinically diagnosed classically as irreversible pulpitis (**Table 4.4**, circled red); 7/37, 18/37 and 12/37 were demonstrated to have mild, moderate, and severe pulpitis, respectively (**Table 4.5**, circled red), when Wolters' criteria were used.

Table 4.4 Assignment of cases according to Classic pulp diagnoses [History: Patient reported symptoms; Clinical: Clinical diagnosis based on routine sensitivity tests; Direct inspection: Direct observation of the exposed pulp; Red circle for emphasis of clinical diagnosis of irreversible pulpitis]

Pulp Diagnosis Classic	Normal	Reversible pulpitis	Irreversible pulpitis	Total necrosis
History	11	5	32	
Clinical	4	7	37	
Direct inspection	8	3	34	3

Table 4.5 Assignment of cases according to Wolters' pulp diagnoses [History: Patient reported symptoms; Clinical: Clinical diagnosis based on routine sensitivity tests; Direct inspection: Direct observation of the exposed pulp; Red circles to emphasise spectrum of irreversible pulpitis, referring to Table 4.4 (above)]

Pulp Diagnosis Wolters'	Normal	Initial pulpitis	Mild pulpitis	Moderate pulpitis	Severe pulpitis	Total necrosis
History	11	4	5	13	15	
Clinical	4	7	7	18	12	
Direct inspection	8	1	5	8	23	3

The measure of agreement between each of the diagnostic parameters (patient history, clinical diagnosis and direct inspection) was carried out using Cohen Kappa statistic. Fleiss Kappa was used to compare all three parameters. The results for the Classic diagnostic parameters are presented in **Table 4.6**, and for Wolters' diagnostic parameters in **Table 4.7**.

Table 4.6 Measures of agreement between diagnostic parameters according to Classic categorisations [History: Patient reported symptoms; Clinical: Clinical diagnosis based on routine sensitivity tests; Direct inspection: Direct observation of the exposed pulp]

Comparisons using Classic Classifications			Cohen's Kappa	Fleiss Kappa
History	Clinical		0.5215	
History	Direct inspection		0.1987	
Clinical	Direct inspection		0.1931	
History	Clinical	Direct inspection		0.3134

Table 4.7 Measures of agreement between diagnostic parameters according to Wolters' categorisations [History: Patient reported symptoms; Clinical: Clinical diagnosis based on routine sensitivity tests; Direct inspection: Direct observation of the exposed pulp]

Comparisons using Wolters' Classifications			Cohen's Kappa	Fleiss Kappa
History	Clinical		0.5987	
History	Direct inspection		0.2116	
Clinical	Direct inspection		0.2569	
History	Clinical	Direct inspection		0.3653

Moderate agreement (Kappa scores ranging from 0.41 - 0.60) was demonstrated when patient history was compared with the clinical diagnosis, using both Classic ($\kappa = 0.5215$) and Wolters' ($\kappa = 0.5987$) criteria. Slight agreement (defined as Kappa scores of 0.10 – 0.20) was demonstrated when direct inspection was compared with patient history ($\kappa = 0.1987$) and with clinical diagnosis ($\kappa = 0.1931$) using Classic definitions. When Wolters' criteria were applied, the agreement improved to a level of fair (defined as scores ranging from 0.21 – 0.40) agreement ($\kappa = 0.2116$ and $\kappa = 0.2569$, respectively).

The Fleiss Kappa analysis resulted in a fair level of agreement when the three diagnosis parameters were contrasted, using Classic ($\kappa = 0.3134$) and Wolter's ($\kappa = 0.3653$) criteria.

4.4.3 Haemorrhage control related to clinical diagnoses

The distribution of cases within each diagnostic category is listed with additional subcategorisation of the ability to achieve haemostasis, as well as the relationship between caries depth with the treatment provided; pulpotomy, or pulpectomy (Tables 4.8, and Table 4.9). Notable findings relate to the clinical (Classic) diagnosis of irreversible pulpitis (Table 4.8, circled red), where pulpal haemorrhage was

possible to control (21 cases). The spectrum of inflammation diagnosed by Wolter's Classification (**Table 4.9**, circled red) included 5 cases of mild pulpitis, 14 cases of moderate pulpitis and two of severe pulpitis. The fact that haemostasis was not possible to achieve in 3 cases diagnosed as normal and reversible pulpitis (**Table 4.8**); or normal and initial pulpitis (**Table 4.9**), underpins some of the diagnostic challenges in accurately determining the pulp status preoperatively, versus *in situ*. Total necrosis was identified exclusively in cases diagnosed with irreversible pulpitis (**Table 4.8**), or moderate-severe pulpitis by Wolters' classification (**Table 4.9**).

Table 4.8 Classic diagnostic distribution catalogued according to Bleeding, Treatment and Caries depth parameters [Dx: Diagnosis; NP: Normal pulp; RP: Reversible pulpitis; IP: Irreversible pulpitis; HA*: Haemostasis achieved (adjusted to include elective pe); HNA: Haemostasis not achieved; TN: Total necrosis; Tx: Treatment; pp: pulpotomy; pe: pulpectomy; Deep: Deep caries (Bjørndal et al. 2019); Ex Deep: Extremely deep caries (Bjørndal et al. 2019); Red circle draws readers' attention to cases, that despite a diagnosis of irreversible pulpitis, haemostasis was achieved]

Classic Pulp Diagnosis				
Dx	NP	RP	IP	Total
Participated	4	8	36	48
HA*	3	6	21	30
HNA	1	2	12	15
TN			3	3
Tx: pp		5	19	24
Deep		1	7	8
Ex Deep		4	12	16
Tx: pe	1	2	16	19
Deep			4	4
Ex Deep	1	1	10	12

Table 4.9 Wolters' diagnostic distribution catalogued according to Bleeding, Treatment and Caries parameters [Dx: Diagnosis; NP: Normal pulp; InP: Initial pulpitis; MiP: Mild pulpitis; MoP: Moderate pulpitis; SeP: Severe pulpitis; HA*: Haemostasis achieved (adjusted to include elective pe); HNA: Haemostasis not achieved; TN: Total necrosis; Tx: Treatment; pp: pulpotomy; pe: pulpectomy; Deep: Deep caries (Bjørndal et al. 2019); Ex Deep: Extremely deep caries (Bjørndal et al. 2019); Red circles draw readers' attention to cases, that despite diagnoses within the spectrum of irreversible pulpitis, haemostasis was achieved]

Wolters' Pulp Diagnosis						
Dx	NP	InP	MiP	MoP	SeP	Total
Participated	4	8	7	17	12	48
HA*	3	6	5	14	2	30
HNA	1	2	1	2	9	15
TN				2	1	3
Tx: PP		5	3	14	2	24
Deep		1	1	6		8
Ex Deep		4	1	9	2	16
Tx: PE	1	2	2	3	11	19
Deep			1	1	2	4
Ex Deep	1	1		2	8	12

A Chi-squared Pearson correlation was conducted comparing Classic and Wolters' pulp diagnoses with the bleeding characteristics (**Tables 4.8, 4.9**), noted as haemostasis achieved, haemostasis not achieved, or total necrosis. No significant differences were detected using the Classic diagnoses of normal pulp, reversible pulpitis and irreversible pulpitis were compared with these bleeding characteristics ($p = .403$ and $p = .801$, when total necrosis was censored). Statistical significance ($p = .025$) was reached when Wolters' classification was correlated with bleeding characteristics. When the parameter of total necrosis was censored, that significance increased substantially ($p = .003$). Data censoring was deemed appropriate in this respect, due to the fact that the test was focused specifically on the assessment of pulpitis, and not necrosis.

4.4.4 Caries depth related to clinical diagnoses and treatment modality

Twenty-four cases were treated by pulpotomy; nineteen of which were clinically diagnosed as irreversible pulpitis (**Table 4.8**), or ranging from mild-to-severe (Wolters et al. 2017) pulpitis (**Table 4.9**), which would traditionally be considered to require root canal treatment. In the pulpotomy cohort, 12/19 cases presented with extremely deep caries and a clinical diagnosis of irreversible pulpitis (**Table 4.8**), or mild-severe pulpitis (**Table 4.9**). Nineteen cases were treated by pulpectomy, three of which were diagnosed classically as normal and reversible pulpitis (**Table 4.8**), or normal and initial pulpitis according to Wolters' criteria (**Table 4.9**). It is notable that two of these cases were classified as presenting with extremely deep caries, and yet were diagnosed as normal, or reversibly inflamed; again, highlighting a commonly occurring diagnostic challenge. The majority (10/12) of extremely deep caries, treated by pulpectomy were diagnosed as irreversible pulpitis (**Table 4.8**), or ranged from mild to severe (Wolters et al. 2017) pulpitis (**Table 4.9**). However, conservative pulp treatment (pulpotomy) was possible in 75% (12/16) of cases diagnosed as irreversible, or mild-severe pulpitis associated with extremely deep caries. This contrasts with 83.3% (10/12) of cases requiring pulpectomy presenting with the same pulp diagnoses and caries depth. Three elective root canal treatments were included in this cohort, one reversible/initial pulpitis and two irreversible/mild pulpitis. Bleeding was possible to control in all instances, where elective root canal treatment was prescribed. Haemorrhage control was not possible in sixteen cases and therefore pulpectomy was necessary.

Caries depth did not correlate significantly with Classic ($p = .477$), or Wolters' ($p = .297$) diagnostic parameters. Chi-squared analyses comparing caries depth

(deep, or extremely deep), with treatment (pulpotomy, or pulpectomy) was also demonstrated not to be significant ($p = .251$).

Table 4.10 Caries depth categorised according to haemorrhage control [HA: Haemostasis achieved; HNA: Haemostasis not achieved; TN: Total necrosis; Deep: Deep caries (Bjørndal et al. 2019); Ex Deep: Extremely deep caries (Bjørndal et al. 2019)]

	HA	HNA	TN
Deep	9	6	0
Ex Deep	15	9	3

Haemorrhage control was not possible to achieve in 6/15 cases presenting with deep caries (**Table 4.10**); in contrast with 15/27 of cases, categorised with extremely deep caries where haemostasis was possible to achieve. Total necrosis was identified exclusively in cases categorised with extremely deep caries. No significant differences were detected when bleeding characteristics were compared with caries depth ($p = .403$). Treatment choice (pulpotomy, or pulpectomy) did not appear to be significantly influenced by Classic diagnostic parameters $p = .311$. When Wolters' diagnostic categories were analysed relative to the treatment chosen, statistical significance was demonstrated ($p = .005$).

4.4.5 Symptoms related to treatment modality and haemorrhage control

Table 4.11 summarises the treatment allocation (pulpotomy, or pulpectomy) and haemorrhage control as they relate to presence of pain. Elective pulpectomy was provided for three cases without pain. Haemorrhage control was possible in all cases (HA $n=10$) presenting without pain. When the presence, or absence of pain was associated with the treatment provided (pulpotomy, or pulpectomy), a significant correlation was not shown ($p = .907$).

Table 4.11 Treatment modality and haemorrhage control categorised according to presence of symptoms [pp: pulpotomy; pe: pulpectomy; HA: Haemostasis achieved; HNA: Haemostasis not achieved; TN: Total necrosis; *: treatment planned for pe]

	pp	pe	HA	HNA	TN
Pain	20	16	20	14	3
No pain	4	3*	10	0	0

4.4.6 Diagnostic accuracy

The potential to introduce haemorrhage control as a reference standard of the level of pulpitis compared with the diagnostic accuracy of clinical diagnosis using index tests based on Classic and Novel definitions was assessed. Haemorrhage control was defined as haemostasis achieved, or haemostasis not achieved. Any cases revealing total necrosis were excluded from the analysis. The index test was defined as health, or disease. Health was defined as normal pulp and reversible pulpitis, whereas disease was defined as irreversible pulpitis in the Classic definition. Strict, or loose criteria were applied to Wolters' definitions. In the strict definition, health was defined as a combination of normal pulp, initial pulpitis, mild pulpitis and moderate pulpitis and disease was defined as severe pulpitis, only. When loose criteria were applied, moderate pulpitis was moved from health to the disease definition. Haemorrhage control was also reviewed in relation to caries depth and symptoms. The raw data is presented in **Appendix XIV**.

The analysis of haemorrhage control provided the highest sensitivity (88.2%) when it was compared using the Classic diagnostic criteria of reversible and irreversible pulpitis only. When normal pulp was included with reversible pulpitis the sensitivity reduced to 83.3%. Specificity was low however in the Classic domain. The negative predictive values (75%) performed better than the positive predictive values

(41.7%). Chi-squared analysis of haemorrhage control compared with Classic diagnosis did not reach significance ($p > .05$).

Wolters' diagnosis produced an acceptable sensitivity of 73.2% when normal pulp was included with initial, mild and moderate pulpitis to denote health, using the loose criteria described. This value decreased substantially however, when the strict criteria were analysed (56.3%), only correctly identifying the presence of disease in just over half of the cases assessed. The application of strict criteria had a positive effect on the test specificity (96.6%), compared with loose criteria (50%). The measurements of positive and negative predictive values scored highly using Wolters' strict criteria (90% and 80%, respectively). Chi-squared analysis of the Wolters' diagnostic accuracy using strict criteria produced highly significant associations ($p < .0001$) and significant agreements using Kappa statistic ($p < .001$). The presence of symptoms was not shown to be a useful reference standard.

4.4.7 Treatment outcome

Treatment outcome (pulpotomy, or pulpectomy) was evaluated clinically and radiographically by the principal operator at time intervals up to 42 months (cut-offs for the purpose of analysis demarcated as: 6, 12, 24, 36 and 42 months). In **Figure 4.9** success and recall rates are reported as percentages with numbers in parentheses. The overall treatment outcomes, independent of time, are summarised in **Table 4.12**.

Pulpotomy had an overall success rate of 83.3% (20/24), with a recall rate of 91.67%. Pulpectomy was successful 100% (19/19) of the time, with a recall rate of 95%. The median follow-up time for the pulpotomy cohort was 28 months, ranging

from 13 days to 45 months. The median follow-up time for the pulpectomy cohort was 32 months, ranging from 2 - 45 months.

Table 4.12 Summary of treatment outcomes [Tx: treatment; pp: pulpotomy; pe: pulpectomy; n: number; %: percentage]

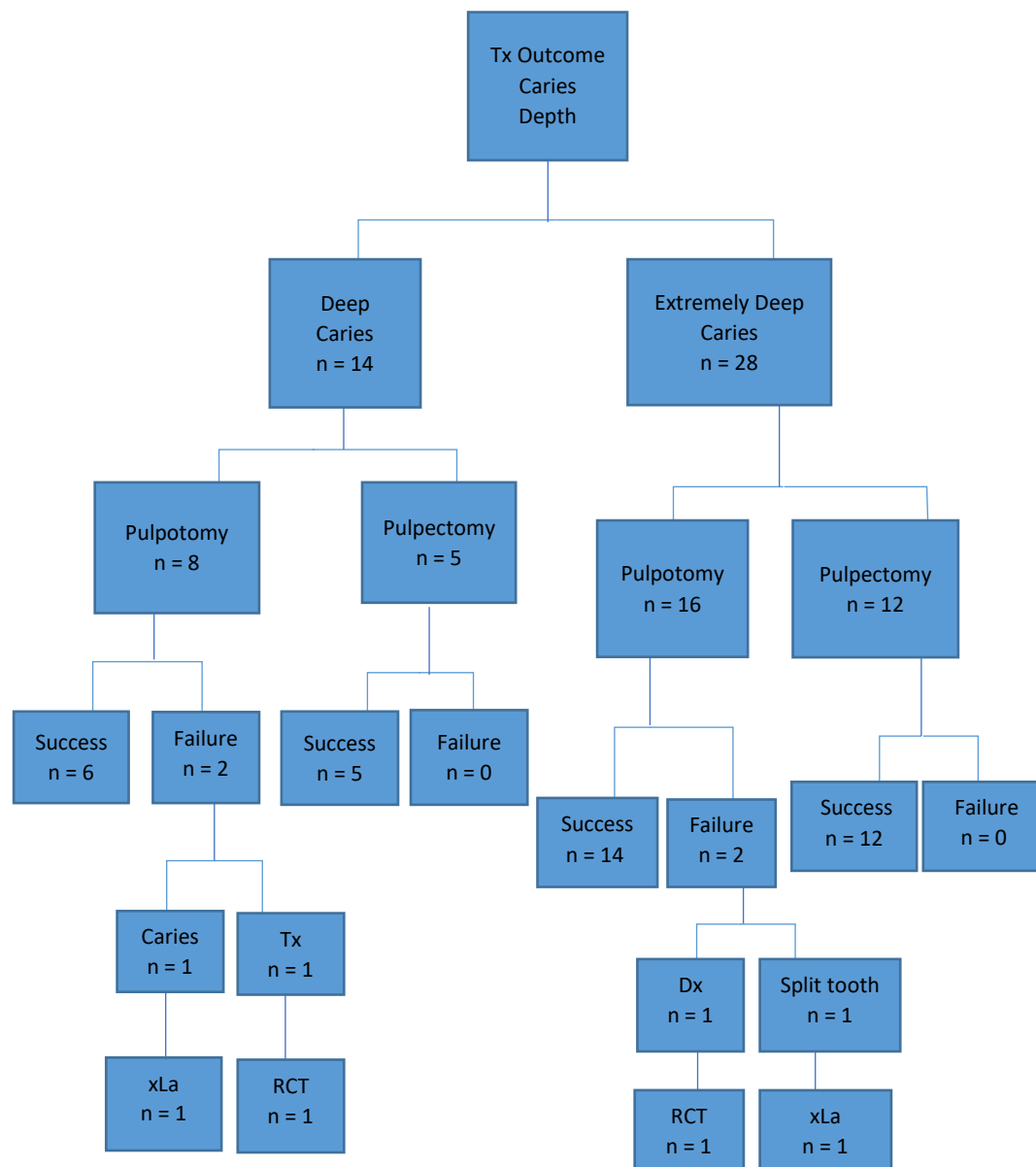
Tx	Success % (n)	Failure % (n)	Follow-up Months Median (Range)	RC Rate % (n)
pp	83.3 (20/24)	16.67 (4/24)	28 (0.33-45)	91.67 (22/24)
pe	100 (19/19)	0 (0)	32 (2-45)	95 (18/19)

4.4.8 Treatment outcome and caries depth

Figure 4.8 illustrates the outcome of treatments (pulpotomy, or pulpectomy) as they relate to caries depth. No failures were identified in the pulpectomy treatment group, in either the deep, or extremely deep caries categories. Eight cases presenting with deep caries were treated by pulpotomy. Two failures occurred, revealing a 25% (2/8) failure rate. Interrogation of the clinical data revealed both cases were late failures at 39 months and 41 months. The modes of failure were secondary caries and apical periodontitis, respectively. The caries failure was unrestorable and extraction was necessary. The failed pulpotomy was possible to treat by root canal treatment.

Sixteen cases presenting with extremely deep caries were treated by pulpotomy. Two failures (12.5%) occurred. One failure was an early, diagnostic failure. The patient returned after 13 days with increased pain and root canal treatment was provided. The second failure presented at 10 months with a split tooth, which was unrestorable. An extraction was provided.

Figure 4.8 Flowchart linking treatment outcome with caries depth [Tx: treatment; n: number; Dx: diagnosis; RCT: Root canal treatment; xLa: extraction]

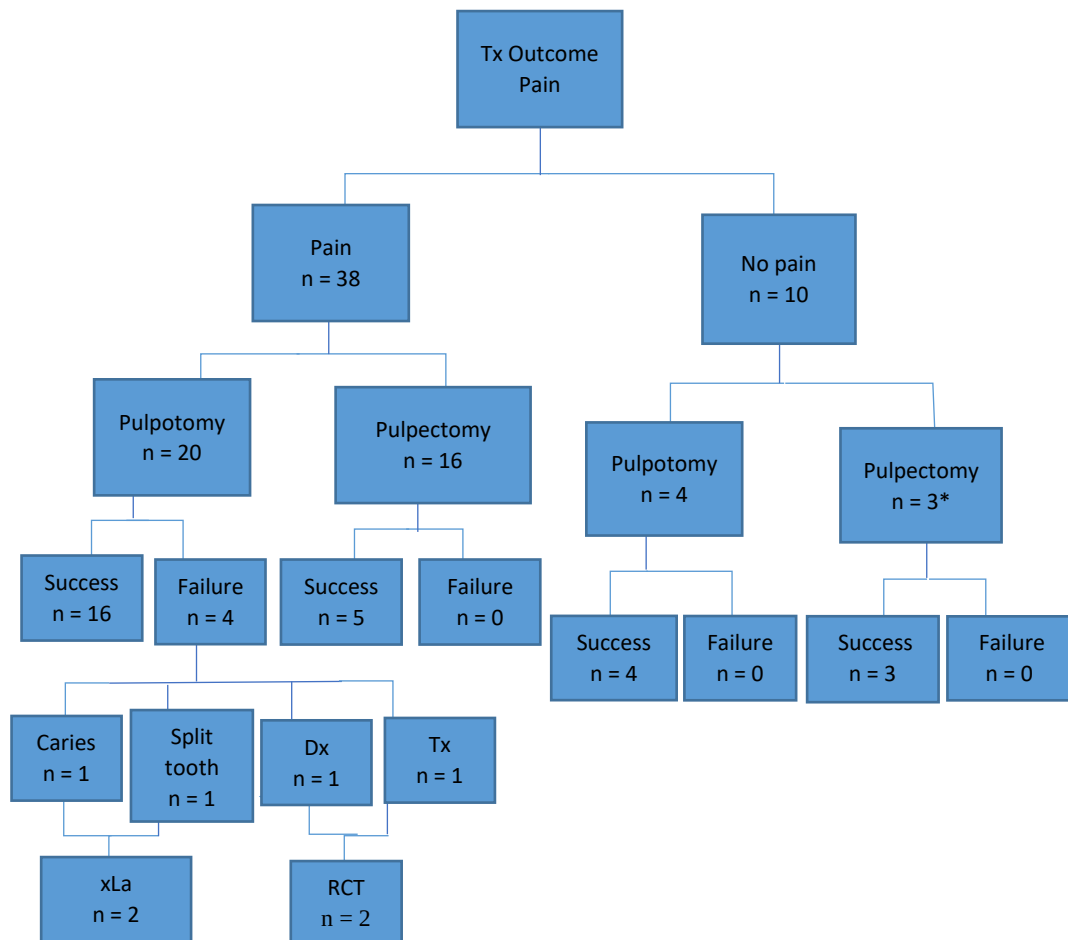


4.4.9 Treatment outcome and the presence of symptoms

A flowchart is presented in **Figure 4.9** outlining the outcomes of the treatments categorised by the presence of pain. No failures were observed in the 10 cases presenting without pain. Of the 38 cases identified with pain, 20 were treated by pulpotomy. An overall success of 80% (16/20) was recorded. Of the four cases that

failed, two were possible to manage by subsequent intervention, namely root canal treatment; extraction was necessary in the other two failures.

Figure 4.9 Flowchart linking treatment outcome with the presence of pain [Tx: treatment; n: number; Dx: diagnosis; RCT: Root canal treatment; xLa: extraction]



Treatment outcome (success, or failure) of the pulpotomy cohort was evaluated relative to the diagnostic parameters (Classic and Wolters'). This data was censored to remove cases with a diagnosis of normal pulp. No significant association could be demonstrated ($p = .099$ and $p = .251$, respectively). Caries depth and the presence, or absence of pain did not influence treatment outcomes of the censored pulpotomy cohort ($p = .515$ and $p = .425$).

4.4.10 Failed pulpotomy details

Details of the four failed pulpotomy cases are summarised in **Table 4.12** and are documented in **Appendix XIII**. All failures occurred in molars. The visual appearance of the pulp was severely inflamed in 3 out of the 4 failed cases, all pulps were designated as irreversible pulpitis ranging from moderate to severe. Caries depth was deep in two cases and extremely deep in the remaining two cases. The patients ranged in age from 18 - 60 years. A 50:50 gender-ratio was noted. Time to failure ranged from 2 weeks to 41 months. The modes of failure presented as structural (caries and split tooth), diagnostic and treatment. The diagnostic failure presented as an early failure and was remedied with root canal treatment. Apical periodontitis developed at 41-month recall and was possible to manage with root canal treatment. The structural failures were catastrophic, rendering the teeth unrestorable and extraction was necessary.

Table 4.13 Details of the failed pulpotomies [FDI tooth numbering system; #: number; RCT: root canal treatment; xLa: extraction, Dx: Diagnosis; DI: Direct inspection, Tx: Treatment]

Tooth #	Age (Years)	Gender	Dx	DI	Time to failure (Months)	Likely mode of failure	Management
37	33	F	IP/MoP	NP	41	Tx failure	RCT
16	18	M	IP/MoP	IP/SeP	10	Split tooth	xLa
47	60	M	IP/MoP	IP/SeP	39	Caries	xLa
37	41	F	IP/SeP	IP/SeP	0.33	Dx failure	RCT

4.5 Discussion

The success rate of primary molar root canal treatment has been reported as 79.9% in a Swedish population (Laukkanen et al. 2019). Thyvalikakath et al. (2022) reported a comparable 5-year survival of root treated molars (74.6%) in a large, American practice-based-network retrospective study. Laukkanen et al. (2021) pragmatically

evaluated the outcome of root canal treatment carried out by general dental practitioners according to tooth type, reported an incidence of suboptimal root fillings in 65% and 49% of maxillary and mandibular molars, respectively. The overall success of molar root canal treatment was shown to be 55.6% (Laukkanen et al. 2021). Barriers relating to the provision of root canal treatment cited by general dentists include technical difficulty (Peciulienė et al. 2009), training, equipment and remuneration (McColl et al. 1999).

In this study, 17 molars designated for pulpotomy (**Table 4.3**), resulted in an overall success rate of 76.5%. The pooled survival of molar pulpotomy, with and without an additional intervention (subsequent root canal treatment), was 88.2%. The evidence to support pulpotomy as an appropriate treatment for mature teeth presenting with irreversible pulpitis is compelling, based on comparable success rates (Asgary et al. 2022, Taha et al. 2023); conservative and biologically-orientated treatment (Duncan et al. 2019b, Lin et al. 2020); as well as a perceived, relative technical ease compared with root canal treatment (Stanley 1989). However, this has not translated into routine uptake in general practice (Edwards et al. 2021), with barriers such as training and contemporary knowledge (Stangvaltaite et al. 2017); as well as equipment and material cost (Chin et al. 2016), being frequently cited. Variations exist in the treatment options selected by operators to manage the inflamed pulp in questionnaire-based research (Careddu et al. 2021, Frisk et al. 2013).

European undergraduate curricula should incorporate and assess competence in the provision of VPTs of their students (De Moor et al. 2013). However, undergraduate students reported high levels of stress at the prospect of exposing the pulp clinically (Chevalier et al. 2021). The provision of practical, pre-

clinical training using 3D-printed teeth, simulating deep caries with bleeding pulps was found to benefit the students in terms of reducing their anxiety towards VPTs, as well as increasing their confidence (Chevalier et al. 2022). Over the ten-year period from 2011-2021, the adoption of VPTs, in preference to root canal treatments, increased in the management of exposed pulps (Stangvaltaite-Mouhat et al. 2022), which was demonstrated to be influenced by the year and site of graduation.

4.5.1 Visual description

The condition of the pulp at the point of exposure was variable. What was visualised under microscopic magnification ranged from an apparent reduced vascularity, to surface necrosis and in certain instances, abscess and finally, total necrosis. The histopathological depiction of similar examples has been comprehensively documented by Ricucci et al. (2019). Unfortunately, in the clinical situation, the transition to healthy pulp is not so clearly discerned. Enhanced magnification and illumination are invaluable adjuncts to provide quality care (Okamoto et al. 2021). Taha et al. (2022) conclude that intraoperative decision-making in relation to pulpal bleeding relates only to colour (uniformly red, without any visibly deconstructed pulp tissue) and secondly, the relative ease with which haemorrhage is controlled.

Students and general practitioners report greater confidence in direct pulp capping rather than partial, or full pulpotomy (Chevalier et al. 2021, Stangvaltaite et al. 2017), perhaps indicating a lack of confidence in handling pulp tissue. In the absence of suitable magnification and illumination however, partial necrosis could be mistaken for haemostasis, particularly when there is a fear of managing pulp tissues, or a providing an unscheduled, technically demanding root canal treatment. In a

randomised controlled clinical trial comparing stepwise excavation (SWE) with partial caries removal (Maltz et al. 2012, Maltz et al. 2018), the methodology revealed that in the first visit, the SWE cohort received an indirect pulp capping (IPC), a more aggressive form of caries removal than is now defined by ESE (Duncan et al. 2019a). It is therefore not surprising that the SWE group (or more accurately, the IPC group) resulted in more failures. It is widely accepted that IPC risks pulp exposure significantly more than SWE (Bjørndal et al. 2017, Hoefler et al. 2016). Extrapolated to general practice, with numerous aforementioned barriers, this information potentially biases the message that pulp exposure is deleterious to the maintenance of pulp vitality.

The fact that pulpal haemorrhage is not considered a prognostic indicator in the outcome of pulpotomy, based on current available evidence (Duncan et al. 2023), is at variance with the fact that the inflammatory status of the pulp does appear to influence the outcome of pulp preservation treatments. Therefore, the manner in which the visual status of the pulp is measured; be that haemorrhage control, or time to haemostasis, could be examples of flawed reference standards, contributing to a misclassification bias (Roever 2016).

Notably, the character, volume and dynamics of pulpal bleeding in this study did not accurately reflect the level of inflammation expected from the combined patient history and clinical findings. One of the limitations of this study should be highlighted, as being a single operator study, not blinded to the clinical information including radiographic images and information from standard endodontic tests. This information may have biased the allocation of cases into pre-defined groups; particularly in the visual inspection category, which was carried out later. The validity

of the proposed classification, could be tested in a larger future study using calibrated examiners and blinding them to the other parameters, such as the clinical findings, or the patient history.

4.5.2 Diagnostic data

The core limitation in clinical pulp diagnosis is the absence of direct access to test the pulp tissues (Newton et al. 2009). Consensus in diagnostic terminology has not yet been agreed between international groups (Azim et al. 2022). Establishing a clear association between patient-reported symptoms and pulpal inflammation has traditionally been difficult to achieve (Hasler & Mitchell 1970, Michaelson & Holland 2002) and this difficulty does appear to be supported by the preliminary findings of this project. It brings into focus the disparity between the traditional clinical diagnostic categorisation of pulpitis and its visual representation. The visual depiction of the exposed pulp is poorly described in the literature and lends credence to the proposal for establishment of an objective measure to analyse the inflamed pulp. This is of particular importance in guiding clinicians to offer treatments tailored towards predicable preservation of pulpal vitality.

4.5.3 Diagnostic reasoning

Three diagnostic reasoning strategies were compared by Coderre et al. (2003) to investigate how various problem-solving strategies influenced diagnostic success. They concluded that the hypothetico-deductive reasoning (a method by which cyclic patterns of reasoning and observation are used to create and test a hypothesis) was weak, ineffective and prone to error. Pattern recognition is not suited to novice

clinicians, as it is based on development of repertoire of matched scenarios, based on similar clinical experiences.

The most significant variables were identified as diagnostic reasoning, expertise and clinical presentation (Coderre et al. 2003). Inductive problem solving used in medical undergraduate training utilise expert schemes to identify, define and solve diagnostic questions can pose problems with the correlation across different strategy choices, not different aspects of the problems *per se* (Eva et al. 1998).

Heuristics, utilising intuition and focusing only on few, select details, is another recognised clinical decision model identified in clinical medicine (Marewski et al. 2012). The potential for bias and resultant diagnostic error has not been widely evaluated in primary care settings (Fernández-Aguilar et al. 2022).

Decision-making in dentistry is influenced by multiple factors. These choices, although strongly influenced by clinical factors, also consider factors such as potential longevity; contextual factors, which include the patient's views, the dentists view, or external circumstances, such as time constraints (Dawson et al. 2021). Enhancement of clinical decision-making accuracy is necessary to improve patient outcomes (Murdoch et al. 2023), particularly in presentations where clinical uncertainty exists (Hatipoğlu et al. 2023).

4.5.4 Correlations between haemorrhage control and caries, or pain related to the decision-making process

The time taken to control pulpal haemorrhage requires consideration. In a clinical setting, multiple clinical steps remain outstanding once haemorrhage control has been achieved. Notwithstanding correct placement of a HSC; ideally, a well-placed

adhesive restoration should be placed immediately after the VPT procedure (Duncan et al. 2019a).

Point-of-care testing has translated effectively in clinical periodontology (Lee et al. 1995, Sorsa et al. 1999, Sorsa et al. 2020). The implementation of Point-of-care testing could simplify elements of clinical decision processes (Florkowski et al. 2017), the application of which could present opportunities in VPT; however, there are no commercially available kits currently available.

Canoglu et al. (2022) cited evidence of bleeding recurring after placement of the capping material as an exclusion criterion. Four cases were excluded in total from this study, based on persistent bleeding within 5 minutes, or after placement of the capping material. It is not clear how many of the exclusions were due to the latter reason, underpinning the subjectivity of judging the inflammatory status of the pulp. Furthermore, as hydraulic calcium silicate materials are hydrophobic in nature they will absorb blood and at least give the appearance of renewed bleeding after material placement.

4.5.5 Outcome evaluation

All cases were followed-up (**Section 2.8.3**) and appropriately reviewed (**Sections 2.8.4, 2.8.4.1 and 2.8.4.2**) in a recognised clinical manner (Barreshi-Nussair & Quidemat 2006, Taha & Abdelkhader 2017, Asgary et al. 2018, Duncan et al. 2019a). Clinical success was defined as resolution of presenting symptoms, lack of tenderness to percussion, or palpation and absence of swelling, or sinus. Sensibility testing was performed for the pulpotomy cases, but absence of a response was not indicative of failure (Duncan et al. 2019a). Radiographic success was determined as the absence

of internal resorption and a PAI score < 2 (Ørstavik et al. 1986). Notably and perhaps surprisingly, equal numbers of failed pulpotomy were noted in the deep caries and extremely deep caries categories; however, the numbers of failure are too low to make meaningful conclusions (**Figure 4.8**).

Failures in the extremely deep caries category occurred substantially earlier (2 weeks, 10 months) than the corresponding deep caries failures (39, 41 months). Presence of pain was a feature of all failed cases (**Figure 4.9**). The relevance of pain and caries depth are regarded as factors likely to influence the outcome of pulpotomy (Duncan et al. 2023). The limitations of this study are however duly noted, being that of a single operator, with limited case numbers and a very small number of failures from which to draw meaningful conclusions

The cases were followed-up for as long as possible, but inevitable erosion occurred over time. Of the 24 cases treated by pulpotomy, 18 cases were reviewed for longer than 12 months and 14 were reviewed for 24 months, or more. Although not considered prospectively in the outcome evaluation, pulp canal sclerosis, or obliteration did not occur in any case up to the 45-month review.

Larger studies, with appropriate long-term follow-up are needed to carefully interrogate the factors that influence the provision of VPT in an adult population. Contemporary published VPT outcome studies relate primarily to university, or specialist settings (Ather et al. 2022) and as a result have limited external validity. There is a need to address whether the level of expertise can influence treatment outcome (Yepes et al. 2020, Duncan et al. 2023) and in which domain; diagnostic elements, or the practical provision of treatment.

4.6 Conclusion

The catalogue of images captured at various stages in the management of exposed pulps show marked variation in the character, volume and ease at which bleeding could be stemmed. As a result, other factors may play a significant role in the decision-making process. Although the ability to arrest pulpal bleeding appears to be a good predictor of pulpal health, it is prone to subjectivity in relation to its acquisition and other operator characteristics, therefore an objective quantifiable biomarker level would be a desirable adjunct.

With a growing emphasis on adopting minimally invasive techniques, such as VPT, perhaps our diagnostic standards may also need to be updated accordingly? The practice of referencing “gold-standard” histologic sections, may not be sufficiently translatable to modern clinical practice, as the true state can only be verified after extraction. As such, developing a suitable reference standard will assist the pursuit of superior diagnostic tests, particularly in the analysis of the inflamed pulp. As this work used pulpal blood and the treatment was not altered, could present a considerable benefit to the current work. In the absence of clinical follow-up, the true reversibility and level of infection cannot be reliably ascertained. The focus should perhaps now shift to adoption of surrogate end-points, which are more biologically-orientated, conservative and clinically relevant i.e. pulpal biomarkers. As such, developing a new more suitable reference standard through biomarker assessment will assist the pursuit of superior diagnostic tests, particularly in the analysis of the inflamed pulp.

Within the limitations of the current study, it appears that the visual appearance of the exposed pulp, caries depth and symptoms have poor correlation

with each other and treatment outcome. This underlines the need for larger, well-controlled studies to analyse these variables; but also, the urgent need for development of objective measurements of pulpal status.

Chapter 5

Cell death mediators as potential pulpal diagnostic biomarkers

5.1 Introduction

A complex, interconnected sequence of molecular events triggered during pulpitis are aimed principally at neutralising invading pathogens (Warfvinge et al. 1985, Hahn & Liewehr 2007a; Farges et al. 2009, Soden et al. 2009). Immune and inflammatory mechanisms, in combination with the selective upregulation of reparative and regenerative processes aim to restore pulpal health (Cooper et al. 2014). Imbalance within these systems through a lack of remedial dental treatment can progress to pulp necrosis (Heyeraas et al. 2001).

Two mechanisms of cell death are recognised, which occur as a result of either a regulated, or an accidental process (Galluzzi et al. 2015). Regulated cell death can function as normal physiological cell turnover, or be stimulated by stressors leading to the release of danger associated molecular patterns (DAMPs), or alarmins that alert the body to potential danger (Bianchi 2007, Nie et al. 2016). Pathogen associated molecular patterns (PAMPs) function to warn the host regarding the invasion of pathogens (Escamilla-Tilch et al. 2013). PRR's (**Section 1.6**) recognise these specific molecular signatures, which trigger the host to respond by activating innate immune networks to react to the threat (Ishii et al. 2008, Brown et al. 2011, Staquet et al. 2011).

Accidental cell death occurs when an overwhelming insult leads to rapid, catastrophic cell death; in contrast to programmed cell death, which accounts for one of many recognised forms of regulated cell death and functions to maintain normal tissue homeostasis in response to an external stimulus (Galluzzi et al. 2012, Hu et al. 2021). Although originally considered to be limited to apoptosis,

programmed cell death is now understood to encompass numerous interconnected molecular events, that are not limited to:

1. *Apoptosis*: Characterised by condensation of chromatin and degradation of DNA.
2. *Autophagy*: Important for normal cell turnover and maintenance of homeostasis, concludes with lysosomal degradation.
3. *NETosis*: Cell death characterised by disintegration of nuclear and granular membranes with release of decondensed chromatin, described as NETs.
4. *Necroptosis*: A form of programmed necrosis dependent on RIPK1 and/or RIPK3 activation (Galluzzi et al. 2012, Nikolettou et al. 2013, Dhuriya & Sharma 2018, Galluzzi et al. 2018, Liu et al. 2018, Wu et al. 2020).

