Dental Treatment and Salivary Antimicrobial Peptide LL-37 in Children with Caries: A Pilot Study

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

‘’Make those who see you bless those who raised you.’’

To my beloved family and friends, I dedicate this to you.

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ABSTRACT

Aims: To investigate the relationship between caries experience and the salivary concentration of LL-37 in children and to determine its role as a diagnostic marker in predicting caries experience in children. **Methods:** At baseline, unstimulated whole saliva was collected from 21 systemically healthy children aged 6-10 years old before dental extraction under general anaesthesia (GA). Eleven children were successfully followed one month after dental treatment and a second saliva sample was obtained from them during the review appointment. Their caries status was recorded before dental treatment. The pre-and post-treatment salivary concentration of LL-37 was determined by enzyme-linked immunosorbent assay (ELISA). **Results:** LL-37 was detected in all saliva samples and its concentration varied widely among the participants. Following extraction of carious teeth, the salivary concentration of LL-37 significantly reduced with a -90.94 ng/ml mean value of concentration change. No correlations between pre-treatment salivary concentration of LL-37 with the numbers of decayed teeth (DT/dt) or surfaces (DS/ds) were observed. No statistically significant differences were found among participants with different DT/dt levels. **Conclusion:** The reduced expression of the salivary concentration of LL-37 following dental extraction in children suggests that the salivary antimicrobial peptide (AMP) LL-37 plays an important role in innate immunity against oral infection and inflammation.

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 Dental caries

The oral cavity harbours complex communities of microorganisms including bacteria, viruses, fungi, mycoplasmas, Archaea and protozoa (Wade, 2013). These diverse and dynamic communities live together and can involve additive, synergistic or antagonistic relationships (Marsh, 2005). However, any disturbances to this ecosystem due to alterations in the host lifestyle, behaviour or health increases the risk of developing infectious oral diseases, of which dental caries and periodontal disease are the most common forms (Roberts and Darveau, 2015, Marsh, 2003).

Dental caries is a chronic, infectious disease that occurs due to the imbalance between the remineralisation and demineralisation process of the dental hard tissue. Keyes (1960) was the first to provide convincing evidence that dental caries is an infectious, transmissible disease in rodents. Dental caries occurs as the result of the interaction of three major factors: the causative microorganisms, a cariogenic diet, and host-related factors (including susceptible tooth, lifestyle, and behavioural aspects) (Marsh et al., 2009). Only a relatively small number of species have been consistently associated with the pathophysiology of dental caries, despite many oral microorganisms whose potential roles have been investigated.

The most widely reported microorganisms to have a strong association with the initiation and development of dental caries in humans are the early colonisers consisting of *Streptococcus mutans* and *Streptococcus sobrinus* (Pitts et al., 2017, Tanzer et al., 2001, Marsh, 1999, Loesche, 1986, VAN and HOUTE, 1980). The virulence properties of these species arise from their ability to adhere to the tooth surface, form dental plaque biofilm, ferment dietary carbohydrates to acids as well as produce extracellular and intracellular polysaccharides (Horiuchi et al., 2009, Selwitz et al., 2007). The production of bacterial acids not only promotes tooth surface demineralisation but also lowers the dental plaque pH which selectively allows the growth and survival of the acid-tolerant bacterial strains (Baker et al., 2017).

The literature widely supports the theory that early acquisition and colonisation, as well as high levels of oral Streptococcus mutans in children, are associated with an increased risk of future development of dental caries (Barsamian-Wunsch et al., 2004, O'Sullivan and Thibodeau, 1996, Thibodeau and O'Sullivan, 1995). However, the microbiological risk factor can be compensated for by the effect of other factors, such as oral hygiene and diet.

Initially, *Lactobacillus* species were considered the major aetiological factor contributing to dental caries (Loesche et al., 1984). There is now an accepted consensus that *lactobacilli* colonise and favour acidophilic dental plaque, therefore, playing a role in facilitating caries progression rather than the initiation of carious lesions (Caufield et al., 2015). Other reports suggest that other acidogenic and acidophilic bacterial species, including *Actinomyces* species and *Bifidobacterium* species, can also contribute to the acidification of the dental plaque and initiation of the caries process in the absence of *Streptococcus mutans* (Van Ruyven et al., 2000, Van Houte et al., 1996, Van Houte, 1994, Sansone et al., 1993). This was supported by a study carried out by Acevedo et al. (2009) that found no significant difference in the frequency of *Streptococcus mutans* in the dental plaque of caries-free and caries-affected children.

Despite the dramatic reduction in the prevalence of dental caries since the introduction of fluoride toothpaste, it remains a major health issue worldwide (König, 2004, Marthaler, 2004). The Global Burden of Disease Study in 2017 estimated that more than 530 million children suffer from caries of the primary dentition, while 2.3 billion people suffer from caries of the permanent dentition (James et al., 2018). The Oral Health Survey of five-year-old children carried out in 2017 reported that although oral health is improving in the United Kingdom, almost 23.4% of five-year-old children have dental caries. According to Public Health England data, extraction due to dental caries is the most common reason for hospital procedures in under 18-year-olds in England with 38,385 extractions due to dental caries in 2017-2018 (GOV.UK, 2019).

Several significant morbidities are associated with dental caries in children including psychological and social issues, increased school absenteeism, as well as speech and sleeping problems (BaniHani et al., 2018, Acs et al., 1999, Low et al., 1999). According to a study carried out by Goodwin et al. (2015), almost half the children waiting for dental treatment under GA experienced pain and a quarter missed an average of three school days due to their dental problems. BaniHani et al. (2018) reported that dental caries not only has a negative impact on the oral health-related quality of life (OHRQoL) of children but also impact their parents/carers. In the previous study, the majority of parents/carers reported that they felt more distressed, guilty and upset due to their children's dental problems, especially pain (BaniHani et al., 2018). In addition, dental diseases continue to compromise a significant portion of the total United Kingdom expenditure on health care. It was estimated that the National Health Service (NHS) spends about £3.4 billion per year in direct treatment costs for children and adults due to dental disease (NHS England, 2014).

1.2 Saliva and dental caries

Saliva is a relatively neutral (pH= 6.2 - 7.6) mucoserous exocrine fluid. This unique biological fluid comprises a mixture of secretions from major salivary glands (the parotid, submandibular and sublingual glands) as well as the minor salivary glands and gingival crevicular fluid (GCF) (Soares Nunes et al., 2015, Humphrey and Williamson, 2001). The whole saliva also contains hormones, electrolytes, antimicrobial compounds, enzymes, blood derivatives, microorganisms, and their products, desquamated epithelial cells, as well as food debris (Tenovuo, 1997).

Saliva plays a major role in providing a protective function for both the soft and hard oral tissues. In addition to protecting the oral tissues, the aiding of food-related functions such as swallowing, tasting, and oral digestion are also important physiological functions of saliva (Kaufman and Lamster, 2002). Saliva plays a crucial role in the maintenance of hard tissue integrity and protection against caries mainly through salivary clearance, the salivary buffering system, balancing the demineralisation and remineralisation process, as well as acting as a delivery mechanism for the antimicrobial activities it contains.

1.2.1 Salivary clearance

The salivary clearance, or the oral clearance capacity, is the process by which saliva dilutes and eliminates microorganisms, desquamated epithelial cells and dietary components from the oral cavity to the gastrointestinal tract (Pedersen and Belstrøm, 2019). This function is achieved by the constant flushing action of saliva and is mainly related to the salivary flow rate, the volume of saliva in the mouth and the swallowing frequency. The average daily flow rate of unstimulated saliva in a healthy person ranges between 0.3-0.4 ml/min (Iorgulescu, 2009). During unstimulated salivary flow, approximately 65% of the salivary volume is contributed to by the submandibular glands, 20% from the parotid glands, 5% to 8% from the sublingual glands and less than 10% from the numerous minor glands (Soares Nunes et al., 2015, Humphrey and Williamson, 2001). Upon stimulation by various factors such as diet and medications, the flow rate can increase to approximately 7ml/min (Cunha-Cruz et al., 2013). It has been shown that a chronically low salivary flow rate is associated with increased caries risk and it has been proposed that this could be attributed to poor salivary resistance against microbial attack. Spak et al. (1994) found that patients diagnosed with xerostomia as a complication following radiotherapy to the head and neck region experienced higher caries activity than patients with normal salivary flow rate.

1.2.2 Salivary buffer capacity and pH

The salivary buffering system aids in neutralizing dietary acids and bacterial acids in the dental plaque and regulating the pH in the oral cavity through the action of the bicarbonate, phosphate, and multiple proteins buffering systems. When the stimulated salivary flow rate rises, the key system to counteract an acidogenic challenge is the bicarbonate system. The concentration of the bicarbonate in saliva and thus the pH is mainly dependent on the salivary flow rate. On the other hand, the inorganic phosphate system is the major buffering agent in resting saliva when salivary flow is low. Although weak, an inverse relationship between the buffering capacity of saliva and caries experience has been established (Hicks et al., 2003, Ericsson, 1959). This demonstrates the protective role provided by the acid-neutralizing effect of saliva.

Demineralisation and dissolution of the tooth surface occur when the pH falls below the critical value of 5.5 - 5.7. At this pH, the hydroxyapatite crystals disintegrate from the tooth structure releasing the phosphate ions into the saliva contributing to restoring the pH balance. Remineralisation is promoted by several salivary proteins, mainly the proline-rich proteins, mucins, histatins, cystatins, and statherins. These proteins have the ability to bind and inhibit the spontaneous precipitation of calcium and phosphate crystals and neutralize the bacterial acids (Lenander-Lumikari and Loimaranta, 2000). However, they also have a role in the formation of the acquired pellicle by facilitating bacterial aggregation and enhancing early bacterial colonisation. For example, proline-rich proteins have been demonstrated to facilitate the binding of bacteria to the tooth surface by adhering to the carboxy-terminal domain of the proline-rich proteins. However, they also neutralize the acids produced by streptococci, hence these proteins can both facilitate and hinder the activity of bacteria (Nireeksha et al., 2017).

1.3 Antimicrobial activities of saliva

1.3.1 Salivary immunoglobulins

One of the important roles of saliva is to provide the first line of defence against microbial infection and invasion as well as help in shaping the diverse resident microflora. These roles are accomplished by several immune and non-immune antimicrobial agents, namely immunoglobulins (Ig), salivary peroxidase systems, lysozyme, lactoferrin and antimicrobial peptides (AMPs). It is well known that these agents act additively or synergistically to inhibit microorganism growth. These agents inhibit oral microorganism invasion by four main methods including aggregation, adherence, microbial cell killing, and interference with microbial glucose uptake and metabolism (Law et al., 2007, Van Nieuw Amerongen et al., 2004b, Tenovuo et al., 1991).

The immunologic salivary agents include different classes of antibodies, IgA, IgG, and IgM and they compose about 5-15% of whole salivary proteins (Van Nieuw Amerongen et al., 2004b). The secretory IgA (s-IgA), a subclass of IgA, constitutes about 60% of the immunoglobulin in saliva. It is synthesized and produced by plasma cells in the connective tissues and translocated through the ductal cells and secreted into saliva via salivary glands. S-IgA exerts its antimicrobial action through several mechanisms including neutralizing, acting as an opsonin, aggregating, and enhancing the elimination of microorganisms (Van Nieuw Amerongen et al., 2004b, McNabb and Tomasi, 1981). In addition, the most important role of s-IgA in the oral cavity is the prevention of microbial adhesion to mucosal and tooth surfaces, thus inhibiting microbial colonisation. It has also been established that IgA enhances the antimicrobial activity of lactoferrin, peroxidase and lysozyme in saliva (Law et al., 2007). IgG and IgM are

present at lower concentrations in saliva and primarily originate from GCF or serum.

Many studies have been carried out to investigate the association between the level of salivary IgA and dental caries. However, currently, no consensus has been reached in the literature regarding the role of salivary immunoglobulin in caries development. A systematic review and meta-analysis were carried out by da Silva Fidalgo et al. (2014) who concluded that the evidence supports the presence of an association between increased levels of salivary IgA and increased caries experience in subjects. Certainly, multiple studies showed that increased concentrations of s-IgA are associated with an increase in caries experience (Letieri et al., 2019, Ranadheer et al., 2011, Bagherian et al., 2008). Malcolm et al. (2014) found that the concentration of bacteria-specific s-IgA, anti-*Streptococcus mutans* s-IgA, and anti-*Streptococcus sanguinis* s-IgA, is significantly higher in 3-year-old children who were culture positive for *Streptococcus mutans* compared to those who were culture negative. The increased antigenic load and bacterial colonization, especially with *Streptococcus mutans*, in caries-active individuals, stimulate an immunological response resulting in enhanced production of s-IgA (Laputková et al., 2018, Hemadi et al., 2017, Parisotto et al., 2011). On the other hand, an inverse relationship between s-IgA levels and dental caries in children has been reported in the literature (Soesilawati et al., 2019, Pandey et al., 2018, Pal et al., 2013). Other researchers did not observe any association between s-IgA and dental caries in children (Giudice et al., 2019, Shifa et al., 2008, Kirstilä et al., 1998). These contradictory results regarding the association between s-IgA and dental caries might be attributed to two different theories that have been proposed in relation to s-IgA action.