Conversely, necrosis is typically characterised by cellular swelling that leads to expulsion of the cell contents following breach of membrane integrity (Vanlangenakker et al. 2008) and usually leads to inflammatory reactions which propagate further tissue damage (Festjens et al. 2006). Accidental cell death; namely necrosis, unlike many forms of regulated cell death is not modulated by enzymes (Fink & Cookson 2005, Tang et al. 2019). Recognised crosstalk between various modes of regulated cell death and necrosis has obscured the identification of a single causative mediator (Nikolettou et al. 2013, Wu et al. 2020, Lu et al. 2021). Therefore, a focus of biomarker research remains to identify surrogate endpoints that indicate cell death (Nahid et al. 2011, Wittwer et al. 2013, Penttilä et al. 2016).

The relationship between cell death and NETosis has been described, linking epigenetic histone modifications with specific cellular functions resulting in up-, or

down-regulation of NET activity (Füllgrabe et al. 2010, Wu et al. 2020). Increased circulating histones have been identified in patients with a range of conditions including cancers, sterile inflammation and infection (Allam et al. 2014). Regulated cell death mechanisms have been reported in pulpitis including apoptosis and NETosis (Huang et al. 2020). During NETosis, DAMPs, in the form of histones, are released into pulp tissues exerting a range of inflammatory and cytotoxic effects, which can mediate extensive tissue damage (Holder et al. 2019). (**Sections 1.8, 1.8.1 and 1.8.2**).

A sensitive assay in the form of an ELISA that was capable of quantifying levels of HMGB₁ was first developed by the Shino-Test Corporation in collaboration with Prof. Maruyama and colleagues (Yamada et al. 2003). Subsequent validation has confirmed the assays reliability (Lehner et al. 2012) and it has since been widely utilised in medical literature (Fahmueller et al. 2013, Wittwer et al. 2013, Liikanen et al. 2015, Laursen et al. 2016). Elevated levels of circulating HMGB₁ have been shown to be associated with the degree of severity of many disease states, including tuberculosis (Zeng et al. 2015), coronary artery disease (Hu et al. 2009), autoimmune disease (Urbonaviciute et al. 2008, Magna & Pisetsky 2014), neurodegenerative disorders (Festoff et al. 2016), liver disease (Laursen et al. 2015), cancer (Kang et al. 2013) and sepsis (Wang et al. 2004, Deng et al. 2019, Li & Lu 2021).

The presence of HMGB₁ has been demonstrated in an *in vitro* pulpitis model (Zhang et al. 2014). Importantly, Zhang et al. (2014) demonstrated immunolocalisation of cytoplasmic HMGB₁ in inflamed pulp tissues exclusively, which was significant as the HMGB₁ was normally confined to the nuclei of healthy specimens. These findings were also validated by Tancharoen et al. (2014).

Macrophages isolated from healthy and inflamed human pulp cells stimulated with HMGB₁ were shown to increase their expression of TLR-2 and TLR-4 in the inflamed groups (Mahmoudi et al. 2016). HMGB₁ stimulated pulp cells increased their mRNA expression of TLR-4 and MAPK (Mahmoud Hashemi et al. 2018). HMGB₁ has also been shown to have a role in proliferation and odontoblastic differentiation of human dental pulp cells (Qi et al. 2013), where its secretion from the nucleus to the cytoplasmic spaces was observed.

Cell death detection assays have been validated in medical research (Salgame et al. 1997, Holdenrieder et al. 2001ii, Kutcher et al. 2012, Ebrahimi et al. 2017, Thålin et al. 2017), but have not been applied to date as a potential diagnostic aid in molecular pulp biology. The fluctuations seen in the levels of inflammatory mediators *in vivo* have proven to be highly variable in human studies, underpinning the complexity and dynamics of the underlying mechanisms and regulatory processes. It is necessary to identify a discriminatory mediator which should ideally not be routinely expressed in health, or more importantly, even in mild inflammation. Specifically, the identification of the transition to the 'point-of-no-return'; namely necrosis (Ricucci et al. 2014), may provide the consistency lacking in the research to date.

The ability of nuclear molecules to perform both regulatory functions and act as immune triggers in host defence, presents certain challenges, as well as opportunities (Pisetsky 2014, Pandolfi et al. 2016). The identification of extracellular nuclear material can serve as novel biomarkers of conditions involving cell injury and death (Magna et al. 2016, Liu et al. 2018, Yang et al 2020b). Targeting such molecules presents an important novel approach to diagnosis, prognosis and therapeutic

strategies (Cooper et al. 2017, Holder et al. 2019), which has not yet been fully exploited in the clinical applications of dental pulp research.

5.2 Study aims and objectives

Biomarkers which can be objectively measured and evaluated, can be utilised as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions (Biomarkers Definitions Working Group 2001, Strimbu & Taavel 2010). Histologically, irreversible pulpitis depicts a focal necrosis within the coronal pulp (Anderson et al. 1981, Lin & Langeland 1981, Ricucci et al. 2014). The identification of the start-point of irreversible damage, namely necrosis, may present a novel target biomarker to differentiate these pulpal states and indicate cases that may not, or are less likely to respond to treatment. An assay targeting circulating extracellular histones was employed in this study to represent pulp cell death (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics), complemented by a quantitative ELISA measuring HMGB₁ (HMGB₁, IBL International GmbH, Hamburg Germany), capable of differentiating regulated from accidental cell death, with a view to developing a discriminatory chairside diagnostic test (Magna & Pisetsky 2016).

5.3 Materials and methods

Patient recruitment, consent, inclusion and exclusion criteria and diagnoses were conducted as described in **Sections 2.2 - 2.9**. Preparation for pulpal blood collection was carried out as reported in **Section 3.3.1**. Pulpal blood was collected as outlined in **Section 3.3.2** and stored at low temperature in advance of analysis **Section 3.3.3**.

5.3.1 Cell Death Detection ELISA^{PLUS}

The Cell Death Detection ELISA^{PLUS} was conducted as described in **Section 3.3.4.3**. Briefly, 20 µl of the test solution was diluted 1:4 in 1% BSA, 0.5% Tween, 1 mM EDTA in PBS, and added to streptavidin-coated microtiter plates, containing biotinylated mouse anti-histone antibody and peroxidase-conjugated anti-DNA antibodies. Peroxidase activity of the retained immune-complexes was developed by incubation with ABTS after standard washing steps and the subsequent colour reaction was then photometrically quantified using a Tecan GENios™ Spectrophotometer at 405 nm. The results were reported as absorbance units (AU). All samples were assayed in duplicate. In total, 23 cases were assayed using a volumetric methodology. The sensitivity of the kits was not stated by the manufacturer.

5.3.2 HMGB₁ ELISA

HMGB₁ in serum and plasma collected from the pulp was quantitatively measured using a sandwich-enzyme immunoassay. The test was conducted in the normal measurement range, in duplicate. Briefly, the wells of the microtiter strips were coated with purified anti-HMGB₁ antibody. HMGB₁ in the sample was bound to the immobilized antibody and incubated before a second enzyme marked antibody was conjugated. Wash steps were carried out according to the test protocol. The HMGB₁ sample concentration was determined using a standard curve constructed from seven serial dilutions of the HMGB₁ standard supplied with the kit (within the normal measurement range) and concentrations were reported in ng/mL. After the substrate reaction was completed, the HMGB₁ concentration was determined by measuring the optical density at 450 nm using Tecan GENios™ Spectrophotometer. In total, 18

cases were assayed using a volumetric methodology. The sensitivity of the kit (standard range) was stated as 1 ng/mL by the manufacturer.

5.3.3 Statistical analysis

Descriptive statistics were prepared using Microsoft Excel 2019 MSO. Maximum and minimum values, means, medians, ranges, standard deviations and quartiles are reported in the relevant sections. Correlation graphs and data charts were constructed using Microsoft Excel 2019 MSO. Further statistical analyses were carried out using SPSS Statistics Version 25 (IBM). Measures of agreement were produced using Cohen's Kappa statistics. Pairwise comparisons were conducted using the Kruskal Wallis Test for non-parametric data. Diagnostic accuracy was reported in percentages relating to sensitivity, specificity, false positive and false negative rates, positive-, and negative-predictive values and disease prevalence. A Chi-squared analysis was conducted to compare qualitative data between the categorical expressions of the biomarkers and diagnostic, treatment and preoperative evaluations of caries depth and the presence of symptoms. Box-and-whisker plots are presented to summarise the threshold data and treatment choices relative to the expression of the biomarkers.

5.4 Results

5.4.1 Quantification of circulating extracellular histones

The full raw data set of the volumetric CDD ELISA^{PLUS} results are presented in **Appendix IXa,b**. Twenty-three cases were assayed. Multiple draws of pulpal blood from each case were averaged with error bars representing the standard deviations

of the absorbance. Circulating extracellular histones, represented by CDD ELISA^{PLUS}, were reliably expressed in all samples tested, including the ‘true’ negative control (**Table 5.1**), case number 12. The shaded rows highlight the cases that were assayed using CDD ELISA^{PLUS} and HMGB₁.

5.4.2 HMGB₁ quantification

Similarly, the raw data depicting the HMGB₁ ELISA results are presented in **Appendix X**. In total, nineteen cases were assayed. Multiple draws were averaged, with error bars representing their standard deviations. HMGB₁ was not expressed in four cases (**Table 5.1**).

Of the four cases that HMGB₁ was not detected, three were diagnosed with a mild-moderate pulpitis and a pulpotomy procedure was possible. One case was diagnosed with initial pulpitis; however, an untreatable crack was discovered upon accessing the case and extraction was required. A single case was diagnosed with severe pulpitis and pulpectomy was necessary (**Table 5.1**).

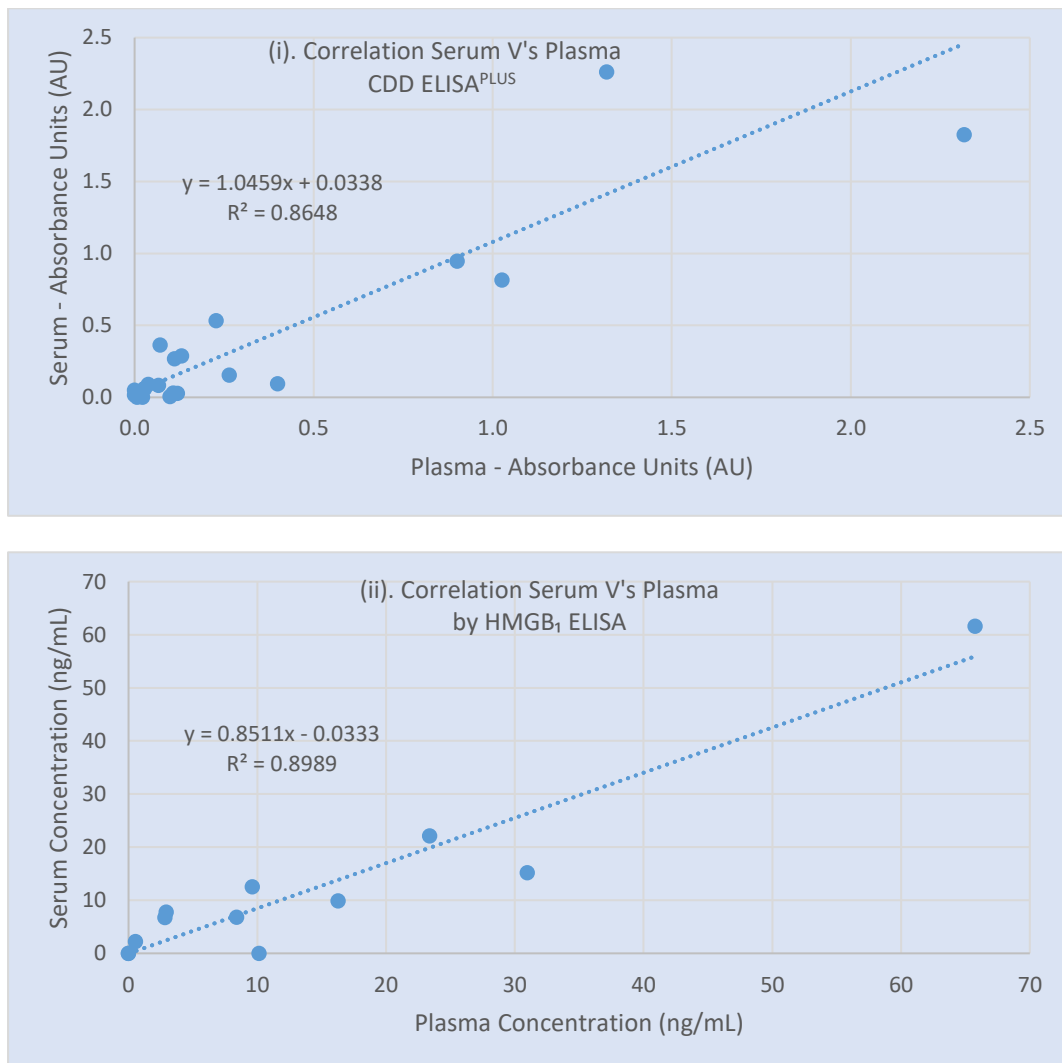
Table 5.1 Summary of biomarkers of cell death compared with clinical parameters: Diagnosis, Haemorrhage Control, Caries depth, Pain and Treatment [CDD: Cell Death Detection Assay; AU: absorbance Units; HMGB₁ Assay; StDev: Standard deviations; Dx: Diagnosis; NP: normal pulp; RP: Reversible pulpitis; IP: Irreversible pulpitis; InP: Initial pulpitis; MiP: Mild pulpitis; MoP: Moderate pulpitis; SeP: Severe pulpitis; xLa: extraction; HC: Haemorrhage control, NC: no caries; D: Deep caries; ExD: Extremely deep caries; Tx: Treatment; pp: pulpotomy; pe: pulpectomy; y: yes; n: no; f: failed; s: success; N/A: not applicable; r/c: recall; *: elective treatment; #: number; Shading highlights case-matched assays]

Case #	CDD (AU) ± StDev	HMGB ₁ (ng/mL) ± StDev	Dx (Classic)	Dx (Wolters')	HC	Caries depth	Pain	Tx	Outcome	Longest r/c (months)
1	0.02 ± 0.04		RP	MiP	y	D	y	pp	f	41
2	0.026		IP	MoP	y	ExD	y	pp	s	39
5	0.06 ± 0.036		IP	MiP	y	NC	n	pe*	s	40
6	0.38 ± 0.46		RP	InP	y	ExD	y	pp	F	10
7	0.22 ± 0.31		IP	SeP	n	ExD	y	pe	s	32
8	0.27 ± 0.34		IP	SeP	y	ExD	n	pp	s	27
9	0.06 ± 0.046	15.17	IP	MiP	y	D	y	pp	s	38
10	0.02 ± 0.034		IP	MoP	y	D	y	pp	s	33
11		0	RP	InP	y	ExD	y	pp	s	33
12	0.03 ± 0.008	13.09 ± 4.5	NP	NP	y	NC	n	xLa*	N/A	N/A
13	0.05 ± 0.029	7.59 ± 1.12	IP	MoP	y	D	Y	pp	s	45
14		23.06 ± 11.17	IP	MoP	y	D	y	pp	s	29
17		0 ± 0	RP	InP	y	ExD	Y	pp	s	45
18	0.04 ± 0.06	0.55 ± 1.19	IP	SeP	n	D	y	pe	s	7
19	2.42 ± 0.35	22.75 ± 0.88	RP	InP	n	ExD	y	pe	s	32
20	0.92 ± 0.82	87.87	IP	SeP	n	D	y	pe	s	45
22	0.23 ± 0.23	23.26	IP	SeP	n	ExD	y	pe	N/A	0
23	0.29 ± 0.25	5.35 ± 3.43	IP	MoP	n	D	y	xLa	N/A	N/A
24	1.07 ± 0.85	11.07 ± 2.07	IP	SeP	n	ExD	Y	pe	s	2
25	0.05 ± 0.10	4.79 ± 2.80	IP	MiP	y	ExD	y	pp	s	22
26	1.89 ± 0.44	63.67 ± 2.92	NP	NP	n	ExD	Y	pe	s	38
27	0.10 ± 0.14	0	RP	InP	n	D	Y	xLa	N/A	N/A
28	0.08 ± 0.13	100.10	IP	MoP	y	D	y	pp	s	42
29	1.79 ± 0.87	97.76	IP	SeP	n	ExD	y	pe	s	43
30	0.06 ± 0.15	0 ± 0	IP	MiP	y	D	y	pp	f	39
31	0.20 ± 0.18	5.07 ± 7.17	IP	MoP	y	ExD	y	pp	s	29

5.4.3 Serum and plasma comparison

A Pearson correlation was performed comparing the serum and plasma readings in both the CDD ELISA^{PLUS} (0.926; $p < .0001$) [Figure 5.1(i).] and the HMGB₁ ELISA (0.948; $p < .0001$) [Figure 5.1(ii).]. Both assays demonstrated strongly positive correlations with a high degree of significance, indicating that plasma, or serum are both suitable substrates for investigation using the specific CDD ELISA^{PLUS} and HMGB₁ ELISA.

Figure 5.1 Correlation graphs comparing serum and plasma results by (i). CDD ELISA^{PLUS} and (ii). HMGB₁ ELISA [R²: Coefficient of determination; Each dot on the graphs represents individual readings]



5.4.4 Threshold values

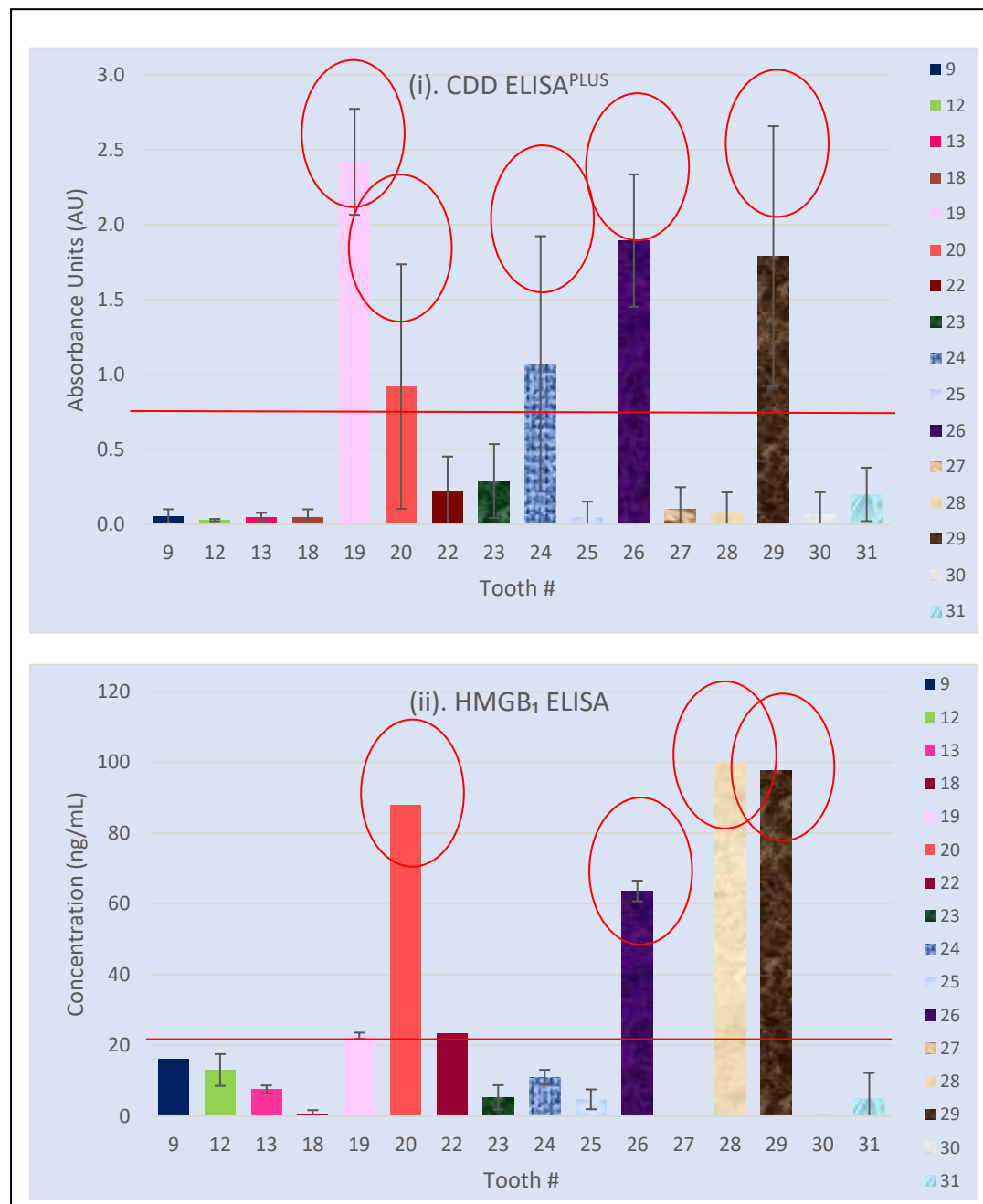
A summary of matched samples depicting the combined average readings for both CDD ELISA^{PLUS} and HMGB₁ ELISA is displayed in **Figure 5.2(i).** and **5.2(ii).** The full data set is in **Appendix IXa,b** and **Appendix X.** Error bars representing standard deviations are included, when repeat assays were possible. As there is no reported clinical reference range for circulating histones, in previous work, Kutcher et al. (2012) dichotomised their results into the highest quartile compared with the combined lower three quartiles (Q3), signifying elevated (High) and non-elevated (Low) threshold expression. Q3 was calculated using Microsoft Excel 2019 MSO and values falling within this range were considered to be Low threshold expression. All values above this range were considered to be High threshold expression.

Table 5.2 Summary of expression values of circulating histones and HMGB₁ ELISA, demarcating High and Low threshold expression [Min: Minimum value; Max: Maximum value; AU: Absorbance units; Q3: Third quartile]

	Min	Max	Q3
CDD ELISA ^{PLUS}	0 AU	2.67 AU	0 - 0.72 AU
HMGB ₁ ELISA	0 ng/mL	100.10 ng/mL	0 – 22.7 ng/mL

Table 5.2 provides a summary of the recorded expressions of both biomarkers. Box-and-whisker plots are presented to give an overview of the categorical (High, or Low threshold expressions, contrasted with the values recorded for the Healthy control sample) data for both circulating histones (**Figure 5.3(i).**) and HMGB₁ (**Figure 5.3(ii).**). These values were used to ascertain if the level of expressions could provide discriminatory information.

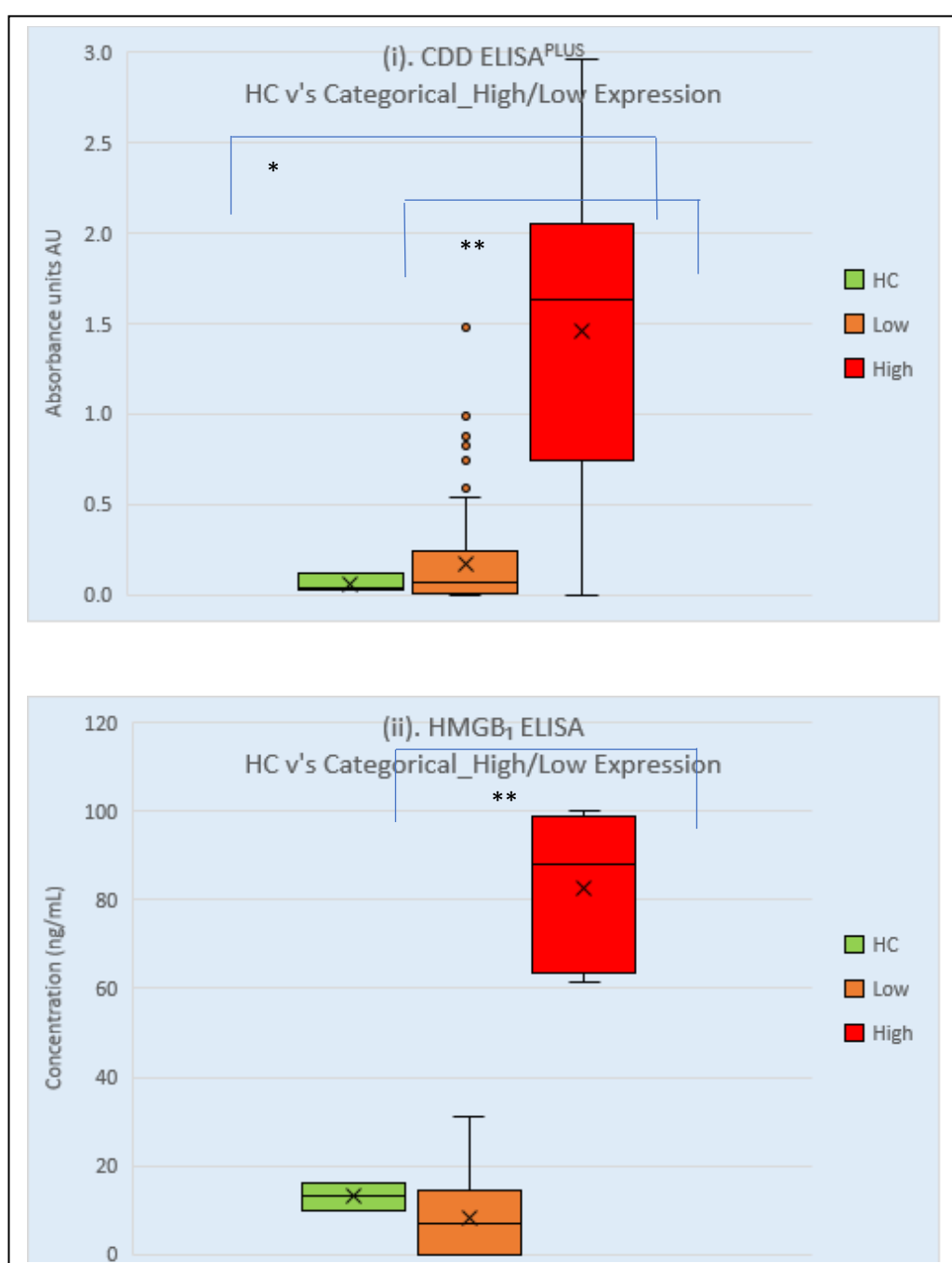
Figure 5.2 Summary of the matched (i). CDD ELISA^{PLUS} and (ii). HMGB₁ ELISA results [#: number, red rings highlighting highest expression; red lines indicate the cut-off (Q3), differentiating High from Low threshold expression for the study groupings]



Pairwise comparisons (Kruskal-Wallis Test) between the three categories of expressions related to circulating histones (**Figure 5.3(i).**), demonstrated statistically significant differences between the 'true' Healthy control and High expression ($p = .033$) and between the Low expression and High expression ($p < .001$), but not

between 'true' Healthy control and Low expression. Apart from four outliers, the Low threshold expression ranges of circulating histones remain below the level of the first quartile values of the High Threshold values (**Figure 5.3(i).**).

Figure 5.3 Box plots depicting range of expression of (i). CDD ELISA^{PLUS} and (ii). HMGB₁ ELISA categorised as defined threshold values (Healthy control provided for reference) [HC: Healthy control (green); Low: Low expression of circulating histones (Q3), shaded orange; High: High expression of circulating histones (> Q3), shaded red; *:p<.05; **: p<.001]



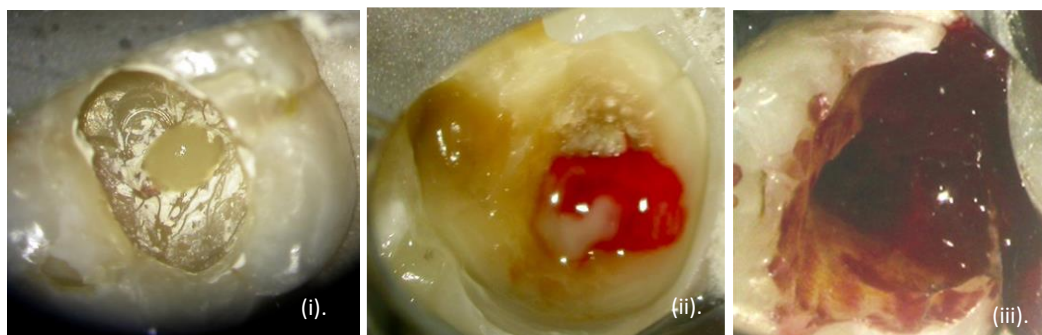
Pairwise comparisons (Kruskal-Wallis test) were made between the categorical ranges of expressions of HMGB₁, demonstrating highly significant differences between High and Low Threshold ranges ($p < .001$), but not between Low threshold and the Healthy control, which does serve to reveal a clinical relevance. The box-and-whisker plot (**Figure 5.3(ii).**) demonstrates no crossover in the delineation of these High and Low Threshold ranges, which may be clinically important.

5.4.5 Discriminatory values

5.4.5.1 CDD ELISA^{PLUS} results

In **Figure 5.2(i)**, five discrete peaks were noted within the highest quartile of the CDD ELISA^{PLUS} expression, circled red. These values all lie within the defined high threshold the range of expressions recorded (**Section 5.4.4, Table 5.2**). The clinical data from these five cases was re-examined and the following findings were noted. The most significant finding was the visual detection of a micro-abscess in 3/5 cases, discovered on initial exposure of the pulp chamber (**Figure 5.4(i, ii).**).

Figure 5.4 Clinical images demonstrating (i, ii). micro-abscess and (iii). profuse haemorrhage



Pus was not observed in two of the five cases, but it could potentially have been washed away by water coolant when using the highspeed handpiece. A

consistent finding in all cases was profuse, persistent, uncontrollable bleeding. Root canal treatment was necessary in all instances due to the inability to control the resultant haemorrhage, within 6 minutes, at the deepest level of amputation (**Figure 5.4(iii).**) and consequently in these cases, the ‘highly expressed’ circulating histones demarcated teeth with consistently the most elevated levels of inflammation.

The decision to enter the pulp was unavoidable based on a number of factors. The dentine overlying the pulp was soft and actively carious (**Figure 5.5 (i).**) [Bjørndal et al. 2019], combined with; a historical, pre-existing pulp exposure being discovered (**Figure 5.5(ii).**), or active caries accompanied by severe symptoms, indicating direct pulp treatment was necessary (**Figure 5.5(iii).**). Radiographic features suggestive of carious exposure were also considered pre-operatively (**Figure 5.6).**

Figure 5.5 Clinical photos depicting active caries (i). Actively progressing; (ii). Actively progressing, old exposure; (iii). Less actively progressing

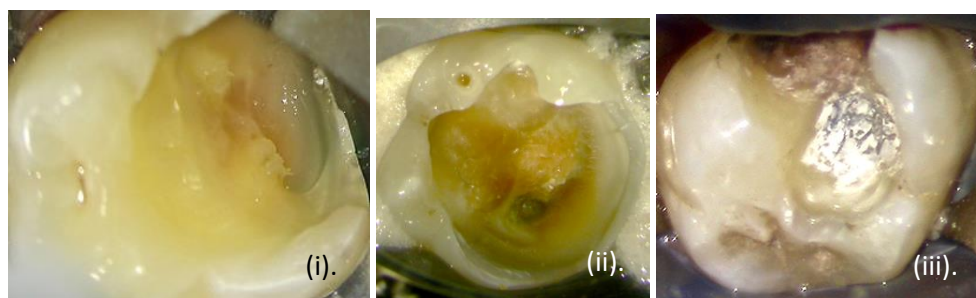


Table 5.3 summarises the qualitative findings relative to the High threshold circulating histones. Haemorrhage control was not possible in all cases. The visual depiction of haemorrhage encountered was described as the most severe (indicating irreversible, or severe pulpitis). Pain was present and pulpectomy was deemed necessary in all instances. Caries depth was judged to be extremely deep, in all but one case.

Figure 5.6 Radiographic appearance suggestive of carious exposure (arrowed yellow)

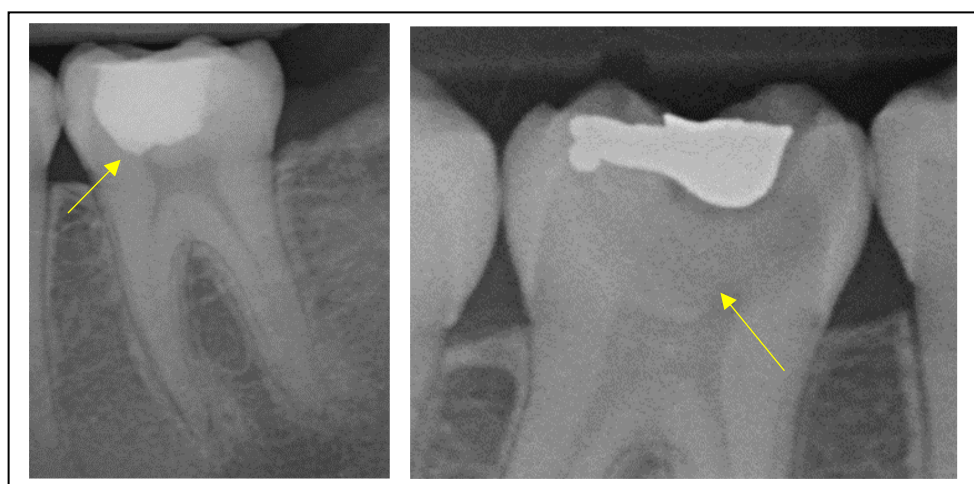


Table 5.3 Summary of the quantitative and qualitative features of the High threshold expressions of circulating histones, contrasted with the corresponding HMGB₁ expressions [#: number; AU: Absorbance units; StDev: Standard deviation; y: yes; n: no; D: Deep caries; ExD: Extremely deep caries; Tx: Treatment; pp: pulpotomy; pe: pulpectomy; HC: Haemorrhage control; DI: Direct inspection; IP: Irreversible pulpitis; NP: Normal pulp; SeP: Severe pulpitis]

Case #	CDD (AU) ± StDev	HMGB ₁ (ng/mL) ± StDev	Intentional exposure	Caries depth	Tx	Pain	HC	DI
19	2.42 ± 0.35	22.75 ± 0.88	n	ExD	pe	y	n	IP/SeP
20	0.92 ± 0.82	87.87	n	D	pe	y	n	IP/SeP
24	1.07 ± 0.85	11.07 ± 2.07	n	ExD	pe	y	n	IP/SeP
26	1.89 ± 0.44	63.67 ± 2.92	n	ExD	pe	y	n	IP/SeP
29	1.79 ± 0.87	97.76	n	ExD	pe	y	n	IP/SeP

5.4.5.1.1 CDD ELISA^{PLUS} threshold correlated with treatment

A cross-tabulation of the categorical expression of circulating histones with treatment decisions is presented in **Table 5.4**. The categorical levels of circulating histones, defined as High, or Low expression, were correlated using Pearson Chi-square analysis were able to demonstrate a highly significant relationship ($p = .008$) with the chosen treatment procedure (pulpotomy, or pulpectomy) [data summarised in **Table 5.5**]. Cohen's Kappa coefficient of agreement was moderate (0.537), also with a high degree of significance ($p = .008$).

Table 5.4 Circulating histones cross-tabulated against treatment [Low: Low expression of circulating histones (Q3); High: High expression of circulating histones (> Q3); Tx: Treatment; pp: pulpotomy; pe: pulpectomy]

		Tx		Total
		pp	pe	
CDD Categorical	Low	11	4	15
	High	0	4	4
Total		11	8	19

Table 5.5 Contingency Table summarising the associations between the categorical expression of circulating histones and Treatment decisions, Clinical Diagnoses, Depth of caries and presence of symptoms [CDD_Cat: Categorical expression of circulating histones, as represented by CDD ELISA^{PLUS}; Tx: Treatment, pp: pulpotomy; pe: pulpectomy; p value: level of significance calculated by Pearson Chi-square; Classic: Classic pulp diagnoses (Seltzer et al. 1963a); Wolters': Wolters' pulp diagnoses (Wolters et al. 2017); CD: Caries Depth; D: Deep caries (Bjørndal et al. 2019), ExD: Extremely deep caries (Bjørndal et al. 2019); Pain: Pre-operative presence of pain including evoked, or spontaneous]

Comparison		Pearson Chi-square	p value
CDD_Cat	Tx (pp/pe)	6.967	.008
CDD_Cat	Dx Classic	4.560	.102
CDD_Cat	Dx Wolters'	7.396	.166
CDD_Cat	CD (D/ExD)	3.685	.055
CDD_Cat	Pain	0.950	.330

Box-and-whisker plots present an overview of the range of expression of the biomarkers (circulating histones and HMGB₁), categorised according to the treatment provided (pulpotomy, or pulpectomy) [Figure 5.7 (i,ii)]. The 'true' Healthy control is presented separately, for reference. Pairwise comparisons of this data (Kruskal-Wallis Test) revealed a statistically significant difference ($p < .001$) between pulpotomy and pulpectomy treatment in the circulating histones, only (CDD ELISA^{PLUS}). The upper extreme of the pulpotomy range of expression, was consistently lower than the median value for the pulpectomy values (Figure 5.7(i)), could provide some clinical relevance.

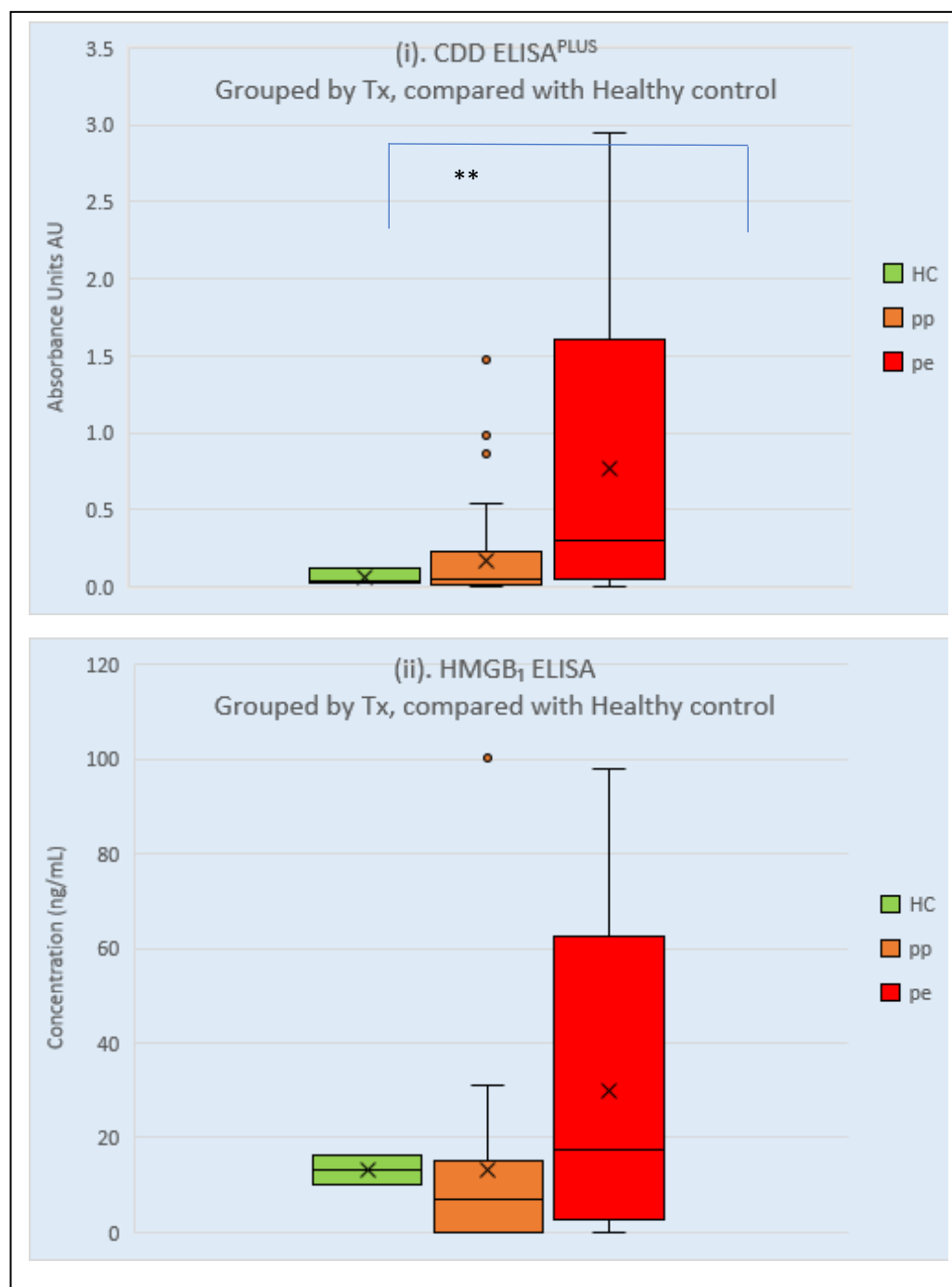
5.4.5.1.2 CDD ELISA^{PLUS} threshold correlated with diagnosis

When the categorical levels of expression of circulating histones were compared with Classic, or Wolters' diagnostic classifications, the following associations were noted (**Table 5.5**). When Classic criteria were applied, Pearson correlation revealed a non-significant relationship at the 5% level ($p = .102$). This association was shown to demonstrate a lower degree of correlation when Wolters' criteria was applied ($p = .166$).

The introduction of novel diagnostic reference standards was considered within this work. In following with the themes developed in **Section 4.4.6**, the categorical expression of circulating histones was proposed as potential novel reference standard against a range of index tests. The index tests included Classic pulp diagnoses, Wolters' pulp diagnoses (applying both strict, or loose criteria) and caries depth (Defined in **Section 4.4.6**). The raw data relating to these diagnostic accuracy calculations is presented in **Appendix XV**. Sensitivity, specificity, positive and negative predictive values as well as false positive and false negative rates were summarised. Taken together, the variations that were noted were likely impacted by the relatively low numbers of cases available for analysis.

Categorical expression of circulating histones compared with Wolters' (Strict) criteria produced the most acceptable sensitivity (60%) and specificity (77.8%). Pearson Chi-squared level of agreement did not reach significance at the 5% level, but nonetheless may be clinically relevant and support a need for additional, broader studies ($p = .104$).

Figure 5.7 Box plots depicting range of expression of (i). CDD ELISA^{PLUS} and (ii). HMGB₁ ELISA categorised according to the treatment groups (Healthy control provided for reference) [Tx: Treatment; HC: Healthy control (green), pp: pulpotomy (orange); pe: pulpectomy (red); **: p<.001]



5.4.5.1.3 CDD ELISA^{PLUS} related to diagnosis and pulpotomy

The CDD ELISA^{PLUS} data is summarised in **Table 5.6** (using Classic diagnostic definitions of patient history [Hx] and clinical diagnosis [Cx]) and **Table 5.7** (using

Wolters' diagnostic definitions of patient history [Hx] and clinical diagnosis [Cx]). Twelve cases were possible to treat conservatively (the pulpotomy data was adjusted to include the negative control, as well as a single case planned for elective pulpectomy) in the CDD ELISA^{PLUS} assays. These diagnostic parameters are stratified according to treatment (pulpotomy, or pulpectomy) and their relationship to the level of expression of circulating extracellular histones (CDD ELISA^{PLUS}), categorised as High, or Low threshold (**Table 5.6** and **Table 5.7**).

Using both Classic (**Table 5.6**) and Wolters' (**Table 5.7**) criteria, no High threshold circulating histones were expressed in teeth treated by pulpotomy (**Tables 5.6, 5.7, circled red**). Fifty eight percent (7/12) and 83% (10/12) of the cases were considered to be irreversibly inflamed according to patient history and clinical findings, respectively; expressed Low threshold circulating histones (**Tables 5.6, yellow circle**) and were possible to treat conservatively by pulpotomy. Of the irreversibly inflamed cases that were possible to treat by pulpotomy, most of these were classified as moderate pulpitis according to Wolters' classification **Table 5.7, circled yellow**.

Table 5.6 Classic diagnostic data (patient history and clinical diagnosis) stratified according to treatments (pulpotomy, or pulpectomy) and the level of expression (High/Low) of circulating histones [Tx: treatment; pp: pulpotomy; n: number; Dx: Diagnosis; Hx: Patient history; Cx: Clinical diagnosis; Low: Low expression of circulating histones (Q3); High: High expression of circulating histones (> Q3); NP: Normal pulp; RP: Reversible pulpitis; IP: Irreversible pulpitis]

Tx: pp n=12	CDD Low		CDD High		Tx: pe n=8	CDD Low		CDD High	
Test/Dx	Hx	Cx	Hx	Cx	Test/Dx	Hx	Cx	Hx	Cx
NP	4	0	0	0	NP	0	0	1	1
RP	1	2	0	0	RP	0	0	0	1
IP	7	10	0	0	IP	3	3	4	3

Table 5.7 Wolters' diagnostic data (patient history and clinical diagnosis) stratified according to treatments (pulpotomy, or pulpectomy) and the level of expression (High/Low) of circulating histones [Tx: treatment; pp: pulpotomy; n: number; Dx: Diagnosis; Hx: Patient history; Cx: Clinical diagnosis; Low: Low expression of circulating histones (Q3); High: High expression of circulating histones (> Q3); NP: Normal pulp; InP: Initial pulpitis; MiP: Mild pulpitis; MoP: Moderate pulpitis; SeP: Severe pulpitis]

Tx: pp n=12	CDD Low		CDD High		Tx: pe n=8	CDD Low		CDD High	
Test/Dx	Hx	Cx	Hx	Cx	Test/Dx	Hx	Cx	Hx	Cx
NP	4	0	0	0	NP	0	0	1	1
InP	1	1	0	0	InP	0	0	0	1
MiP	1	4	0	0	MiP	0	0	1	0
MoP	5	6	0	0	MoP	0	0	0	0
SeP	1	1	0	0	SeP	3	3	3	3

5.4.5.1.4 CDD ELISA^{PLUS} related to diagnosis and pulpectomy

Analysis of the cases treated by pulpectomy are also summarised in **Table 5.6** and **Table 5.7**. Eight cases treated by pulpectomy had the circulating levels of extracellular histones (CDD ELISA^{PLUS}) analysed. Low expression of circulating histones was not seen in any cases diagnosed as normal/asymptomatic, or reversible pulpitis (**Table 5.6, circled green**). Of the three cases that were shown to express low levels of extracellular histones and requiring pulpectomy; all were categorised as irreversibly, or severely pulpitic (**Tables 5.6, 5.7, blue oval**). Indeed, the majority of the cases expressing High threshold circulating histones were seen only in the most severely inflamed categories (**Tables 5.6, 5.7, blue oval**). It is possible that the single case designated as a normal/asymptomatic pulp (Tooth 26) may have been a so-called 'silent' or asymptomatic pulpitis (**Tables 5.6, 5.7, grey oval**). This serves to highlight the discrepancies that can occur even with standard diagnostic testing.

5.4.5.1.5 CDD ELISA^{PLUS} threshold correlated with symptoms and caries depth

The categorical expression of circulating histones was compared with caries depth and the pre-operative presence of pain (**Table 5.5**). Caries depth (dichotomised as Deep, or Extremely deep) approached statistical significance with the categorical level of expression of circulating histones ($p = .055$); however, the presence of pain did not appear to correlate significantly with the level of expression of circulating histones ($p = .350$).

5.4.5.2 HMGB₁ ELISA results

As can be seen in **Figure 5.2 (ii)**., four discrete peaks (circled red) were noted in the HMGB₁ ELISA chart; of which, three were common to the CDD ELISA^{PLUS} High threshold expression (Tooth numbers 20, 26 and 29), also circled red, in **Figure 5.2(i)**.

Tooth 28 produced a high reading that was not in keeping with the clinical findings, or the CDD ELISA^{PLUS} data (**Table 5.1**). The qualitative findings relating to this single case, included the ability to achieve haemostasis, a normal visual appearance of the exposed pulp and pulpotomy was possible. Pain was present and the depth of caries was deep.

5.4.5.2.1 HMGB₁ ELISA threshold correlated with treatment

Although there was evidence of a trend between treatment and the categorical expression of HMGB₁ (**Table 5.8**), significance at the 5% confidence level was not demonstrated ($p = .146$) [**Table 5.9**]. The influence of low sample numbers cannot be discounted; however, the trend may be clinically relevant. The direction of the pattern of frequencies, although not statistically significant, does demonstrate a

potential relationship with HMGB₁ in relation to decision-making and treatment (**Table 5.8**). A greater proportion of cases expressing Low threshold HMGB₁ were possible to treat conservatively by pulpotomy. Equally, a greater proportion of the highly expressed HMGB₁ necessitated pulpectomy.

Table 5.8 HMGB₁ cross-tabulated against treatment [Low: Low expression of HMGB₁ (Q3); High: High expression of HMGB₁ (> Q3); Tx: Treatment; pp: pulpotomy; pe: pulpectomy]

		Tx		Total
		pp	pe	
HMGB ₁ Categorical	Low	8	4	12
	High	1	3	4
Total		9	7	16

Returning to the box-and-whisker plots in **Figure 5.7(ii)**., Kruskal-Wallis non-parametric testing did not demonstrate any statistically significant difference between the ranges of expression of HMGB₁. However, the upper quartile of the pulpotomy values do remain below the median value of the pulpectomy cohort.

5.4.5.2.2 HMGB₁ ELISA threshold correlated with diagnosis

When the categorical levels of expression of HMGB₁ were compared using Classic, diagnostic criteria, the relationship was not shown to be statistically significant ($p = .135$) [**Table 5.9**]. This association was also not significant when Wolters' diagnostic criteria were applied ($p = .231$). Again, the low sample numbers may have diminished the opportunity to provide statistical significance in these instances.

Table 5.9 Contingency Table summarising the associations between the categorical expression of HMGB₁ and Treatment decisions, Clinical Diagnoses, Depth of caries and presence of symptoms
 [HMGB₁_Cat: Categorical expression of HMGB₁, as represented by HMGB₁ ELISA; Tx: Treatment, pp: pulpotomy; pe: pulpectomy; p value: level of significance calculated by Pearson Chi-square; Classic: Classic pulp diagnoses (Seltzer et al. 1963a); Wolters': Wolters' pulp diagnoses (Wolters et al. 2017); CD: Caries Depth; D: Deep caries (Bjørndal et al. 2019), ExD: Extremely deep caries (Bjørndal et al. 2019); Pain: Pre-operative presence of pain including stimulated or spontaneous]

Comparison		Pearson Chi-square	p value
HMGB ₁ _Cat	Tx (pp/pe)	2.116	.146
HMGB ₁ _Cat	Dx Classic (incl NP)	4.0	.135
HMGB ₁ _Cat	Dx Wolters' (incl NP)	5.6	.231
HMGB ₁ _Cat	CD (D/ExD)	0.085	.771
HMGB ₁ _Cat	Pain	0.762	.383

Diagnostic accuracy relating to the categorical expression of HMGB₁ compared with Classic and Wolters' diagnostic criteria, produced non-significant findings, hampered again, possibly by low test samples for comparative analysis (**Appendix XV**).

5.4.5.2.3 HMGB₁ ELISA related to diagnosis and pulpotomy

Ten cases, treated by pulpotomy, were assayed using HMGB₁ ELISA and these results are stratified according to Classic (**Table 5.10**) and Wolters' (**Table 5.11**) diagnostic criteria (patient history and clinical findings). The expression of HMGB₁ was also dichotomised as either High, or Low threshold. A single case (Tooth 28) diagnosed as irreversible, or severe pulpitis and treated by pulpotomy, demonstrated high expression of HMGB₁ (**Tables 5.10, 5.11, circled red**).

Table 5.10 Classic diagnostic data (patient history and clinical diagnosis) stratified according to treatments (pulpotomy, or pulpectomy) and the level of expression (High/Low) of HMGB₁ [Tx: treatment; pp: pulpotomy; n: number; Dx: Diagnosis; Hx: Patient history; Cx: Clinical diagnosis; Low: Low expression of HMGB₁ (Q3); High: High expression of HMGB₁ (> Q3); NP: Normal pulp; RP: Reversible pulpitis; IP: Irreversible pulpitis]

Tx: pp n=10	HMGB ₁ Low		HMGB ₁ High		Tx: pe n=9	HMGB ₁ Low		HMGB ₁ High	
Test/Dx	Hx	Cx	Hx	Cx	Test/Dx	Hx	Cx	Hx	Cx
NP	3	1	0	0	NP	0	1	1	1
RP	1	2	0	0	RP	1	2	0	0
IP	5	6	1	1	IP	5	4	2	2

Table 5.11 Wolters' diagnostic data (patient history and clinical diagnosis) stratified according to treatments (pulpotomy, or pulpectomy) and the level of expression (High/Low) of HMGB₁ [Tx: treatment; pp: pulpotomy; n: number; Dx: Diagnosis; Hx: Patient history; Cx: Clinical diagnosis; Low: Low expression of HMGB₁ (Q3); High: High expression of HMGB₁ (> Q3); NP: Normal pulp; InP: Initial pulpitis; MiP: Mild pulpitis; MoP: Moderate pulpitis; SeP: Severe pulpitis]

Tx: pp n=10	HMGB ₁ Low		HMGB ₁ High		Tx: pe n=9	HMGB ₁ Low		HMGB ₁ High	
Test/Dx	Hx	Cx	Hx	Cx	Test/Dx	Hx	Cx	Hx	Cx
NP	3	1	0	0	NP	0	0	1	1
InP	1	2	0	0	InP	1	2	0	0
MiP	2	2	0	0	MiP	1	0	0	0
MoP	3	4	0	0	MoP	1	1	0	0
SeP	0	0	1	1	SeP	3	3	2	2

The expression of circulating histones was shown to be low in the same case (Tooth 28) in the CDD ELISA^{PLUS} results (**Table 5.1**). Within the pulpotomy cohort, high expression of HMGB₁ was not shown in any case diagnosed as normal, or reversible pulpitis, according to Classic criteria (**Table 5.10**); or in normal-moderate pulpitis (**Table 5.11**), using Wolters' criteria.

Most of the remaining 9 cases, treated by pulpotomy and expressing low levels of HMGB₁, were diagnosed as irreversible pulpitis (**Table 5.10, circled yellow**), which included predominantly mild and moderate pulpitis cases (**Table 5.11, circled yellow**) when Wolters' criteria were applied.

5.4.5.2.4 HMGB₁ ELISA related to diagnosis and pulpectomy

Nine cases requiring pulpectomy, were analysed using HMGB₁ ELISA. Low expression of HMGB₁ was mostly seen the inflamed categories [Irreversible pulpitis (**Table 5.10, circled green**), or Mild-Severe pulpitis (**Table 5.11, circled green**)]. It is conceivable that the single case (Tooth 26) expressing high levels of HMGB₁ and categorised as normal/asymptomatic (**Tables 5.10, 5.11, circled grey**) again as with circulating histones, could have been a 'silent' pulpitis, suggesting that all cases expressing high levels of HMGB₁ and requiring pulpectomy, were clinically severely inflamed.

5.4.6 Correlation/agreement between ELISAs

A total of sixteen matched cases were assayed using both CDD ELISA^{PLUS} and HMGB₁ ELISA (**Figure 5.2**). **Table 5.3** summarises the quantitative and qualitative findings relating to the highest levels of expression of both biomarkers. **Table 5.12** summarises the quantitative and qualitative results as they relate to their expressions within the first quartile (Q1) expression. The data has been adjusted to compensate for the negative control and one instance of elective root canal treatment (Cases 12 and 5 respectively, classed as pulpotomy for ease of analysis).

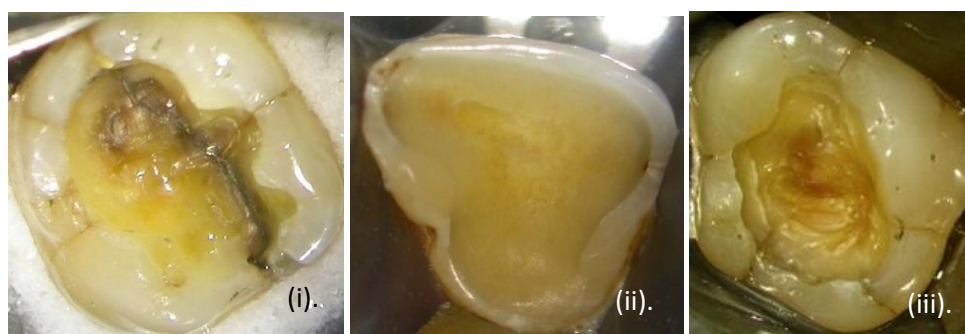
A common theme noted within the first quartile (Q1) of the biomarkers' expression, was the clinical decision to intentionally expose the pulp. **Figure 5.8(i-iii)**. depicts a number of situations where the decision to enter the pulp was influenced by clinically significant cracks (**Figure 5.8(i)**.), treatment planning (**Figure 5.8(ii)**.), or presence of severe symptoms (**Figure 5.8(iii)**.). This serves to underpin the sometimes, complex decisions in pulp diagnostics, warranting further investigation,

to understand if the Low level of expression of biomarkers could direct VPT in preference to RCT.

Table 5.12 Summary of quantitative and qualitative features of lowest threshold (Q1) expression of circulating histones, contrasted with corresponding HMGB₁ expression [Q1: First quartile; #: number; AU: Absorbance units; StDev: Standard deviation; y: yes; n: no; D: Deep caries; ExD: Extremely deep caries; Tx: Treatment; pp: pulpotomy; pe: Pulpectomy; pe*: indicates elective root canal treatment; xLa*: indicates orthodontic extraction of Healthy control; HC: Haemorrhage control; DI: Direct inspection; IP: Irreversible pulpitis; NP: Normal pulp; SeP: Severe pulpitis; 1)shading indicates case-matched analysis with CDD ELISA^{PLUS} and HMGB₁, Bold font indicates where CDD ELISA^{PLUS} and HMGB₁ do not concur]

Case #	CDD (AU) ± StDev	HMGB ₁ (ng/mL) ± StDev	Intentional exposure	Caries depth	Pain	Tx	Outcome	Time	HC	DI
1	0.02 ± 0.04		y	D	Y	pp	f	41	y	NP
2	0.026		y	ExD	Y	pp	s	39	y	IP/SeP
5	0.06 ± 0.036		y	NC	n	pe*	s	40	y	NP
9	0.06 ± 0.046	15.17	y	D	y	pp	s	38	y	IP/MiP
10	0.02 ± 0.034		y	D	Y	pp	s	33	y	NP
11		0	y	ExD	Y	pp	s	33	y	NP
12	0.03 ± 0.008	13.09 ± 4.5	y	NC	n	xLa*	-	-	y	NP
13	0.05 ± 0.029	7.59 ± 1.12	y	NC	Y	pp	s	45	y	IP/MiP
18	0.04 ± 0.06	0.55 ± 1.19	y	D	y	pp	s	38	y	IP/SeP
23	0.29 ± 0.25	5.35 ± 3.43	y	D	y	xLa	-	-	n	IP/SeP
25	0.05 ± 0.10	4.79 ± 2.80	y	ExD	y	pp	s	22	y	IP/MiP
27	0.10 ± 0.14	0	y	D	y	xLa	-	-	n	IP/SeP
28	0.08 ± 0.13	100.10	y	D	Y	pp	s	42	y	NP
30	0.06 ± 0.15	0 ± 0	y	D	y	pp	f	39	y	IP/SeP
31	0.20 ± 0.18	5.07 ± 7.17	y	ExD	Y	pp	s	29	y	IP/MoP

Figure 5.8 Clinical images depicting caries/dentine removal prior to intentional pulp exposure (i). Selective caries removal, significant, stained crack, (ii). Dentine removal just prior to intentional exposure, (iii). Selective caries removal to firm dentine prior to intentional exposure



Categorical expression of circulating extracellular histones (CDD ELISA^{PLUS}) was shown to agree with HMGB₁ 81.8 % of the time (**Tables 5.1**). Correlation statistics revealed a Pearson Chi-Square value of 5.605 ($p = .018$) and a moderate Cohen's Kappa coefficient of 0.586, which demonstrated highly significant ($p = .018$) associations.

Table 5.3 provides a focused view exclusively of highly expressed circulating histones and/or HMGB₁ in combination with the treatment provided. Agreement was noted as 66.67%. **Table 5.12** demonstrates the instances where circulating histones and HMGB₁ were expressed within the lowest (Q1) threshold category. Eighty percent of these cases (8/10) were possible to treat conservatively by pulpotomy (as before, data adjusted to include one case of elective root canal treatment and a single healthy control). The High and Low threshold data for both assays is depicted/summarised in **Figure 5.3(i,ii)**.

Categorical expression of HMGB₁ demonstrated no significant association with caries depth (deep, or extremely deep), or the presence of pain.

5.5 Discussion

Neutrophil extracellular traps (NETs), are recognised innate defences that function to combat microbial infection (Brinkmann et al. 2004). NETs are described as web-like structures composed of decondensed nuclear chromatin and granule proteins, which interact with the cell membrane of bacteria, trapping them and ultimately killing the target by inducing further inflammatory mechanisms (Brinkmann et al. 2004). From a diagnostic perspective, the easiest means of *in vivo* NET detection involves measurement of NET-associated products in the blood such as circulating

cell-free DNA, citrullinated Histone-3 (citH3), neutrophil elastase (NE), and myeloperoxidase (MPO) (Snoderly et al. 2019). Expression of NET-associated molecules has recently formed a particular focus in pulp biology (Cooper et al. 2017, Holder et al 2019, Huang et al. 2020). Their usefulness in many clinical applications from diagnosis, monitoring of disease progression and as prognostic indicators is now widely accepted (Lim et al. 2020, Guo et al. 2021, Mutua & Gershwin 2021). Interactions between HMGB₁ and TLR-4 also potentiate NET formation and are useful markers of the inflammatory process (Tadie et al.2013).

Baseline serum levels for circulating HMGB₁ have not been established in the medical literature. Equally, there is no known reference range for circulating histones. Therefore, the categorisation of threshold expression of HMGB₁, as well as circulating histones, as high (above Q3), or Low (Q3) is considered an acceptable reporting standard (Kutcher et al. 2012, Liikanen et al. 2015, Kim et al. 2022). The delineation between High and Low expression of HMGB₁ (**Figure 5.3 (ii).**) was clearer than for circulating histones (**Figure 5.3(i)**), in that no overlap was noted in the box and whisker plots depicted by HMGB₁, suggestive of a distinct cut-off in the threshold ranges. Despite this, statistically significant differences were demonstrated by both methods when High and Low expression was compared ($p < .001$).

Critically, 2/5 of the cases clinically diagnosed as normal, or reversibly inflamed (**Table 5.1, Table 5.3**); could have been scheduled for conservative, indirect pulp therapy, which would have almost certainly resulted in treatment failure.

Furthermore, the pre-operative radiographic examination revealed the presence of extremely deep caries, indicating inevitable carious exposure (Bjørndal

et al. 2019) (**Figure 5.6**), which, combined with a patient history reflective of irreversible pulpitis; indicated that pupal exposure would be unavoidable.

The highest expression of the biomarkers investigated did correlate positively with poor outcomes (with regard to pulp survival, in this case indicating that pulpectomy was necessary), which is in keeping with the findings of similar studies in medical research (Wittwer et al. 2013, Beltrán-García et al. 2021, Kim et al. 2023). The potential for biomarker expression to direct treatment decision-making based on prognosis offers opportunity for point-of-care testing

The ability to demonstrate statistically significant differences between the categorical expressions of the biomarkers is particularly relevant clinically. What is lacking in the diagnostic literature to date, is a diagnostic test that provides discriminatory values within the spectrum of pulpitis. In particular, the relevance of discriminating between moderate and severe pulpitis; as opposed to the common situation where mild (reversible pulpitis) is compared with the more severe end of the inflammatory spectrum (irreversible pulpitis).

The expression of the selected cell death mediators, was compared with treatment provided, i.e. either pulpotomy, or pulpectomy (**Tables 5.6, 5.7 and Tables 5.10, 5.11**). In the pulpotomy cohort, it is interesting to note the large numbers of cases diagnosed as irreversible pulpitis (**Table 5.6, Table 5.10, circled yellow**); or moderate/severe pulpitis (**Table 5.7, 5.11, circled yellow**); where partial pulp preservation (pulpotomy) remained possible. Traditionally, reaching such a diagnosis would automatically be designated for pulpectomy. More data will be necessary to determine if a consistent finding of low circulating histone expression correlates with the ability to provide more conservative pulp preservation procedures; however,

these preliminary results are promising.

A single case, treated by pulpotomy, expressed high levels of HMGB₁ was shown to express low levels of circulating histones. When the clinical details were reviewed, it was notable that a deep pulpotomy was necessary, due to difficulty in handling the pupal tissue and controlling haemorrhage. Wolters et al. (2017) recommend pulpotomy in cases of severe pulpitis, but include pulpectomy as a reasonable treatment parameter, when haemorrhage cannot be controlled with deeper amputation. Many factors need to be considered by the operator in the decision-making process (**Section 4.5.3**), many of which are subjective. The relatively low numbers of cases should be emphasized in terms of a recognised limitation of the study.

When considering the pulpectomy data, all of the low expressions of circulating histones were confined to the irreversible pulpitis (**Table 5.6, blue oval**), or severe pulpitis (**Table 5.7, blue oval**) categories. This finding agrees with treatment suggestions proposed by Wolters et al. (2017), that some cases diagnosed as severe pulpitis may in fact, require pulpectomy. The cases demonstrating low CDD expression were uniformly categorised as irreversibly inflamed; moreover, they all presented with the most severe form of irreversible pulpitis according to patient history and clinical findings. The final decision to remove the entire pulp was clinically determined by the inability to attain acceptable haemostasis at the deepest level of amputation. Future research will be necessary to establish why certain cases diagnosed as irreversibly inflamed, in particular the most severe category, were possible to treat conservatively (**Table 5.6, Table 5.7, yellow circle**), whilst others were not (**Table 5.6, 5.7, blue oval**).

The presence of pre-operative symptoms, presenting as either evoked, or spontaneous pain, is often relied on as an important clinical indicator of advanced pulpal injury (Bestall et al. 2020, Richert et al. 2022). Nevertheless, the absence of symptoms does not reliably exclude 'silent' pulpitis (Hasler & Mitchell 1970). This is supported by the fact that pain did not reveal significant associations with the level of expression of the selected biomarkers. In reevaluating index tests better suited to the diagnosis of pulpitis, the association between High threshold expression of circulating histones CDD was significantly linked with caries depth. This points to caries depth as another potentially important variable to consider.

One of the limitations of the CDD ELISA^{PLUS} assay is the inability to calculate the concentration of the analytes using a standard curve, which obscured the inter-assay comparisons (Beltrán-García et al. 2021). Therefore, additional markers of cell death (HMGB₁) were introduced (Tancharoen et al. 2014) to supplement these findings, with a view to verifying the preliminary results.

Low expression of both selected mediators (circulating extracellular histones and HMGB₁) was expressed in almost all cases that were possible to treat conservatively by pulpotomy (**Table 5.12**). It is recognised that differential expression of cell death markers is indicative of apoptosis, whereas secondary necrosis as a result of elevated levels apoptosis, leads to the release of alarmins, which results in immune stimulation and tissue damage (Melcher et al. 1999). Low levels of circulating cell death markers can be considered physiological, or immunologically silent (Jorch & Kubes 2017). Indeed, vital NETosis appears more commonly in the context of infectious disease, whereas lytic NETosis is observed in sterile injury (Jorch & Kubes 2017).

5.6 Conclusion

A number of cases produced high expressions of selected cell death mediators. The cases related to the presence of micro-abscess in more than half of these samples and a uniform inability to obtain sufficient haemostasis to provide pulp preservation procedures, which could not have been determined prior to accessing the pulp.

Of the cases that were treated by pulpotomy, none demonstrated high expression of circulating histones, which included cases, despite being clinically diagnosed with severe pulpitis, were still possible to treat conservatively.

Cases requiring pulpectomy and expressing low levels of circulating extracellular histones, were exclusive to the most severe category of pulpitis. As a matter of importance, future analysis and cases will be necessary to ascertain why certain cases diagnosed with severe pulpitis and requiring pulpectomy, produced high expression of circulating histones and HMGB₁, while others produced low readings.

The value of investigating multiple biomarkers associated with the interrelated process of inflammation, coagulation and sepsis may assist in the clinical decision making, as well as prognostic information in terms of disease management (Wittwer et al. 2013, Liikanen et al. 2015, Beltrán-García et al. 2021, Kim et al. 2022). Circulating histones and selective DAMPs present novel diagnostic targets and potential prognostic indicators. However, more studies are necessary with larger patient numbers and multiple, calibrated clinical operators.

Chapter 6

High-throughput analysis of pulpal blood to identify novel inflammatory diagnostic targets

6.1 Introduction

Molecular diagnosis has been defined as a technique that analyses the genome, or proteome by applying molecular biology to medical testing (Fiehn 2001). These strategies have revolutionized clinical medicine, particularly in the fields of infectious diseases and oncology amongst others (Tang et al. 1997, Sokolenko & Imyanitov 2018). Testing is based on expressions of biomarkers in blood, or other bodily fluids, which have been used objectively as indicators of physiologic health, or pathogenic processes. The first step toward molecular diagnosis of dental pulp disease is identification of key inflammatory mediators that are over or under-expressed during the disease process, which will increase our understanding of their role in inflammation and regeneration. Due to the complex regulation of inflammatory pulp disease, it is unlikely that a candidate approach will provide sufficient information to define the threshold of irreversible inflammatory change (Rechenberg et al. 2016a, Hirsch et al. 2017, Zaninni et al. 2017).

Utilisation of a profiling approach via an antibody array or other high-throughput technologies will allow simultaneous analysis of multiple mediators and processes, while maximising the information gathered from minute volumes of substrate available (Galicía et al. 2016). The introduction of broader analysis using Multiplex technology of pulpal blood substrates aims to highlight specific trends within the inflammatory spectrum, which has not been documented in the endodontic literature to date. In order to combat known limitations of multiplex analysis, such as analytical variability, specificity and cross-reactivity (Tighe et al. 2015), a combined candidate approach of selected mediators, could further interrogate the validity of some of the microarray findings.

6.2 Study aims and objectives

To analyse the expression of an extensive panel of established inflammatory and regenerative mediators using a commercially available multiplex array in pulpal serum from healthy, 'sound' teeth; compared with teeth exhibiting signs and symptoms indicative of severe pulpitis. A secondary aim was to validate these findings on a selection of candidate biomarkers using ELISA assays.

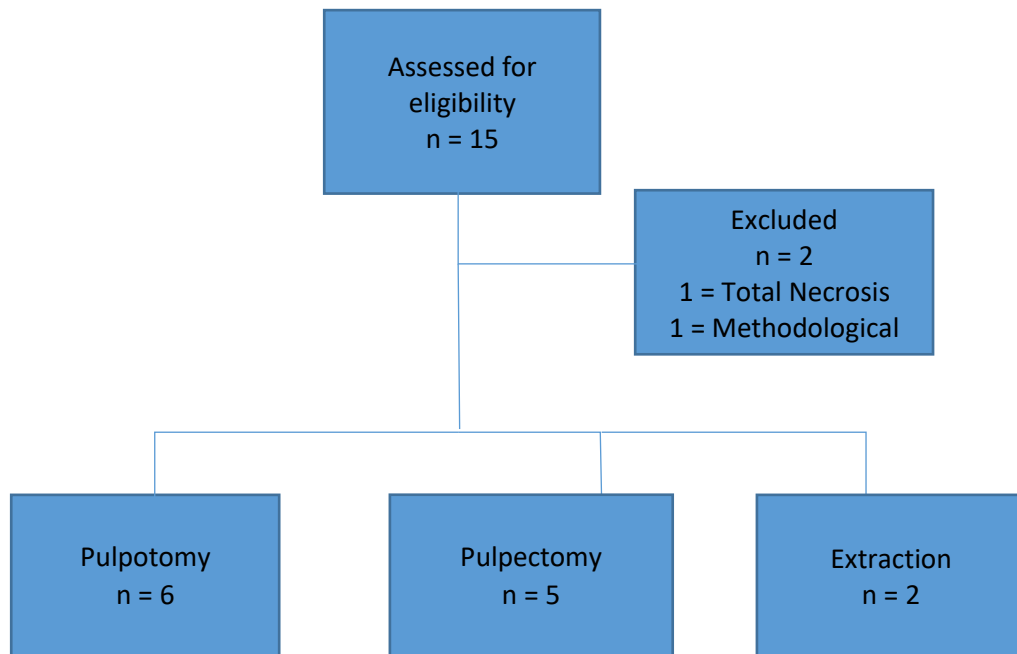
6.3 Materials and methods

Fifteen patients presenting in DDUH outpatient clinics were recruited to this arm of the study (**Figure 6.1**). The recruitment, consent, diagnostic steps and clinical procedures are reported in greater detail in **Sections 2.2 - 2.8**.

Briefly, when suitable patients were identified in the Restorative Dentistry, Endodontic, Periodontology, or Orthodontic clinics (**Section 2.2**), the purpose and nature of the study was explained to them. Patient information leaflets were provided to each patient and their informed, valid consent obtained.

The patient's symptoms and the results of the clinical and radiographic examinations were recorded in a standardised manner (**Figure 2.2**), before a diagnosis was made and treatment options discussed. The collection of blood for this study did not interfere, or change the treatment that had already been decided with the patient (**Section 2.8.1, 2.8.2**).

Figure 6.1 Flowchart High-throughput patient cohort [n: number]



In a modification to the previously described laboratory preparation for blood collection (**Section 3.3.1**), the blood serum collection protocol (**Section 3.3.1.4**) was altered as follows: Twenty-five microliters of pulpal blood, collected by microcapillary tubes (Hirschmann), were expressed into pre-prepared, coded 0.5 mL Safe-Lock Tube (Eppendorf AG) containing 100 µl PBS and 15 µl protease inhibitor (**Figure 6.2**). Pulpal blood was collected after the access cavity preparation had been completed; under local anaesthesia and dental dam isolation (**Section 2.8.2**). A second collection of 25 µl of pulpal blood was attempted in every case, but the additional collection of the full volume was not always predictably achieved. The samples were stored on ice on the clinic as before, until the clinical procedure was completed. Two healthy controls ('sound' teeth) were sourced from the Orthodontic Department. Those teeth underwent blood collection in an identical manner to diseased teeth, with their subsequent extraction completed, on the same day, in the

oral surgery clinic, once the blood samples had been collected (**Section 2.8.2.3**). A further, seven teeth were recruited from patients undergoing pulpotomy (**Section 2.8.2.1**), one of which was subsequently excluded from analysis due to loss of sample during transfer to the laboratory. Finally, six patients were recruited who were to undergo pulpectomy (**Section 2.8.2.2**); one of which, was excluded intraoperatively due to the discovery of total necrosis on accessing the root canal system.

Figure 6.2 Updated dilution protocol providing 1:6 dilutions

ALL SAMPLES Dilute all samples with 110 µl PBS	ALL SAMPLES Protease inhibitor {cOmplete ULTRA Tablets, Mini, EDTA- free EASYpack 05 892 791 001} Stock solution dissolve one tablet in 1.5ml redistilled water (x7 Solution) Store 4 weeks -20°C USE 15 µl

Once the treatment was completed, samples were transferred to the laboratory, before being spun in a chilled centrifuge at 4°C at 13,552 x g for 15 minutes. The supernatant was aliquoted into a 0.5 mL Safe-Lock Tube with a known volume of 120 µl and stored in a -80°C freezer prior to analysis.

6.3.1 Pilot study

A pilot study was initially conducted to determine if the small volumes of serum collected from human pulp were sufficient to conduct the desired analysis and if the dilution protocol was suitable for analysis. Three, well-characterised samples (**Table 6.1**) were prepared for transport to an external laboratory for a multiplex discovery

assay (**Appendix XVIII**), Human Cytokine Array/Chemokine Array 71-Plex Panel (EveTechnologies, Alberta, Canada). The samples were clinically characterized (**Section 2.6**) as two moderately inflamed samples requiring pulpotomy and a single, severely inflamed sample requiring pulpectomy.

After thawing, the samples were homogenised by vortexing at 1800 x g in a Heidolph REAX top (Heidolph Instruments GmbH & Co. KG) for 20 seconds. Thereafter, 75 µl of serum from each sample were coded in 0.5 mL Safe-Lock tubes and shipped on dry ice to the external laboratory for analysis.