1.3.2 Salivary peroxidase, lysozyme and lactoferrin

The glandular acinar cells produce salivary peroxidase that includes sialoperoxidase, lactoperoxidase, myeloperoxidase, and hydrogen peroxide. These peroxidases interact with thiocyanate and hydrogen peroxide to form hypothiocyanite and cyanosulfurous acid. They have a fundamental role in the oxidization of the bacterial sulfhydryl groups and in inhibiting glucose metabolism (Hicks et al., 2003). In the oral cavity, salivary lysozyme originates mainly from salivary glands and to a lesser extent from the GCF and it is stored in the lysosomal granules of neutrophils and macrophages (Callewaert and Michiels, 2010). It exerts a unique enzymatic activity that results in hydrolysing the bacterial cell wall peptidoglycan leading to the destruction and inhibition of bacterial growth (Callewaert and Michiels, 2010, Pollock et al., 1987). Besides its antibacterial action, it was demonstrated that lysozyme has antifungal and antiviral activities (Fabian et al., 2012).

Lactoferrin has a very different mode of action and is produced in the intercalated ductal cells of the salivary glands. Since iron is essential for bacterial metabolism, lactoferrin exerts its bacteriostatic activity by depriving and depleting iron from the oral environment (Van Nieuw Amerongen et al., 2004a). This phenomenon of starving bacteria of vital nutrients is called nutritional immunity (Mandel, 1976). It has been reported that the level of salivary lactoferrin is negatively correlated with the number of subgingival *Aggregatibacter actinomycetemcomitans* in patients diagnosed with periodontitis (Groenink et al., 1999).

1.3.3 Cationic antimicrobial peptides

AMPs provide protection early and throughout potential infections and are considered an essential component of innate immunity against microbial infection. Forms of AMPs have been identified and evolved across a broad spectrum of organisms from plants and insects through to vertebrates including human beings (da Silva et al., 2012, Hancock, 2001). In 1980, cecropins were the first AMPs to be characterised and they were isolated from the haemolymph of the moth Hyalophora cecropia (Cederlund et al., 2011). This discovery answered an important longstanding question of how insects defend themselves while they lack adaptive immune systems. Hence, it emphasized the importance of investigating and exploring those defence molecules in other species. Indeed, recently, they have been a point of interest for many researchers due to their recognized implications in oral as well as general health (Sierra et al., 2017).

In general, cationic AMPs encompass diverse groups of positively charged (+2 or more) short peptides (Schneider, 2005, Hancock, 2001). Cationic AMPs differ in terms of their amino acid composition, conformational structure, and size, although they are commonly 12-50 amino acids in length (da Silva et al., 2012, Hancock and Diamond, 2000). Most cationic AMPs are amphipathic having both hydrophobic and hydrophilic domains with an average of 40-50% hydrophobic residues (Cederlund et al., 2011, Klotman and Chang, 2006). This amphipathic nature allows these peptides to interact electrostatically as well as integrate with and potentially damage the integrity of the microbial cell membrane (Khurshid et al., 2016). They are considered a crucial component of innate immunity due to their multiple modes of biological activity including inhibition of nucleic acid or protein synthesis as well as induction of membrane poration and cell death. Overall, these peptides insert into the microbial cell membrane leading to

membrane permeability by several models, including the barrel-stave model, toroidal-pore model, or carpet model (Brogden, 2005). In the barrel-stave model, peptides are inserted into the microbial membrane forming a bundle with a central lumen. The hydrophobic regions of the peptide interact with the lipid core (acyl chains) of the microbial membrane and their hydrophilic regions lining the interior region of the pore (Yang et al., 2001, Ehrenstein and Lecar, 1977). In the carpet model, peptides act like a detergent where they accumulate and orient parallel to the microbial membrane surface leading to the formation of non-lamellar micelles or small peptide-lipid aggregates (Ladokhin and White, 2001, Shai, 1999). Finally, in the toroidal (or worm-hole) model, the inserted peptides aggregate and form pores by inducing bending of the lipid monolayer, and hence the pore is lined by both the peptide and the lipid headgroups (Yang et al., 2001, Matsuzaki et al., 1996). It has been shown that the mode of interaction between antimicrobial peptides and microorganisms depends on several factors, including the concentration of the peptide, the sequence and distribution of hydrophilic and hydrophobic amino acids as well as the peptide's secondary structure. Also, the physicochemical properties and lipid composition of the microbial membrane can also influence the mode of interaction (Schreier et al., 2014). AMPs also play an important role as immunomodulatory agents in both the innate and adaptive immune functions by inducing the production of pro-inflammatory cytokines, acting as chemokines, and enhancing the activity of the immune cells (Klotman and Chang, 2006, Brogden, 2005, Scott et al., 2002).

The expression pattern of AMPs varies across the different tissues and ecological niches within the human body. In humans, at least three families of antimicrobial peptides can be distinguished in saliva: defensins, histatins and cathelicidins.

1.3.3.1 Salivary defensins

Human defensins are cationic peptides that are divided into two main subfamilies: α- and β-defensins, due to differences in the spacing and pairing of the six conserved cysteine amino acids (Fabian et al., 2012). Their β-sheet-rich fold is stabilized by two or three intramolecular disulphide bonds linking cysteine residues. In humans, α-defensins consist of six types and are further subdivided into two groups: Human neutrophil peptide (HNP)-1 to -4, and human (enteric) defensins (HDs)-5 and -6. HNPs-1-4 are stored in the primary (azurophilic) granules of neutrophils, while HDs-5 and -6 are mainly found in mucosal Paneth's cells of the small intestine and epithelial cells of the female urogenital tract (Pisano et al., 2005, Dale et al., 2001, Quayle et al., 1998, Ouellette and Selsted, 1996). HNPs-1-4 constitute approximately 30-50% of the total proteins in the neutrophil's azurophilic granules, and nearly 5-7% of the total protein in the entire cell (Diamond et al., 2008, Rice et al., 1987). Human β-defensins (hBDs) are expressed by epithelial cells of various tissues throughout the body, such as oral mucosa, skin, respiratory tract, gastrointestinal tract, genitourinary tract, and kidneys (Gorr, 2012, Sahasrabudhe et al., 2000, Mathews et al., 1999, Zhao et al., 1996). HBD-1 is constitutively expressed, whereas hBD-2 and hBD-3 are upregulated and induced by microbial insults and proinflammatory cytokines, such as L1-1β, TNF- α, lFN-γ (Dale and Fredericks, 2005, García et al., 2001, Mathews et al., 1999). This might confirm the role of hBD-1 in inhibiting the transition to pathogenic microflora, while hBD-2 and hBD-3 may be more effective against pathogens (Dale and Fredericks, 2005). Despite several hBDs had been identified, only four have been investigated elaborately: hBD-1, hBD-2, hBD-3, and hBD-4.

Genes encoding both α-defensins and β-defensins have been located in a cluster on chromosome 8p23, except HNP-2, which is a truncated product of either HNP-1 or HNP-3 (Linzmeier et al., 1999, Liu et al., 1997, Sparkes et al., 1989). θ-defensins are only found in leukocytes of rhesus macaque monkeys and it has been proposed that they evolved from a mutation in α-defensins (Yi-Quan et al., 1999).

In the human oral cavity, only HNPs-1-4 and hBDs-1-3 are expressed. HNPs-1-4 are found in saliva, GCF, and junctional epithelium mainly deriving from the circulating neutrophils (Kohlgraf et al., 2010, Dale et al., 2001, McKay et al., 1999). Fanali et al. (2008) found that edentulous patients express lower levels of HNPs-1-3 in the whole saliva compared to dentate patients and it is most likely due to a lack of GCF. In contrast, hBDs-1-3 are detected in the differentiated layers of the gingival epithelium, tongue, salivary glands, and ducts, as well as the palate and buccal mucosa (Lu et al., 2005, Dunsche et al., 2002, Dale et al., 2001, Mathews et al., 1999).

The innate immunity function of defensins as broad-spectrum antibacterial peptides against both Gram-positive and Gram-negative bacteria arises from their positive net charge, which allows them to interact and damage the integrity of the negatively charged bacterial cell membrane (Fabian et al., 2012). These peptides are also known to exhibit antimicrobial activities against fungi, protozoa, and some strains of enveloped and non-enveloped viruses (Lehrer and Lu, 2012, Hancock and Diamond, 2000). In addition to their role in innate immunity, defensins promote adaptive immunity by recruiting leukocytes, such as dendritic cells and T-cells (Yang et al., 2000, Yang et al., 1999). Niyonsaba et al. (2001) evaluated the effects of hBD-1, hBD-2 and LL-37 on mast cell functions and found

that hBD-2 and LL-37 stimulate mast cells to release histamine and/or generate prostaglandin D2 (PGD_2).

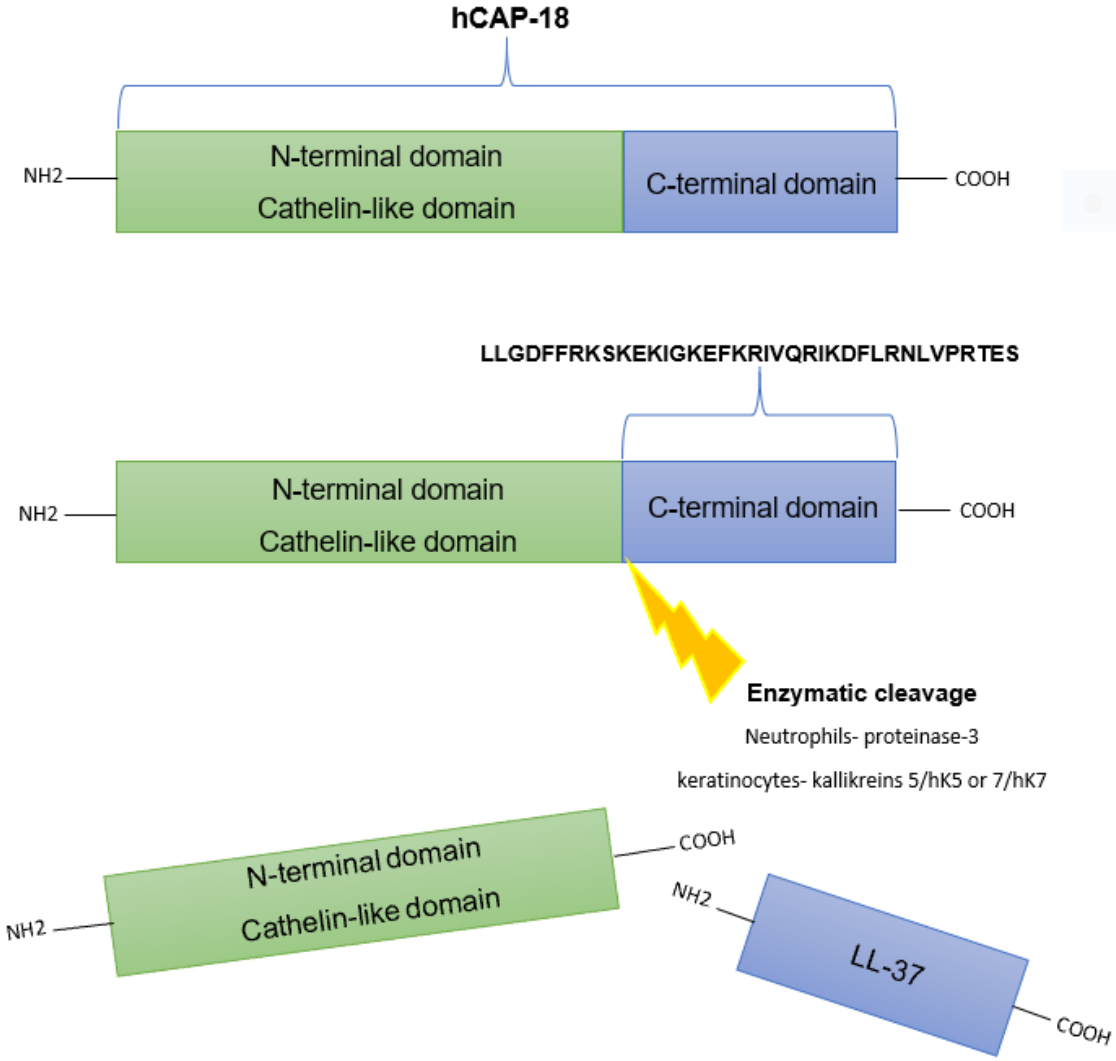
1.3.3.2 Salivary histatins

Histatins are a family of salivary proteins that range from 7-38 amino acids in length and contain at least 12 histidine residues. Those small cationic peptides are mainly synthesized and secreted by the parotid and the submandibular salivary duct cells. In 1988, histatins (HST)-1,-3,-5 were isolated from human saliva and they are the most common forms found in humans and higher primates and account for about 85% of the total histatin proteins in saliva (Khurshid et al., 2016, Kavanagh and Dowd, 2004, Oppenheim et al., 1988). Although they are well known for their broad-spectrum antibacterial, antiviral, and antifungal actions, HST-5 has been demonstrated to be primarily most effective against commensal fungi - Candida Albicans (Edgerton et al., 1998, Oppenheim et al., 1988). It was shown that HST-5 exerts its antifungal activity through binding to specific receptors on the fungal cell wall and targeting the mitochondria leading to enhanced production of reactive oxygen species and hence to cell lysis (Helmerhorst et al., 2001). In addition to their antimicrobial action, they also contribute to the formation of the acquired enamel pellicle, thus playing a role in bacterial colonisation on the tooth surface (MacKay et al., 1984)