Table 6.1 Pilot data summary data [Dx: Diagnosis; Tx: Treatment; pe: pulpectomy; pp: pulpotomy; IP: Irreversible pulpitis; SeP: severe pulpitis; MoP: Moderate pulpitis]

Sample	Dx	Tx	Volume	Replicate
32	IP/SeP	pe	75 µl	Single
33	IP/MoP	pp	75 µl	Single
34	IP/MoP	pp	75 µl	Single

6.3.2 High-throughput analysis

The three experimental groups were carefully sourced to demonstrate a range of states of the inflammatory process, including a healthy control (**Table 6.2**). The samples were prepared for shipping in the same manner as that of the pilot data (**Section 6.3.1**). After discussion with the laboratory and on the basis of the pilot data, the agreed volume of serum required to provide duplicate analysis was 100 µl. The laboratory applied a low volume strategy to analyse these samples in duplicate, which led to an unforeseen methodological variation, precluding direct comparison with the pilot data. Due to alterations in international shipping, it was not possible to ship the remaining 9 samples for high-throughput analyses.

Table 6.2 High-throughput data summary [Dx: Diagnosis; Tx: Treatment; pe: pulpectomy; pp: pulpotomy; xLa: extraction; IP: Irreversible pulpitis; SeP: severe pulpitis; MoP: Moderate pulpitis; NP: Normal pulp; *: indicates low volume strategy]

Sample	Dx	Tx	Volume	Replicate	*
37	IP/SeP	pe	100 µl	Duplicate	Low volume strategy
41	IP/MoP	pp	100 µl	Duplicate	Low volume strategy
44	NP	xLa	100 µl	Duplicate	Low volume strategy

6.3.3 Validation ELISA assays

Four commercial ELISA kits were used to evaluate two markers and to ascertain if the information obtained could substantiate and reproduce the data acquired from the high-throughput analysis. The candidate markers selected were IL-8 and M-CSF, which was based on statistically significant differences obtained in the high-throughput data. An observed upregulation of IL-8 was noted in the high throughput analysis from health, that progressively increased with inflammatory condition. In contrast, a significant downregulation was observed in the expression of M-CSF from the most severe inflammation, when compared with the healthy samples.

All samples were assayed using a volumetric method in duplicate with total protein not calculated. To provide sufficient substrate, all serum samples were further diluted prior to assay. After thawing, 700 µL of PBS was added to the samples before homogenising by vortexing at 1800 x g in a Heidolph REAX for 20 seconds.

6.3.3.1 IL-8 validation ELISA's

Two human IL-8 ELISA kits were sourced from different manufacturers (**Table 6.3**). A high sensitivity (HS) assay was sourced from R&D (R&D Systems, Abbingdon, UK) and

a standard ELISA kit from Abcam (Abcam, Cambridge, UK). The assays were carried out according to the manufacturer's instructions.

Briefly, standards and samples were pipetted into the provided 96-well format and IL-8 present in a serum sample bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-Human IL-8 antibody was added. After washing away any unbound biotinylated antibody, HRP-conjugated Streptavidin was pipetted to the wells. An additional step in the high sensitivity (HS) assay protocol, included the addition of an amplifier solution to the wells. The colour development was stopped at a recommended time point and the intensity of the colour was then measured at 450-490 nm using a NanoDrop™ 2000/2000c Spectrophotometer (ThermoFisher Scientific).

6.3.3.2 M-CSF validation ELISA's

Two human M-CSF ELISA quantitative immunoassay kits were also sourced (**Table 6.3**), from R&D and Abcam and carried out according to manufacturer's instructions. The R&D protocol involved pre-coated microplates with monoclonal antibody specific for human M-CSF. Standards and samples were pipetted into the wells and any M-CSF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human M-CSF was added to the wells. Following washing to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of M-CSF bound in the initial step. The colour development was stopped and the intensity of the colour is measured at 450 nm using a spectrophotometer.

Table 6.3 Validation assays: Summary details

Analyte	Manufacturer	Sample Volume	Stated sensitivity (mean MDD)
Human IL-8 Quantikine HS ELISA	R&D	100 µL	0.13 pg/mL
IL-8 Human ELISA	Abcam	100 µL	< 1 pg/mL
Human M-CSF Quantikine ELISA	R&D	100 µL	11.2 pg/mL
Human M-CSF Simple Step ELISA	Abcam	50 µL	3.8 pg/mL

The Abcam protocol differed from the R & D protocol in that samples and standards were first added to the wells prior to the incubation with the capture/analyte detector antibody complex. Washing then removed any unbound complex, before the addition of a chromagen substrate, 3,3',5,5'-tetramethylbenzidine (TMB) development solution and HRP conjugation. Stop solution was added at an established time point prior to reading the optical density at 450 nm using a spectrophotometer.

6.3.4 Statistical analysis

Descriptive statistics were produced using Microsoft Excel 2019 MSO. One-way analysis of variance with Tukey *post hoc* tests were used ($P < 0.05$) to determine the influence of inflammation on the levels of detectable cytokines and chemokines in human pulp sera using IBM SPSS Statistics 25 software (IBM).

6.4 Results

6.4.1 High-throughput analysis - Pilot data findings

A multiplex array (Human Cytokine Array/Chemokine Array 71-Plex Panel (Eve Technologies) analysis was conducted on samples of collected human pulp serum

(Section 6.3.1). Eighty one percent of the analytes tested in the pilot study (Section 6.3.1) produced readings within the range of detection of the assay. Nineteen percent of the analytes were out of range of the assay, most often below the limit of detection of the analyte, are presented in **Table 6.4**, or potentially not expressed. Of a total of 142 separate readings, 24 were out of range (below the 5 Parameter Logistic Standard Curve) in the moderate pulpitis category and 21/71 were in the severe pulpitis category. The range of observed concentrations obtained from the moderate pulpitis samples was 0.02 pg/mL – 1133.85 pg/mL and from the severe pulpitis samples was 0.06 pg/mL – 704.10 pg/mL. The raw data is charted in **Appendix XVIII** [The data is divided into three separate charts (numbered i, ii, iii) due to the scale].

Table 6.4 Details of analytes that were not detected in a multiplex discovery assay which tested 71 inflammatory-related biomarkers: Pilot samples [MoP: Moderate pulpitis; SeP: Severe pulpitis; &: and; Human Cytokine Array/Chemokine Array 71-Plex Panel (Eve Technologies)]

Diagnosis	MoP	SeP	MoP & SeP	All
Analyte	EGF	IFN- α 2	TNF β	IL-3
	BCA-1	IL-12p70	IL-2	IL-22
		MIP-1 β	IL-13	IL-23
		LIF	IL-16	IL-28A
			IL-20	
			IL-21	
			IL-33	
			MCP-3	
			MIP-1 α	
			Eotaxin-3	
			SCF	
			TPO	
			TSLP	

6.4.2 Investigation of inflammatory mediators in human pulp serum

The same multiplex array (Human Cytokine Array/Chemokine Array 71-Plex Panel (Eve Technologies) as used in the pilot study, was conducted on samples of collected human pulp serum (Section 6.3.2). The raw experimental data is presented, for

reference in **Appendix XIX**. Overall, 72% of the analytes tested demonstrated valid readings that were within the stated thresholds. The mediators that were undetected in the multiplex array are shown in **Table 6.5**. The mediators are categorised in **Table 6.5** based on absence of detection across all samples; or absence of detection in 2/3 of the categories; or absence of expression in a single category only. The table also specifies in which category they failed to be detected.

Table 6.5 Details of analytes that were not detected in a multiplex discovery assay which tested 71 inflammatory-related biomarkers: Test samples [NP: Normal pulp; MoP: Moderate pulpitis; SeP: Severe pulpitis; Human Cytokine Array/Chemokine Array 71-Plex Panel (Eve Technologies)]

Analyte	No reading x 3	Analyte	No reading x 2	Analyte	No reading x 1
IFN γ	ALL	GM-CSF	MoP, SeP	IL-2	NP
IL-15	ALL	IL-4	MoP, SeP	IL-12p40	MoP
IL-23	ALL	IL-21	MoP, SeP	IL-13	SeP
IL-28A	ALL	IL-9	NP, SeP	IL-17F	NP
IL-33	ALL	IL-10	NP, MoP	MCP-4	MoP
Eotaxin-3	ALL	IL-12p70	NP, MoP	MIP-1 β	NP
TPO	ALL	IL-16	NP, MoP	EGF	NP
TSLP	ALL	IL-17A	NP, MoP		
		sCD40L	NP, MoP		

6.4.3 High-throughput data analysis

Details of the levels of expression are documented in **Table 6.6**. The mean expression in pg/mL for each mediator in pulpal serum obtained from normal pulp, moderate pulpitis and severe pulpitis is presented along with any statistical significance between the diagnostic groups. In the normal pulp category 43/142 analytes produced results out of range of the limit of detection of the array. For the moderate pulpitis category, this number was 44/142 and for severe pulpitis 31/142. The range of concentrations for all analytes produced in the normal category were 0.13 pg/mL-

5289.27 pg/mL; moderate pulpitis ranged from 0.07 – 7869.89 pg/mL and severe pulpitis 0.00 – 4138.61 pg/mL.

Panel A, Table 6.6 depicts a group of mediators that were shown to demonstrate statistically significant differences between normal pulps and those teeth exhibiting signs and symptoms indicative of moderate pulpitis; as well as between normal pulp and severe pulpitis; and between moderate pulpitis and severe pulpitis. EGF demonstrated the greatest significance (p -value $< .0001$) across all comparisons. $GR\alpha$, IL-1RA, IL-6, PDGF-AA, FLT-3L and Eotaxin-2 demonstrated significance between all groups tested, and reached highly significant (p -value $< .0001$) differences in 2/3 of the listed comparisons. G-CSF $TGF\alpha$, IP-10, MCP-1, M-CSF, and FGF-2 demonstrated statistically significant differences across the three comparisons with p -values ranging from $<.0001$ to $<.05$.

Panel B, Table 6.6 depicts those mediators that demonstrated statistically significant differences between normal pulp and moderate pulpitis; and between normal pulp and severe pulpitis only.

Panel C, Table 6.6 shows the mediators that reached statistical significance between normal pulp and moderate pulpitis and between moderate pulpitis and severe pulpitis only, while **Panel D** lists the mediators that were statistically different when normal pulp and severe pulpitis; and when moderate pulpitis and severe pulpitis were compared. Of interest, are those mediators that demonstrated the highest significance ($p < 0.0001$) in both categories and these included IL-2, IL-8, IL-16, MDC and ENA-78.

IL-1 β and IL-27 only reached significant in one of the three comparators (**Panel E**). The mediators that did not demonstrate any significant differences are listed in **Panel F**.

Table 6.6 Expression of inflammatory mediators in serum collected from normal pulps, moderate and severe pulpitis [*p<.05; †p≤.001; ‡p<.0001; NS: Not statistically significant; Statistical significance: - A: All groups; B: NP-MoP & NP-SeP; C: NP-MoP & MoP-SeP; D: MoP-SeP & NP-SeP; E: One group; F: No statistical significance; se: standard error]

Panel	Molecule	NP mean ± se (pg/mL)	MoP mean ± se (pg/mL)	P value NP v's MoP	SeP mean ± se (pg/mL)	P Value NP v's SeP	P Value MoP v's SeP
A	G-CSF	2.06 ± 2.06	22.88 ± 3.27	*	93.31 ± 3.53	‡	†
	GROα	1.67 ± 0.19	41.52 ± 0.33	‡	6.38 ± 0.26	*	‡
	TGFα	51.23 ± 1.70	20.29 ± 0.58	†	14.42 ± 1.29	†	*
	IL-1RA	9.80 ± 0.21	5.90 ± 0.33	†	28.67 ± 0.12	‡	‡
	IL-6	0.05 ± 0.07	2.15 ± 0.19	*	7.41 ± 0.14	‡	‡
	IP-10	19.32 ± 0.49	5.84 ± 0.15	‡	9.54 ± 0.43	†	*
	MCP-1	16.29 ± 0.57	20.60 ± 0.33	*	30.36 ± 0.28	‡	†
	M-CSF	306.87 ± 21.35	113.65 ± 4.63	†	4.76 ± 0.92	†	*
	PDGF-AA	66.67 ± 3.27	327.41 ± 1.15	‡	352.02 ± 1.19	‡	*
	EGF	0.00 ± 0.00	15.69 ± 0.28	‡	7.77 ± 0.00	‡	‡
	FGF-2	5183.82 ± 104.30	6295.34 ± 255.46	*	4116.38 ± 22.16	*	†
	FLT-3L	79.10 ± 1.08	5.50 ± 0.12	‡	2.93 ± 0.17	‡	*
	Eotaxin-2	134.30 ± 1.28	26.86 ± 2.06	*	594.04 ± 18.58	‡	‡
B	IL-3	8.79 ± 0.69	1.24 ± 0.13	*	0.22 ± 0.13	‡	NS
	IL-4	0.29 ± 0.08	0.00 ± 0.00	*	0.00 ± 0.00	*	NS
	IL-18	43.46 ± 0.75	84.60 ± 5.26	*	68.38 ± 0.43	*	NS
	MIP-1α	49.58 ± 4.17	12.49 ± 6.13	*	20.30 ± 2.15	*	NS
	PDGF-AB/BB	271.79 ± 18.88	1250.20 ± 34.49	†	1182.52 ± 52.64	†	NS
	VEGF-A	11.19 ± 1.07	3.84 ± 0.72	*	2.31 ± 0.10	*	NS
	Fractalkine	173.82 ± 8.75	45.44 ± 13.06	*	13.33 ± 15.60	*	NS
	CTACK	3.92 ± 1.09	23.66 ± 1.56	*	20.41 ± 0.99	*	NS

C	IL-7	0.75 ± 0.04	1.91 ± 0.25	*	0.36 ± 0.09	NS	*
	IL-9	0.00 ± 0.00	2.92 ± 0.51	*	0.00 ± 0.00	NS	*
	MCP-3	3.75 ± 0.21	10.75 ± 0.97	*	6.22 ± 0.28	NS	*
	MIG/CXCL9	453.42 ± 8.10	342.54 ± 5.42	*	423.04 ± 7.47	NS	*
D	IL-1α	316.03 ± 7.50	270.08 ± 18.26	NS	178.67 ± 0.59	*	*
	IL-2	0.00 ± 0.00	0.10 ± 0.03	NS	1.79 ± 0.09	‡	‡
	IL-8	1.10 ± 0.29	0.63 ± 0.19	NS	57.47 ± 1.04	‡	‡
	IL-12p40	7.64 ± 4.95	0.00 ± 0.00	NS	92.49 ± 4.65	†	†
	IL-16	0.00 ± 0.00	0.00 ± 0.00	NS	350.05 ± 17.05	‡	‡
	IL-17F	0.00 ± 0.00	1.04 ± 0.21	NS	3.55 ± 0.70	*	*
	MDC	49.05 ± 1.44	56.24 ± 2.72	NS	119.54 ± 0.48	‡	‡
	MIP-1β	0.00 ± 0.00	3.37 ± 2.38	NS	11.56 ± 0.09	*	*
	sCD40L	0.00 ± 0.00	0.00 ± 0.00	NS	10.19 ± 2.15	*	*
	ENA-78	33.89 ± 2.64	71.22 ± 1.08	NS	581.07 ± 17.62	‡	‡
E	TARC	2.05 ± 0.13	2.34 ± 0.01	NS	2.85 ± 0.02	*	*
	IL-1β	2.05 ± 0.36	0.00 ± 0.00	NS	3.41 ± 0.33	NS	*
F	IL-27	23.08 ± 23.08	82.33 ± 19.16	NS	139.98 ± 5.34	*	NS
	IFN-α2	50.80 ± 2.00	24.21 ± 13.70	NS	15.31 ± 6.51	NS	NS
F	TNFα	5.98 ± 2.08	6.95 ± 0.56	NS	6.26 ± 0.97	NS	NS
	TNFβ	0.72 ± 0.31	0.00 ± 0.31	NS	0.21 ± 0.21	NS	NS
	IL-5	0.45 ± 0.05	0.33 ± 0.07	NS	0.80 ± 0.07	NS	NS
	IL-10	0.00 ± 0.00	0.00 ± 0.00	NS	0.04 ± 0.08	NS	NS
	IL-12p70	0.00 ± 0.00	0.00 ± 0.00	NS	0.00 ± 0.14	NS	NS
	IL-13	15.17 ± 16.22	11.16 ± 11.16	NS	0.00 ± 0.00	NS	NS
	IL-17A	0.00 ± 0.00	0.00 ± 0.00	NS	0.05 ± 0.58	NS	NS
	IL-17E/IL-25	86.07 ± 92.65	193.75 ± 8.43	NS	382.69 ± 22.39	NS	NS
	IL-20	498.46 ± 83.37	455.76 ± 44.34	NS	508.70 ± 51.01	NS	NS
	IL-21	2.35 ± 1.75	0.00 ± 0.00	NS	0.00 ± 0.00	NS	NS

	IL-22	47.17 ± 11.07	44.14 ± 7.40	NS	50.49 ± 11.79	NS	NS
	MCP-2	17.98 ± 11.71	17.98 ± 6.89	NS	26.18 ± 1.09	NS	NS
	MCP-4	2.93 ± 2.93	0.00 ± 0.00	NS	2.93 ± 2.93	NS	NS
	MIP-1δ	181.19 ± 17.04	190.66 ± 20.84	NS	296.88 ± 24.43	NS	NS
	RANTES	2070.72 ± 15.08	5022.66 ± 2078.78	NS	1990.56 ± 132.16	NS	NS
	Eotaxin	5.50 ± 0.55	5.03 ± 0.48	NS	6.37 ± 0.50	NS	NS
	6CKine	72.22 ± 32.83	59.49 ± 10.75	NS	59.49 ± 10.75	NS	NS
	I-309	0.46 ± 0.16	0.43 ± 0.13	NS	0.50 ± 0.04	NS	NS
	LIF	3.71 ± 0.00	1.68 ± 0.87	NS	4.59 ± 3.20	NS	NS
	SDF-1α+β	534.04 ± 14.59	541.31 ± 7.24	NS	590.54 ± 68.69	NS	NS
	TRAIL	18.39 ± 0.57	18.63 ± 0.81	NS	17.90 ± 0.73	NS	NS

The mean expression of the mediators reported in **Panel A** are depicted in **Figure 6.3**. A constant upregulation of G-SCF, IL-6, MCP-1 and PGDF-AA is noted from the normal healthy control to the moderate pulpitis and finally, to severe pulpitis. TGF α , M-CSF and FLT-3L were consistently downregulated from normal pulp to severe pulpitis. A fluctuating, less predictable pattern between the groups of expression is evident with the following mediators: GRO α , EGF, FGF-2, IL-1RA, IP-10 and Eotaxin-2.

Comparison between normal pulp and severe pulpitis and between moderate pulpitis and severe pulpitis, demonstrated statistically significant differences ($p < .05$) in every mediator listed in **Panel D**. These results are presented in chart form in **Figure 6.4**. Consistent upregulation from NP-SeP and from MoP-SeP is noted in every instance.

It is notable, that although EGF demonstrated the most impressive statistical significance, when each of the pulp states were compared, it was undetected in the normal pulp (**Tables 6.5, Table 6.6**). It was also undetected in one of the moderate pulpitis samples used in the pilot data (see **Appendix XVIII**). It is beyond the scope of this study to assess whether this was due to the mediator not being expressed, or the expression being lower than the threshold of the multiplex assay. Other mediators that displayed statistical significance (**Panel D, Table 6.6**) but failed to generate readings in one category, included the following: IL-2, IL-12p40, IL-17F and MIP-1 β ; and in two categories: IL-16, sCD40L. Of the mediators that did not demonstrate any statistical significance (**Panel F, Table 6.6**), the following were not expressed in one category: IL-13 and MCP-4; and in two categories: IL-10, IL-12p70, IL-17A and IL-21.

Figure 6.3 (See over) Charts depicting the mediators which demonstrated statistically significant differences ($p < .05$ – $p < .0001$) between all comparators in a Multiplex Array [1: Normal pulp; 2: Moderate pulpitis; 3: Severe pulpitis; Values shown are mean values, error bars are standard deviations; All significantly different]

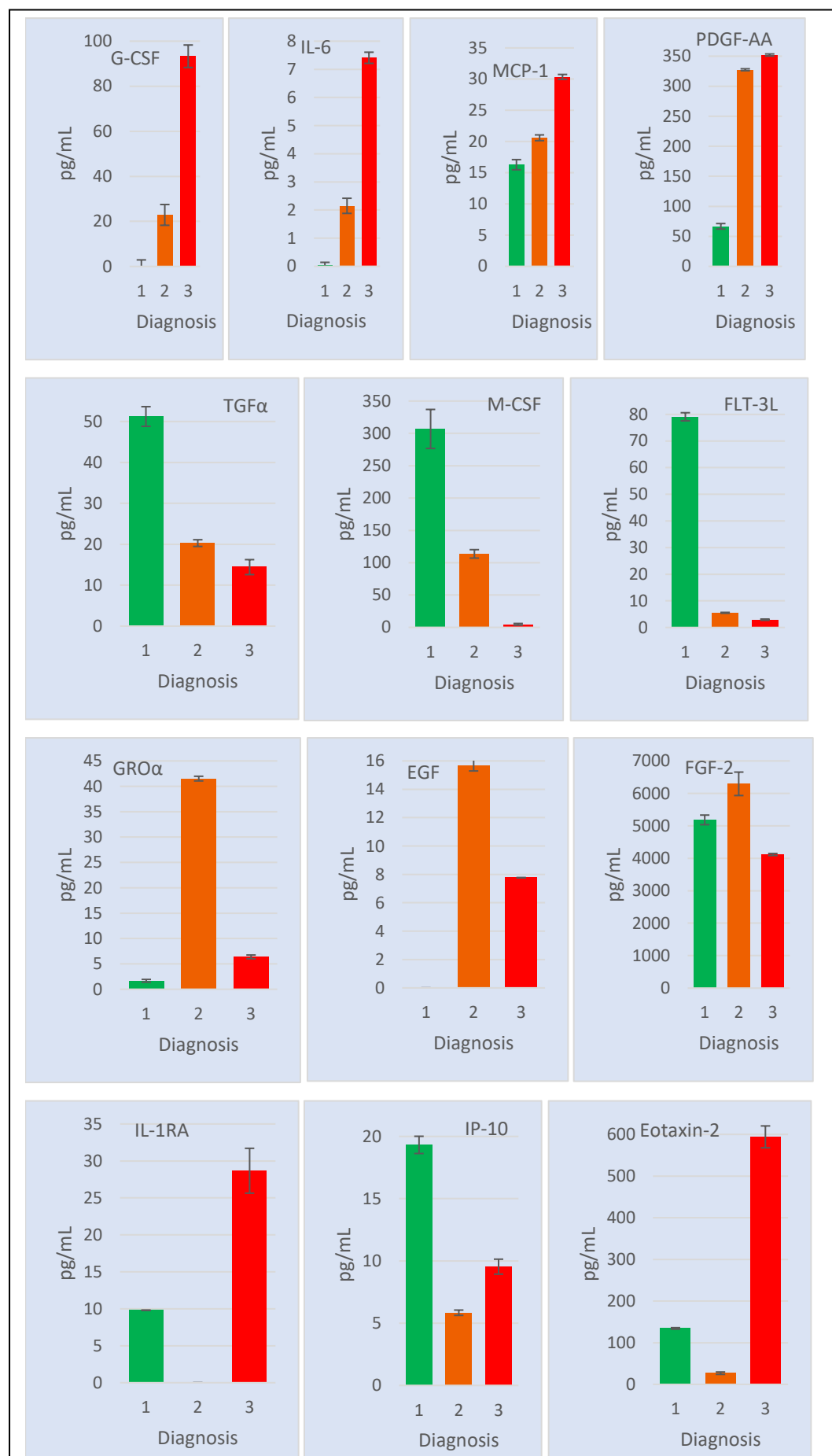
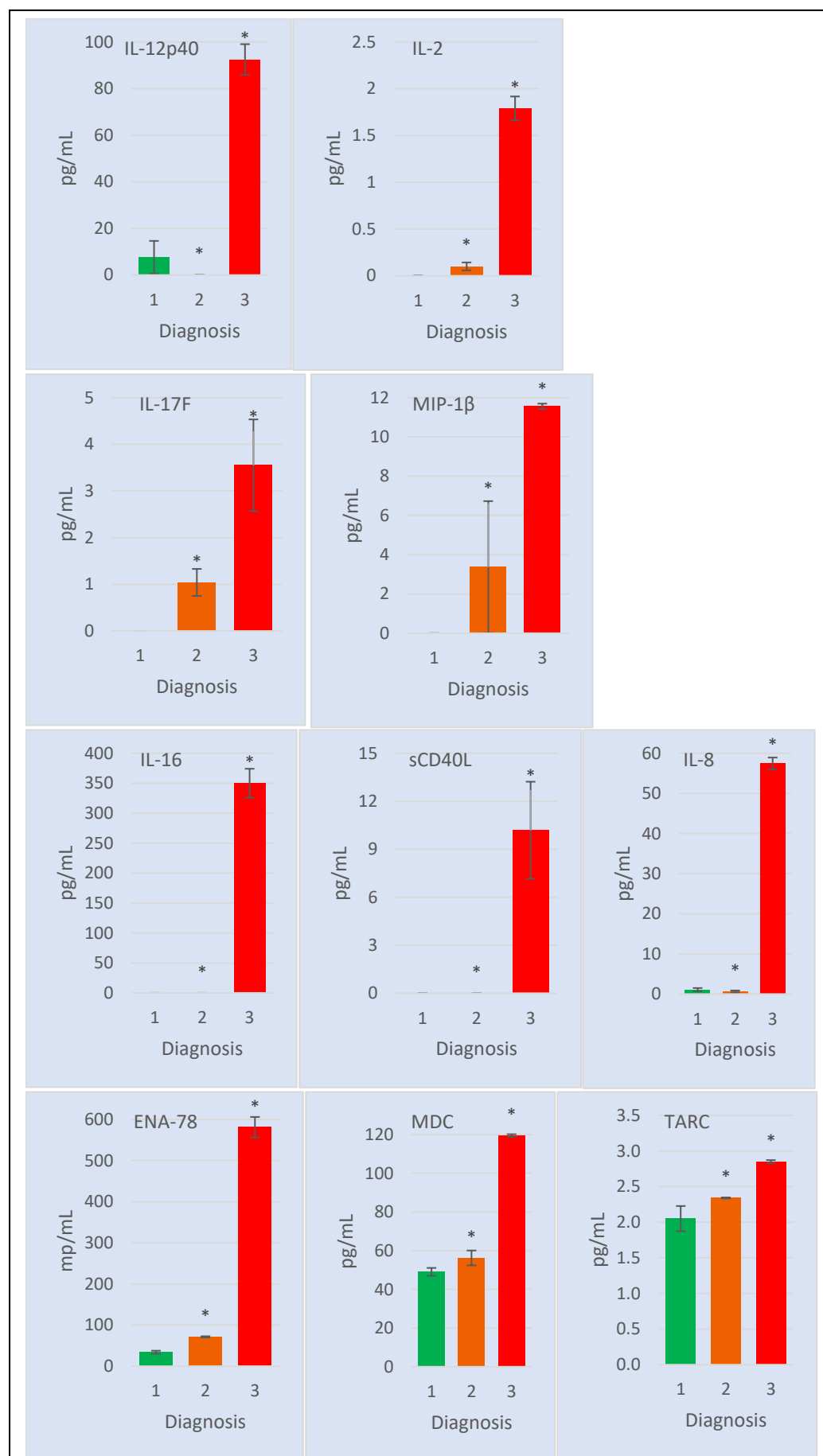


Figure 6.4 (See over) Charts depicting the mediators which demonstrated significant differences (*) between NP-SeP and MoP- SeP ($p < .05$) in a Multiplex Array [1: Normal pulp; 2: Moderate pulpitis; 3: Severe pulpitis; Values shown are mean values, error bars are standard deviations]



6.4.4 Validation ELISA – Pilot findings

6.4.4.1 IL-8 ELISA

Two analytes were selected to validate the findings presented in the high-throughput analysis (**Section 6.3.3**). **Figure 6.5 (i-iii)**, charts the comparison of the IL-8 high-throughput and the corresponding ELISA data. Multiple assays have been averaged and their standard deviations represented by error bars. The trend visualised, represents a marked reduction in the expression from moderate to severe pulpitis across all assays. The selected candidate ELISA's appear to validate the high throughput pilot findings. Importantly, all assays (High-throughput and validation ELISA's) produced readings, within the detection limit of the kits used.

6.4.4.2 M-CSF ELISA

Figure 6.6 (i-iii), summarises the findings of the high-throughput assay (M-CSF) with the corresponding validation M-CSF ELISA (R&D, Abcam). Multiple assays have been averaged and their standard deviations represented by error bars. A reduction in expression of M-CSF from moderate pulpitis to severe pulpitis is mirrored in the high-throughput and R&D M-CSF ELISA. Conversely, although a similarly high expression of M-CSF was evident in both samples an increase in expression is noted from moderate to severe pulpitis using the Abcam M-CSF ELISA.

Figure 6.5 High-throughput IL-8 results compared with corresponding validation IL-8 ELISA: Pilot data (i). IL-8: EveT; (ii). IL-8: R&D; (iii). IL-8: Abcam [#: number; Dx: Diagnosis; MoP: Moderate pulpitis; SeP: Severe pulpitis; EveT: Human Cytokine Array/ Chemokine Array 71-Plex Panel (Eve Technologies): Multiple assays have been averaged with error bars representing their standard deviations]

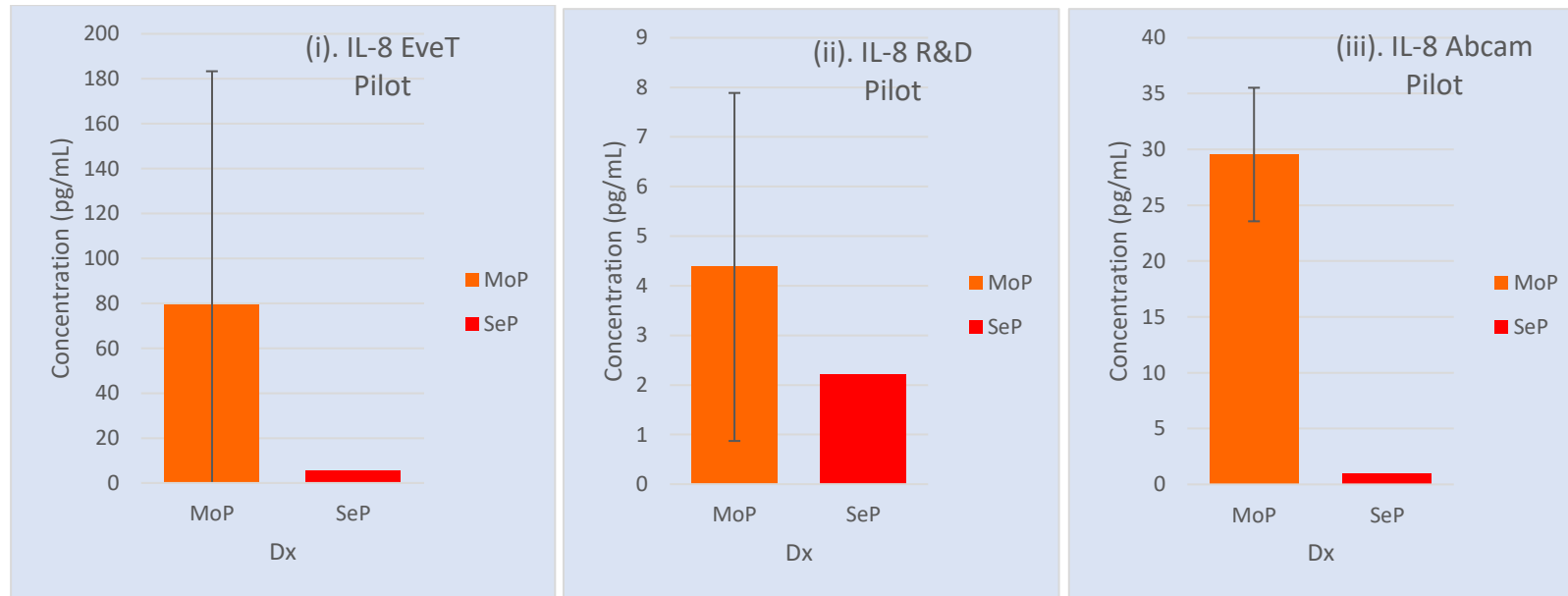
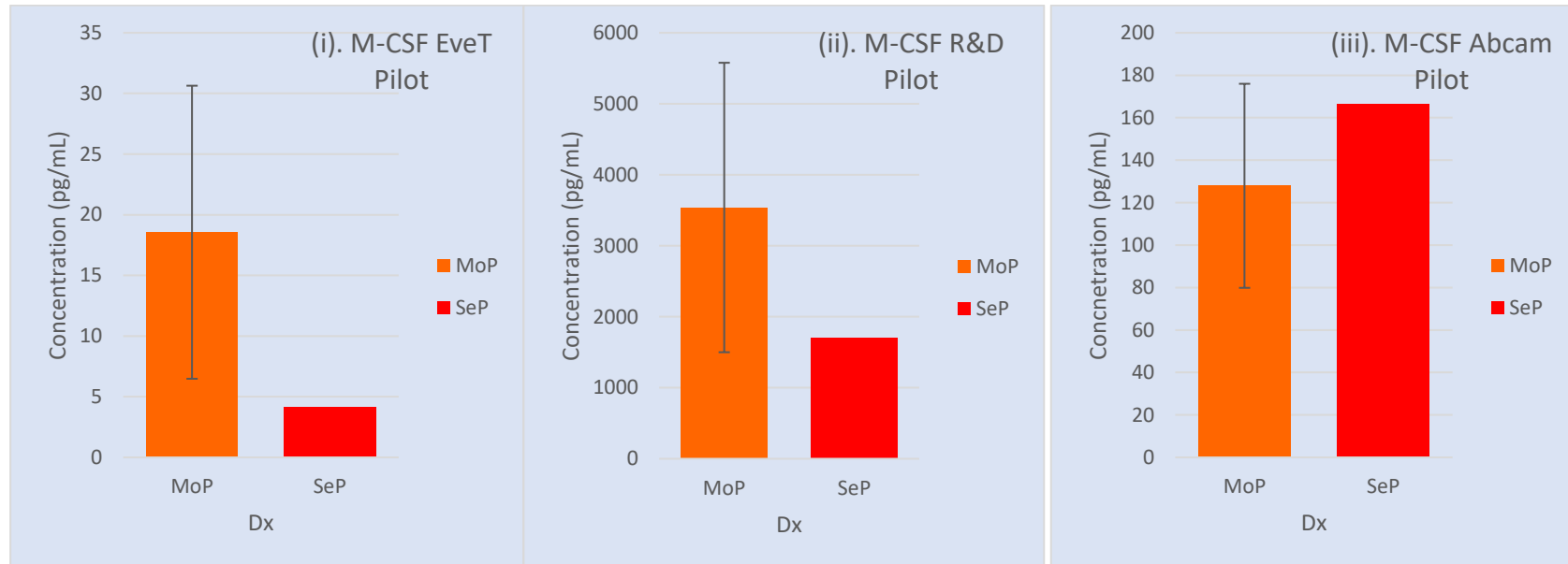


Figure 6.6 High-throughput M-CSF results compared with corresponding validation M-CSF ELISA: Pilot data (i). M-CSF: EveT; (ii). M-CSF: R&D; (iii). M-CSF: Abcam [#: number; Dx: Diagnosis; MoP: Moderate pulpitis; SeP: Severe pulpitis; EveT: Human Cytokine Array/ Chemokine Array 71-Plex Panel (Eve Technologies): Multiple assays have been averaged with error bars representing their standard deviations]



6.4.5 Validation ELISA – Test findings

6.4.5.1 IL-8 ELISA

Figure 6.7 (i-iii), charts the results of the high-throughput findings for IL-8 compared with the two, commercial validation ELISA's (R&D, Abcam). The array demonstrated increases between normal and severe pulpitis; and between moderate pulpitis and severe pulpitis. The trend was not replicated in the IL-8 R&D ELISA and the Abcam ELISA (IL-8) failed to produce any reading.

6.4.5.2 M-CSF ELISA

The results of the comparisons between the high throughput analysis of M-CSF and the corresponding validation ELISA's are presented in **Figure 6.8 (i-iii)**. The microarray demonstrates a reduction in expression of M-CSF from normal pulp to moderate pulpitis and from moderate pulpitis to severe pulpitis. With the R&D ELISA a reduction is noted from normal pulp to moderate pulpitis and from normal pulp to severe pulpitis. The M-CSF Abcam ELISA reveals increases from normal pulp and from moderate pulpitis, to severe pulpitis.

Figure 6.7 High-throughput IL-8 results compared with corresponding validation IL-8 ELISA: Test data (i). IL-8: EveT; (ii). IL-8: R&D; (iii). IL-8: Abcam [Dx: Diagnosis; NP: Normal pulp; MoP: Moderate pulpitis; SeP: Severe pulpitis; EveT: Human Cytokine Array/ Chemokine Array 71-Plex Panel (Eve Technologies): Multiple assays have been averaged with error bars representing their standard deviations]

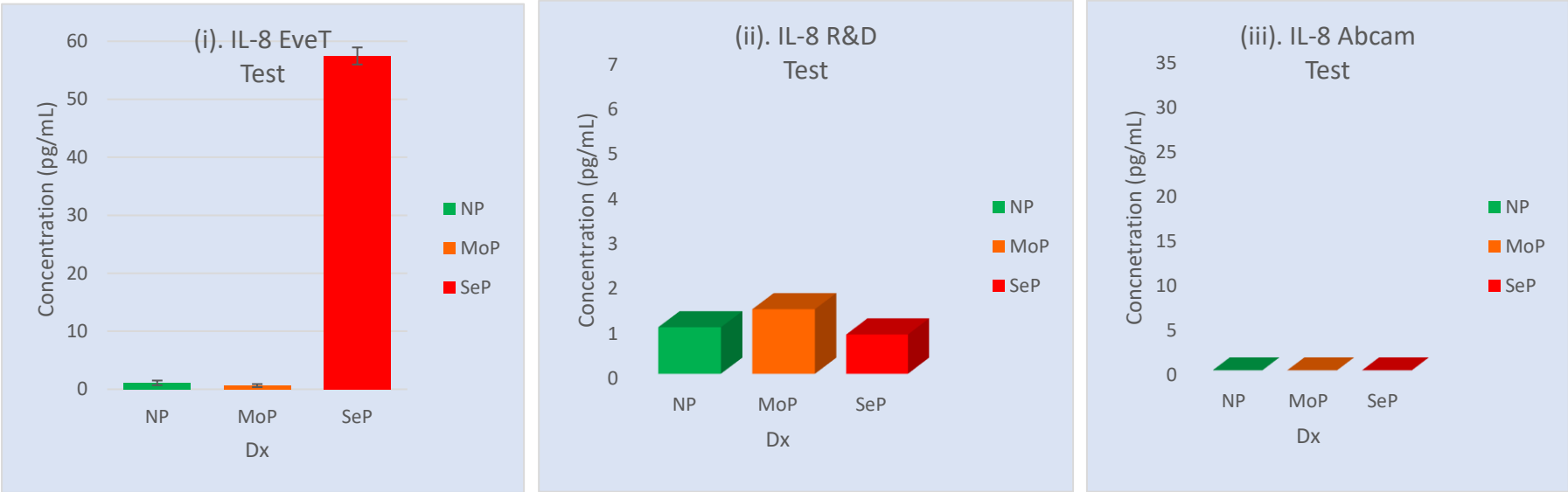
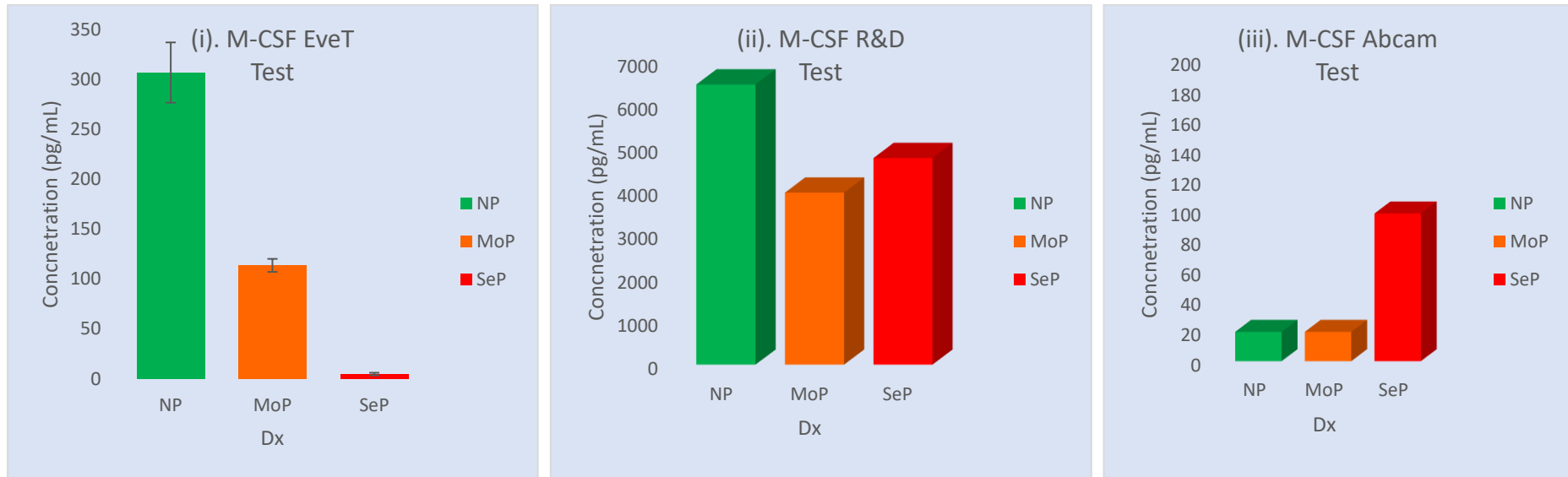


Figure 6.8 High-throughput M-CSF results compared with corresponding validation M-CSF ELISA: Test data (i). M-CSF: EveT; (ii). M-CSF: R&D; (iii). M-CSF: Abcam [Dx: Diagnosis; NP: Normal pulp; MoP: Moderate pulpitis; SeP: Severe pulpitis; EveT: Human Cytokine Array/ Chemokine Array 71-Plex Panel (Eve Technologies): Multiple assays have been averaged with error bars representing their standard deviations]



6.4.6 Validation ELISA analysis

Sufficient collected serum was available from the same pilot and test samples to run validation ELISA assays with the selected kits, as described in **Section 6.3.3**.

The results of the combined validation data are presented in **Figure 6.9** and **Figure 6.10**. The IL-8 ELISA charts (R&D, Abcam) in **Figure 6.9 (i, ii)** appear symmetrical. The reduction in expression from moderate to severe pulpitis is also mirrored in both the pilot antibody array (**Figure 6.5 (i)**.) and the pilot ELISA data (**Figure 6.5 (ii, iii)**.).

The pattern demonstrated by the combined validation ELISA M-CSF (R&D) results (**Figure 6.9 (iii)**.) is mirrored in the array results (**Figure 6.8 (i)**.) and the corresponding pilot data (**Figure 6.6 (i, ii)**.). The increasing expression obtained using M-CSF ELISA (Abcam), **Figure 6.9 (iv)**., is replicated in the test data **Figure 6.8 (iii)**., and the corresponding pilot data (**Figure 6.6 (iii)**.).

In **Figure 6.10** the distribution of the cases used in the validation ELISA's can be viewed. The cases are arranged according to Wolter's diagnostic criteria (**Section 2.6.2**) and demonstrate the corresponding concentrations of the selected analytes in pg/mL. The mediators were all relatively expressed within the detection limit of the kit, with the exception of three normal samples using M-CSF ELISA [Abcam] (**Figure 6.10 (iv)**.).

Figure 6.9 Combined validation IL-8, M-CSF ELISA (R&D, Abcam) data (i). IL-8: R&D; (ii). IL-8: Abcam; (iii). M-CSF: R&D; (iv). M-CSF: Abcam [Dx: Diagnosis; NP: Normal pulp; MoP: Moderate pulpitis; SeP: Severe pulpitis; Multiple assays have been averaged with error bars representing their standard deviations; * Indicates statistically significant difference NP-MoP $p < .05$]

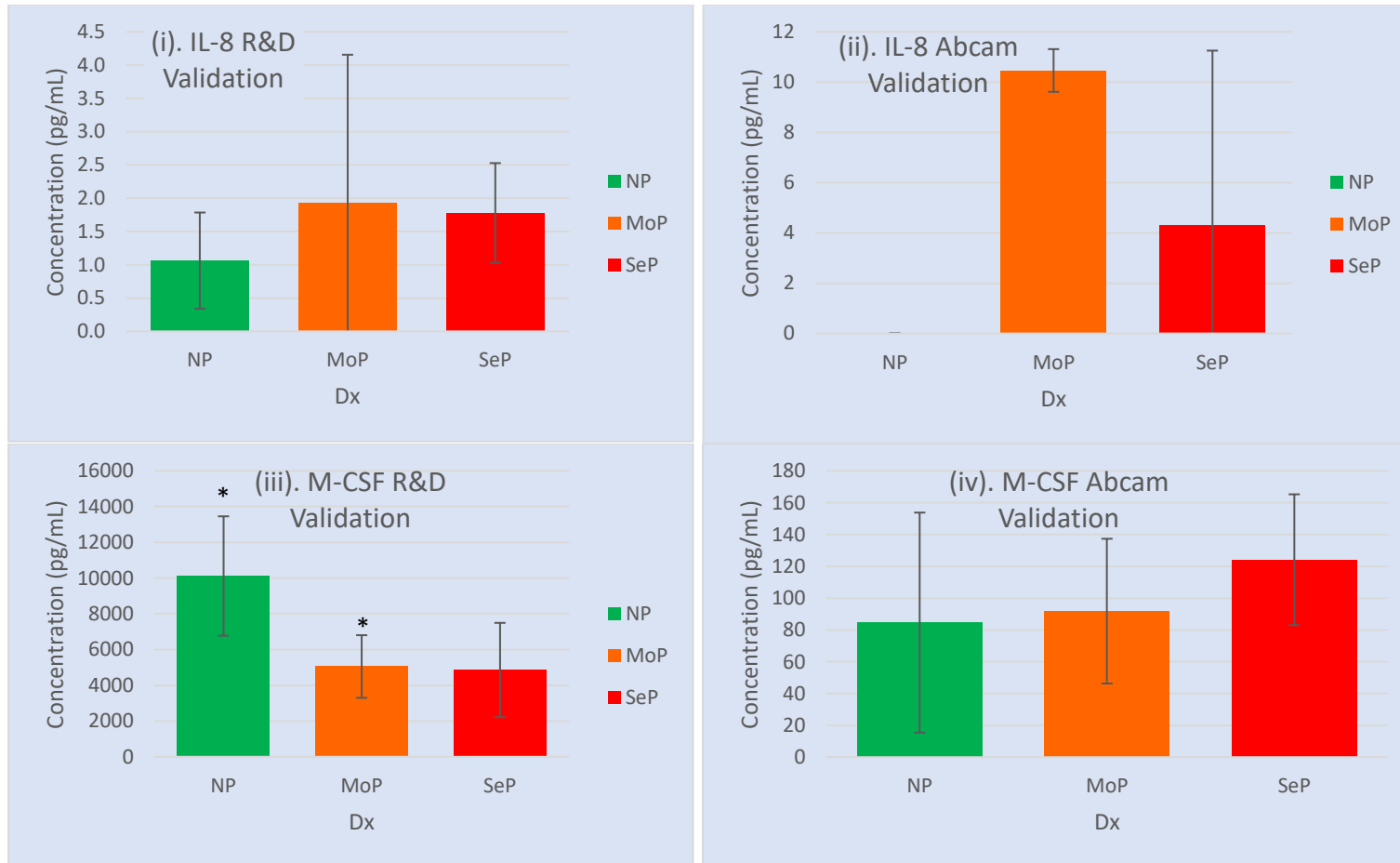
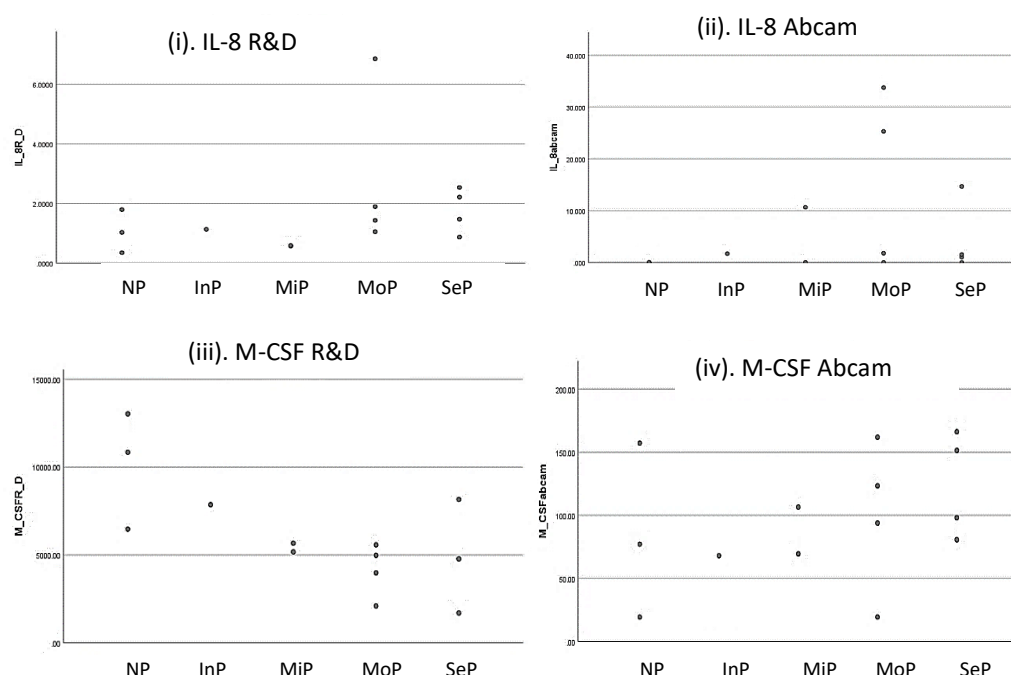


Figure 6.10 Dot plots depicting the distribution of selected mediators by ELISA categorised by diagnosis (i). IL8: R&D; (ii). IL-8: Abcam; (iii). M-CSF: R&D; (iv). M-CSF: Abcam [NP: Normal pulp; InP: Initial pulpitis; MiP: Mild pulpitis; MoP: Moderate pulpitis; SeP: Severe pulpitis]



6.5 Discussion

In this chapter a profiling method that analyses a broad panel of inflammatory cytokines in serum extracted from human pulpal blood is presented. Validation of the multiplex findings was based on a selection of clinically relevant candidate markers.

Interrogation of the data presented in **Table 6.6** revealed many interesting and novel analytes. Statistically significant differences were demonstrated between all diagnostic categories (normal pulp, moderate pulpitis and severe pulpitis) in **Panel A, Table 6.6**. In considering which analyte to select for the validation ELISA's, not only was it important to select a marker that demonstrated statically significant differences between each of the three domains, but also, it was important that the analyte provided a reading in all of the domains.

EGF (epidermal growth factor), was considered as a potential analyte, particularly due to the highly significant ($p < .0001$) differences between each of the diagnostic categories. One concern, however was that it failed to produce a reading in normal samples. It was unclear if this was due to the analyte being below the detection limit of the assay, or due to technical reasons. A further concern was the undulant pattern produced from normal through severe pulpitis (**Figure 6.3**). A linear gradient seemed preferable, particularly when we are trying to identify a threshold.

In the candidate selection process, markers that demonstrated statistically significant differences and either increasing, or decreasing gradients in expression from normal to severe pulpitis were preferred to those presenting undulant patterns, which were ruled out [GRO- α , EGF, FGF-2, IL-1Ra, IP-10, Eotaxin-2] (**Figure 6.3**). Although an increasing gradient was demonstrated by G-CSF and IL-6, both failed to produce consistent readings in the normal category. In excluding MCP-1, the levels of expression were very close in value between all three diagnostic categories, while PDGF-AA produced expressions very close in value between moderate and severe pulpitis.

Three analytes remained, which fulfilled the selection criteria described; TGF α , M-CSF, or FLT-3L. M-CSF (macrophage colony-stimulating factor) is a haematopoietic growth factor with functions in homeostasis and inflammation (Ushach & Zlotnik 2016, Mun et al. 2020), presents a novel target in understanding its role in the progression of pulp inflammation (Iwasaki et al. 2011). Consistent downregulation of M-CSF was observed in both the pilot (**Figure 6.6 (i).**) and test (**Figure 6.8 (i).**) antibody arrays. This was supported by the findings of the pilot (**Figure 6.6 (ii, iii).**) and the validation findings (**Figure 6.9 (iii).**).

One of the limitations noted in previous studies focused on biomarker identification relates to diagnostic categorisation, as generally, only the disease extremes are contrasted (Nguyen et al. 2020, Shmueli et al. 2020, Sharma et al. 2021). However, in a clinical setting, discrimination within the spectrum of inflammation would be more useful, to potentially inform potential conservative treatment strategies. The relevance of demonstrating significant discrimination between moderate and severe pulpitis could be exploited clinically to determine the limit of VPT. In **Figure 6.4** ten mediators demonstrating significant discrimination ($p < .05$) between moderate and severe pulpitis are presented. Using a similar rationale to that outlined above, six mediators that failed to register a consistent reading were excluded from consideration (IL-12p40, IL-2, IL-17F, MIP-1 β , IL-16, sCD40L).

Four candidates remained for consideration. TARC (thymus-and activation-regulated chemokine) was excluded based on a lower statistical significance ($p < .05$), compared with the remaining three candidates ($P < .0001$). Any of the three remaining candidates could have provided a useful target for analysis (IL-8, MDC, or ENA-78). The decision to select IL-8 was perhaps most influenced by the existing previous literature (Huang et al. 1999, Isett et al. 2003, Karapanou et al. 2008, Abd Elmeguid et al. 2013) and the fact that a novel target had already been selected (M-CSF) for validation. A new link has been identified between the cytokines IL-8 and IL-6, neutrophil activation, and the release of H3Cit in the blood stream (Yang et al. 2020a). IL-8 and IL-6 have also been shown to induce NET formation *in-vitro* (Thålin et al. 2018).

IL-8 produced the most consistent readings with the R&D ELISA; however, the Abcam ELISA failed to produce readings in the normal category. Unfortunately, direct

comparisons cannot be drawn with the existing literature, due to the substantial variation in their methodologies (**Section 1.5**). In line with ElSalhy et al. (2013), statistical significance ($p < .0001$) was demonstrated between normal and moderate pulpitis, as well as between moderate and severe pulpitis groups.

Notably, this work represents the first presentation of biomarker identification using a novel diagnostic classification (Wolters et al. 2017). Further work is necessary to validate these preliminary findings and correlate the biomarker level with treatment outcome.

Although some encouraging trends were demonstrated within the Pilot validation ELISA's (**Figure 6.5 (i,ii,iii)**. and **Figure 6.6 (i,ii)**.), some discrepancies were also noted in the pattern of expressions in the corresponding Test data (**Figure 6.7 (i,ii,iii)**., **6.8(i,ii,iii)**.). This serves to highlight the importance of maintaining a constant dilution protocol within the study samples. Firstly, the volume strategy applied by the external laboratory, was not consistent (in order to provide duplicate analysis of the test data, the application of a low volume strategy was unforeseen and unavoidable). The addition of dilutions blood substrates may not behave in a linear manner due to a matrix effect (Chiu et al. 2010, Zhou et al. 2017). Limited volumes of available substrate are a challenge seen only in rare circumstances within medical diagnostics (Proytcheva 2009). In order to provide sufficient substrate to conduct the subsequent validation assays, additional diluents were added to the remaining samples. Conceivably, the analytes may have degraded, or homogenation may have been insufficient to uniformly disperse the analytes for the additional assays. Finally, it must be acknowledged that a drawback of the study, relates to the fact that it was conducted by a single operator, and therefore neither calibration, nor intra-

operator agreement could be reliably assessed, in the diagnostic realm. The discrepancies could reflect the challenges relating to the accurate clinical assessment of the inflamed pulp.

Limitations of this study include the exploratory nature of the work with only low numbers of study samples, which can impact on the conclusions drawn from this preliminary work. As expected, variation in the levels of expression of the analytes was also noted. That said, good validation was achieved with M-CSF ELISA [R&D] (**Figure 6.9 (iii).**) with the preliminary high-throughput data (**Figure 6.8 (i).**).

ELISA has long been considered the most validated and standard tool available for cytokine measurement method; however, there remains an experimental concern that performance appears to be highly dependent on antibody quality, kit manufacturer and handling – possibly influencing the outcome, if not strictly adhered to. That aside, ELISA still appears to be the most accurate, sensitive, and specific test for molecular evaluation of dental pulp inflammation at chairside as other techniques such as qRT-PCR are not applicable in this context.

Within each diagnostic subset a wide range of expression was evident (**Figure 6.10 (i-iv).**). These variations could point to technical error, or diagnostic limitations in appropriately classifying the inflamed pulp, in the absence of direct access to the tissues. Due to the inherent limitation in availability of substrate (pulpal serum), addition of diluents was unavoidable and necessary. Dilutions have been shown to affect serum and plasma matrices in a non-linear manner and may differentially affect the expression of various cytokines (Rosenberg-Hasson et al. 2014).

6.6 Conclusion

Multiplex analysis of inflammatory cytokines in serum extracted from pulpal blood, obtained from carefully characterised samples representing health through progressive inflammation, provides a reliable, cost effective screening tool to inform useful targets for further investigations using candidate ELISA analyses.

Initial validation assays revealed promising results with several markers identifying significant differences in expression between the diagnostic categories. A significant drawback in the analysis of pulpal blood is the limited availability of substrate to provide multiple analyses. Preliminary operating procedures should take account of the collected volumes of substrate necessary to provide standardised dilution across a range of assays.

Within the limitations of the model presented, an opportunity exists to direct larger studies combining a profiling approach with candidate validation, to direct future standards of biomarker identification in pulpal blood that can direct clinical treatment.

Chapter 7

General discussion and future research

7.1 Discussion

Establishing an accurate determination of the level of pulpal inflammation is central to guiding clinicians towards appropriate, predictable treatments. Barriers to adopting contemporary, minimally-invasive vital pulp treatments include the limitations of traditional sensibility testing and patient history to accurately identify the degree of inflammation present prior to accessing the pulp. These flawed techniques and tests influence the treatment options considered by treating clinicians. The concept that the exposure of the pulp is a negative prognostic factor is historically engrained in the dental psyche. Educational bias, expertise, anxiety, prognosis, lack of training and uncertain longevity, specialised equipment and materials, as well as cost and time, have all been cited as obstacles to the adoption of pulp preservation techniques in routine clinical practice.

Routine clinical practice, requires clinicians to formulate often complex diagnoses. This diagnosis may be provisional with the clinician remaining open-minded regarding the potential treatment options, until a final, *in-situ* diagnosis can be reached, once the exposed pulp has viewed and the ability to control the resultant haemorrhage can be assessed. If suitable for VPT expert tissue handling, careful disinfection, cavity cleaning, placement of a HSC and a definitive adhesive restoration are ideally provided during the treatment appointment.

Furthermore, the visual assessment of the exposed pulp is not widely discussed or detailed in contemporary literature; certainly not, from the perspective of education. The inability to identify surface necrosis, in the absence of suitable light and magnification, could contribute to the erroneous provision of direct pulp capping (DPC). Equally, the understanding that profuse haemorrhage should not preclude the

potential to provide VPT, may not be widely appreciated. The question of what is an appropriate time to achieve haemostasis remains unanswered.

Numerous clinical decisions are required, which could be influenced by a number of factors, including operator, or patient factors; all of which, are subjective. The use of heuristics (i.e. mental shortcuts to facilitate decisions) in clinical decision-making in practice and whether this influences the potential for diagnostic error (Fernández-Aguilar et al. 2021), has not been widely investigated in the dental literature (McGeown et al. 2022). Heuristics is influenced by intuition and cognitive bias, but also the aversion to uncertainty in relation to outcomes (Feller et al. 2020), as well as treatment failure that is likely to influence future treatment choices (Braddock et al. 1999). Hence, there is potential to select less conservative treatment options moving forward (Careddu et al. 2021, Edwards et al. 2021, Chevalier et al. 2022, Hatipoğlu et al. 2023). In better understanding these processes, strategies could be devised to affect positive changes (Fouillen et al. 2023).

The purpose of this work was to identify a threshold between teeth amenable to VPT and those that require RCT, potentially to redefine true 'reversibility' of pulpitis in a clinical setting, without resorting to histologic assessment, which is impractical and precludes treatment outcome evaluation.

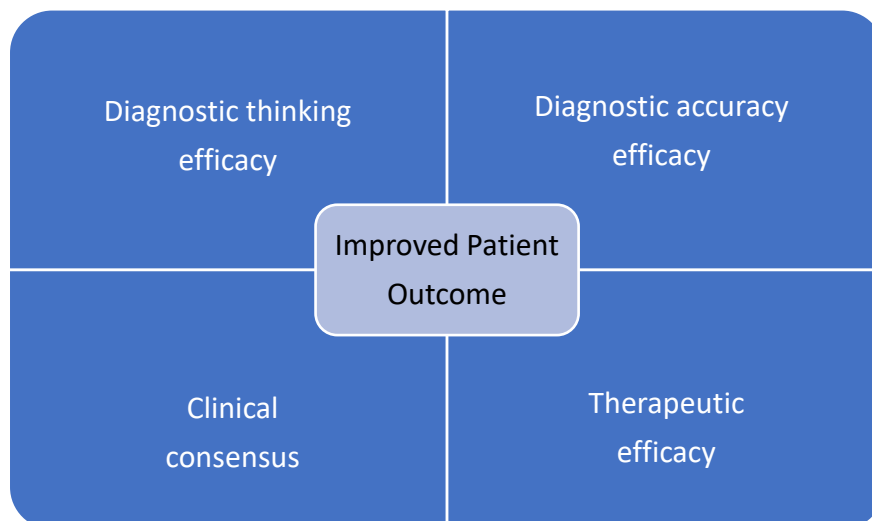
Point-of-care (or chairside) testing presents an opportunity to reduce the burden of certain diagnostic decisions, that could be prone to subjectivity (Lee et al. 1995). In applying a new test, consideration of various factors is necessary. The purpose of introducing a new test could include:

1. A form of screening to avoid more expensive tests;
2. To replace an existing test (with a better, cheaper, or more effective test); or

3. To complement existing tests, to improve their diagnostic accuracy (Beuhler et al. 2019).

In recommending a new test, it is also necessary to understand how it may influence patient outcomes (Duncan et al. 2021). The diagnostic performance of the test compared with treatment outcomes could be evaluated using a tailored modification (**Figure 7.1**) of Fryback and Thornberry's model of diagnostic efficacy (Fryback & Thornbury 1991).

Figure 7.1 Modified model of diagnostic efficacy, adapted from Fryback & Thornbury 1991



Fryback & Thornbury (1991) present a hierarchal model, defining specific domains or levels which could be extrapolated into pulp diagnostics and the development, or appraisal of the effectiveness of novel biomarkers in clinical decision-making.

Principally, a test must measure what it purports to measure. An inherent limitation of diagnostic accuracy studies that aim to measure the level of pulpitis present, relates not only to the obvious absence of direct access to the tissues, but also the routine reliance on imperfect index tests and perhaps highlights a need to

link with a suitable surrogate end-point, namely pulp survival. This area requires appropriate and robust investigation (Fleming & De Mets 1996).

Diagnostic accuracy efficacy could be reliably informed by the adoption of defined outcome measures that identify what, how and when to measure (Duncan et al. 2021). Perhaps, most could be learned by careful interrogation of the modes of VPT failure. These domains should include time to failure; and critically, dynamic of failure, whether diagnostic, treatment, structural, or influenced by the level of expertise of the operator (Yepes et al. 2020, Duncan et al. 2023).

Clinical consensus is currently lacking in many domains relating to the provision of VPTs. This does not only apply to diagnostic terminology (Rechenberg & Zehnder 2020, Azim et al. 2022, Ahmed et al. 2023); but critically also in position statements from leading global organisations (Schwendicke et al. 2016, Duncan et al. 2019a, AAE 2021).

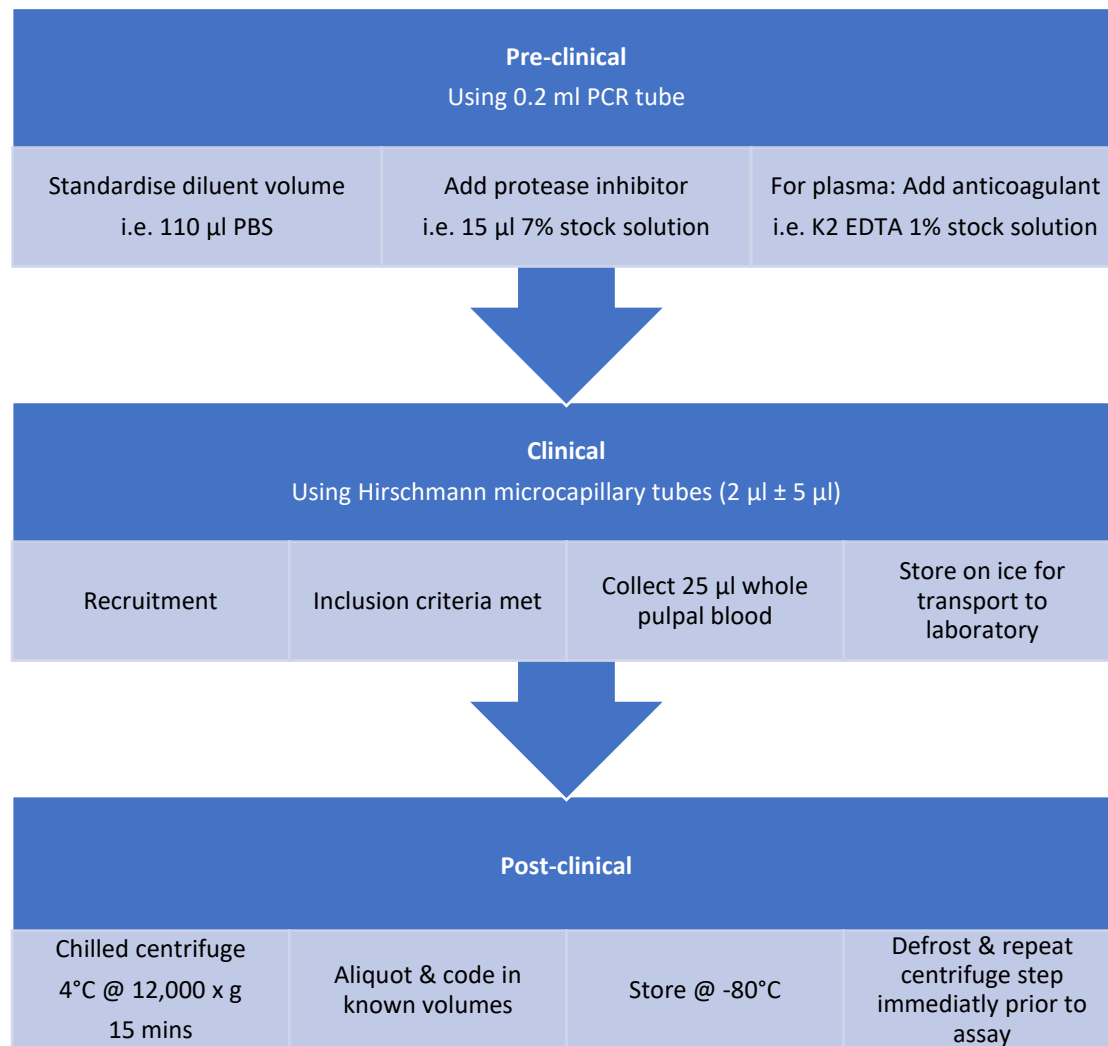
The therapeutic efficacy of VPT is well established, however many of the steps leading up to selection of this treatment modality are subject to multiple external influences. Regarding the clinical validation of the tests, assessment of whether they enhance clinical decision-making could be considered in conjunction with the manner in which they improve patient outcomes.

This work proposes a first step in a hierarchical pyramid of evidence-based laboratory medicine (Florkowski et al. 2017). In this thesis a robust method of collecting and handling pulpal blood is presented (**Figure 7.2**).

Proof of concept in relation to candidate markers of cell death, present a novel direction in the search to identify suitable objective biomarkers, as diagnostic

adjuncts to direct future therapeutic decisions related to the maintenance of pulpal vitality.

Figure 7.2 Proposed protocol for the collection of pulpal blood for molecular analysis [mins: minutes; @: at]



7.2 Future Research

In order to improve generalizability, the next steps would propose further technical validation of the methodology presented, using a wider range of operators and samples. The calibration of operators, diagnostic accuracy and management outcomes in the form of cross-sectional studies could contribute significantly to the

current evidence. The need for consistent and structured methodological reporting in the form of checklist and flowcharts enables more transparency and reproducibility with the result that many working groups have developed guidelines and developing reporting standards in a wide range of relevant dental domains (Nagendrababu et al. 2019, Al-Natour et al. 2021, Nagendrababu et al. 2021, Cushely et al. 2022, ElKarim et al. 2023). This should assist researchers at the outset of study design, to produce research capable of feeding into a repository of higher-level evidence.

In designing studies in the future, continued collaboration enshrined in clinician-scientist partnerships will enhance the quality of information gleaned. The scarcity of available substrate demands expertise in laboratory handling, to minimise the potential for technical error, as the potential to repeat assays is not readily possible. The standardisation of planned, necessary addition of diluents should be universally maintained as should standardised centrifuge conditions immediately prior to conducting assays that will help to ensure uniform equilibration of analytes. Cost-effectiveness is of course, an important consideration. Although a candidate approach offers certain advantages over a profiling one, a detailed interrogation of multiplex platforms may be worth considering to better inform the most suitable candidate analytes for validation tests. It may be, an indeed seems likely, that cassettes of markers rather than signal markers may be the best way to identify the level of pulpitis.

7.3 Conclusions

The work presented in this thesis describe, a comparison of methodologies and collection methods for sampling pulpal blood, highlighting challenges and solutions to enable standardised testing in the future. Furthermore, this project describes clinical parameters including visual appearance, pulpal bleeding and symptoms and relates them to outcome. For the first time, markers of cell death are analysed as potential diagnostic targets in pulpitis. Finally, a broad approach was employed to highlight the role of established and novel biomarkers in identifying the threshold of inflammation using an established and novel diagnostic classification. While much information remains to be elucidated regarding the expression of relevant biomarkers in dental pulp disease and their relation to outcome and repair, continued research will allow for a comprehensive understanding of their utility in a clinical setting. Within the limitations of the work presented, novel targets and substrates are presented in the search to identify suitable biomarkers of pulp inflammation with a view to developing point-of-care testing. New questions are raised in relation to future directions in the diagnosis of pulpitis; both in terms of the choice of index tests and potential rethinking of suitable reference standards.

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Appendices

Appendix I – Decision rules for Risk of Bias assessment

No.	Criterion	Decision rule (y=1, n=0)	Score
Selection			
1.	Selection of the cohort	a) Defined recruitment process y b) Insufficient detail recorded/mixed primary secondary dentition n	
2.	Condition of the cohort	a) Clearly defined inclusion/exclusion criteria y b) Insufficient information regarding population demographics n	
Comparability			
3.	Diagnostics of cases and controls	a) Clearly defined history, clinical & radiologic parameters differentiating health and disease y b) Insufficient data to reliably inform cases from controls n	
4.	Pulpal inflammation confirmed histologically	a) Gold standard y b) Reference standard?? y c) No reference n	
5.	Quality of the controls	a) True –ve control, stipulating health, caries-free, perio etc y b) Proxy control Defined normal may be asymptomatic /elective tx indicated n c) No control n d) Insufficient detail reported to classify quality of control n	
6.	Calibration/blinding? of examiners	a) Details of calibration recorded y b) Single operator?? n? c) No detail of operator calibration n	
Outcome			
7.	Assessment of outcome	a) Detailed/defined measure of outcome y b) Outcome assessment insufficiently reported n	
8.	Same tests performed for cases and controls	a) y b) n	
9.	Standardisation of method	a) Detailed information of methodology/reporting/statistics/results y b) Lacking sufficient detail in methodology/reporting/results n	
10.	Intra-study comparability	a) Methodology/assay comparable with >1 other study y b) Methodology comparable with <1 other study n	

Appendix II – Official supporting documentation of received ethical approval (REC REF: 2017-07 Chairman's Action [16])

THIS NOTEPAPER MUST NOT BE USED FOR
PRESCRIPTIONS OR INVOICING PURPOSES

SJH/AMNCH Research Ethics Committee Secretariat
Claire Hartin Ph: 4142199
email: claire.hartin@amnch.ie



**THE ADELAIDE & MEATH
HOSPITAL, DUBLIN**
INCORPORATING
THE NATIONAL CHILDREN'S HOSPITAL

TALLAGHT, DUBLIN 24, IRELAND
TELEPHONE +353 1 4142000

Dr. Síle Lennon
Assistant Professor/Lecturer in Endodontics
Dublin Dental University Hospital
Lincoln Place,
Dublin 2

25th July 2017

Re: An investigation of molecular markers in progressive pulpal inflammation and their potential use as a diagnostic tool.

REC Reference: 2017-07 Chairman's Action (16)
(Please quote reference on all correspondence)

Dear Dr. Lennon,

The SJH/AMNCH Research Ethics Committee is in receipt of your recent application in which you queried ethical approval for the above named study.

The Chairman, Dr. Peter Lavin, on behalf of the Research Ethics Committee, has reviewed your correspondence and grants ethical approval for this study.

Yours sincerely,

Claire Hartin
Secretary
SJH/AMNCH Research Ethics Committee

The SJH/AMNCH Joint Research and Ethics Committee operates in compliance with and is constituted in accordance with the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations

Appendix III – Official supporting documentation of amendment to ethical approval (2018-10 List 33[16])

SJH/TUH Research Ethics Committee Secretariat
email: researchethics@tuh.ie

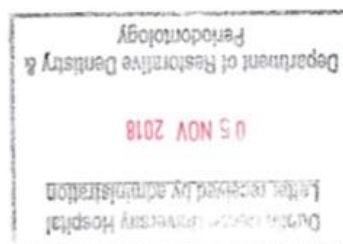


Tallaght
University
Hospital

Ospidéal
Ollscoile
Thamhlachta

An Academic Partner of Trinity College Dublin

Dr Sile Lennon,
Assistant Professor/Lecturer in Endodontics,
Division of Restorative Dentistry and Periodontology,
Dublin Dental University Hospital,
Lincoln Place
Dublin 2



30th October 2018

RE: An Investigation of Molecular Markers in Progressive PULPAL Inflammation and their Potential Use as a Diagnostic tool

REC REF: 2018-10 List 33 (16)

ORIGINAL REC REF: 2017-07 Chairman's Action (16)

(Please quote reference on all correspondence)

Date of valid submission to REC: 19.10.2018

Date of Ethical Review: 26.10.2018

Dear Dr Lennon,

Thank you for your recent correspondence to SJH/TUH Research Ethics Committee in which you requested an amendment in relation to the above referenced study.

The Chairman, Prof. Richard Deane, on behalf of the Research Ethics Committee, has reviewed this request and grants permission for this amendment.

Documentation reviewed and approved:

- Standard application form 16.10.2018, Version 2 Amendment Request
- Non-clinical research amendment request form, dated 19.10.2018
- Patient Information Leaflet Version 2 Autumn 2018

Yours sincerely,

REC Officer – Dr Sadhbh O'Neill - SJH/TUH Research Ethics Committee

The SJH/TUH Joint Research and Ethics Committee operates in compliance with and is constituted in accordance with the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004 & ICH GCP guidelines.

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Tallaght University Hospital is a registered
business name of 'The Adelaide and Meath
Hospital, Dublin Incorporating The National
Children's Hospital'.

Appendix IVa - Patient Information Leaflet: Minors

Version 3 Date Autumn 2021

PARTICIPANT INFORMATION LEAFLET

HOSPITAL: Dublin Dental University Hospital

DEPARTMENT: Division of Restorative Dentistry & Periodontology

STUDY TITLE: Investigation of molecular markers of pulpal inflammation and cell death and their potential use as a diagnostic tool.

NAME OF PRINCIPAL INVESTIGATORS:

Dr Hal Duncan BDS, FDS RCS, MClint Dent, MRD RCSEd, PhD;
Dr Síle Lennon B.Dent.Sc., M.Clin.Dent., MRD RCSEd

You are being invited to join in our research study. Thank you for taking time to read this.

WHAT IS THE PURPOSE OF THE STUDY? We want to look very closely at the 'living centre' of your tooth (called the 'pulp'). We want to learn what happens before the tooth is damaged by tooth decay (a cavity). We hope this will help us understand which toothaches can be fixed with simple treatment, rather than removing the tooth, or carrying out root canal treatment.