1.3.3.3 Cathelicidin

All cathelicidins in the animal kingdom share a similar structure characterized by a highly conserved N-terminal domain of approximately 100 amino acid residues and a C-terminal domain that contains antimicrobial and immunomodulatory properties (Greer et al., 2013). Cathelicidin is synthesized as a pro-peptide and stored as a biologically inactive form in the granules of some cells, such as in the specific (secondary) granules in neutrophils. Upon stimulation, the inactive precursor is released from the intracellular environment and is enzymatically cleaved to produce the active, mature form of the peptide. In humans, hCAP-18 (human cationic antimicrobial peptide) is the only known member of the cathelicidin family that has been identified and has an approximate molecular weight of 18 kDa. It is encoded by the cationic antimicrobial peptide (CAMP) gene, located at chromosome 3p2, and consists of four exons. The active peptide LL-37 is derived by the extracellular cleavage of hCAP-18 by serine proteases such as proteinase-3 in neutrophils, which cleaves between the alanyl and leucyl residues, while kallikreins 5/hK5 or 7/hK7 in keratinocytes produce shorter fragments of the peptide, such as RK-31 and KS-30 (Figure 1) (Yamasaki et al., 2006, Sørensen et al., 2001, Sørensen et al., 1997). Cathelicidins are constitutively expressed at low levels by most epithelial cells, while their expression in keratinocytes is induced (Frohm et al., 1997). In leukocytes, the expression of cathelicidin is variable and complex depending mainly on the type of cell that is stimulated.

Figure 1-1: Basic structure of the human hCAP-18 cathelicidin peptide and formation process of LL-37 peptide.



1.3.3.3.1 Salivary LL-37

LL-37 is composed of 37 amino acid residues starting with two N-terminal leucine residues. It is a linear, amphipathic, cationic peptide with a positive net charge of +6 under physiological conditions (pH 7.0) (Xhindoli et al., 2016, Henzler Wildman et al., 2003). Upon interaction with the bacterial cell membrane, the active peptide folds into an amphipathic α-helical structure (Johansson et al., 1998). LL-37 is expressed in various types of cells throughout the human body, including the epithelial cells of the intestine, respiratory system, genitourinary system, and gastrointestinal tract (Hans and Madaan Hans, 2014, Fabian et al., 2012). In addition to its expression by the epithelial cells, LL-37 is produced by neutrophils, monocytes, macrophages, natural killer cells, dendritic cells, and mesenchymal stem cells (Bandurska et al., 2015). In the oral cavity, Dale et al. (2001) detected this peptide within the connective tissue and the junctional epithelium. A recent study has found that desquamated salivary oral epithelial cells express hCAP18 that is confined to a peripheral part of the cytoplasm corresponding to the cell plasma membrane (Aidoukovitch et al., 2020). In addition, it was reported that LL-37 is detected in the whole saliva at concentrations ranging from 0.14–3 µg/ml, and the secretion rate of this peptide was not influenced by the rate of salivary flow (Bachrach et al., 2006, Tao et al., 2005).

It has been established that 1,25-dihydroxy vitamin D (vitamin D_3), pro-inflammatory cytokines, insulin-like growth factor-1 (IGF-1), butyrate, hypoxic conditions, and microbial products can upregulate cathelicidin expression (Cederlund et al., 2011, Peyssonnaux et al., 2008, Hase et al., 2002, Wu et al., 2000). Vitamin D_3 induces hCAP-18 synthesis in various cell types by binding to the vitamin D receptor (VDR) that triggers the vitamin D response elements (VDRE) region in the CAMP gene promoter sequence (Dixon et al., 2012, Liu et al., 2007, Gombart et al., 2005).

1.3.3.3.2 LL-37 mechanism of action

The direct antibacterial action of LL-37 is mediated by binding and integrating into the bacterial cell membrane bilayer, resulting in electrostatic interactions and hence bacterial cell lysis (Turner et al., 1998). Electrostatic attractions, as for all cationic peptides, occur between the lipopolysaccharide (LPS) in the outer membrane of the Gram-negative bacteria or the lipoteichoic acids in the Gram-positive bacteria, as well as phospholipids in the bacterial cytoplasmic membranes (Schneider et al., 2005). Henzler Wildman et al. (2003) suggested that the bactericidal mechanism of LL-37 arises from its ability to cover the bacterial cell membrane and form toroidal pores by inducing positive curvature strain or forming less well-defined membrane defects. In contrast, the mammalian cells were found to be protected from the action of AMPs due to the presence of cholesterol that rigidifies the plasma membranes and increases the bilayer thickness, thus impeding the penetration of peptides (Matsuzaki et al., 1995). In addition, the membranes of mammalian cells are mainly composed of zwitterionic phosphatidylcholine and sphingomyelin phospholipids which do not electrostatically interact with cationic AMPs. Thus, AMPs will preferentially interact with the negatively charged bacterial membranes. However, it was shown

that cathelicidins, unlike other helical antimicrobial peptides, only have moderate selectivity for anionic bacterial cells. In addition, at higher concentrations compared to the bactericidal activity, it can exert cytotoxic and apoptotic effeconrds different eukaryotic cells *in vitro* (Henzler Wildman et al., 2003, Johansson et al., 1998).

LL-37 exerts pleiotropic effects and in addition to the biological activities, this peptide also has antimicrobial properties. It is known to have broad-spectrum activity against Gram-negative and Gram-positive bacteria, viruses, parasites as well as fungi (Xhindoli et al., 2016, Kai-Larsen and Agerberth, 2008). At sub-bactericidal concentrations, LL-37 was shown *in vitro* to enhance the uptake of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* by neutrophils and macrophages via opsonization and agglutination (Sol et al., 2013). Nizet et al. (2001) found that cathelicidin-related antimicrobial peptide (mCRAMP) knockout mice were more prone to a necrotic skin infection caused by group A streptococcus. In addition, Pütsep et al. (2002) reported that patients with Morbus Kostmann disease, severe congenital neutropenia, suffer from the early-onset and recurrent periodontal disease despite being treated by a recombinant granulocyte-colony stimulating factor (GCSF). Treatment with recombinant GCSF restores the neutrophil levels, however, these patients remain deficient in LL-37 and α-defensin HNP-1 which results in the overgrowth of *Aggregatibacter actinomycetemcomitans* (Pütsep et al., 2002). Another syndrome associated with early-onset periodontitis is Papillon-Lefèvre syndrome in which the genetic defect is a mutation in the lysosomal protease cathepsin C gene (Hart et al., 1999). These patients lack active neutrophil serine protease that is necessary for the generation of LL-37 from hCAP-18, thus leading to LL-37 deficiency and consequently robust proliferation of *Aggregatibacter*

actinomycetemcomitans (Eick et al., 2014). Hence, the previous findings demonstrate the importance of this peptide in combating pathological microorganisms as well as its role in modulating both innate and adaptive immunity.

High salt concentrations and other host or microbial factors were found to impair most oral AMPs' bactericidal activities including those of LL-37 (Nagaoka et al., 2000). It was shown that the concentration of LL-37/hCAP-18 in the airway surface fluid of cystic fibrosis patients correlated positively with the degree of pulmonary inflammation and disease severity (Chen et al., 2004). However, the antibacterial activity of LL-37 is significantly impaired in these patients due to altered ionic and hydration states as well as direct interaction with anionic polymers of DNA and filamentous (F)-actin which leads to the formation of bundled aggregates (Bucki et al., 2007, Weiner et al., 2003, Vasconcellos et al., 1994). Overhage et al. (2008) demonstrated *in vitro* that LL-37 at very low concentrations affects the development of and the pre-grown *Pseudomonas aeruginosa* biofilms. This is the most prevalent pulmonary pathogen in patients with cystic fibrosis that can eventually leads to fatal lung disease. The anti-biofilm effects of LL-37 are mainly achieved by stimulating bacterial surface motility and reducing the adherence of bacterial cells to surfaces via increasing the twitching motility of Pseudomonas aeruginosa (Overhage et al., 2008). However, several studies have demonstrated that numerous pathogens, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*, are capable of degrading and inactivating AMPs, including LL-37, via producing proteinases (Sieprawska-Lupa et al., 2004, Schmidtchen et al., 2002). These findings represent one of the measures by which bacterial pathogens can evolve to resist the action of the innate immune system mediated by AMPs.

LL-37 has also been reported to have roles in angiogenesis (Koczulla et al., 2003), re-epithelization of wounds (Ramos et al., 2011), stimulation of an immune response and acting as a chemoattractant (Davidson et al., 2004), neutralizing bacterial LPS as well as regulating apoptosis (Coffelt et al., 2009). LL-37 has contradictory and complex roles in tumorigenesis and the mechanism is tissue specific. Overexpression of LL-37 was found to induce tumorigenesis in ovarian, lung and breast cancers (Wu et al., 2010). In contrast, LL-37 displays an anti-tumour effect in colon cancer and gastric cancer (Ren et al., 2012).

Finally, it is known that LPS are large molecules that are found in the outer membrane of Gram-negative bacteria. These molecules are the principal virulence factors that cause massive cytokine release and hence septic shock. Nagaoka et al. (2001) found that LL-37 inhibits the production of the pro-inflammatory cytokine tumour necrosis factor α (TNF)-α by mononuclear phagocytes by blocking the binding of LPS to $CD14^{+}$ on these cells. The same group of researchers investigated the effects of LL-37 on the LPS-induced endothelial cell apoptosis *in vitro* and *in vivo* and they found that LL-37 suppresses the assembly of the LPS receptor complex, thus preventing endothelial cell apoptosis (Suzuki et al., 2011). Their ability to block macrophage activation and suppress host cell apoptosis suggests that they have a role in the prevention of an overwhelming immune response that can lead to sepsis and subsequently death. LL-37 and defensins also have been shown to promote the migration of monocytes, T-cells, neutrophils and mast cells into the site of inflammation by acting as a chemoattractant, thus enhancing the adaptive immune response against microbial threats (Niyonsaba et al., 2002b, Niyonsaba et al., 2002a, Chertov et al., 1996).

1.3.3.4 Synergistic actions of LL-37 and other AMPs

Although AMPs have different roles and mechanisms of action against microorganisms, they act in concert with other defence mechanisms to combat infection and inflammation. A synergistic interaction has been demonstrated previously between LL-37 and HNP-1 leading to a higher efficiency microbicidal activity against *E. coli* and *Staphylococcus aureus* (Nagaoka et al., 2000). Also, Bedran et al. (2014) showed that LL-37 and HBD-3 act in synergy to reduce the secretion of cytokines by an LPS-stimulated three-dimensional (3D) co-culture model of gingival epithelial cells and fibroblasts. It was found *in vitro* that any combination of HBD-2, HBD-3 or LL-37 increased the antimicrobial activity against *Streptococcus mutans*, suggesting an additive interaction between these peptides (Phattarataratip, 2010). The previous finding agrees with a study done by Singh et al. (2000), which reported an additive interaction between β-defensins and LL-37 against *E. coli*.