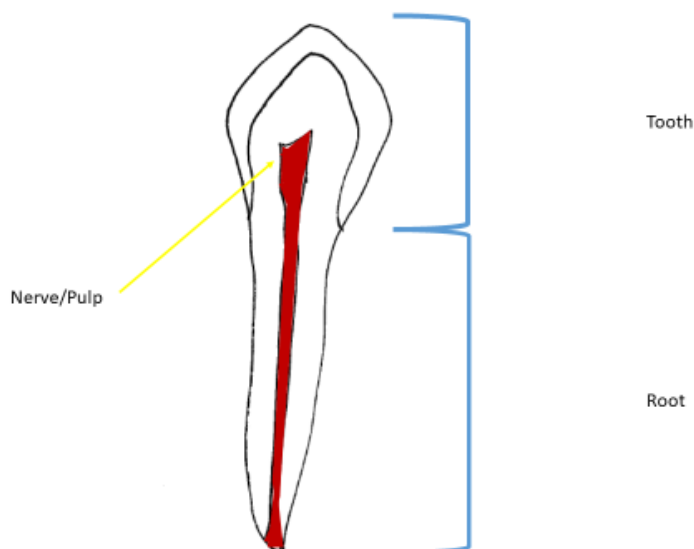


Figure 1 Tooth picture

WHY HAVE I BEEN CHOSEN TO PARTAKE IN THIS STUDY? You are going to have a healthy tooth (or teeth), removed as part of your orthodontic treatment plan. We would like to ask your permission to examine the pulp (inner aspect) of this tooth closely just before the tooth is removed, in the Dublin Dental University Hospital clinic. This is a simple pain-free procedure which will not affect your treatment.

WHAT WILL HAPPEN IF I VOLUNTEER TO PARTICIPATE? You and your parents will meet Dr Síle Lennon, the dentist involved in carrying out the research. She will talk with you and your parents about the research study and answer any questions that you may have about your treatment. There are some things about this study you should know. She will ask some questions about your health to make sure you are able to join our study. She will look at your tooth and the teeth next to it. She will gently check it. She will measure the gum around it and test if it can feel any cold. She will look at your x-ray (dental picture of your tooth) to ensure your suitability.

Orthodontic extraction: -

Some anaesthetic gel will be placed next to your tooth to numb the gum before placement of the dental anaesthetic. This will make sure the area will be totally frozen and you will not feel any discomfort while your tooth is being treated. Before your tooth is removed, a rubber sheet (dam) will be placed over your tooth (Figure 2) and secured with a metal button/clamp. The living centre (pulp) is reached using a dental drill with water. You will have a chance to see the drill and hear the noise it makes before Dr Lennon works on your tooth. Dr Lennon will use a tiny tube to sample the blood inside the surface of pulp (inner core). A few samples of the pulp blood will be taken using the tiny tubes and this will take only a few minutes. This will conclude your participation in the research aspect of the study. Your extraction(s) will then be completed by our colleagues in the oral surgery team immediately after.

Once your tooth has been removed, you and your parents will be given information on what to do and what not to do when you get home. The dentist will make sure that you are ok before letting you go home. Your parents will be given the contact numbers to the hospital, or research team to get more advice if anything unexpected happens. You may be offered further appointments with your dentist here in the hospital to have the remainder of your extractions carried out, or advised to return to your orthodontist for your continuing dental care, once all the planned extractions have been completed.

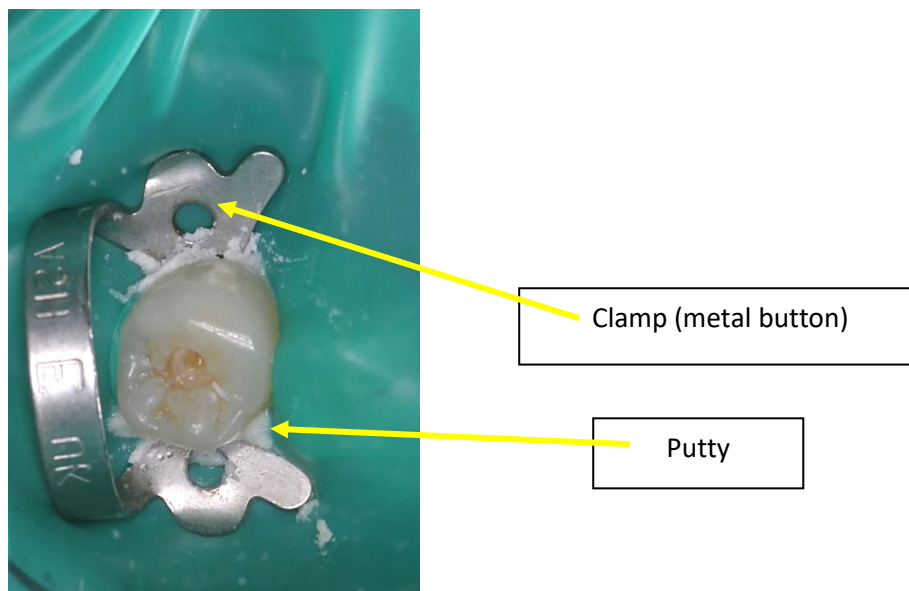


Figure 2 Dental dam (sheet)

ARE THERE ANY RISKS INVOLVED IN PARTICIPATING? We don't think joining in our study will put you at risk. A risk means that something bad could happen. We want to help you and fix your tooth problem. While doing this, we would like to explore/investigate what goes on inside the nerve of the tooth before any decay damages it. The only extra step involves taking a little blood sample from inside your tooth before it is extracted. If you, or your parents were worried about anything afterwards you would be able to talk to us, or come back to see us to make sure everything is ok.

ARE THERE ANY BENEFITS INVOLVED IN PARTICIPATING? Not everyone who takes part in this study will benefit. A benefit means that something good happens to you. We think these benefits might be curing a toothache without needing to take away all of the nerve tissue, which may mean keeping a stronger tooth for longer.

WHAT HAPPENS IF I DO NOT AGREE TO PARTICIPATE? You do not have to be in this study if you do not want to be. Your parents know about the study too.

WILL MY PARTICIPATION OR WITHDRAWAL HAVE ANY IMPACT ON MY ROUTINE CARE? No. You do not have to be in this study if you do not want to be. If you decide to stop after we begin, that's okay too.

WILL MY PARTICIPATION BE CONFIDENTIAL? Yes. When we are finished with this study we will write a report about what was learned. This report will not include your name, or that you were in the study.

INDEMNITY: Your dentists are insured by the State Claims Insurance Service.

WHO IS ORGANISING AND FUNDING THIS RESEARCH? Dublin Dental University Hospital Further Education Scheme

HAS THIS STUDY REVIEWED BY AN ETHICS COMMITTEE? Yes. St. James' Hospital/Tallaght Hospital Joint Research and Ethics Committee. ID: 2018-10 List 33 (16); Original REC REF: 2017-07 Chairman's action (16) Date: 06/11/2018

PATIENT CONTACT DETAILS

Name:

Address:

Phone:

If you are happy to participate in this study, please contact our administration team and we will be happy to arrange an appointment with Dr Síle Lennon.

Tel: 01 6127359/ 086 3130946

Email: sile.lennon@dental.tcd.ie

Appendix IVb - Patient Information Leaflet_Adults

Version 02 Date Autumn 2018

PARTICIPANT INFORMATION LEAFLET

HOSPITAL: Dublin Dental University Hospital (DDUH)

DEPARTMENT: Division of Restorative Dentistry & Periodontology

STUDY TITLE: Investigation of molecular markers of pulpal inflammation and cell death and their potential use as a diagnostic tool.

NAME OF PRINCIPAL INVESTIGATORS:

Dr Hal Duncan BDS, FDS RCS, M Clin Dent, MRD RCSEd, PhD;

Dr Síle Lennon B.Dent.Sc., M.Clin.Dent., MRD RCSEd

You are being invited to participate in a research study. Thank you for taking time to read this.

WHAT IS THE PURPOSE OF THE STUDY? To analyse the molecular mediators (signals) of inflammation and necrosis (cell death) due to inflammation/irritation because of decay in human teeth. We plan to examine the pulp, or nerve tissue (inner core of the tooth consisting of tiny blood vessels and nerves) after removal of tooth (extraction), or removal of some (root canal therapy); or all (root canal treatment) of the nerve tissue. This is a normal procedure to treat toothache as a result of deep decay in a tooth. The nerve tissue will be stored in a storage material and examined in the laboratory after your treatment has been completed and you have been made comfortable. The laboratory analysis of the nerve tissue/samples will be compared with a peripheral blood sample (a standard blood test) to find out if the mediators (signal molecules) within the nerve are detectable in the bloodstream also.

WHY HAVE I BEEN CHOSEN TO PARTAKE IN THIS STUDY? The Dublin Dental University Hospital (DDUH) is strongly committed to improving health, in particular oral health, in Ireland through research by furthering our knowledge and understanding of Dentistry and Oral Biology. As a patient attending the DDUH, you have been identified as having a tooth matching our research question. You have been offered and agreed to have your tooth removed (extracted), or a root canal treatment/therapy to relieve pain, or for another reason. We would like to ask your permission to examine the nerve tissue after your tooth has been removed, or the nerve tissue removed during root canal therapy/treatment.

WHAT WILL HAPPEN IF I VOLUNTEER TO PARTICIPATE? Dr Síle Lennon, the lead investigator, will meet with you to explain the purpose of the study. If you agree, she will review your answers in your medical questionnaire to ensure there is no medical reason to exclude you from the research study. She may need to examine your tooth again, asking questions about your toothache (if any), checking how your tooth responds to cold/hot testing, tapping on the tooth, looking for swellings or excessive looseness of your tooth and assessing the gum around your tooth. You may already have had a radiograph (a standard dental x-ray) taken of your tooth and Dr Lennon will examine this. She will discuss the diagnosis for your dental problem with you and all the relevant options for treating it.

1. Extraction group: Your tooth will be numbed and carefully removed according to your agreed treatment plan. Prior to the removal of your tooth, a rubber sheet (dam) will be placed over your tooth and secured with a metal button/clamp. The inner core (pulp) is

reached using a dental drill with water. A sample of the pulp blood will be taken using a tiny tube. A small portion, or all the pulp tissue will be removed and the samples will be stored in a transport fluid, or frozen for transport to the laboratory in the DDUH. This will conclude your participation in the research study. Once your tooth has been removed/extracted, standard post-operative instructions will be given verbally and in writing. As with any dental extraction, you may expect some mild discomfort after the anaesthetic has worn off. You will be given advice regarding appropriate pain relief. Some swelling, bleeding or bruising can occur. You will be informed how to contact the hospital, or research staff if any complications arise after you return home. You may be offered further appointments with your allocated clinician to have the remainder of your dental treatment carried out, or discharged back to your family dentist for your continuing dental care.

2. Pulp Therapy/ Treatment group: Your tooth will be numbed with a dental anaesthetic. A rubber sheet (dam) will be placed over your tooth and secured with a metal button/clamp. The decay in your tooth will be removed just like having a filling carried out, using a dental drill with water. When the inner core (pulp) is reached, a sample of the pulp blood will be taken using a tiny tube. A small portion, or all the pulp tissue will be removed as determined by the level of inflammation encountered in your specific tooth and the samples will be stored in a transport fluid or frozen for transport to the laboratory in the DDUH. Your tooth will be filled with a specialised cement (pulp therapy) followed by a standard dental tooth filling; or a root canal filling (root canal treatment) will be performed, followed by a standard dental filling. This will conclude your participation in the research study. You will be offered follow-up and review appointments as a matter of routine, to ensure the treatment has been successful, this aspect does not form part of the research study. You may be offered further appointments with your allocated clinician to have the remainder of your dental treatment carried out, or discharged back to your family dentist for your continuing dental care.

ARE THERE ANY RISKS INVOLVED IN PARTICIPATING? No significant risks are foreseen through your participation in this study. Specific risks regarding your treatment will be discussed with you prior to attending for your agreed treatment. You will be informed how to contact the hospital, or research team should any post-operative concerns, or complications arise.

ARE THERE ANY BENEFITS INVOLVED IN PARTICIPATING? Your participation in this study will potentially offer new scientific insight to determining when root canal treatment is unavoidable, or when more conservative options could be predictably offered to dental patients in the future.

WHAT HAPPENS IF I DO NOT AGREE TO PARTICIPATE? Your dental care will be provided, according to current best practice, regardless of your inclusion in this study. You will be offered a diagnosis for your presenting problem, with a number of options with regard to how best to remedy the problem. This information will respect your decision to accept, or reject the options offered and your voluntary participation although greatly appreciated, will have no bearing in the provision of your dental care to the best standards.

WILL MY PARTICIPATION OR WITHDRAWAL HAVE ANY IMPACT ON MY ROUTINE CARE? No. Your treatment will be carried out according to current best practice within the acceptance criteria with the DDUH. You will be free to withdraw your participation at any stage during the study.

WILL MY PARTICIPATION BE CONFIDENTIAL? Yes. Your clinical records will be accessed by the lead investigator, Dr Síle Lennon; in order to compare the clinical findings at check-up {medical history, tapping of tooth, response to cold, gum health, x-ray findings: -how deep the decay (if any) reaches inside your tooth, presence/ absence of swellings, mobility} with the laboratory analysis/results. The data/information will be anonymised, coded and encrypted on secure PC's within the DDUH.

INDEMNITY: Your dentists are insured by the State Claims Insurance Service.

WHO IS ORGANISING AND FUNDING THIS RESEARCH? Dublin Dental University Hospital Further Education Scheme

HAS THIS STUDY REVIEWED BY AN ETHICS COMMITTEE? Yes. St. James' Hospital/Tallaght Hospital Joint Research and Ethics Committee. ID: 2018-10 List 33 (16); Original REC REF: 2017-07 Chairman's action (16) Date: 06/11/2018

CONTACT DETAILS

Name:

Address:

Phone:

Appendix V - Patient assent form

PATIENT CONSENT FORM

Title: Investigation of molecular markers in progressive pulpal inflammation and their potential use as a diagnostic tool.

Name of principal researcher: Dr Hal Duncan, Consultant in Endodontics, Dublin Dental University Hospital (DDUH), Trinity College Dublin (TCD)

Co-investigator: Dr Síle Lennon, Lecturer in Endodontics, Dublin Dental University Hospital (DDUH), Trinity College Dublin (TCD)

Please tick the responses:

I have read, or had read to me, the information leaflet for this project and I know what it means. Yes No.....

I have had the chance to ask questions and talk about the study. Yes No.....

I am happy that all my questions have been answered. Yes No.....

I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. Yes No.....

I know that I can opt out of the study at any time without this affecting my future care. Yes No.....

PARTICIPANTS NAME:

CONTACT DETAILS:

PARTICIPANT'S or PARENT/GUARDIAN'S SIGNATURE (Circle to select):

..... **Date:**.....

Statement of investigator's responsibility: I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

INVESTIGATOR'S SIGNATURE:..... **Date:**.....

Appendix VI - Microcapillary tube details

1. Hirschmann® Minicaps® 20µl plain (Hirschmann Laborgeräte GmbH & Co.KG, Eberstadt, Germany)
2. Hirschmann® Minicaps® 10µl plain; Hirschmann® Minicaps® 5µl plain, Hirschmann® Minicaps® 2µl plain
3. Hirschmann® Minicaps® 10µl Na-Hep
4. Minivette® POCT 20µl K3E. (SARSTEDT AG & Co.KG, Nümbrecht, Germany)
5. End-to-End Capillary 20µl K2E SARSTEDT AG & Co.KG
6. Hirschmann® ringcaps® 10 µl plain
7. Hirschmann Pipetting aid for capillaries 0.5-100µl



1.



2.



3.



4.



5.



6.



7.

Appendix VII – Raw data: Bradford assay

Tooth #	Sample ID (Draw #)	Protein (µg)	Protein (µg)	Protein (µg)	Tooth #	Sample ID (Draw #)	Protein (µg)	Protein (µg)	Protein (µg)
		50	30	20			50	30	20
1	P5(p3)	19.05	11.43	7.62					
	P5(p3)	18.51	11.11	7.40					
2	P7(p1)	30.09	18.06	12.04	2	S5(s2)	17.71	10.62	7.08
3	P11(p1)	36.53	21.92	14.61	3	S7(s1)	101.11	60.67	40.44
	P11(p1)	28.41	17.04	11.36		S7 (s1)	41.69	25.01	16.68
	P14(p2)	39.31	23.59	15.72		S10(s2)	42.70	25.62	17.08
4	P18(p1)	34.43	20.66	13.77	4	S15(s2)	20.34	12.20	8.14
	P20(p2)	22.08	13.25	8.83					
	P21(p2)	21.87	13.12	8.75					
	P22(p3)	23.89	14.33	9.55					
7	P45(p2)	11.99	7.19	4.80	7	S40(s2)	9.85	5.91	3.94
	P47(p2)	11.65	6.99	4.66		S42(s2)	13.72	8.23	5.49
					8	S49(s4)	12.56	7.54	5.02
						S56(s1)	8.81	5.29	3.53
						S56(s1)	12.79	7.67	5.12
					9	S64(s2)	8.88	5.33	3.55
12	P76(p3)	7.02	4.21	2.81	12	S78(s3)	19.77	11.86	7.91
	P78(p4)ppt	6.21	3.73	2.49		S79(s3)	11.81	7.09	4.72
						S83(s4)ppt	13.35	8.01	5.34
					13	S94(5)ppt	10.58	6.35	4.23
14	P83(p2)	18.72	11.23	7.49	14	S96(s1)	12.73	7.64	5.09
15	P89(p1)	32.21	19.33	12.89	15	S104(s1)	44.86	26.92	17.94
	P93(p3)	32.33	19.40	12.93		S105(s1)	59.46	46.64	23.78
	P94(p3)	82.39	49.43	32.96					
	P95(p3)	42.71	25.63	17.09					
16	P100(p1)	3.39	2.03	1.36	16	S120(s2)	13.06	7.84	5.22
	P102(p2)	9.30	5.58	3.72		S121(s2)	6.25	3.75	2.50
	P102(p2)	8.11	4.87	3.25		S124(s5)	19.88	11.93	7.95
	P103(p2)~	2.18	1.31	0.87		S125(s5)~	5.50	3.30	2.20
	P105(p3)	6.07	3.64	2.43		S129(s6)~	5.83	3.50	2.33
	P107(p3)	2.02	1.21	0.81					
	P112(p6)~	2.74	1.64	1.10					
	P116(p7)~	5.69	3.42	2.28					
17	P119(P1)	6.47	3.88	2.59	17	S132(s1)	31.48	18.89	12.59
	P122(p2)	70.33	42.20	28.13		S135(s2)	13.88	8.33	5.55
	P124(p3)~	6.59	3.95	2.64		S141(s4)	15.41	9.24	6.16
	P126(p4)~	6.78	4.07	2.71		S143(s5)	20.02	12.01	8.01
	P127(p5)~	11.47	6.88	4.59		S145(s6)	16.43	9.86	6.57
	P128(p6)ppt	2.67	1.60	1.07					

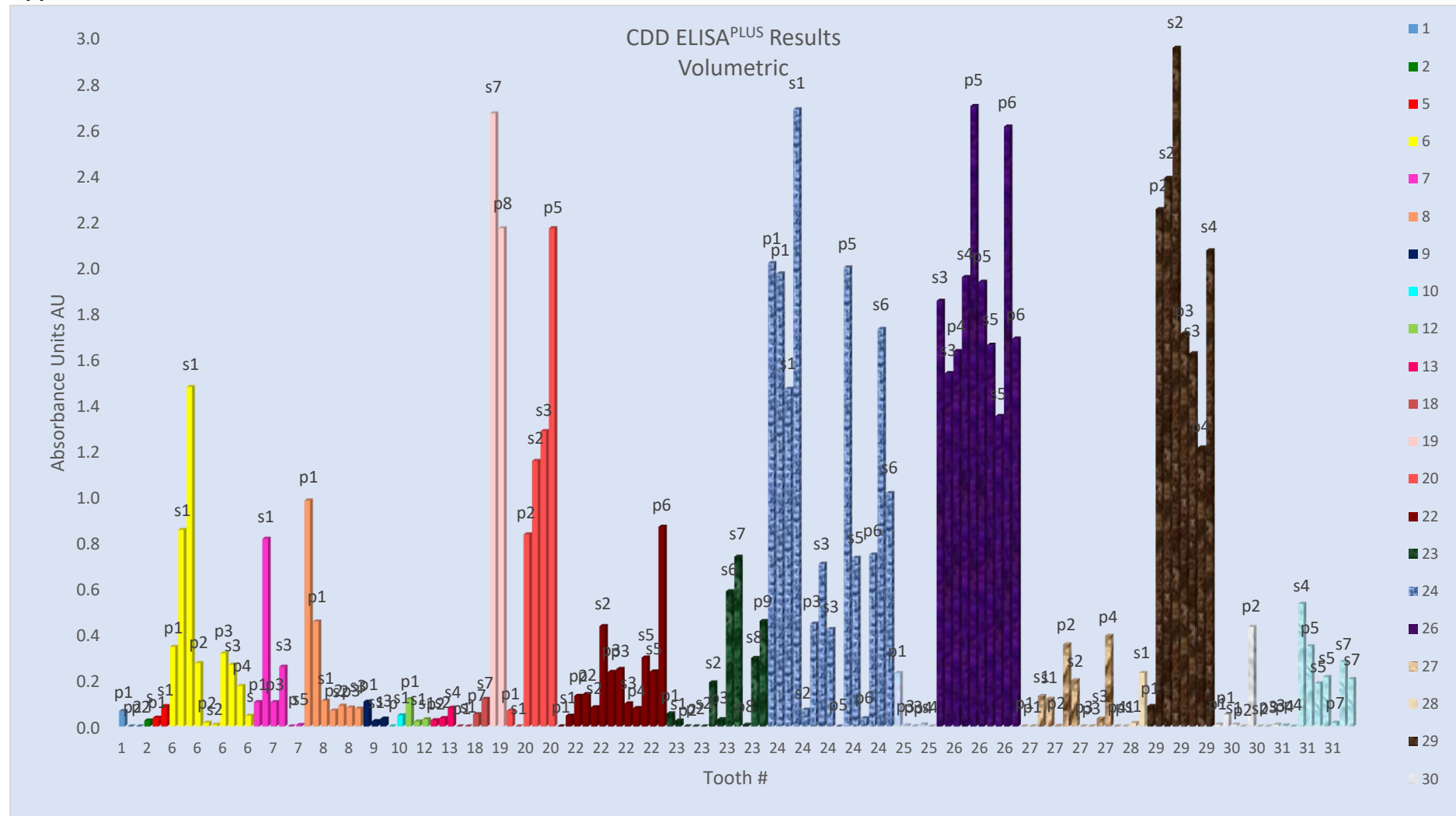
[#: number; P: Plasma; S: Serum; ID: Identification; ~: haemolysed specimen; ppt: paper point; Darker shading highlights samples with insufficient volume to provide 30 µg protein concentration, therefore 20 µg was used]

Appendix VIII – Raw data: Blood collection

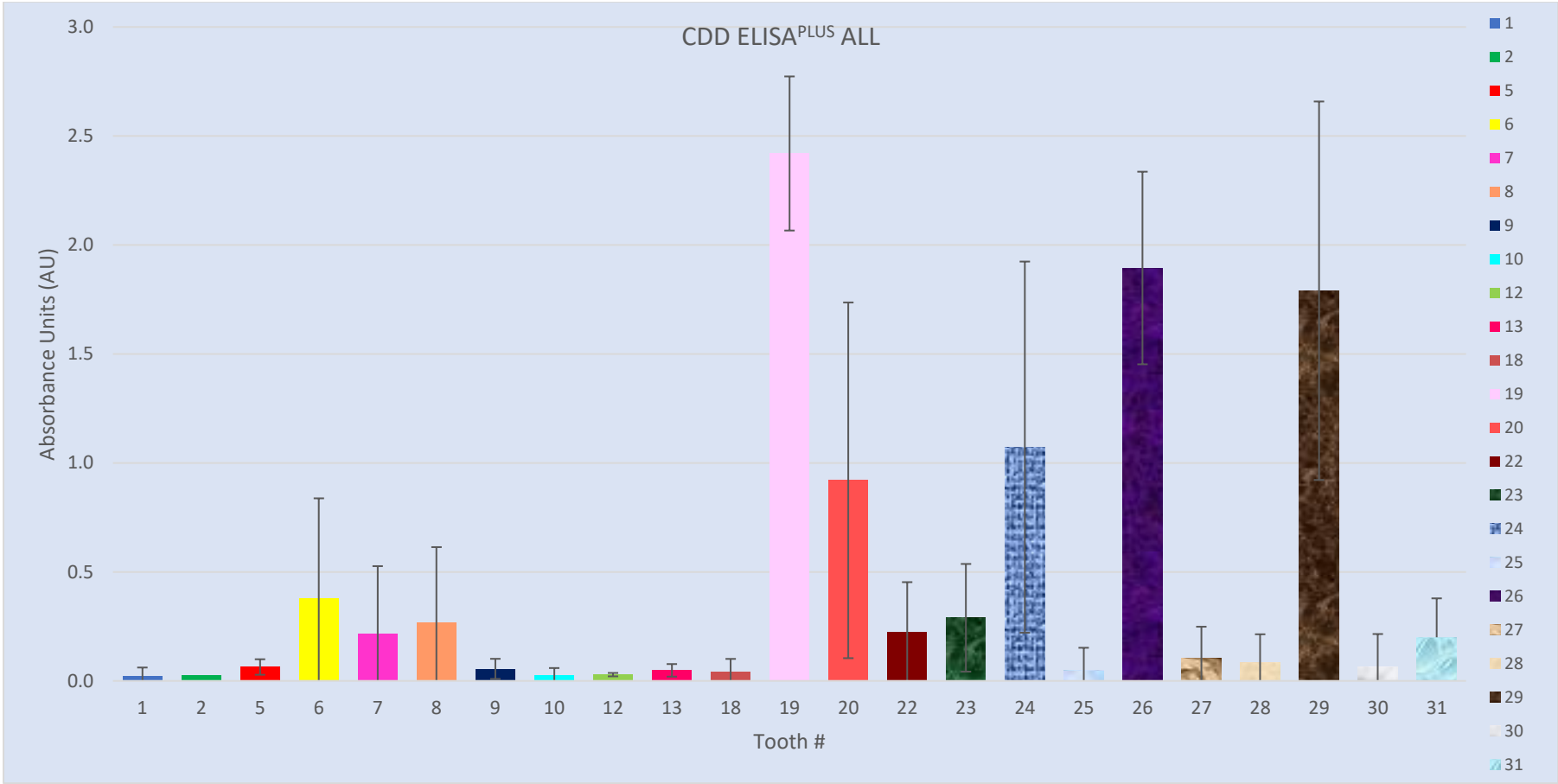
Case #	Total (µL)	Draws (Serum + Plasma)	Vehicle	Whole blood for Serum analysis (µL)	Whole blood for Plasma analysis (µL)
1	13.7	3	MiVe 20	-	13.7
2	ukv	2 + 2	MiVe & NaHep	4.6, 2 nd draw ukv	* clot
3	ukv	2 + 2	Na Hep		Diluted w op water
4	?	3 + 3	NaHep/pl 10	?	?
5	3.75	1 + 1	PI10	1.875	1.875
6	69.07	4 + 4	PI 10	34.84 / 4	34.22
7	90.8	5 + 5	PI 10	48.3/5	42.51/5
8	38.6	3 + 3	PI 5/10	18.9/3	19.69/3
9	23.44	3 + 2	PI 5/10	13.44/3	10/2
10	8.84	3 + 3	PI 5/10 ppt	6.92/2, 1ppt	4.92/2, ppt
11	15.8	2 + 2	PI 5, ppt	7.5/2, pptx2	8.3/2
12	17.8	3 + 3	PI 2, 5, 10, ppt	8.8/3, ppt	9/3, ppt
13	22.63	4 + 2	PI5, 10, ppt	14.23, ppt	8.4
14	26.74	2 + 3	PI5, ppt	9.15/2, ppt	17.59/3, ppt
15	8.08	2 + 3	PI 5/2 ppt	2.78, pptx2	5.3/3, ppt
16	78	6 + 7	PI 2/5/10	30.88/6	47.12
17	34.44	6 + 5	PI 2/5	16.38/6	18.06/5, ppt
18	91.6	7 + 7	PI 5/10	44.54/7	47.06/7
19	32.13	5 + 7	PI 2/5/10, ppt	14.41/5, pptx3	17.72/7, pptx2
20	54.94	3 + 6	PI 2/5/10, ppt	21.22/3, ppt	33.72/6, ppt
21	TN				
22	50.13	5 + 6	PI 2/5/10, ppt	22.5/5, ppt	27.6356, ppt
23	114	10 + 11	PI 2/5/10, ppt	48.93/10, ppt	65.06/11
24	75.39	5 + 5	PI 5/10	37.66/5	37.73/5
25	31.65	4 + 4	PI 5	13.75/4	17.9/4
26	95.13	8 + 9	PI 2/5/10	45.57/8	49.56/9
27	35	3 + 4	PI 5	15/3	20/4
28	10	1 + 1	PI 5	5/1	5/1
29	42	5 + 4	PI 2/5	22/5	20/4
30	54	5 + 5	PI 2/5/10, ppt	27/5, pptx2	27/5, ppt
31	94.44	7 + 7	PI 5/10, ppt	47.88/7, ppt	46.56/7, ppt
32	60	3	PI	60	
33	100	4	PI	100	
34	42.5	2	PI	42.5	
35	50	2	PI	50	
36	30	2	PI	30	
37	50	2	PI	50	
38	TN				
39	25	1	PI	25	
40	50	2	PI	50	
41	25	1	PI	25	
42	25	1	PI	25	
43	25	1	PI	25	
44	25	1	PI	25	
45	25, ppt	1, ppt	PI	25	
46	25	1	PI	25	

[ppt: paper point collection; PI: Plain Hirschman Microcapillary tube; MiVe: Minivette; ukv: unknown volume; ?: Sample contaminated with operative water; TN: Total necrosis, x: multiple; w: with; op: operative]

Appendix IXa - Raw data CDD ELISA^{PLUS} - Volumetric

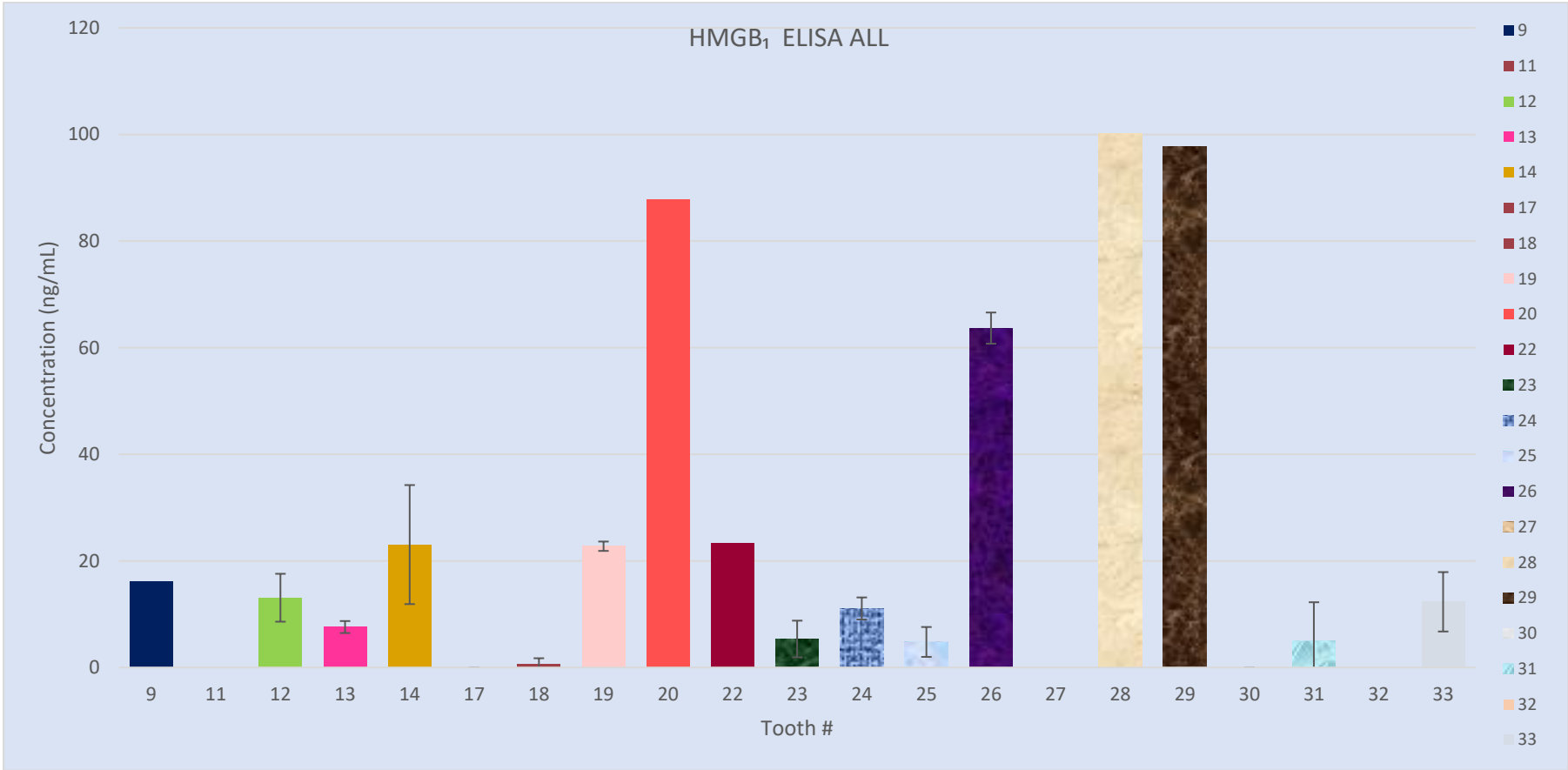


Appendix IXb - All data CDD ELISA^{PLUS} - Volumetric



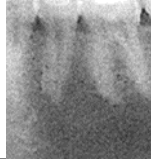
[Error bars depict standard deviation; #: Number]

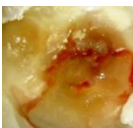
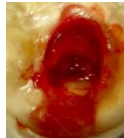
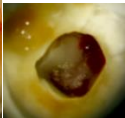
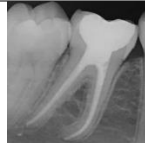
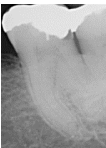
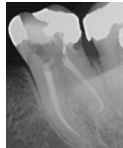
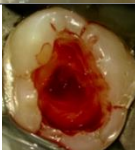
Appendix X – All data HMGB₁ ELISA

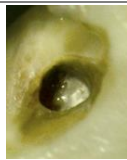
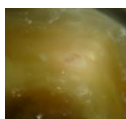
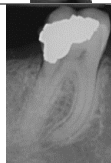
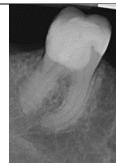
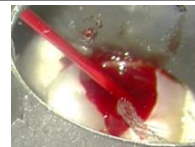
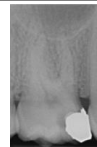
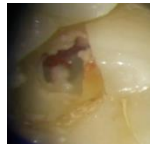
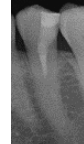


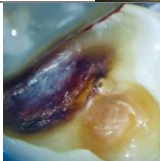
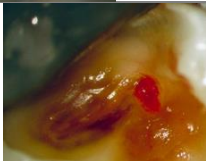
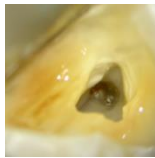
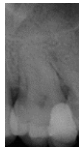
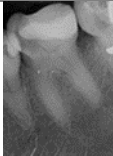
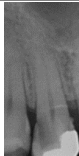
[Error bars depicting standard deviations; #: Number]

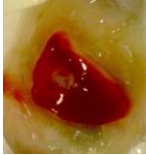
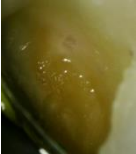
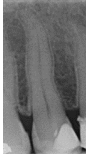
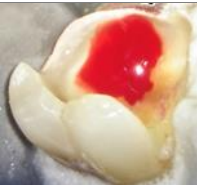
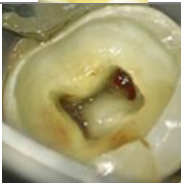
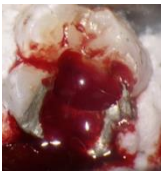
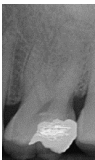
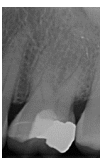
Appendix XI – Complete log of visual data catalogued according to diagnosis and treatment [Dx: Diagnosis, #: Number; Tx: Treatment; Pre: Pre-operative radiograph; Post: Post-operative radiograph; NP: Normal pulp, RP: Reversible pulpitis; IP: Irreversible pulpitis; InP: Initial pulpitis, MiP: Mild pulpitis, MoP: Moderate pulpitis, SeP: Severe pulpitis; HA: Haemostasis achieved; y: yes, n: no; TN: Total necrosis; pe: pulpectomy; pp: pulpotomy; xLa: extraction; *: tx planned RCT; D: Deep caries; ExD: Extremely deep caries; S: Success, mo: months; EF: Endo Frost; +: positive response, -: negative response; ?: uncertain]

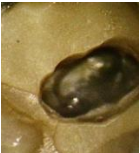
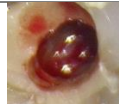
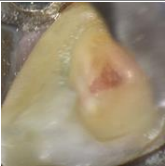
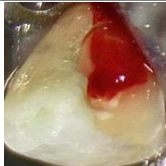
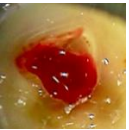
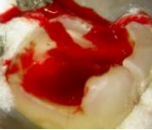
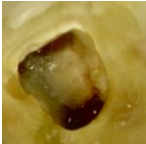
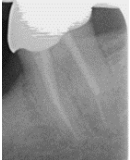
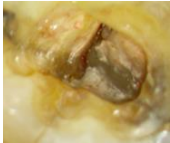
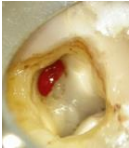
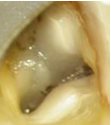
Case #	Pain?	Dx	HA	Tx	Caries Depth			Pre	Post
12	N	NP	y	xLa	No Caries				
44	N NP	NP	Y	xLa	No caries	 			
45	N NP	NP	y	xLa	No caries	 			
3	AsP/NP	RP/InP	y	pp	ExD	Narrative			

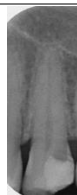
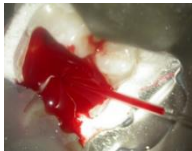
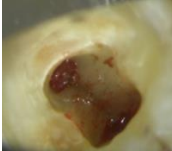
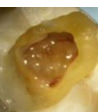
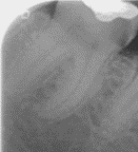
4	AsP/NP	IP/MiP	y	pe*	No caries	Narrative					
5	AsP/NP	IP/MiP	y	pe*	No Caries	Narrative					
8	AsP/NP	IP/SeP	y	pp	ExD	Narrative					
26	Y SeP	NP	n	pe	ExD						
42	N	RP/InP	y	pe*	D						
27	Y InP	RP/InP	n	xLa	D						