1.4 Dental caries and salivary AMPs

Several previous studies have investigated the relationship between dental caries and salivary AMPs, however, there is a lack of conclusive information, and no consensus has been reached to date. Regarding α-defensins, Tao et al. (2005) reported low levels of salivary HNPs-1-3 in caries active 11- to 15-year-old participants. However, the levels of LL-37 and HBD-3 did not correlate with caries experience. In addition, caries active children did not demonstrate high levels of salivary *Streptococcus mutans*, and salivary levels of HNPs-1-3 did not correlate with salivary *Streptococcus mutans* counts in the previous study. In contrast, Malcolm et al. (2014) showed that higher salivary concentrations of the HNPs-1–3 and LL37 correlated positively with higher *Streptococcus mutans* counts. Davidopoulou et al. (2012) found low salivary levels of LL-37 in patients with dental caries and suggested that this peptide could be used as a prognostic tool or therapeutic agent for patients with caries. Two further studies did not find any significant differences between the salivary levels of LL-37, hBD-2 and hBD-3 in patients with dental caries compared to caries-free subjects and they did not support the measurement of salivary antimicrobial peptide levels as a reliable tool to predict caries risk (Colombo et al., 2016, Phattarataratip, 2010). However, Colombo et al. (2016) reported a positive correlation between salivary HST-5 levels and *Streptococcus mutans* counts. Ribeiro et al. (2013) found that the presence of HNP-3 and HBD-3 showed a reduction in the incidence of early childhood caries (ECC) when evaluating the salivary peptide profiles in 10- to 71-month-old children. A different report showed no statistically significant difference in the salivary level of HNPs-1-3 between caries-active and caries-free 3 to 5-year-old children (Toomarian et al., 2011). The salivary levels of HBD-2 and HST-5 were significantly higher in children with ECC compared to the caries-free

controls and the levels correlated with the severity of dental caries (Jurczak et al., 2015). Sun et al. (2016) evaluated the salivary profiles of children aged 3-5 years with severe early childhood caries (S-ECC) at different time points before, one and four weeks after dental treatment, utilizing western blot and magnetic bead (MB)-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The results of this study showed that the levels of salivary HST-1 were significantly higher in children with S-ECC for four weeks after dental treatment compared with pre-treatment. Similarly, a longitudinal study that utilized the MALDI-TOF MS technique for peptide analysis found that increased concentration of salivary histatin correlated with increased ECC incidence in children (Ao et al., 2017). Differences in the results among studies can be attributed to multiple factors, such as sample size, participants' demographics, the index used for dental caries diagnosis and assessment, as well as the method of AMPs detection and analysis.

1.5 Fasting and salivary concentration of AMPs

It is well known that food ingestion is a strong stimulus for the secretion of saliva. However, information regarding the effect of fasting on the concentration of salivary proteins, including AMPs, is scarce. A recent systematic review reported that salivary flow rate decreased by 10% during Ramadan in fasting subjects due to the lack of food and mastication stimulation (Besbes et al., 2022). Moreover, a study conducted on a group of adults fasting during Ramadan showed that total salivary protein and salivary acidity significantly increased after breaking the fast (Illahi et al., 2016). Another study found that food intake between 2 p.m. and 4 p.m. was associated with an increase in the salivary concentration of proline-rich proteins and satethrin (Brandão et al., 2014). The previous authors concluded that variations in salivary protein concentration throughout the day are related to fasting and fed states of subjects, as well as a circadian rhythm rather than the salivary flow rate.

1.6 Research aim and hypotheses

1.6.1 Aim

The present study aimed to investigate the relationship between caries experience and the salivary concentration of LL-37 in children and to determine its role as a diagnostic marker in predicting caries experience in children.

1.6.2 Objectives

- To assess and compare the effect of dental treatment on the salivary concentration of LL-37 using a commercially available LL-37 human enzyme-linked immunosorbent assay (ELISA) kit.
- To assess if changes in the salivary concentration of LL-37 pre- and post-dental treatment reflect the changes in caries experience in children.

1.6.3 Null hypothesis

There are no differences in salivary concentrations of the AMP LL-37 in children prior to and after dental treatment to remove their carious teeth.

2 MATERIALS AND METHODS

2.1 Study design

This research was designed to be a pilot study involving the measurement of salivary levels of the AMP LL-37 in children receiving dental extractions under GA. All eligible participants were asked to provide a saliva sample on the day of their dental treatment prior to undergoing GA. The participants were asked to provide a second saliva sample one month after their dental treatment during their routine dental review appointment following the treatment. Salivary level of LL-37 was measured using ELISA and changes in its concentration pre- and post-dental treatment (dental extractions) were assessed in relation to changes in the number of decayed primary or permanent teeth (DT/dt) and decayed surfaces (DS/ds).

2.2 Ethical and regulatory aspects

The study protocol and written consent forms were approved by the Research Ethics Committee (REC) committee of Yorkshire (Appendix A) & The Humber - Leeds West Research Ethics Committee and Health Research Authority (HRA) (Appendix B). The REC reference number is 19/YH/0119. Following this, the study received approval from the Leeds Research and Innovation (R&I) committee for it to be conducted at Leeds Teaching Hospitals NHS Trust (LTHT R&I number: DT19/124003 (Appendix C).

The chief investigator (FA) ensured that this study was conducted in full conformance with United Kingdom laws and regulations in which the research was conducted and as per the World Medical Association Declaration of Helsinki. The written consent and assent forms were all signed by the participant child and his/her parents/guardians before entering the study.

2.3 Training

Prior to the commencement of the study, the chief investigator (FA) obtained health and safety induction, genetically modified organisms (GMOs) online training and ELISA training.

2.4 Recruitment and selection of participants

Once identified, potential participants were initially invited to take part in the study by posting the child and parents/guardians' information sheets (Appendices D & E) with the GA appointment letter. These were sent at least one week prior to the appointment, giving parents and children sufficient time to consider the information regarding the study and their decisions about whether to take part. The chief investigator (FA) was the person responsible for identifying potential participants and recruitment to the study. Informed assent and consent documents (Appendices F&G) were also provided to each participant and his/her parents/guardians respectively. Potential participants could be recruited to this study based on fulfilling the following inclusion and exclusion criteria:

2.4.1 Inclusion criteria

- Age: 6-10 years of age at recruitment.
- General health: Medically fit and healthy.
- Dental examination:
    - Presence of caries in primary and/or permanent teeth.
- Treatment plan to receive dental extractions under GA in Leeds dental institute (LDI).
- Participants can fully understand the procedure involved in the study and are likely to comply, as evidenced by voluntary written informed assent.

Previous literature found that salivary LL-37 shows different expressions in every stage of human growth. Davidopoulou et al. (2012) reported a significant correlation between the salivary concentration of LL-37 and age. Also, the previous authors suggested that this correlation is influenced by the type of dentition (primary vs permanent). Hence, the age group of the participant in this

study was selected to ensure that all children recruited were in their mixed dentition stage. Therefore, variations in the salivary concentration of LL-37 could be only related to the caries experience in these children.

Children assigned to receive dental extractions under GA require to fast for 6 hours before the procedure. Also, food and drink have a potent influence on the integrity of the saliva, such as having the potential to influence salivary pH levels and viscosity. Research participants, therefore, were recruited from this group of children to ensure that no food or drink was consumed before saliva sample collection.

2.4.2 Exclusion criteria

- Age: < 6 or >10 years-of-age.
- Children with any pre-existing medical condition.
- Signed informed consent not obtained from parents.
- Children with a dental or oral condition other than caries.
- Food consumption within 60 min before saliva sample collection.
- Antibiotic treatment within 28 days before saliva collection.
- Non-steroidal anti-inflammatory drugs (NSAIDs) treatment within 24 hours before saliva collection.
- Children with severe learning difficulties who would be unable to participate and provide saliva samples or give assent even when supported.

2.5 Dental examination and data collection

Saliva samples were obtained from participants by the chief investigator (FA) before undergoing GA for multiple dental extractions at LDI. Participants were invited to attend a second appointment, as part of their dental review appointment, one month after the completion of the dental treatment during which a second saliva sample was obtained and participants were provided with appropriate oral hygiene advice and instructions.

Participants had been previously examined in the paediatric dental department by members of staff for dental caries using the standard protocol used within the LDI. All examinations were performed in well-equipped dental clinical facilities with good lighting conditions. Caries status was determined by the number of DT/dt and DS/ds, however, no information was recorded regarding lesions' cavitation status. All charted dental caries, including cavitated and non-cavitated enamel and dentin caries, were included and recorded.

Participants' demographic data, medical history, and dental diagnosis were collected by the chief investigator (FA) from their electronic dental files. In addition, information regarding the extent of dental caries, number of DT/dt and DS/ds, presence of dental abscess as well as evidence of peri-radicular radiolucency was confirmed from the participants' radiographic examination. The radiographic examination also confirmed the presence of cavitated occlusal and proximal dental caries. This information was anonymised and each participant in the study was assigned a study number code.

2.6 Participant withdrawal criteria

Participants had the right to withdraw from the study at any time, for any reason and this was explained during the consenting process and in the patient information sheet. The chief investigator (FA) also had the right to withdraw subjects from the study in the event of current illness, protocol deviations, administrative reasons, or other reasons. It was understood by all concerned that an excessive rate of withdrawal of subjects could render the study underpowered; therefore, unnecessary withdrawal of subjects was avoided.

2.7 Sample size determination

Statistical advice was sought from Dr Jing Kang, a statistician and lecturer in biostatistics at the University of Leeds. The literature search before commencing the study revealed that there had been no previous studies with similar study designs in the published literature that could provide appropriate information regarding the statistical values needed for sample size determination such as effect size, standard deviation, median, and interquartile range. This study was therefore designed as a pilot study to determine and generate initial data to inform the design of larger future studies. According to Lancaster et al. (2004), at least 30 participants are needed to estimate a parameter when designing pilot studies. As we set the lost rate to 20% for each time point, at least 36 participants were required to meet the required numbers as an estimate.

2.8 Changes in response to the COVID-19 pandemic

The recruitment process began in September 2019 and 90 potential participants were identified and invited to the study. However, the recruitment process was prematurely terminated in mid-March 2020 due to the COVID-19 pandemic when changes to the provision of NHS dental care at the LDI had to be made. There was also a halting of student clinical and laboratory research activity as set down by the University of Leeds regulations. The recruitment process was originally proposed to terminate in July 2020. This resulted in the original target number of participants needed to meet the pre-planned sample size not being met.

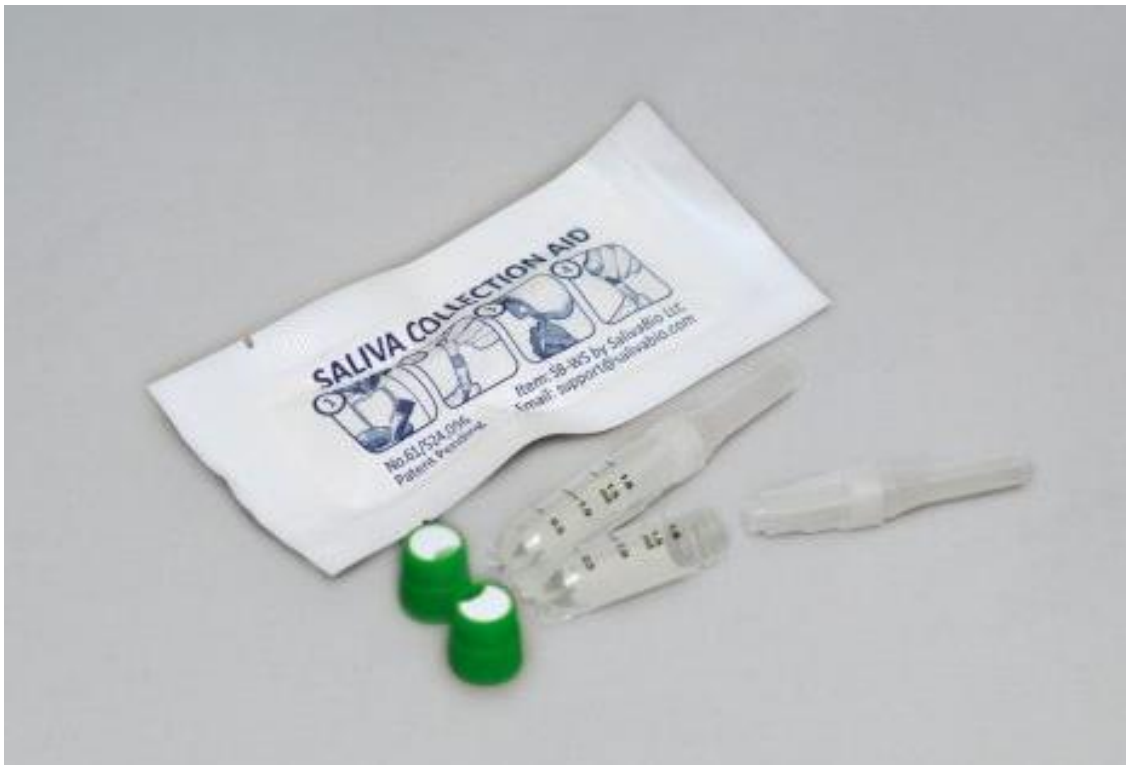
2.9 Sample collection and storage

Sampling was performed in the afternoon immediately before participants underwent GA for dental treatment (around 1 pm), to avoid a possible variation in peptide concentration due to the circadian rhythm in salivary flow rate. Participants were asked to refrain from eating, drinking (other than plain water), chewing gum, or brushing teeth within 60 minutes before sampling. As children were unable to eat or drink for two hours before the GA this condition was met for all participants.

Unstimulated whole saliva (2ml) was collected from 21 participants by the passive drool technique for a maximum of 5 min utilizing the SalivaBio Collection Aid (figure 2-1) and 2ml SalivaBio cryovials (Salimetrics, Newmarket, UK). They were supervised by the chief investigator (FA). Samples were then immediately stored in a container filled with dry ice and transported immediately to a laboratory within 1 hour. Saliva samples were then aliquoted. Each aliquot was labelled with the number code and immediately frozen on dry ice before storage in a -80°C freezer until subjected to ELISA.

The second salivary sample was collected approximately one month after the completion of the dental treatment under GA in a similar manner as explained earlier with the first sample. This appointment was arranged as part of the patients' review dental appointment during which the child participant received a comprehensive dental examination, oral health education advice and instructions as well as topical fluoride varnish application. To ensure participants attend the second appointment, the chief investigator (FA) arranged this appointment in agreement with the participants' parents/guardians on the day of their GA appointment. In addition, the chief investigator (FA) contacted the participants' parents/guardians via phone 1 week before the scheduled second appointment to provide them with the pre-saliva collection instructions as mentioned earlier.

Figure 2-1: SalivaBio Collection Aid (Salimetrics, Newmarket, UK) utilised for increased ease of saliva collection from participants.



2.10 Preliminary experiments

For validating and assessing the accuracy of the ELISA assay used in our study, preliminary recovery experiments were performed by adding recombinant LL-37 peptide in saliva samples collected from the main investigator (FA) with a variable dilution of 1:5, 1:10, 1:20 and 1:50. These recovery experiments resulted in high peptide recovery rates, however, 1:20 dilution factor generated a linear dilution and equivalent quantification of the peptide across the standard curve range. Furthermore, we tested the stability of the salivary LL-37 stored for approximately 1 month at -80°C freezer and following single freeze-thaw cycle. Saliva samples collected from the chief investigator (FA) underwent single freeze-thaw cycle and then tested by ELISA. The test confirmed that salivary LL-37 concentration remained stable following prolonged period of storage at -80°C freezer and single freeze-thaw cycle.

2.11 Analysis of saliva samples

2.11.1 Preparation of saliva samples

To avoid inter-assay variation, saliva samples were stored for batch analysis. Aliquots of saliva samples were thawed on ice and cleared by centrifugation for 10 min at 4^{o}C and then the supernatant was separated for testing.

2.11.2 The enzyme-linked immunosorbent assay (ELISA)

The concentration of LL-37 peptide was measured by the human LL-37 ELISA (Hycult Biotechnology, Uden, the Netherlands) and was performed according to the manufacturer's instructions. The ELISA analysis does not discriminate between hCAP18 and LL-37. All steps were performed at room temperature. Briefly, the wash/dilution buffer preparations were performed (according to the manual provided with the Kit) before the ELISA procedure. The 96-well ELISA

plate is pre-coated with a solid bound specific antibody that captures human LL-37/hCAP18. The standards were prepared by a 1:3 serial dilution with the supplied wash/dilution buffer. The saliva samples were diluted with supplied wash/dilution buffer 1:20. This dilution factor was chosen following preliminary experiments using control saliva to ascertain the optimal dilution for the saliva samples. The standards, blanks and saliva samples were analysed in duplicate, and the mean values were calculated. 100µL of diluted standards, blanks and saliva samples were transferred into the appropriate, designated wells and the plate was incubated for one hour at room temperature. After incubation, the plate was washed manually with wash/dilution buffer four times using a multichannel pipette to remove any excess unbound reagents that might otherwise cause inaccuracy. Next, 100 µl of diluted tracer (biotinylated tracer antibody) was added to each well and the plate was incubated for 1 hour at room temperature during which the biotinylated tracer antibody binds to the captured human LL-37/hCAP-18. After incubation, the plate was washed as previously described. Then, 100 µl of diluted streptavidin-peroxidase conjugate that binds to the biotinylated tracer antibody was added to each well and the plate was incubated for 1 hour at room temperature. After incubation, the plate was washed as previously described. Next, 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate that reacts with streptavidin-peroxidase conjugate was added to each well and the plate was incubated for approximately 15 minutes at room temperature with inspecting the reaction on the plate regularly. The plate was covered with aluminium foil to prevent exposing the plate to direct sunlight. Finally, the reaction was stopped with the addition of 100 µl of stop solution (oxalic acid). Using a plate reader, the plate was read within 30 minutes of adding the stop solution at a wavelength of 450 nm.

Figure 2-2: Schematic flowchart of planned human LL-37 ELISA from experiment to data analysis.

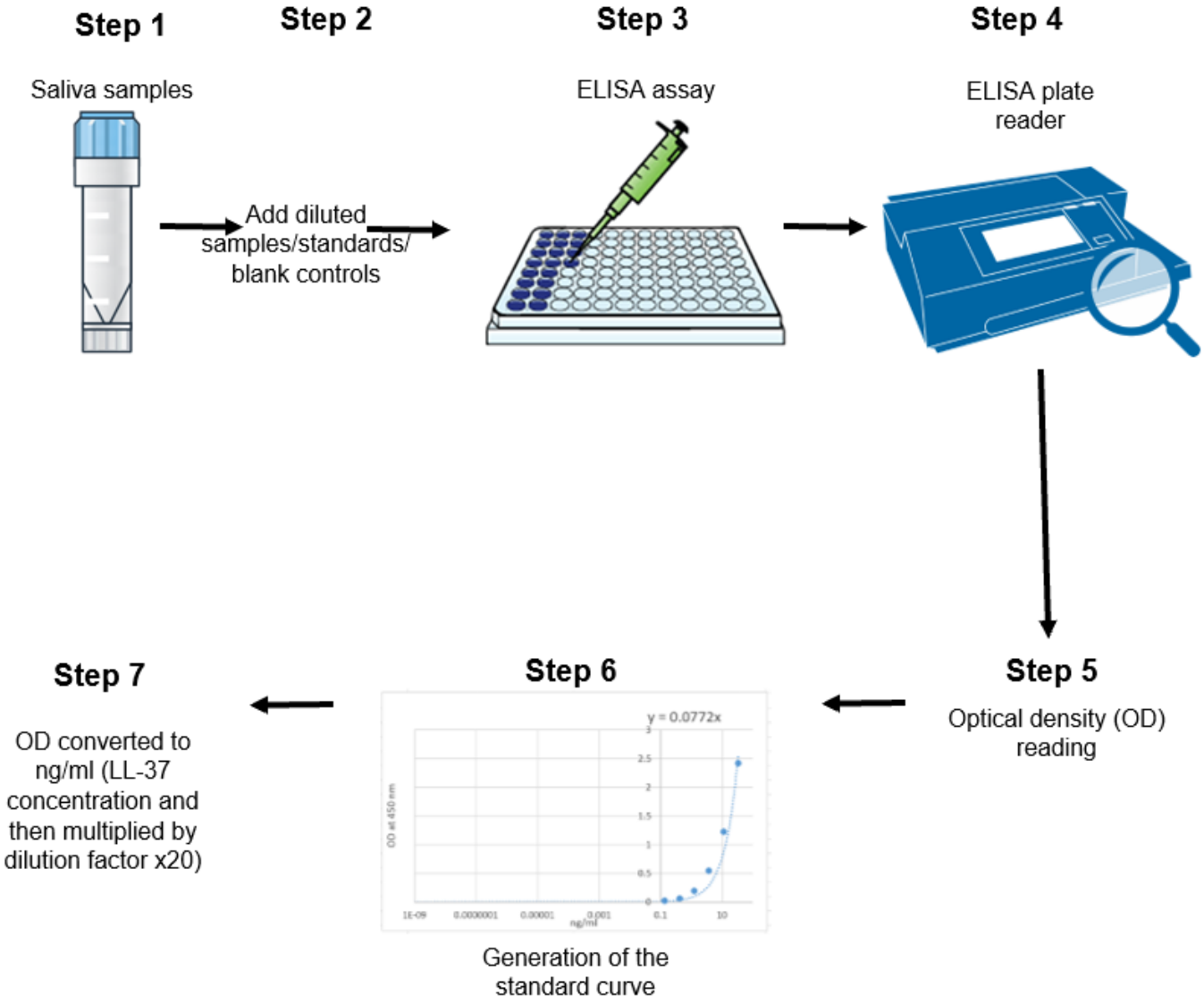


Figure 2-3: Summary of human LL-37 ELISA protocol.