17	Y InP	RP/InP	y	pp	ExD					
11	Y MiP	RP/InP	y	pp	ExD					
6	Y SeP	RP/InP	y	pp	ExD					
19	Y MiP	RP/InP	n	pe	ExD					
1	Y IP/SeP	RP/MiP	y	pp	D	Narrative				
9	N	IP/MiP	y	pp	D					

30	Y MiP	IP/MiP	y	pp	D						
25	Y MiP	IP/MiP	y	pp	ExD						
46	Y MiP	IP/MiP	y	pp	ExD						
2	Y IP/MoP	IP/MoP	y	pp	ExD	Narrative					
34	N	IP/MoP	y	pp	ExD						
10	Y MoP	IP/MoP	y	pp	D						

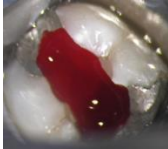
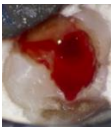
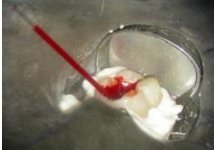
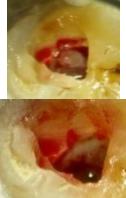
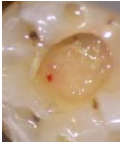
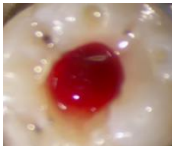
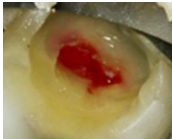
13	Y MoP	IP/MoP	y	pp	D				
14	Y MoP	IP/MoP	y	pp	D				
15	Y MoP	IP/MoP	y	pp	D	 	 		
28	Y MoP	IP/MoP	y	pp	D				
31	Y MoP	IP/MoP	y	pp	ExD				
33	Y MoP	IP/MoP	y	pp	ExD				

41	Y MoP	IP/MoP	y	pp	ExD			 			
43	Y MoP	IP/MoP	y	pp	ExD						
16	Y MoP	IP/MoP	n	pe	D						
39	Y MoP	IP/MoP	n	pe	ExD						
23	Y MoP	IP/MoP	n	xLa	D crack						
40	Y SeP	IP/MoP	n	pe	D			 			

000	NRP/InP	IP/MoP	TN	pe	ExD	Narrative					
0	Y IP/SeP	IP/MoP	y	pp	ExD	Narrative					
21	Y SeP	IP/MoP	TN	pe	ExD						
37	Y MiP	IP/SeP	n	pe	ExD						
35	Y SeP	IP/SeP	y	pp	ExD						
36	Y SeP	IP/SeP	y?	pp	ExD						

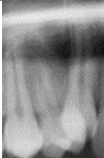
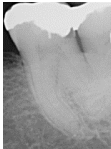
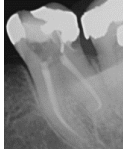
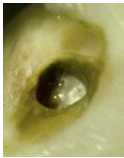
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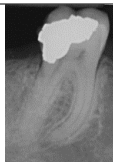
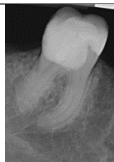
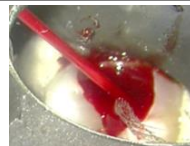
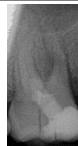
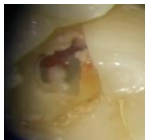
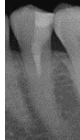
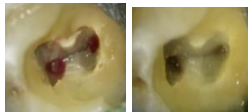
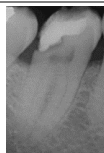
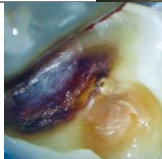
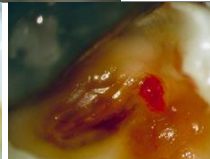
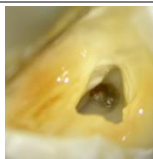
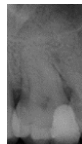
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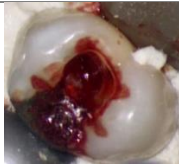
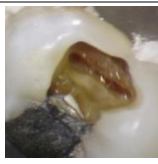
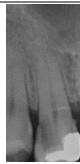
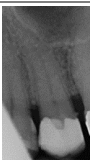
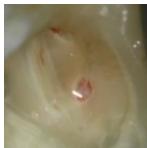
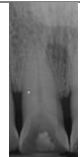
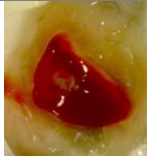
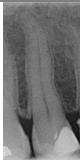
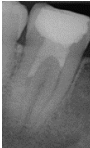
18	Y SeP	IP/SeP	n	pe	D				
20	Y SeP	IP/SeP	n	pe	D		 	 	
7	Y SeP	IP/SeP	n	pe	ExD				
22	Y SeP	IP/SeP	n	pe	ExD	 			
24	Y SeP	IP/SeP	n	pe	ExD	 			
29	Y SeP	IP/SeP	n	pe	ExD				

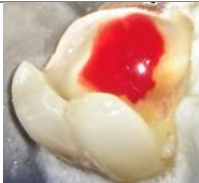
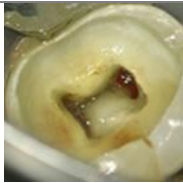
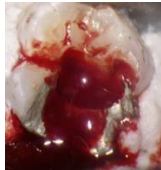
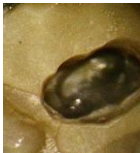
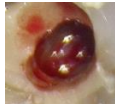
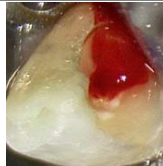
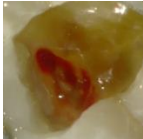
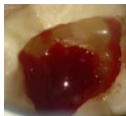
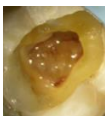
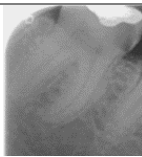
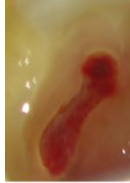
32	Y SeP	IP/SeP	n	pe	ExD				
38	Y SeP	IP/SeP	TN	pe	ExD				

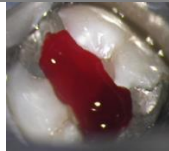
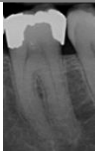
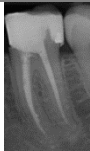
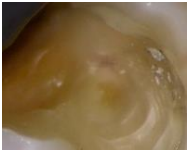
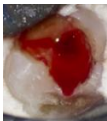
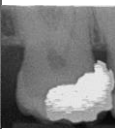
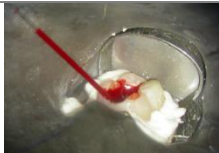
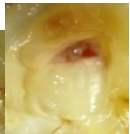
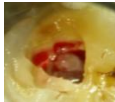
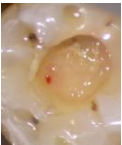
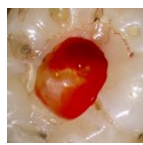
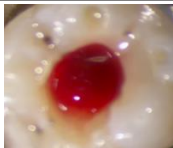
Appendix XII – Complete log of visual data ordered by haemostasis [Dx: Diagnosis, #: Number; Tx: Treatment; Pre: Pre-operative radiograph; Post: Post-operative radiograph; NP: Normal pulp, RP: Reversible pulpitis; IP: Irreversible pulpitis; InP: Initial pulpitis, MiP: Mild pulpitis, MoP: Moderate pulpitis, SeP: Severe pulpitis; HA: Haemostasis achieved; y: yes, n: no; TN: Total necrosis; pe: pulpectomy; pp: pulpotomy; xLa: extraction; *: tx planned RCT; D: Deep caries; ExD: Extremely deep caries; S: Success, mo: months; EF: Endo Frost; +: positive response, -: negative response; ?: uncertain]

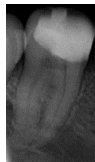
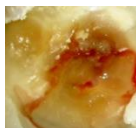
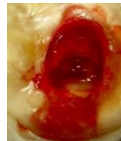
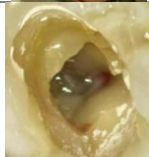
Case #	Pain?	Dx	HA	Tx	Caries Depth				Pre	Post
12	N	NP	y	xLa	No Caries					
44	N NP	NP	Y	xLa	No caries					44
45	N NP	NP	y	XLa	No caries					45
42	N	RP/InP	y	pe*	D					
17	Y InP	RP/InP	y	pp	ExD					

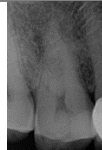
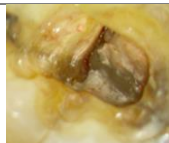
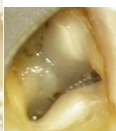
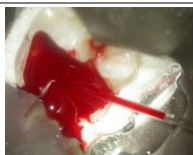
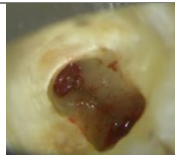
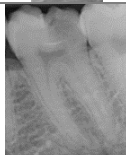
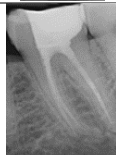
11	Y MiP	RP/InP	y	pp	ExD					
6	Y SeP	RP/InP	y	pp	ExD					
9	N	IP/MiP	y	pp	D					
30	Y MiP	IP/MiP	y	pp	D					
25	Y MiP	IP/MiP	y	pp	ExD					
46	Y MiP	IP/MiP	y	pp	ExD					

34	N	IP/MoP	y	pp	ExD						
10	Y MoP	IP/MoP	y	pp	D						
13	Y MoP	IP/MoP	y	pp	D						
14	Y MoP	IP/MoP	y	pp	D						
15	Y MoP	IP/MoP	y	pp	D						
28	Y MoP	IP/MoP	y	pp	D						

31	Y MoP	IP/MoP	y	pp	ExD						
33	Y MoP	IP/MoP	y	pp	ExD						
41	Y MoP	IP/MoP	y	pp	ExD						
43	Y MoP	IP/MoP	y	pp	ExD						
35	Y SeP	IP/SeP	y	pp	ExD						
36	Y SeP	IP/SeP	y?	pp	ExD						pp fail 10/7

18	Y SeP	IP/SeP	n	pe	D				
20	Y SeP	IP/SeP	n	pe	D		 	 	
7	Y SeP	IP/SeP	n	pe	ExD				
22	Y SeP	IP/SeP	n	pe	ExD	 	 		
24	Y SeP	IP/SeP	n	pe	ExD	 			
29	Y SeP	IP/SeP	n	pe	ExD				

32	Y SeP	IP/SeP	n	pe	ExD					
38	Y SeP	IP/SeP	TN	pe	ExD					
26	Y SeP	NP	n	pe	ExD					
27	Y InP	RP/InP	n	xLa	D					
19	Y MiP	RP/InP	n	pe	ExD					
16	Y MoP	IP/MoP	n	pe	D					

39	Y MoP	IP/MoP	n	pe	ExD						
23	Y MoP	IP/MoP	n	xLa	crack						
40	Y SeP	IP/MoP	n	pe	D						
21	Y SeP	IP/MoP	TN	pe	ExD						
37	Y MiP	IP/SeP	n	pe	ExD						

Appendix XIII – Details of failed pulpotomies

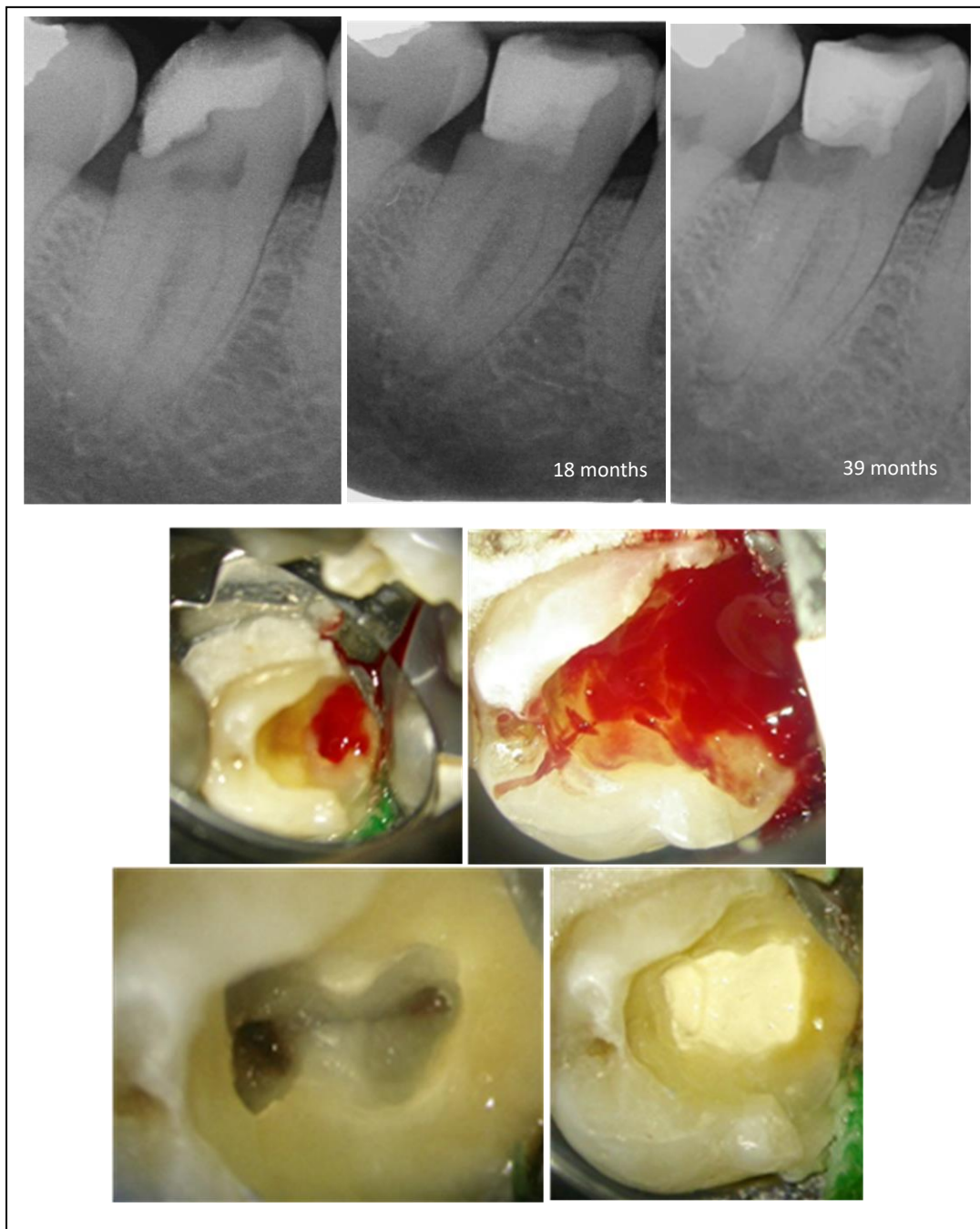
Case 1. Treatment failure – Apical periodontitis at 41 months



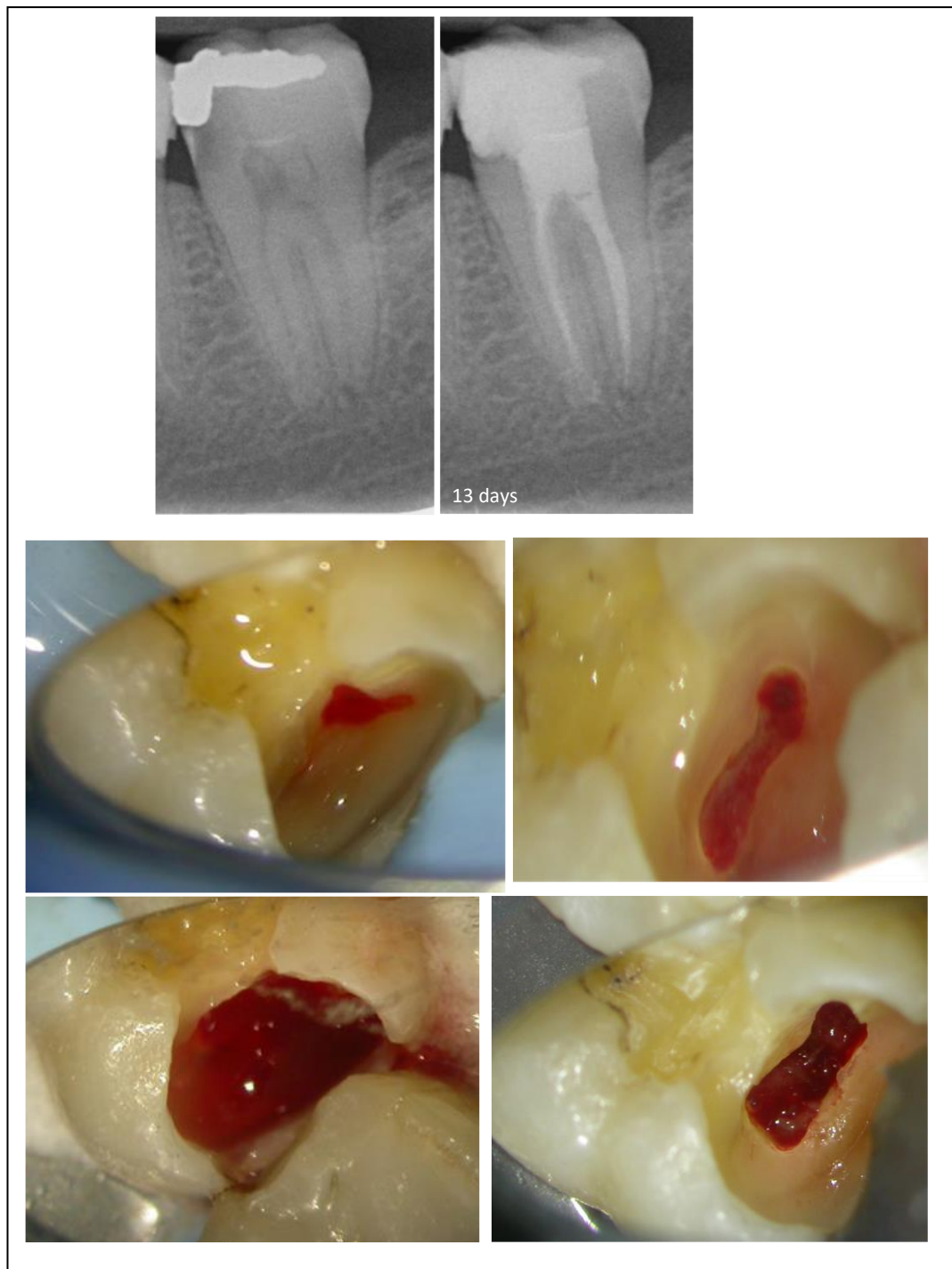
Case 2. Structural failure- Split tooth at 10 months



Case 3. Structural failure - Caries at 39 months



Case 4. Diagnostic failure – Symptoms increased at 13 days



Appendix XIV – Raw data Diagnostic Accuracy: Chapter 4

Comparison between tests		Sensitivity % (n)	Specificity % (n)	False positive % (n)	False negative % (n)	Positive predictive value % (n)	Negative predictive value % (n)	Disease prevalence % (n)	Pearson Chi-Square	p value	Measure of agreement Kappa (p)
Reference Standard	Index Test										
HC	Dx Classic excl NP	88.2 (15)	22.2 (6)	77.8 (21)	11.8 (2)	41.7 (15)	75 (6)	38.6(17)	0.767	.381	0.087 (.381)
HC	Dx Classic NP+RP=Health*	83.3	30	70	16.7	41.7 (15)	75 (6)	37.5	1.067	.302	0.111 (.302)
HC, excl TN	Dx Wolters Strict, incl NP	56.3 (9)	96.6(28)	3.4 (1)	43.8(7)	90(9)	80(28)	35.6(16)	16.633	<.0001	0.576 (<.0001)
HC excl TN	Dx Wolters Loose incl NP	73.2 (11)	50(15)	50(15)	26.7(4)	42.3 (11)	78.9 (15)	33.3 (15)	2.232	.135	0.197 (.135)
HC	Caries Depth	75 (4)	29.4 (5)	70.6 (12)	25 (1)	20 (3)	83.3(5)	19 (4)	0.31	.861	0.022 (.861)
HC	Symptoms	80 (12)	30 (9)	70 (21)	20(3)	36.4 (12)	75 (9)	33.3 (15)	0.511	.475	0.077 (.475)
Symptoms	HC	36.4 (12)	75 (9)	25 (3)	63.6 (21)	80 (12)	30 (9)	73.3 (33)	0.511	.475	0.077 (.475)
Caries Depth	Dx Classic	61.8 (21)	33.3 (3)	66.7 (6)	38.2 (13)	77.8 (21)	18.8 (3)	79.1 (34)	0.073	.787	-0.038 (.787)
Caries Depth	Dx Wolters Strict	54.4 (6)	30 (9)	70 (21)	45.5 (5)	22.2 (6)	64.3 (9)	26.8 (11)	0.855	.355	-0.106 (.355)
Caries Depth	Dx Wolters Loose	62.5 (15)	36.8 (7)	63.2 (12)	37.5 (9)	55.6 (15)	43.8 (7)	55.8 (24)	0.002	.965	-0.007 (.965)
Symptoms	Dx	0 (0)	0 (0)	100 (12)	100 (36)	0 (0)	0 (0)	75 (36)	4.8	.000	-0.600 (.000)
Symptoms	Dx Wolters Strict	100 (12)	33.3 (12)	66.7 (24)	0 (0)	33.3 (12)	100 (12)	25 (12)	5.333	.021	0.20 (.021)
Symptoms	Dx Wolters Loose	100 (29)	63.2 (12)	36.8 (7)	0(0)	80.6 (29)	100 (12)	60.4 (29)	24.421	.000	0.674 (.000)

[HC: Haemorrhage control; n: Number; Dx: Clinical Diagnosis; NP: Normal Pulp; RP: Reversible pulpitis; IP: Irreversible pulpitis; TN: Total necrosis; incl: including; excl: excluding]

Appendix XV – Raw data Diagnostic Accuracy: Chapter 5

Comparison between tests		Sensitivity % (n)	Specificity % (n)	False positive % (n)	False negative % (n)	Positive predictive value % (n)	Negative predictive value % (n)	Disease prevalence % (n)	Pearson Chi-Square	p value	Measure of agreement Kappa (p value)
Reference Standard	Index Test										
CDD_Cat	Dx Classic RP v's IP	75 (3)	17.6(3)	82.4 (14)	25 (1)	17.6 (3)	75 (3)	19(4)	0.114	0.763	-0.033 (.736)
CDD_Cat	Dx Classic NP + RP v's IP	60(3)	22.2(4)	77.8(14)	40(2)	17.6 (3)	66.7(4)	21.7 (5)	0.641	0.423	-0.95 (.423)
CDD_Cat	Dx Wolters Strict	60(3)	77.8 (14)	22.2(4)	40(2)	42.9 (3)	57.1(4)	21.7 (9)	2.638	0.104	0.330 (.104)
CDD_Cat	Dx Wolters Loose	60(3)	38.9 (7)	61.1 (11)	40 (2)	21.4(3)	77.8(7)	21.7(5)	0.002	0.964	-0.007 (.964)
CDD_Cat	Caries depth	80(4)	50 (8)	50(8)	20(1)	33.3 (4)	88.9(8)	23.8 (5)	1.4	0.237	0.203 (.237)
HC	CDD_Cat	55.6(5)	100 (14)	0(0)	44.4 (4)	100(5)	77.8(14)	39.1(9)	9.938	0.002	0.603 (.002)
HMGB ₁ _Cat	Dx Classic RP v's IP	100(3)	42.9(6)	57.1(8)	0(0)	27.3 (3)	100 (6)	17.6 (3)	1.987	0.159	0.209 (.159)
HMGB ₁ _Cat	Dx Classic NP + RP v's IP	75 (3)	29.4 (5)	70.6 (12)	25 (1)	20 (3)	83.3 (5)	19 (4)	0.031	0.861	0.022 (.861)
HMGB ₁ _Cat	Dx (Classic) NP+RP v's IP	75(3)	29.4(5)	70.6(12)	25(1)	20(3)	83.3(5)	19(4)	0.31	0.861	0.022 (.861)
HMGB ₁ _Cat	Dx Wolters Strict	50 (2)	76.5(13)	23.5(4)	50(2)	33.(2)	86.7(13)	19(4)	1.112	0.292	0.222 (.292)
HMGB ₁ _Cat	Dx Wolters Loose	73.3 (11)	50 (15)	50 (15)	26.7 (4)	42.3(11)	78.9 (15)	33.3 (15)	2.232	0.135	0.197 (.135)
HMGB ₁ _Cat	Caries Depth	25 (1)	43.8(7)	56.3(9)	75(3)	10 (1)	70 (7)	20 (4)	1.250	0.264	-0.20 (.264)
HC	HMGB ₁ _Cat	22.2(2)	81.8 (9)	18.2 (2)	77.8 (7)	50 (2)	56.3 (9)	45 (9)	0.051	.822	0.043 (.821)

[HC: Haemorrhage control; n: Number; Dx: Clinical Diagnosis; NP: Normal Pulp; RP: Reversible pulpitis; IP: Irreversible pulpitis; TN: Total necrosis; incl: including; excl: excluding; CDD: Cell Death Detection ELISA^{PLUS}; HMGB₁: HMGB₁ ELISA, Cat: Categorical value (High/Low); v's: versus]

Appendix XVI – Raw data: ELISA Data ALL assays

Tooth #	Dx	DI	Tx	Conc IL-8 R&D	StDev	Conc IL-8 abcam	StDev	Conc M-CSF R&D	StDev	Conc M-CSF abcam	StDev	CDD (Vol)	StDev	HMGB ₁	StDev	HS IL-6 (TotPr)	StDev	CDD (TotPr)	StDev
1	RP/MiP	NP	pp									0.02	0.04			0.27		0.01	0.02
2	IP/MoP	IP/SeP	pp									0.03				1.11		0.04	
3	RP/InP	IP/SeP	pp													1.17		0.00	0.00
4	IP/MiP	RP/InP	pe													0.01		0.01	0.01
5	IP/MiP	NP	pe									0.06	0.04			0.00			
6	RP/InP	IP/SeP	pp									0.38	0.46			0.00			
7	IP/SeP	IP/SeP	pe									0.22	0.31			1.37		0.00	0.00
8	IP/SeP	IP/MoP	pp									0.27	0.35			3.68		0.00	0.00
9	IP/MiP	IP/MiP	pp									0.06	0.05	16.17		4.24		0.00	
10	IP/MoP	NP	pp									0.02	0.03			0.00			
11	RP/InP	NP	pp											0.00		0.00			
12	NP	NP	xLa									0.03	0.01	13.09	4.49	2.52		0.02	0.04
13	IP/MoP	RP/MiP	pp									0.05	0.03	7.59	1.12	3.08		0.07	0.08
14	IP/MoP	RP/MiP	pp											23.06	11.17	0.61		0.00	
15	IP/MoP	IP/SeP	pp													2.20		0.01	
16	IP/MoP	IP/SeP	pe													2.03		0.11	0.20
17	RP/InP	IP/MoP	pp	3.26		0.00		8658.84		1.88				0.00	0.00	2.23		0.01	0.01
18	IP/SeP	IP/SeP	pe	0.67	0.11	15.69	5.92	3191.25	1366.03	30.62	6.39	0.04	0.06	0.55	1.19				
19	RP/InP	IP/SeP	pe	0.44		281.49		4483.56		60.21		2.42	0.35	22.75	0.88				
20	IP/SeP	IP/SeP	pe	2.22	0.23	13.05	18.30	5179.44	1687.07	46.11	31.19	0.92	0.82	87.87					
22	IP/SeP	IP/SeP	pe									0.23	0.23	23.26					
23	IP/MoP	IP/SeP	xLa	1.34	0.56	16.91	23.17	2886.28	4999.18	21.40	21.39	0.29	0.25	5.35	3.44				
24	IP/SeP	IP/SeP	pe	1.26		3.21		6770.02		23.45		1.07	0.85	11.07	2.07				
25	IP/MiP	IP/MiP	pp									0.05	0.10	4.79	2.80				
26	NP	IP/SeP	pe	18.93	13.64	125.05	27.11	2495.33	2783.52	32.09	47.31	1.89	0.44	63.67	2.92				
27	RP/InP	IP/SeP	xLa									0.10	0.14	0.00					
28	IP/MoP	NP	pp									0.08	0.13	100.10					
29	IP/SeP	IP/SeP	pe									1.79	0.87	97.76					
30	IP/MoP	IP/SeP	pp	0.89		0.00		4582.97		21.04		0.06	0.15	0.00	0.00				
31	IP/MoP	IP/MoP	pp	0.81	0.31	4.75	4.51	3340.32	2089.23	18.69	8.72	0.20	0.18	5.07	7.17				
32	IP/SeP	IP/SeP	pe	2.22		1.03		1700.04		166.25				0.00					
33	IP/MoP	IP/MoP	pp	1.90		25.32		2097.69		161.91				1.29	1.83				
34	IP/MoP	IP/MoP	pp	6.86		33.77		4980.61		93.95									
35	IP/MiP	IP/SeP	pp	0.57		10.66		5179.44		106.60									
36	IP/SeP	IP/SeP	pp	2.54		14.68		4781.79		80.69									
37	IP/SeP	IP/SeP	pe	0.87		0.00		4781.79		98.16									
39	IP/SeP	IP/SeP	pe	1.48		1.53		8161.78		151.42									
40	IP/MiP	IP/SeP	pe	0.59		0.00		5676.49		69.48									
41	IP/MoP	IP/SeP	pp	1.44		0.00		3986.50		19.48									

42	RP/InP	IP/MiP	pe~	1.14		1.70		7863.54		68.04								
43	IP/MoP	IP/MoP	pp	1.06		1.78		5577.08		123.35								
44	NP	NP	xLa	1.04		0.00		6471.79		19.48								
45	NP	NP	xLa	1.08	1.02	0.00	0.00	11939.41	1546.48	117.20	56.58							

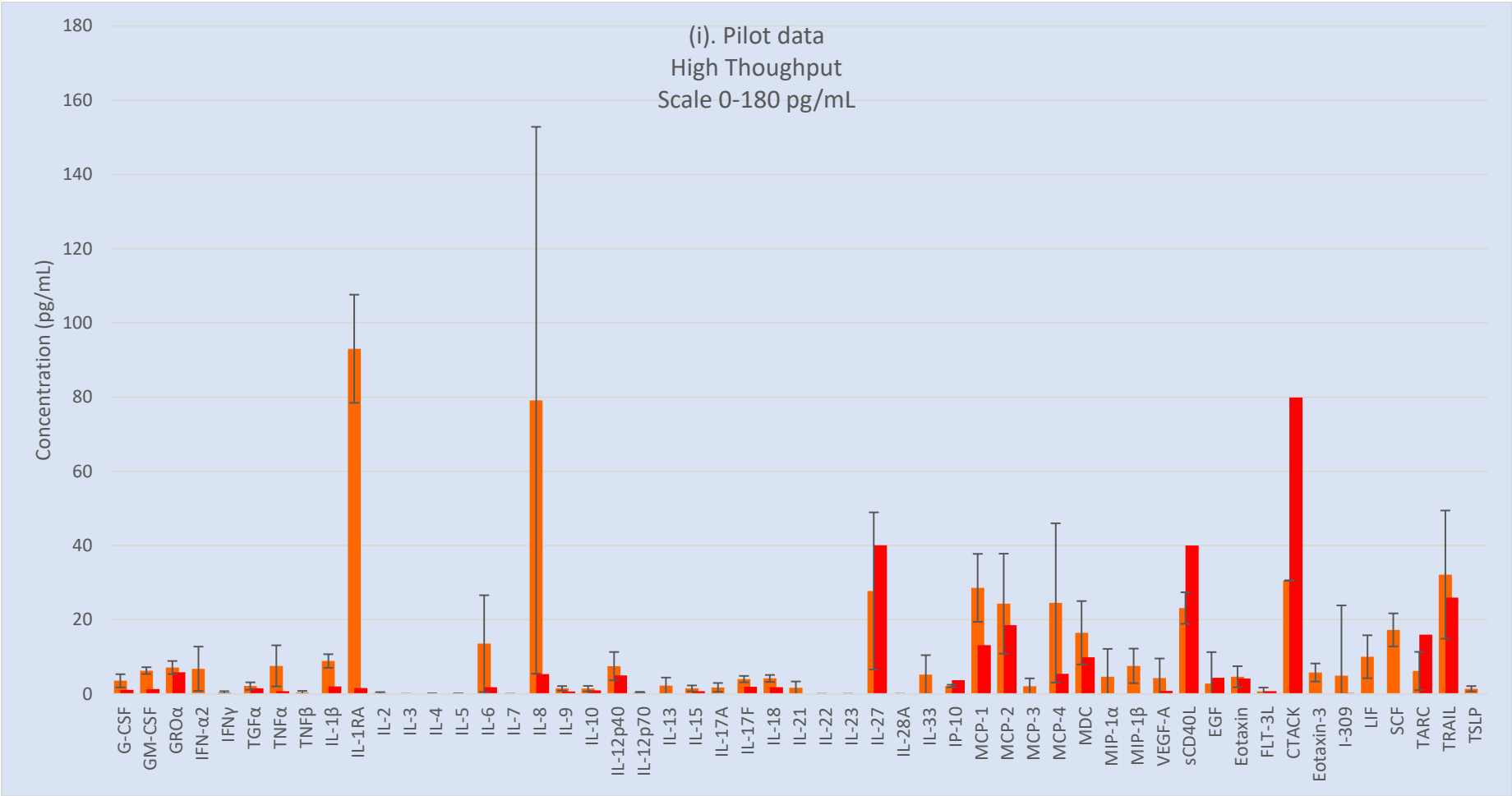
Appendix XVII - List of abbreviations and names of multiplex discovery assay, Human Cytokine Array/ Chemokine Array 71-Plex Panel (EveTechnologies)

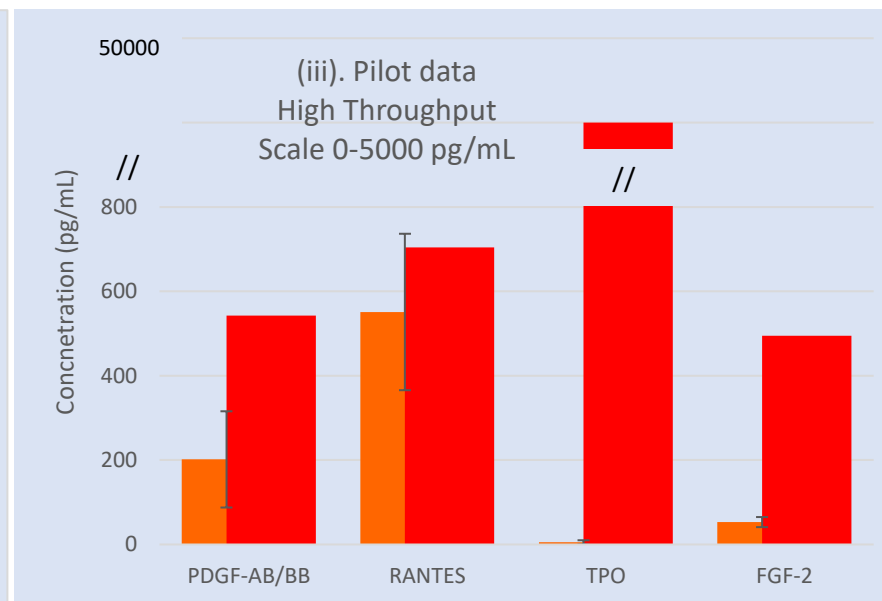
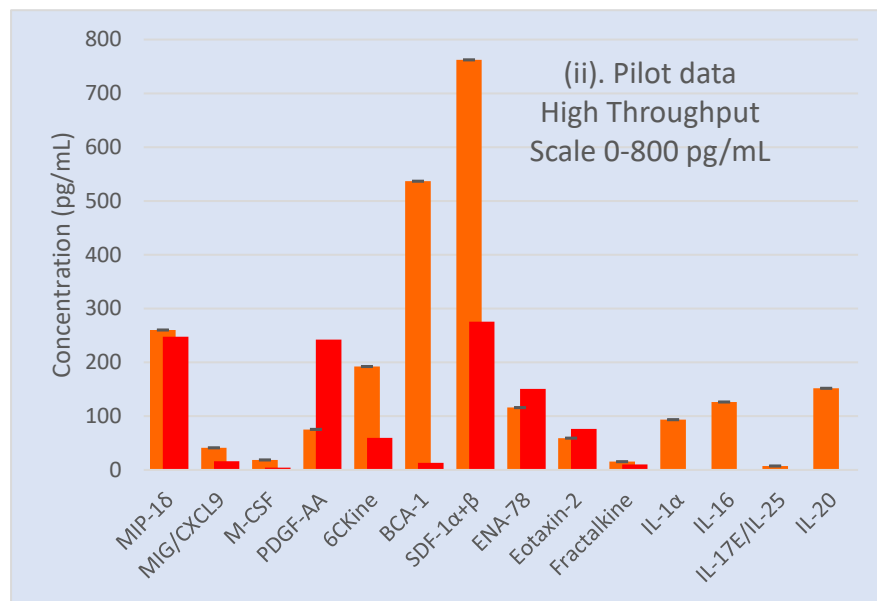
Abbreviation	Protein definition	Cytokine grouping
G-CSF	Granulocyte-colony stimulating factor	7
GM-CSF	Granulocyte-macrophage colony-stimulating factor	7
GRO α	Growth regulated oncogene alpha	
IFN- α 2	Interferon alpha-2	
IFN γ	Interferon gamma	
IP-10	Interferon gamma-induced protein 10 kDa	
TGF α	Pro-transforming growth factor alpha	1
TNF α	Tumour necrosis factor alpha	
TNF β	Tumour necrosis factor beta	
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand	
IL-1 α	Interleukin-1 alpha	
IL-1 β	Interleukin-1 beta	
IL-1RA	Interleukin-1 receptor antagonist	
IL-2	Interleukin-2	
IL-3	Interleukin-3	
IL-4	Interleukin-4	
IL-5	Interleukin-5	
IL-6	Interleukin-6	
IL-7	Interleukin-7	
IL-8	Interleukin-8	
IL-9	Interleukin-9	
IL-10	Interleukin-10	
IL-12p40	Interleukin-12 subunit p40	
IL-12p70	Interleukin-12 subunit p70	
IL-13	Interleukin-13	
IL-15	Interleukin-15	
IL-16	Interleukin-26	
IL-17A	Interleukin-17	
IL-17E/IL-25	Interleukin-17E, or Interleukin-25	
IL-17F	Interleukin-17F	
IL-18	Interleukin-18	
IL-20	Interleukin-20	
IL-21	Interleukin-21	
IL-22	Interleukin-22	
IL-23	Interleukin-23	
IL-27	Interleukin-27	
IL-28A	Interleukin-28A	
IL-33	Interleukin-33	
MCP-1	Monocyte chemoattractant protein-1	
MCP-2	Monocyte chemoattractant protein-2	
MCP-3	Monocyte chemoattractant protein-3	
MCP-4	Monocyte chemoattractant protein-4	
M-CSF	Macrophage colony-stimulating factor	7
MDC	Macrophage-derived chemokine	
MIG/CXCL9	Monokine induced gamma interferon/Chemokine (C-X-C motif) ligand 9	
MIP-1 α	Macrophage inflammatory protein-1 alpha	
MIP-1 β	Macrophage inflammatory protein-1 beta	
MIP-1 δ	Macrophage inflammatory protein-1 delta	
PDGF-AA	Platelet-derived growth factor – Two A subunits	4
PDGF-AB/BB	Platelet-derived growth factor – AB/Two B subunits	4
RANTES	Regulated on activation, normal T cell expressed and secreted	
VEGF-A	Vascular endothelial growth factor A	4
sCD40L	Soluble CD-40-ligand	
EGF	Pro-epidermal growth factor	6
Eotaxin	Eosinophil-specific chemokine	
FGF-2	Fibroblast growth factor 2	2
FLT-3L	Fms-like tyrosine kinase 3	
Fractalkine	Fractalkine (CX ₃ C chemokine)	
6CKine	Chemokine ligand 21 (CCL21)	
BCA-1	B Lymphocyte Chemoattractant/Chemokine ligand 13 (CXCL13)	

CTACK	Cutaneous T cell-attracting chemokine	
ENA-78	Epithelial-derived neutrophil-activating peptide 78 (CXCL5)	
Eotaxin-2	Eosinophil chemotactic protein 2 (CCL ₂₄ and MPIF-2)	
Eotaxin-3	Eosinophil chemotactic protein 3 (MIP-4α Macrophage inflammatory protein-4 alpha)	
I-309	Chemokine ligand 1 (CCL1)	
LIF	Leukaemia inhibitory factor	
SCF	Stem cell factor, c-Kit ligand	4
SDF-1α+β	Stromal cell-derived factors 1-alpha and 1-beta	
TARC	Thymus- and activation-regulated chemokine	
TPO	Thrombopoietin	
TSLP	Thymic stromal lymphopoietin	

[Functional cytokine groupings (1) TGF superfamily members, (2) fibroblast GF-related, (3) neurotrophic GF-related, (4) angiogenic GF-related, (5) insulin-related GFs and cytokines, (6) other GF-related members and (7) Colony-stimulating factors (M-CSFR)]

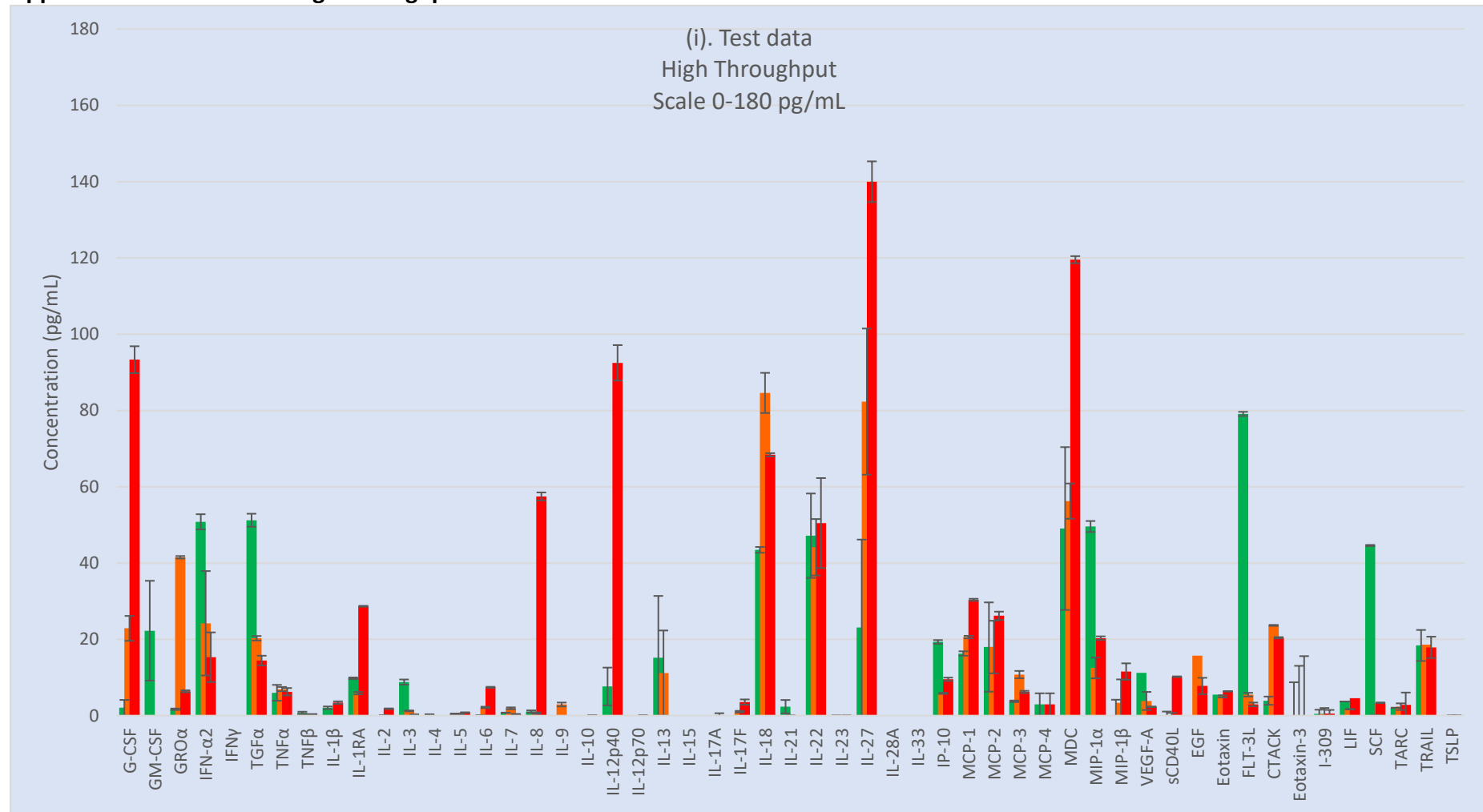
Appendix XVIII – Raw data: High-throughput Pilot

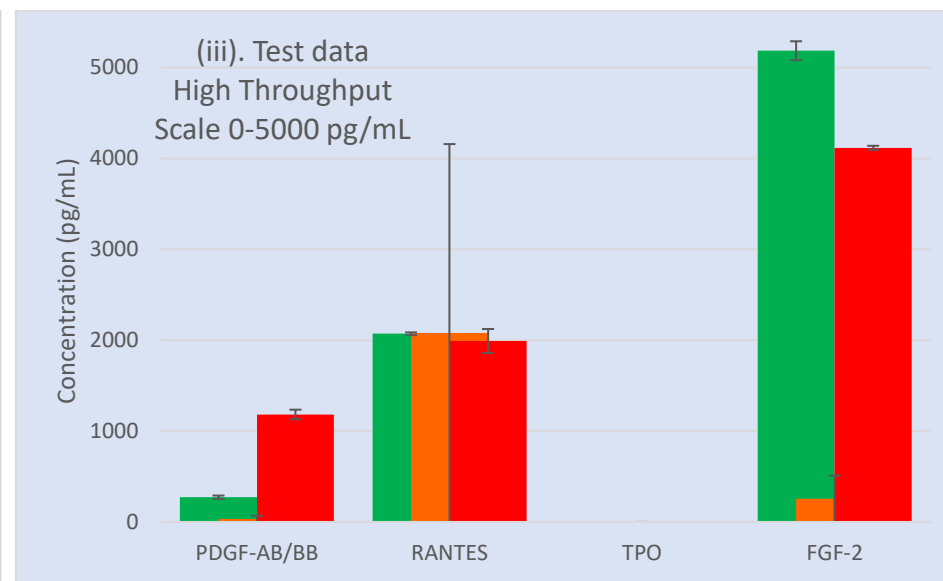
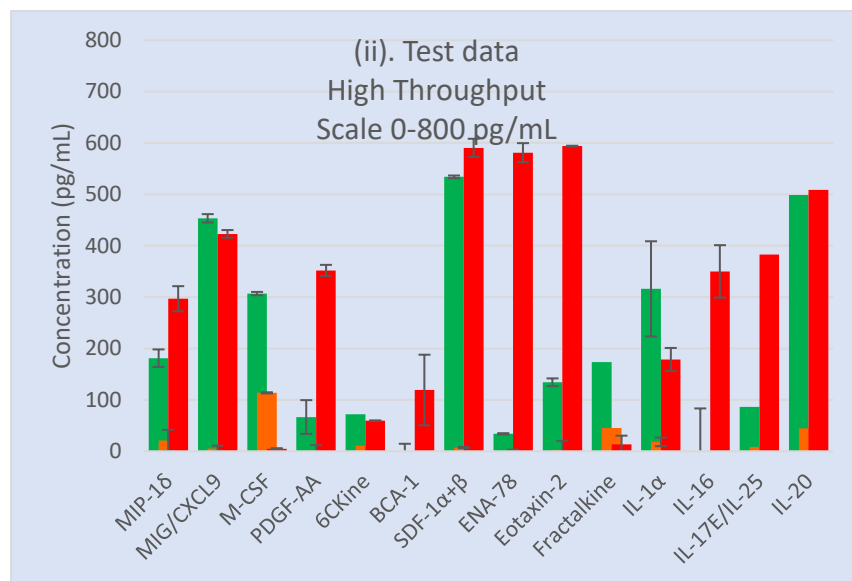




[Orange shading indicates Moderate pulpitis samples; Red shading indicates Severe pulpitis samples; Duplicate samples were averaged with error bars demonstrating standard error]

Appendix XIX – Raw data: High-throughput Test





[Green shading indicates normal pulp (healthy control) samples; Green shading indicates normal pulp (healthy controls) samples; Orange shading indicates Moderate pulpitis samples; Red shading indicates Severe pulpitis samples; Duplicate samples were averaged with error bars demonstrating standard error]

Appendix XX – Presentations, publications, additional responsibilities

Internal presentations

- **Lennon, S.** Curriculum Mapping Project-Pain topic. Staff training and development day. Board Room, DDUH, Dublin, Ireland. August 2017.
- **Lennon, S.** An investigation into molecular biomarkers of pulp inflammation and cell death and their potential use as a diagnostic tool. Postgraduate Research Seminar Presentations, LLT, DDUH Dublin, Ireland. May 2018.
- **Lennon, S.** Next generation diagnostics in the inflamed pulp. Staff training and development day. Zoom, DDUH, Dublin Ireland. April 2021.
- **Lennon, S.** An investigation into molecular biomarkers of pulp inflammation and cell death and their potential use as a diagnostic tool. Postgraduate Research Seminar Presentations, LLT, DDUH Dublin, Ireland. May 2022.

Publications

- **Lennon, S.** & Duncan, H. Minimally invasive endodontics – pulp fact or pulp fiction? Journal of the Irish Dental Association, 66: 134-138; 2020.
- Chevalier, V., Dessert, M., Fouillen, K.J., **Lennon, S.** & Duncan, H.F. (2022) Preclinical 3D-printed laboratory simulation of deep caries and the exposed pulp reduced student anxiety and stress, while increasing confidence and knowledge in vital pulp treatment. International Endodontic Journal, 55, 844– 857.

Memberships

- President, Irish Endodontic Society 2018-2021
- Committee, Irish Endodontic Society 2013-2023
- Restorative Advisory Committee, Irish Committee for Specialist Training in Dentistry 2015-2022
- Teachers of Endodontology Group, British Endodontic Society 2018-2022

Positions of responsibility

- Examiner Undergraduate Dental Science Years 2-5 2016-2023
- Clinical Academic Advisor 2016-2023
- Portfolio Supervisor 2016-2023
- Dissertations Provider 2016-2023
- Dissertation Supervisor 2016-2022

Conference organisation

- Irish Endodontic Society Annual Scientific Meetings, Dublin, Ireland. 2017-2022

Appendix XXI- Non-thesis related activities

Course/Activity	Date	Time	Venue	Certificate (Yes/No)	Facilitator's Name
ESE Biennial Congress	17-19/09/2015	18 hrs	Barcelona, Spain	Y	Prof C Löst/P Dummer
Senior Staff Research Away Day	08/04/2016	6 hrs	Dublin, Ireland	N	Dr Gary Moran
Dental Council Bench Test Preparatory Course	12/04/2016	3 hrs	CSL, DDUH	N	DDUH
Postgraduate Research Seminar Presentations	03/05/2016	3 hrs	LLT, DDUH	N	Dr Gary Moran
Postgraduate Research Seminar Presentations	13/05/2016	3 hrs	LLT, DDUH	N	Dr Gary Moran
Postgraduate Research Seminar Presentations	25/04/2016	3 hrs	LLT, DDUH	N	Dr Gary Moran
Presenter, Metropolitan Branch IDA	22/09/2016	3 hrs	Dublin, Ireland	N	Dr Una Lally
ESE Research Meeting on Endodontology	07/10/2016	8 hrs	Amsterdam, Holland	Y	Dr Patrick Sequiera-Byron
Research Seminar Programme	10/10/2016	1 hr	LLT, DDUH	N	Prof Orla Shields
Research Seminar Programme	27/10/2016	1 hr	LLT, DDUH	N	Dr Evelina Kratunova/Prof Tim Wright
Research Seminar Programme	16/11/2016	1 hr	LLT, DDUH	N	Prof Jeffrey Dean
Research Seminar Programme	28/11/2016	1 hr	LLT, DDUH	N	Dr Jimmy Walker
Research Seminar Programme	23/01/2017	1 hr	LLT, DDUH	N	Dr Gary Moran/Prof S Dancer
Irish Endodontic Society ASM	26-27/01/2017	8 hrs	Dublin, Ireland	Y	Dr S Lennon/ Dr C Durack
Research Seminar Programme	09/02/2017	1 hr	LLT, DDUH	N	Dr Gary Moran/Prof W Wade
AHA, IHF BLS Basic Life support	07/03/2017	5 hrs	DDUH, Dublin	Y	IHF
IES Case night	23/03/2017	2 hrs	Dublin, Ireland	N	Dr Síle Lennon
Postgraduate Research Seminar Presentations	28/04/2017	3 hrs	LLT, DDUH	N	Dr Gary Moran
Postgraduate Research Seminar Presentations	09/05/2017	3 hrs	LLT, DDUH	N	Dr Gary Moran
Staff training & development days	28-29/08/2017	8 hrs	Board Room, DDUH	N	Dr Denise McCarthy
ISTAPS Internal Examiner & Preparation of Report	12/09/2017	6 hrs	DDUH	N	Ms Catherine Creagh
ESE Biennial Congress	14-16/09/2017	19 hrs	Brussels, Belgium	Y	Prof C Löst/ P Dummer
Microbiology Lab Orientation	27/10/2017	1 hr	Division IV, DDUH	N	Dr Mary O'Donnell
Irish Endodontic Society ASM	25-26/01/2018	8 hrs	Dublin, Ireland	Y	Dr S Lennon
An introduction to Children First	20/02/2018	1 hr	Online	Y	HSELand, Pat O'Bpyle
MMI Interviews Selection Dental Science	10/04/2018	3 hrs	Clinic 2 DDUH	N	Dr M O'Sullivan
DDUH Staff Research Away Day	12/04/2018	5 hrs	Dublin, Ireland	N	Dr Jeff O'Sullivan

Postgraduate Research Seminar Presentations	25/04/2018	3 hrs	LLT, DDUH	N	Dr Michael O'Sullivan
University Chemical Safety Workshop	26/04/2018	5 hrs	TBSI, TCD	N	-
Postgraduate Research Seminar Presentations	01/05/2018	3 hrs	LLT, DDUH	N	Dr Michael O'Sullivan
Postgraduate Research Seminar Presentations	11/05/2018	3 hrs	LLT, DDUH	N	Dr Michael O'Sullivan
Dentsply Sirona Clinical Education Programme	26-28/09/2018	16 hrs	Ballaigues, Switzerland	Y	Drs. A Castellucci & W Dias
DDUH Research Distinguished Seminar Series	04/10/2018	1 hr	LLT, DDUH	N	Prof Glen Clarke
DDUH Research Distinguished Seminar Series	11/10/2018	1 hr	LLT, DDUH	N	Prof Roseanne Mulligan
ESE Research & Clinical Meeting on Endodontology	26-27/10/2018	14 hrs	Amsterdam, Holland	Y	Dr H Duncan
BES Teachers of Endodontology Meeting	07/12/2018	5 hrs	Manchester, UK	Y	Prof Alison Qualtrough
Irish Endodontic Society ASM	24-25/01/2019	8 hrs	Dublin, Ireland	Y	Dr S Lennon
Reviewer JIDA	13/02/2019	2 hrs	Dublin Ireland	Y	Dr Ciara Scott
MMI Interviews Selection Dental Science	09/04/2019	3 hrs	Clinic 2 DDUH	N	Dr M O'Sullivan
Postgraduate Research Seminar Presentations	21/05/2019	3 hrs	LLT, DDUH	N	Dr Ioannis Polyzois
Postgraduate Research Seminar Presentations	31/05/2019	3 hrs	LLT, DDUH	N	Dr Ioannis Polyzois
AHA, IHF BLS Basic Life support	22-27/08/2019	5 hrs	DDUH, Dublin	Y	IHF
Reviewer JIDA	10/09/2019	2 hrs	Dublin Ireland	Y	Dr Ciara Scott
New Orofacial Pain Classification ICOP-1 beta	11/09/2019	4 hrs	Vienna, Austria	Y	Dr M Pigg
ESE Biennial Congress	12-14/09/2019	19 hrs	Vienna, Austria	Y	Prof P Dummer
Reviewer JIDA	18/10/2019	2 hrs	Dublin Ireland	Y	Dr Ciara Scott
National Oral Health Policy Dental Education Strategy Consultation	24/10/2019	1 hr	LLT, DDUH	N	Dr Dympna Kavanagh, CDO
CRDI Course Techniques & Strategies in Molecular Medicine	02-03/12/2019	13 hrs	TBSI, TCD, Dublin	Y	SECTS Gaél Parent
Irish Endodontic Society ASM	23-24/01/2020	9 hrs	Dublin, Ireland	Y	Dr S Lennon
Endodontics - Safer Practice Series	21/02/2020	45 min	Online	Y	Dental Protection
Staff training and Development Day	16/04/2021	6 hrs	Online/Zoom DDUH	N	Dr Michael O'Sullivan
Postgraduate Research Seminar Presentations	20/05/2021	3 hr	LLT, DDUH	N	Dr Jeff O' Sullivan
How to thrive at work Module 1	08/09/2021	1 Hr	Online	Y	Dental Protection
Dental Conference Dental Protection	05/09/2020	3 hrs	Online	Y	Dental Protection
Display Screen Equipment: User Awareness	14/10/2020	1 hr	Online DDUH	Y	HSEland, Pat O'Boyle
Dental Conference Dental Protection	10/10/2021	3 hrs	Online	Y	Dental Protection
How to thrive at work Module 2	13/10/2021	1 Hr	Online	Y	Dental Protection

Learning from Cases Dental Protection	04/11/2021	1 hr	Online	Y	Dental Protection
Reviewer JIDA	16/11/2021	2 hrs	Dublin Ireland	Y	Dr Ciara Scott
How to thrive at work Module 3	10/11/2021	1 hr	Online	Y	Dental Protection
How to thrive at work Module 4	08/12/2021	1 hr	Online	Y	Dental Protection
Good research conduct	21/01/2022	1 hr	Online	Y	Epigeum Online Course
Risk Management Training	07/02/2022	1 Hr	Online DDUH	Y	HCI, Patricia Fernandes
Irresponsible research practices	18/02/2022	1 hr	Online	Y	Epigeum Online Course
Planning your research	18/02/2022	1 hr	Online	Y	Epigeum Online Course
Managing and recording your research	18/02/2022	1 hr	Online	Y	Epigeum Online Course
BES Teachers of Endodontology Meeting	25/02/2022	6 hrs	Manchester, UK	Y	Prof A Qualtrough
Dental Council Bench Test Preparatory Course	05/04/2022	3 hrs	CSL, DDUH	N	DDUH
Postgraduate Research Seminar Presentations	18/05/2022	3 hrs	LLT, DDUH	N	Dr Hal Duncan
Postgraduate Research Seminar Presentations	19/05/2022	3 hrs	LLT, DDUH	N	Dr Hal Duncan
An introduction to Children First	13/12/2022	1 hr	Online DDUH	Y	HSELand, Pat O'Boyle
Cyber security awareness	13/12/2022	1 hr	Online DDUH	Y	HSELand, Pat O'Boyle
Dignity at work	13/12/2022	1 hr	Online DDUH	Y	HSELand, Pat O'Boyle
Behaviours that challenge awareness	14/12/2022	1 Hr	Online DDUH	Y	Voluntary Healthcare Agency Risk Management ELearning Programme
Strategic Plan	16/12/2022	4 hrs	TCD Business School	N	Colm Murphy
Irish Endodontic Society ASM	20/01/2023	6 hrs	Dublin, Ireland	Y	Dr M Egan
Reviewer Clinical & Experimental Dental Research	15/04/2023	2 hrs	Dublin, Ireland	Y	Prof A Stavropoulos
Salud User Group	2016-2022	150 hrs	DDUH	N	Ms S Bushe/TTH
Website Development Project Div II rep	2016-2020	30 hrs	DDUH	N	Ms Helen McCabe
Curriculum Mapping Project	2017	25 hrs	DDUH	N	Prof B O Connell
Pre-clinical undergraduate practical endodontic teaching- supervisor	2016	60 hrs	CSL, DDUH	N	Dr Hal Duncan
Pre-clinical undergraduate practical endodontic teaching- Co-lead	2017-2022	360 hrs	CSL, DDUH	N	Dr Hal Duncan
Clinical supervision undergraduate endodontics	2016-2023	800 hrs	DDUH	N	-

[ESE: European Society of Endodontology; AHA: American Heart Foundation; IES: Irish Endodontic Society; ASM: Annual Scientific Meeting; IDA: Irish Dental Association; BES: British Endodontic Society; hrs: hours; Y: Yes; N: No; DDUH: Dublin Dental University Hospital; TCD: Trinity College Dublin; LLT: Large lecture

theatre; CSL: Clinical skills Lab; Div II: Division II, Department of Restorative Dentistry and Periodontology, School of Dental Science; DIV IV: Division 4, Department of Oral Biosciences, School of Dental Science]

Appendix XXII - Invited Clinical Feature

Minimally invasive endodontics – pulp fact or pulp fiction?

Could pulpotomy offer a reliable treatment strategy to manage advanced pulpal inflammation as an alternative to root canal treatment?

Abbreviations: GIC: glass ionomer cement; RM-GIC: resin-modified glass ionomer cement; HSC: hydraulic silicate cement; VPT: vital pulp therapy; IPT: indirect pulp therapy; MTA: mineral trioxide aggregate; RCT: root canal treatment; DPC: direct pulp cap

Introduction

The European Society of Endodontology (ESE) recently published a position statement on the management of deep caries and the exposed pulp.¹ That statement outlined the development of vital pulp treatment (VPT) in cases with advanced pulpal inflammation. The following feature aims to outline the clinical

steps involved in providing coronal pulpotomy to manage a mature permanent molar with signs and symptoms indicative of irreversible pulpitis. Traditionally, pulpotomy was regarded as a treatment reserved for traumatic pulp exposure.² It was subsequently accepted for the management of carious exposure in the primary dentition,³ as well as in the immature permanent carious teeth,⁴ and the advantages of this remain irrefutable. The shift towards adopting a biologically based approach to preserve pulpal health continues to gain support.⁵ Bacterial penetration of the pulp tissue with discrete tissue necrosis are the histological indicators of an irreversibly inflamed pulp.⁶ However, beyond the zone bounded by acute inflammatory infiltrate, normal or relatively uninfamed pulp is present.⁶ By removing the severely inflamed tissue and providing an appropriate restoration, the regenerative capacity of the pulp can be exploited.⁷

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FIGURE 1:
A periapical radiograph demonstrating deep caries affecting the LR7. A band of dentine is noted between the caries front and the pulp chamber.

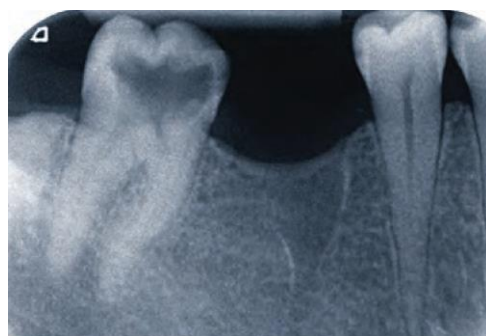


FIGURE 2:
A periapical radiograph demonstrating extremely deep caries affecting the LR7. The carious lesion appears to extend to the pulp. Pulp exposure may be unavoidable.

Terminology

The radiographic appearance of deep caries is illustrated in **Figure 1**, and that of extremely deep caries in **Figure 2**. Extremely deep caries extends through the full depth of the dentine, where pulp exposure during operative treatment is inevitable.⁸ Currently accepted diagnostic terminology describing the irreversibly inflamed pulp does not adequately inform the clinician regarding the spectrum of inflammation likely to be encountered, or the inherent reparative capacity of the involved tissues.⁷ Root canal treatment (RCT) is regarded as the definitive treatment for irreversible pulpitis, or carious exposure. Novel classifications of pulpitis have been proposed recently, to guide the clinician towards potentially less-invasive strategies (**Table 1**).⁹

Diagnosis

Although the limitations in accurately diagnosing the level of pulp inflammation are well recognised, the distinctions between initial and severe pulpitis remain useful clinical tools to support the decision to initiate direct pulp therapy.⁹ Interpretation of sensibility testing should focus on the threshold to the stimulus (normal, heightened, or diminished), as well as its sensitivity (character and duration), to guide the diagnosis. More severe symptoms include prolonged intense pain, which may be provoked or spontaneous. At the most severe end of the inflammatory spectrum, the reparative potential of the pulp may have been surpassed, in which case pulpectomy and subsequent RCT will be necessary.

Treatment procedure

Rubber dam isolation is essential to ensure a predictable outcome (**Figures 3 and 4**).^{1,8} Non-selective (complete) caries removal is completed at the

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Table 2: Comparison of vital pulp therapy studies.

Author	Tx	Material	Numbers	Success rate (%)	Follow-up (months)	% recall
Asgary and Egbahl, 2013 ¹⁷	Pulpotomy	MTA, CEM	413	95; 92	12	84
Asgary, <i>et al.</i> , 2015 ¹³	Pulpotomy	CEM	205	77.5	60	67
Awawdeh, <i>et al.</i> , 2018 ¹⁸	DPC, pulpotomy	BD, MTA	68	92; 96	36	72
Barrieshi-Nusair and Qudeimat, 2006 ¹⁹	Partial pulpotomy	MTA	31	100	24	25
Galani, <i>et al.</i> , 2018 ²⁰	Pulpotomy	MTA	27	85	18	96
Kang, <i>et al.</i> , 2017 ²¹	Partial pulpotomy	MTA	104	95	12	75
Linsuwanont, <i>et al.</i> , 2017 ²²	Pulpotomy	MTA	66	87	48	82
Nosrat, <i>et al.</i> , 2013 ²³	Pulpotomy	MTA, CEM	51	100	12	96
Taha and Khazali, 2017 ¹⁵	Pulpotomy	MTA	52	92	36	80
Taha and Abdelkader, 2018 ²⁴	Pulpotomy	BD	64	98	12	92

MTA: mineral trioxide aggregate; CEM: calcium-enriched material (alternative hydraulic silicate cement); BD: Biodentine; Tx: treatment.

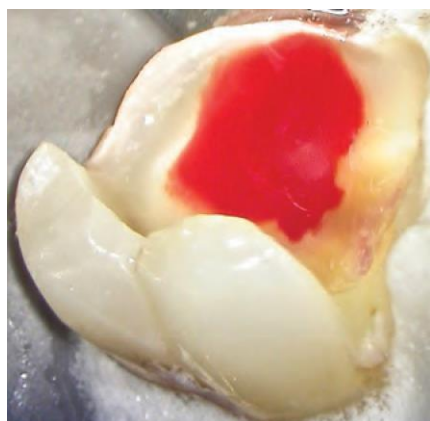


FIGURE 8: Pulpal haemorrhage after unroofing the pulp chamber.



FIGURE 9: Further pulp amputation can be completed using sharp hand instruments to the level of the orifice to the root canal system. Magnification and illumination enhance operator vision.



FIGURE 10: A cotton pellet soaked in disinfectant is applied to the orifice of the root canal system.

The volume of bleeding encountered once the pulp has been exposed can range from minimal to profuse (**Figure 8**). Careful handling of the pulp tissues is critical to provide a suitable wound surface. The inflamed pulp tissues visible throughout the pulpal floor (**Figure 9**) need to be further amputated to the level of the orifice to the root canals in a full pulpotomy. This can be achieved using small-diameter diamond burs under constant water coolant, or sharp hand instruments, to produce a clean cut surface to the pulp.

Antimicrobial irrigation using sodium hypochlorite will enhance antisepsis. Alternatively, a cotton pellet soaked with sodium hypochlorite can be placed over the pulp (**Figure 10**). The application of light pressure facilitates haemostasis. Consensus has not been reached regarding the timeframe within which haemostasis should be achieved. Control of pulpal bleeding is one of the final clinical criteria necessary to provide VPT (**Figure 11**). The pulp chamber and cavity must be clean of all traces of blood residue to eliminate the risk of tooth discolouration.

The superiority of hydraulic silicate cements (HSCs) over calcium hydroxide cement in direct pulp therapy has been demonstrated both histologically¹² and through clinical outcome studies (**Table 2**).¹³⁻¹⁶ As such, these are now

regarded as the materials of choice in the provision of VPT.¹ Once haemostasis has been established (up to five to six minutes, sometimes longer), the HSC (**Figure 12**) can be placed into the pulp chamber (**Figures 13 and 14**). The materials can be tapped gently into place using pluggers (**Figure 15**) or inverted paper points.

Although definitive restoration is recommended as a one-step procedure, significant questions remain to be answered regarding the best method to achieve this. Etching with 37% phosphoric acid has been shown to cause both structural and chemical changes to Biodentine (Septodont; Saint-Maur-des-Ditch Cedex, France).²⁵ Due to the extended setting time of ProRoot mineral trioxide aggregate (MTA) (Dentsply Sirona; Ballaigues, Switzerland),²⁶ wash-out of the material will occur during adhesive restoration unless a suitable liner is used. Interactions between MTA and various materials revealed that chemical-cured glass ionomer cement (GIC) absorbed water from the hydration of MTA, leading to increased porosity of the material.²⁷ Overlaying Biodentine with a resin-modified GIC (RM-GIC) was not shown to affect the setting reaction of the material.²⁸ However, *in-vitro* analysis has demonstrated significant reduction in the shear bond strength, as well as gaps at the material

Table 1: Novel classification of pulpitis (adapted from Wolters, et al.).⁹

Traditional classification	Reversible pulpitis		Irreversible pulpitis	
Wolters' classification ⁹	Initial pulpitis	Mild pulpitis	Moderate pulpitis	Severe pulpitis
Clinical features	Heightened response to cold, not lingering, no spontaneous pain.	Heightened and lingering response to cold/heat/sweet stimuli up to 20s, resolve. May be percussion sensitive	Heightened prolonged reaction to cold, lingering minutes, may be percussion sensitive, spontaneous pain.	Severe spontaneous pain, warm and cold stimuli, range from sharp to dull throbbing, disturbed sleep often, percussion sensitive
Suggested treatment	IPT	IPT	Coronal pulpotomy – partial or complete	Coronal pulpotomy or RCT

s: seconds; IPT: indirect pulp therapy; RCT: root canal treatment.



FIGURE 3: Selected products, including OraSeal (Ultradent Products GmbH; Cologne, Germany) and Rubber Dam Liquid (Cerkamed; Stalowa Wola, Poland), enhance moisture control during the procedure.

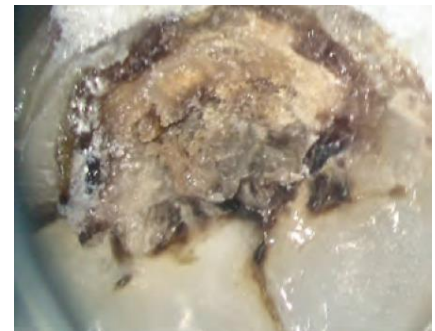


FIGURE 4: A cavitated, grossly carious lower molar, isolated with rubber dam and OraSeal.



FIGURE 5: Non-selective caries removal to hard dentine at the cementoenamel junction.



FIGURE 6: Selective caries removal to firm dentine overlying the pulpal aspect.



FIGURE 7: Left: Selection of burs useful for unroofing the pulp chamber. Right: Series of small-diameter, round burs suitable to use during partial pulpotomy or pulp amputation, ideally used with magnification and illumination.

periphery of the cavity to hard dentine (**Figure 5**), to provide an optimal environment for bonding and sealing of the cavity.¹

Selective caries removal to firm dentine (**Figure 6**) is characteristically resistant to excavation using hand instruments. The assessment of the level of activity of the carious lesion is subjective.⁸ Actively progressing lesions tend to be light brown in colour, contain a higher degree of moisture and are easily disrupted with a sharp probe.⁸ As the level of activity reduces, the colour becomes darker and the moisture content reduces.⁸ Non-selective caries removal poses a known risk of unintentional pulp exposure in the presence of deep caries.¹⁰ Treatment strategies aimed at avoiding pulp exposure include one-stage selective caries removal, or two-stage stepwise excavation, usually to firm or soft dentine, respectively. Indirect pulp capping generally requires caries

removal to hard dentine, with a reported exposure rate of 35.5%¹⁰ now regarded as overtreatment.¹¹ Indirect pulp therapy (IPT) should be reserved for cases where caries has extended no deeper than the pulpal quarter and has been diagnosed as reversibly inflamed.¹ Strategies aimed at avoiding pulp exposure are recommended.¹

In cases presenting with a history and clinical findings indicating moderate to severe pulpitis,⁹ with a radiographic appearance of extremely deep caries, partial or full pulpotomy could offer a conservative alternative to traditional pulpectomy and RCT (**Table 2**). Magnification and illumination optimise visibility. Prior to unroofing the pulp chamber, under constant water coolant, new sterile burs should be selected (**Figure 7**). The use of rosehead burs, or ultrasonic tips is not recommended, as they are likely to damage the pulp tissues.

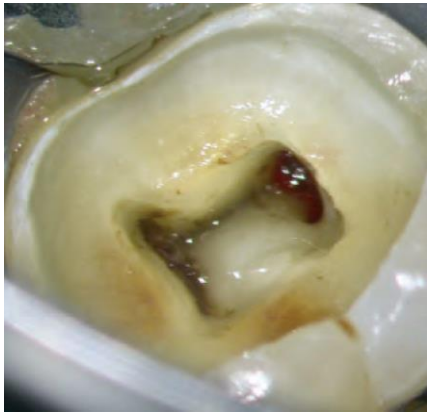


FIGURE 11: Haemostasis achieved over the mesial root canal system (a small bleed may occur on removal of the cotton pellet, which should arrest with gentle rinsing).



FIGURE 13: A hydraulic silicate cement (Biodentine) has been placed incrementally into the pulp chamber.

interface with GICs.²⁹ GICs are not advocated as the definitive restorative material over HSCs. The implications of providing immediate restorations over HSCs (Figures 16 and 17) have not been conclusively answered in the materials research to date. Alternatively, delaying the permanent restoration for up to two weeks after placing the HSC could allow sufficient maturation of the matrix to withstand contraction forces during subsequent adhesive restoration (Figure 17).^{30,31}

Follow-up

Clinical review should be carried out within six months, noting any symptoms and responses to sensibility tests.¹ After one year, a periapical radiograph is recommended in addition to the clinical examination, bearing in mind that no response to thermal stimuli could be expected in teeth that have had full coronal pulpotomy. The prognosis of coronal pulpotomy in the management of irreversible pulpitis is reported to range between 75% and 95%.^{13,14,16} More

high-quality comparable studies are necessary to clarify gaps in the research to date and practitioners are advised to keep updated in this rapidly evolving area.

Summary/key points

- A thorough, documented review of the patient history and interpretation of appropriate sensibility tests, as well as radiographs, remains key to guiding the clinician towards indirect or direct pulp therapy.
- The patient should be informed regarding the possibility and implications of pulpal sequelae, either intra- and/or postoperatively, before initiating treatment.



FIGURE 12: Two hydraulic silicate cements: ProRoot MTA (Dentsply Sirona) and Biodentine (Septodont).



FIGURE 14: A selection of carriers suitable for hydraulic silicate cement placement (from left): Amalgam carrier, MTA+ Applicator (Cerkamed; Stalowa Wola, Poland), Micro Apical Placement (MAP) System (Dentsply Maillefer; Vevey, Switzerland).



FIGURE 15: Machou Hand Pluggers (Dentsply Sirona; Charlotte, USA).

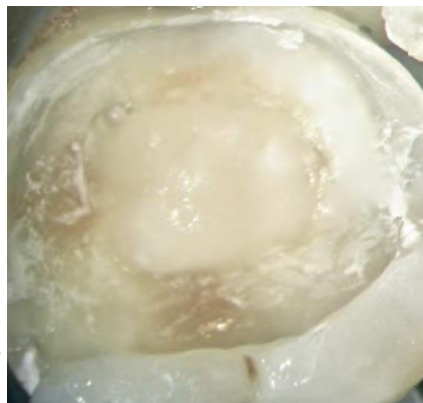


FIGURE 16: Glass ionomer cement overlaid onto the hydraulic silicate cement.



FIGURE 17: Completed composite restoration.

- An aseptic technique, using suitable antiseptic strategies and careful tissue handling, is essential to enhance the outcome of VPTs.
- Magnification and illumination are strongly recommended.
- If haemorrhage control cannot be achieved within a reasonable timeframe, consider a deeper level of amputation (in partial pulpotomy) or, during a full pulpotomy, extension to pulpectomy may be necessary.
- HCSs are the materials of choice for direct pulp therapy.
- Definitive restoration is recommended as soon as possible.
- Clinical review should be carried out within six months, supplemented with radiographic examination at one year (sooner, should symptoms arise).

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