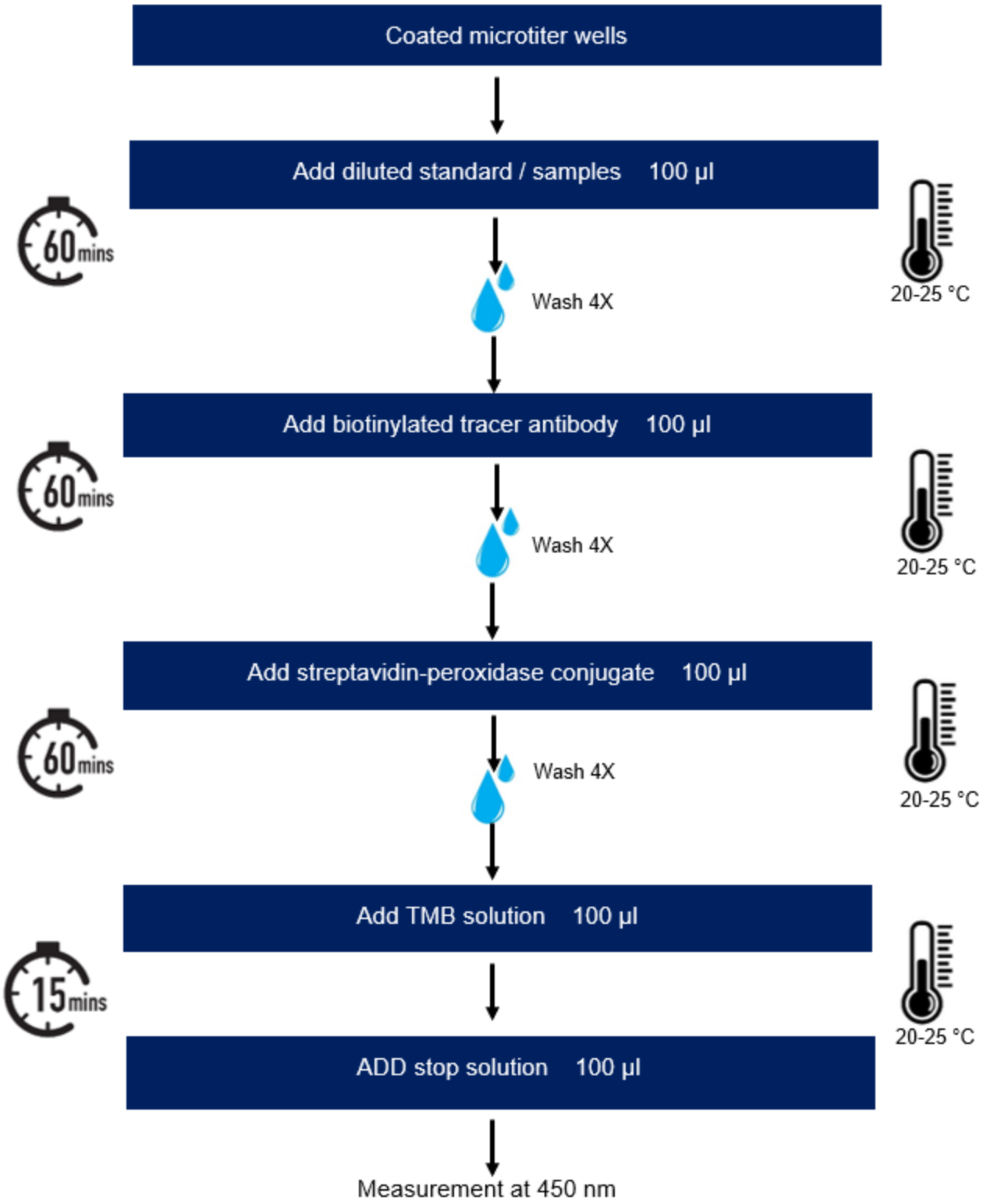
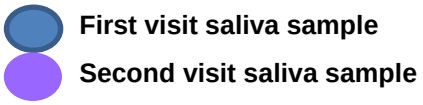
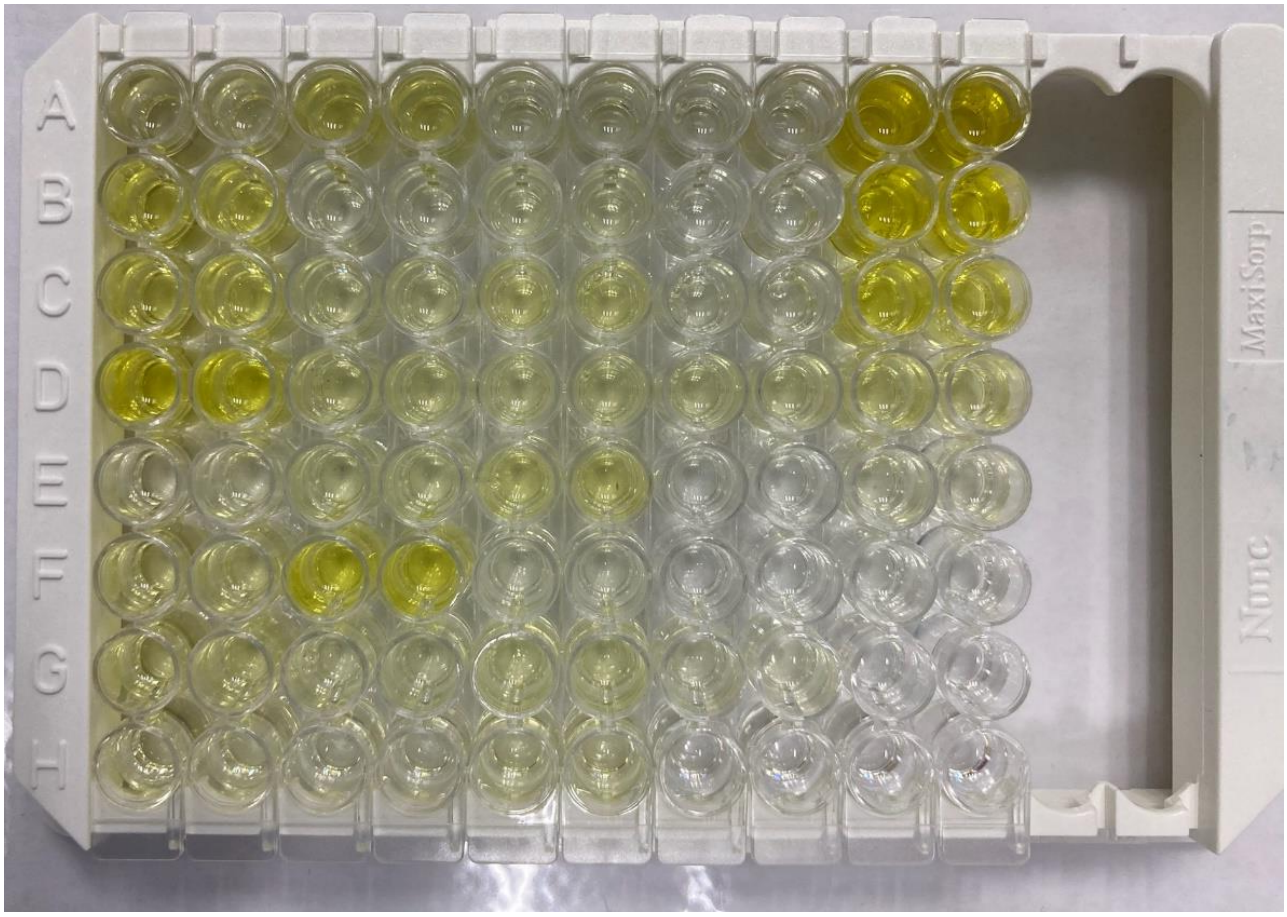


Figure 2-4: Mapping of 96-well LL-37 ELISA plate layout.



	1	2	3	4	5	6	7	8	9	10	11	12
A	Code 1	Code 1	Code 9	Code 9	Code 18	Code 18	Code 4	Code 4	Standard 100 ng/ml	Standard 100 ng/ml		
B	Code 2	Code 2	Code 10	Code 10	Code 22	Code 22	Code 5	Code 5	Standard 33.3 ng/ml	Standard 33.3 ng/ml		
C	Code 3	Code 3	Code 11	Code 11	Code 24	Code 24	Code 7	Code 7	Standard 11.1 ng/ml	Standard 11.1 ng/ml		
D	Code 4	Code 4	Code 12	Code 12	Code 26	Code 26	Code 9	Code 9	Standard 3.70 ng/ml	Standard 3.70 ng/ml		
E	Code 5	Code 5	Code 13	Code 13	Code 29	Code 29	Code 10	Code 10	Standard 1.23 ng/ml	Standard 1.23 ng/ml		
F	Code 6	Code 6	Code 15	Code 15	Code 1	Code 1	Code 11	Code 11	Standard 0.41 ng/ml	Standard 0.41 ng/ml		
G	Code 7	Code 7	Code 16	Code 16	Code 2	Code 2	Code 12	Code 12	Standard 0.14 ng/ml	Standard 0.14 ng/ml		
H	Code 8	Code 8	Code 17	Code 17	Code 3	Code 3	Code 13	Code 13	Standard / Blank 0 ng/ml	Standard / Blank 0 ng/ml		

Figure 2-5: LL-37 ELISA plate displaying the enzymatic colour reaction following the addition of the stop solution. The yellow colour indicates that LL-37/hCAP18 is present and the more intense the yellow colour, the higher the concentration of LL-37/hCAP18.



2.12 Statistics and data analysis

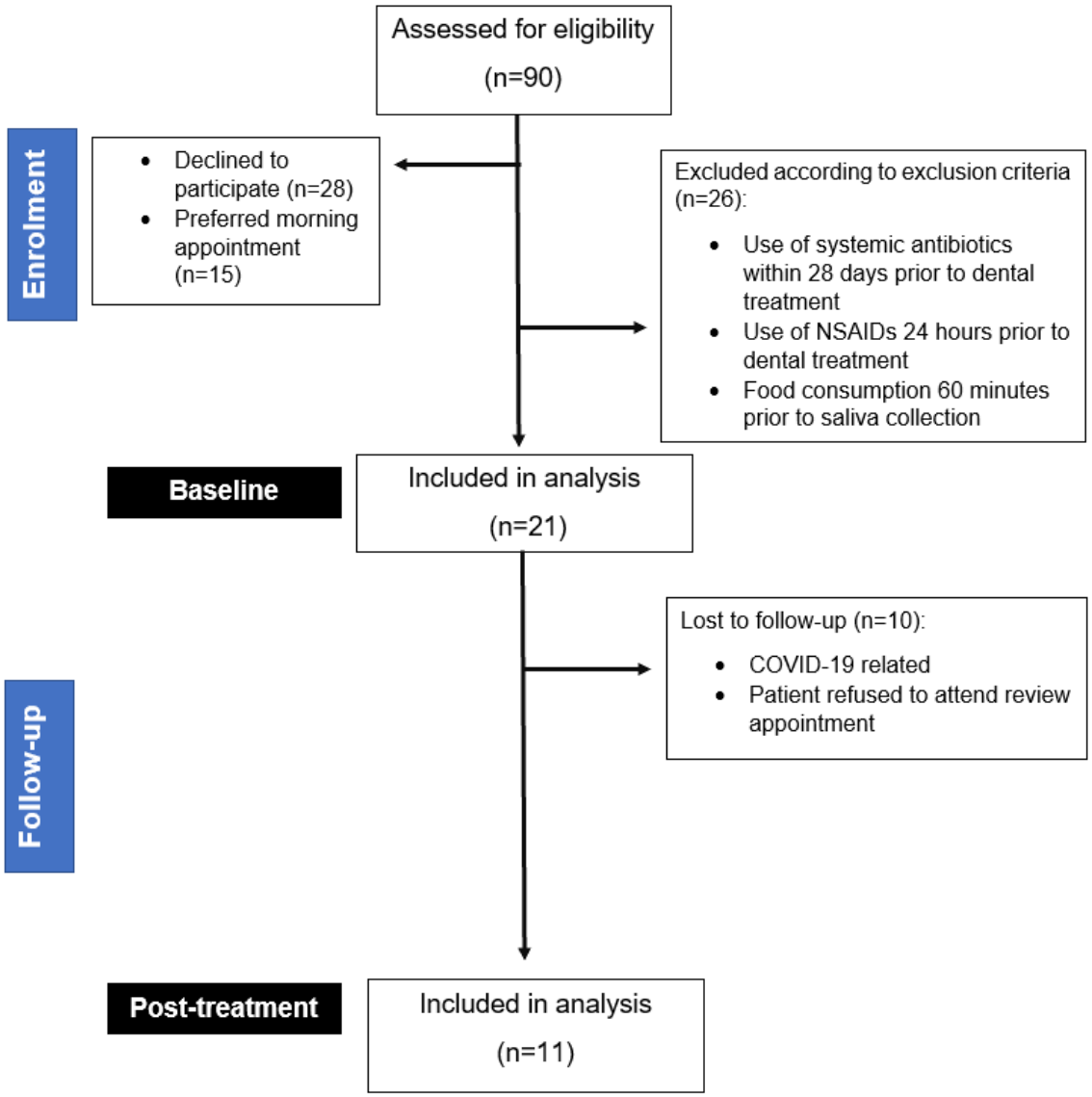
All the collected raw data were entered into Microsoft Office Excel 365 spreadsheets, including participants' demographics, clinical measurements, and lab samples of the two experimental replicates from each participant. Data were analysed using GraphPad Prism for Windows, version 9.1.2 software, and IBM® SPSS® Statistics Version 27. Descriptive statistics were used to calculate the mean, median, range, and standard deviation of all recorded data. All continuous data were checked for normality using the Shapiro-Wilk test and Kolmogorov-Smirnov test. The lab measurement of salivary LL-37 concentration from each participant was taken as the average of the two experimental repeats, at each time point (baseline and follow-up). Non-parametric statistical methods were preferred because the normality of pre-treatment salivary concentration of LL-37 was not confirmed. Hence, statistical comparisons between groups of children were performed with Mann–Whitney U test. Spearman's correlation coefficient was used to evaluate the linear relationship between the pre-treatment salivary concentration of LL-37 and the number of DT/dt, DS/ds, and age. Paired samples t-test or Wilcoxon signed-rank test was to be conducted to determine whether a statistically, significant difference existed between the mean salivary concentration of LL-37 pre-and post-dental treatment, should the data follow the normal or non-normal distribution. Profile plots were performed to demonstrate the change of each participant's salivary concentration of LL-37 from baseline to post-treatment. The significance level of all statistical tests was set at 0.05.

3 RESULTS

3.1 Participant demographics

The initial study plan was to collect two saliva samples, one saliva sample taken pre-and one post-dental treatment, from all participants who agreed to participate in the study and fulfilled the inclusion and exclusion criteria (refer to Materials & Methods chapter). Twenty-one participants were recruited from LDI, and the pre-treatment saliva samples were successfully obtained from these participants. However, we were unable to collect post-treatment saliva samples from eight participants due to COVID-related issues and the closure of the routine clinics in the dental hospital for several months during the first wave of the pandemic. Moreover, two participants dropped out of the study and did not provide a second saliva sample as their parents/legal guardians preferred to continue with the follow-up/ review dental appointments with their general dental practitioner (GDP) because of accessibility reasons. Hence, we collected the second saliva sample from 11 participants only (figure 3-1).

Figure 3-1: Flow chart showing the participants' flow from eligibility to analysis.



At baseline, of the 21 participants who provided the pre-treatment saliva samples, 13 were females and 8 were males. Their mean age was 7.95 (SD 1.49) years, range (6–10 years). The mean number of decayed teeth (DT/dt) was 6.24 (SD 1.84), range (2-10 teeth) and mean decayed surfaces (DS/ds) was 9.33 (SD 3.06), range (3-14 surfaces). Caries status was grouped according to the number of DT/dt: two participants had Low DT/dt levels (1-3), 10 had moderate DT/dt levels (4-6), and nine had high DT/dt levels (>6). All participants were medically fit and well with no current health conditions, except for two participants who had mild controlled asthma. In addition, only six participants had been prescribed antibiotic treatment within the three to six months before their GA appointment. None of the enrolled participants had NSAIDs treatment within the last three months before collecting the first saliva sample. All participants were in their mixed dentition stage.

Table 3-1: Descriptive statistics of the study participants at baseline.

	N	Mean/frequency*	SD/%*
Age	21	7.95	1.49
Gender, female	21	13	62%
DT/dt	21	6.24	1.84
Low (1-3)	2	2.5	0.71
Moderate (4-6)	10	5.6	0.7
High (>6)	9	7.78	1.09
DS/ds	21	9.33	3.06

*Mean (SD) is presented if the variable is continuous and frequency (%) is presented if the variable is categorical.

3.2 LL-37 antimicrobial peptide in saliva

To measure the salivary concentration of the AMP LL-37, an ELISA assay was performed using saliva from participants planned for dental extraction under GA. The ELISA assay for LL-37 (Hycult Biotechnology, Uden, The Netherlands) is commercially available and can readily be used to measure the peptide concentration in saliva. The AMP LL-37 was detected in all saliva samples. The pre-and post-treatment salivary concentrations of LL-37 varied considerably among study participants. The mean pre-treatment concentration of LL-37 was 132 ng/ml (SD= 92.42) whereas, the mean post-treatment concentration of LL-37 was 41.06 ng/ml (SD= 35.43).

Figure 3-2: Bar chart showing the pre-treatment salivary concentration of LL-37 (ng/ml) of each participant as measured by ELISA. Data presented is the mean generated from n=2 assays and standard error of the mean (SEM), each performed in duplicate**.**

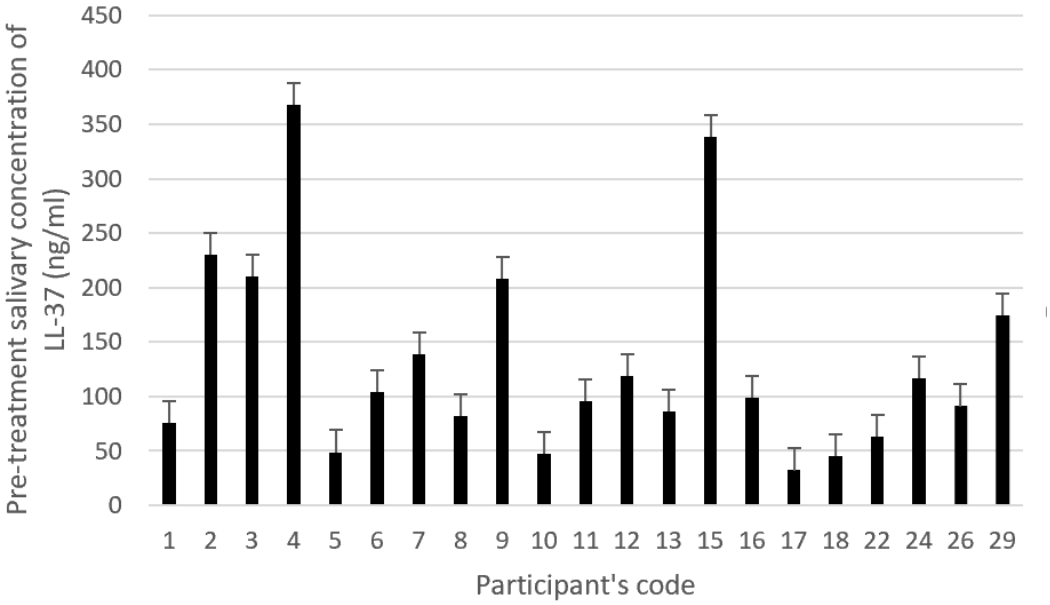


Figure 3-3: Bar chart showing the post-treatment salivary concentration of LL-37 (ng/ml) of each participant as measured by ELISA. Data presented are the mean generated from n=2 assays and the standard error of the mean (SEM), each performed in duplicate**.**

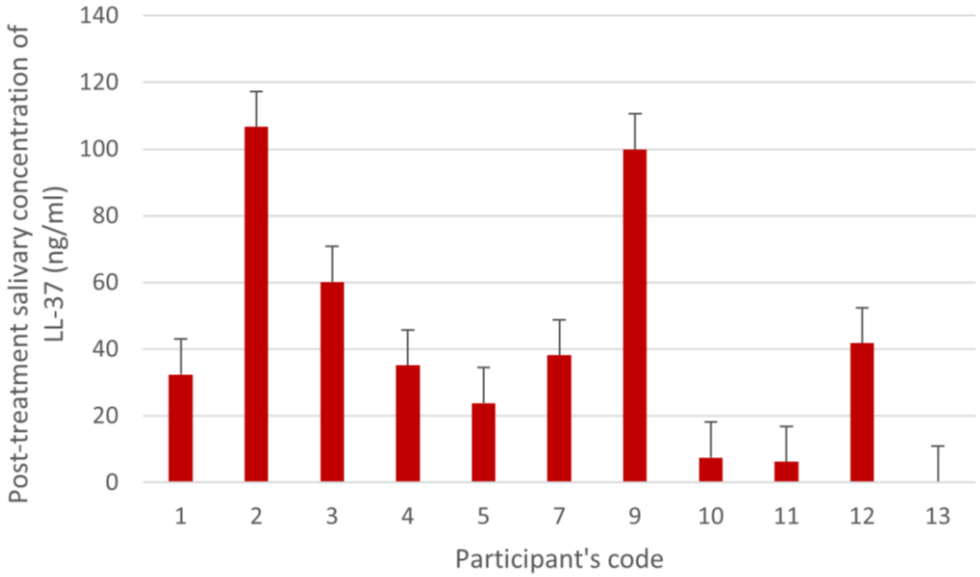


Table 3-2: Summary of average pre-and post-treatment salivary concentration of LL-37.

	Pre-treatment	Post-treatment
Participant's code	Average The salivary concentration of LL-37 (ng/ml)	Average The salivary concentration of LL-37 (ng/ml)
1	75.88	32.38
2	230.14	106.6
3	209.89	60.15
4	368	35.13
5	48.86	23.77
6	104.05	-
7	138.28	38.16
8	81.82	-
9	207.93	99.87
10	47.1	7.45
11	96.00	6.19
12	119.07	41.76
13	86.43	0.23
15	338.05	-
16	98.39	-
17	32.1	-
18	45.21	-
22	63.28	-
24	116.56	-
26	90.94	-
29	174.08	-

3.2.1 Normality of the salivary concentration of LL-37 data

Shapiro-Wilk and Kolmogorov-Smirnov[a] tests were used to assess the data normality of the pre-treatment salivary concentration of LL-37 of all participants. The tests gave a *p*-value of <0.05 which indicated that the data were not normally distributed and therefore, non-parametric tests were used. Spearman's correlation was used to test for statistically significant correlations between the pre-treatment salivary concentration of LL-37 and the number of DT/dt, DS/ds, and age. Mann-Whitney U test was used to determine whether there were any statistically significant differences between the defined two groups. Also, Kruskal-Wallis test was used to determine whether there are any statically significant differences among participants with different DT/dt scores. However, when the same tests were used to assess the data normality of the difference between the pre-treatment and the post-treatment salivary concentration of LL-37, the Kolmogorov-Smirnov[a] test gave a *p*-value of >0.05, and the Shapiro-Wilk test gave a *p*-value of <0.05. However, due to the small sample size, it was decided to consider that the data are not normally distributed. Therefore, the non-parametric Wilcoxon signed-rank test was used to determine whether there were any statistically significant differences between pre-and post-treatment salivary concentration of LL-37. The statistical significance level was set at a *p*-value < 0.05.

Table 3-3: Tests of normality.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistics	df	Sig.	Statistics	df	Sig.
Pre-treatment concentration of LL-37	0.22	21	0.01	0.84	21	0.003
Post-treatment concentration of LL-37	0.22	11	0.15	0.89	11	0.13
The difference between pre-and post-treatment concentrations of LL-37	0.24	11	0.08	0.77	11	0.004

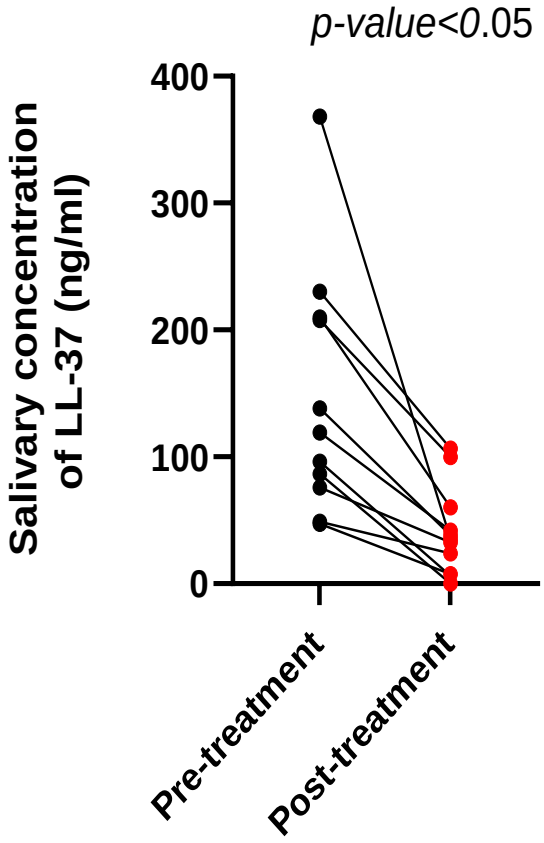
3.3 Difference between the pre-and post-treatment salivary concentration of LL-37

Wilcoxon Signed rank test was performed to test the hypothesis that the median difference between the pre-and post-treatment salivary concentration of LL-37 is equal. The null hypothesis of equal median pre-and post-treatment salivary concentration of LL-37 was rejected (Z= -2.93, *p*-value<0.05) as the test showed there was a significant difference between the pre-and post-treatment salivary concentration of LL-37. The median concentration for pre-treatment salivary LL-37 was 98.39 compared to 35.13 for post-treatment salivary LL-37. Thus, we can conclude that the salivary concentration of LL-37 significantly decreased after dental treatment.

Table 3-4: Summary of Wilcoxon Signed rank test indicates that the median salivary concentration of LL-37 decreased significantly after dental treatment.

	N	Median	Interquartile (IOR)	Z	*p*-value
Pre-treatment salivary concentration of LL-37	11	98.39	121.42	-2.93	<0.05
Post-treatment salivary concentration of LL-37	11	35.13	52.702		

Figure 3-4: Profile plot of the salivary concentration of LL-37 change for each participant. Wilcoxon Signed rank test displaying the difference of the salivary concentration of LL-37 pre- and post-dental treatment, *p*-value <0.05.

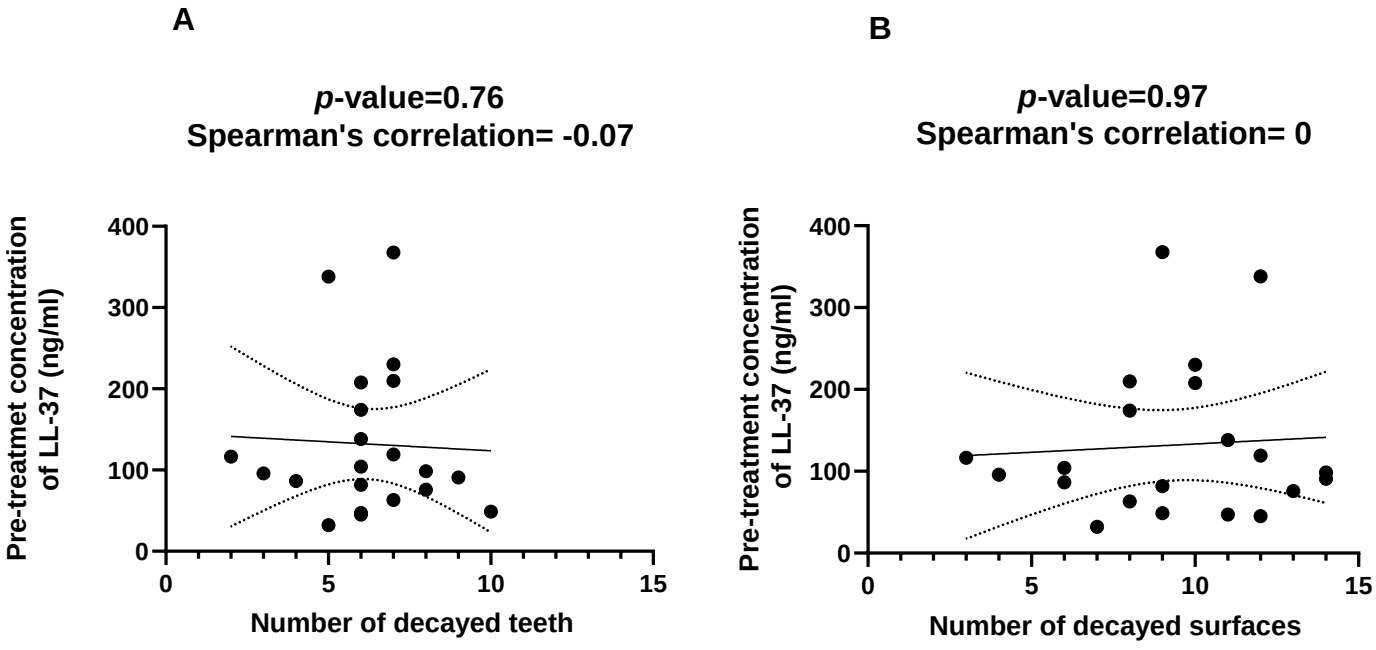


3.4 Salivary concentration of LL-37 and participants' caries experience

3.4.1 Correlations between the pre-treatment salivary concentration of LL-37 and the number of decayed teeth and surfaces

To assess the relationship between the pre-treatment salivary concentration of LL-37 and caries experience in children, Spearman's rank correlation analysis was used because the normality of the pre-treatment salivary concentration of LL-37 was not confirmed. No correlations were found between the salivary concentration of LL-37 and the number of DT/dt nor with the number of DS/ds (correlation coefficient= -0.07, n= 21, *p*-value= 0.76), (correlation coefficient= 0, n=21, *p*-value= 0.97), respectively.

Figure 3-5: No correlations between pre-treatment salivary concentration of LL-37 and the number of (A) decayed teeth and (B) surfaces.



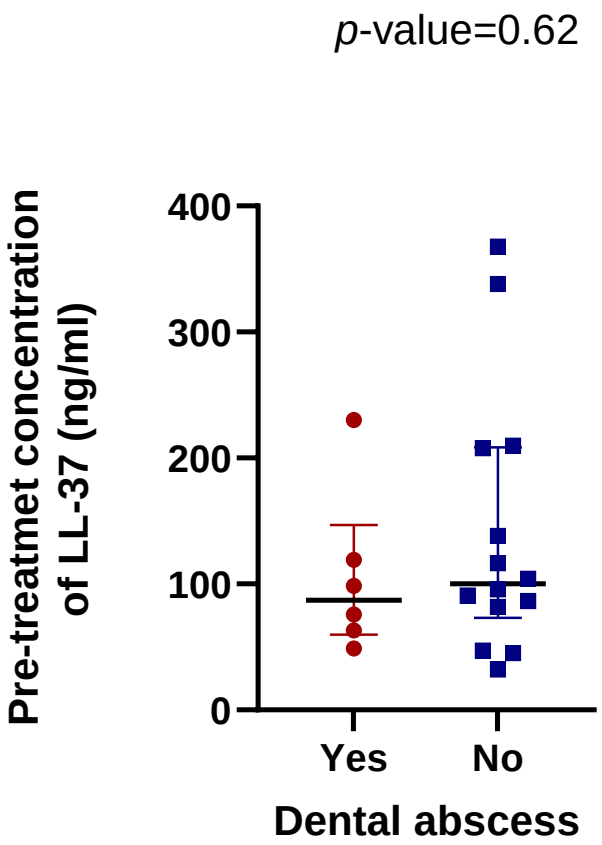
3.4.2 Relationship between the pre-treatment salivary concentration of LL-37 and the presence of a dental abscess

A Mann-Whitney U test was conducted to determine if there was a statistically significant mean difference in the pre-treatment salivary concentration of LL-37 between patients who presented with a dental abscess on the day of the dental examination and those without. Six participants presented with dental abscesses and 15 with no abscesses on examination. The results of the test showed that there was not a statistically significant difference, U=38, *p*-value=0.62, between the pre-treatment salivary concentration of LL-37 for participants with dental abscesses compared to those without.

Table 3-5: Summary of Mann-Whitney U test indicates no statistically significant difference was found between participants with and without dental abscess in the mean pre-treatment salivary concentration of LL-37.

Dental abscess	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Sig. (2-tailed)
Yes	6	9.83	59	38	0.62
No	15	11.47	172		

Figure 3-6: Scatter plot for the distribution of the pre-treatment concentration of LL-37 in participants with dental abscess compared to those without, demonstrating the median value and interquartile range.



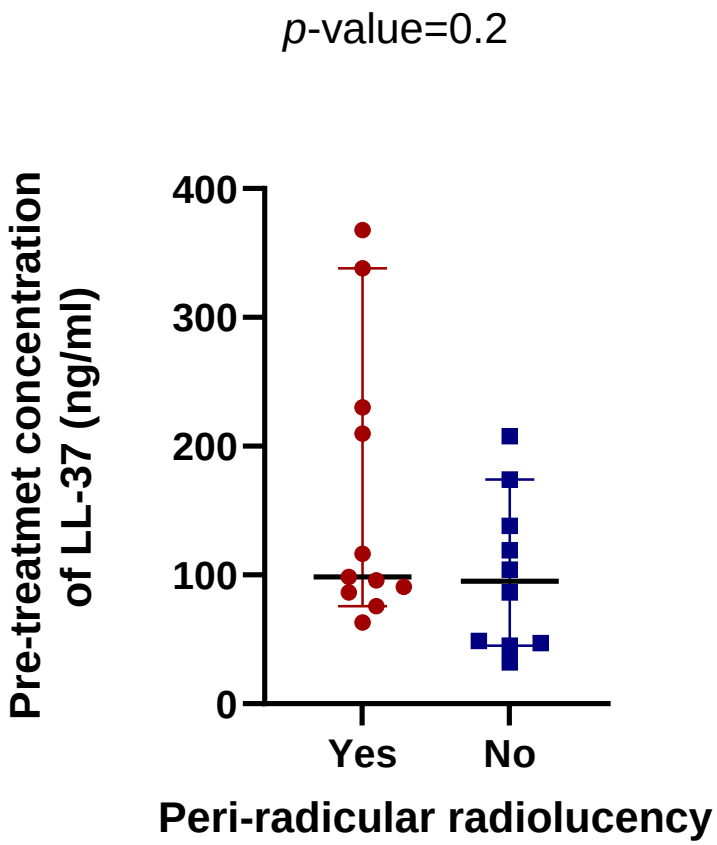
3.4.3 Relationship between the pre-treatment salivary concentration of LL-37 and the presence of peri-radicular radiolucency

Out of the total 21 participants, 11 participants presented radiographic evidence of peri-radicular radiolucency. Hence, the Mann-Whitney U test was conducted to determine if there was a statistically significant mean difference in the pre-treatment salivary concentration of LL-37 between participants who presented with radiographic evidence of peri-radicular radiolucency on the day of the dental examination and those without. The difference was not statistically significant, U=37, p-value=0.2. These results suggest that the presence of peri-radicular radiolucency (as determined by radiographic evidence) does not influence the salivary concentration of LL-37.

Table 3-6: Summary of Mann-Whitney U test indicates no statistically significant difference was found between participants with and without per-radicular radiolucency in the mean pre-treatment salivary concentration of LL-37.

Peri-radicular radiolucency	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Sig. (2-tailed)
Yes	11	12.64	139	37	0.2
No	10	9.2	92		

Figure 3-7: Scatter plot for the distribution of the pre-treatment concentration of LL-37 in participants with peri-radicular radiolucency compared to those without, demonstrating the median value and interquartile range.



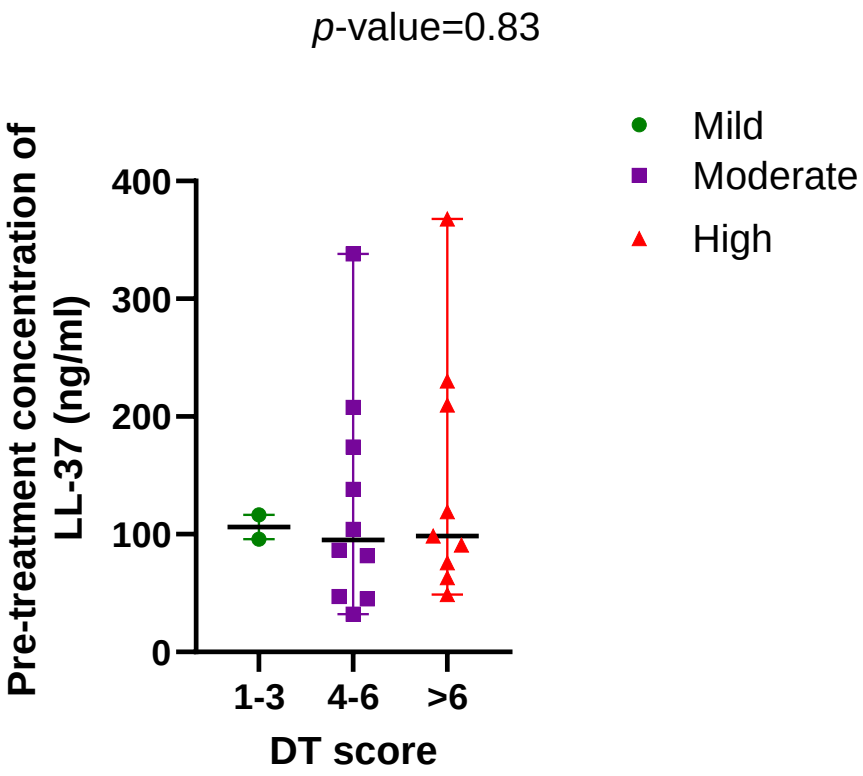
3.4.4 Pre-treatment salivary concentration of LL-37 among participants with different DT/dt levels

The median pre-treatment salivary concentration of LL-37 was highly variable among participants with low, moderate, and high DT/dt levels. Therefore, the non-parametric Kruskal-Wallis test was conducted due to the small sample size in each DT/dt group to determine if the difference in the median pre-treatment salivary concentration of LL-37 is statistically significant between the 3 groups. The results showed that the median difference in the pre-treatment salivary concentration of LL-37 is not statistically significant between the 3 groups, *p*-value=0.83.

Table 3-7: Descriptive statistics of pre-treatment salivary concentration of LL-37 among participants with different DT levels.

DT/dt	N	Median	Interquartile (IOR)
Low (1-3)	2	106.3	20.6
Moderate (4-6)	10	95.24	135.87
High (>6)	9	98.39	150.42

Figure 3-8: Scatter plot displaying Kruskal-Wallis test results (median value and interquartile range) for pre-treatment salivary concentration of LL-37 among mild, moderate, and high caries groups.

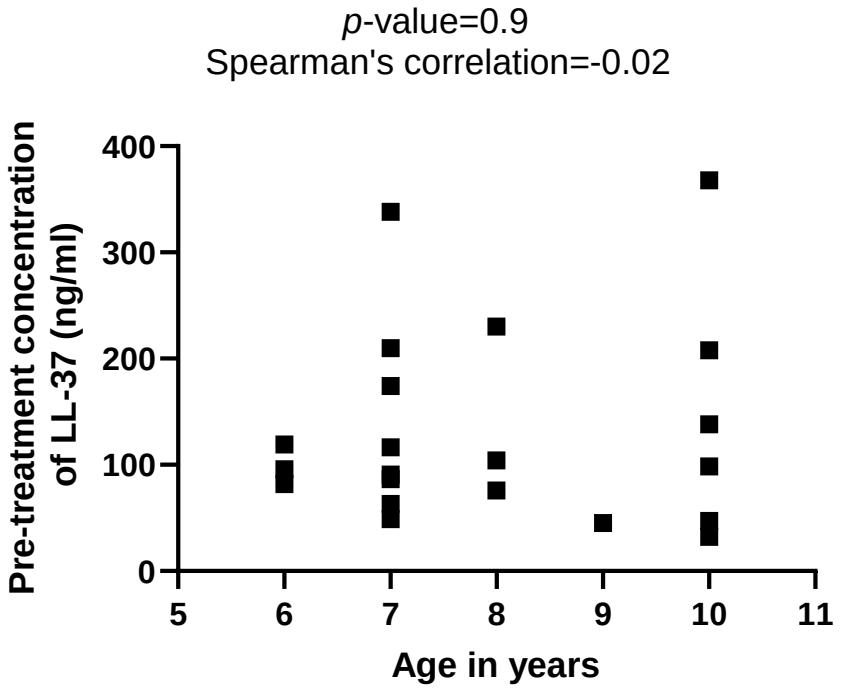


3.5 Salivary concentration of LL-37 and participant demographics

3.5.1 Correlation between the pre-treatment salivary concentration of LL-37 and age

To assess the relationship between the pre-treatment salivary concentration of LL-37 and participant age, Spearman's correlation analysis was used. A non-significant correlation was found between the salivary concentration of LL-37 and the age of the participants (correlation coefficient=-0.02, n=21, *p*-value=0.9).

Figure 3-9: No Correlation between pre-treatment salivary concentration of LL-37 and age in years.



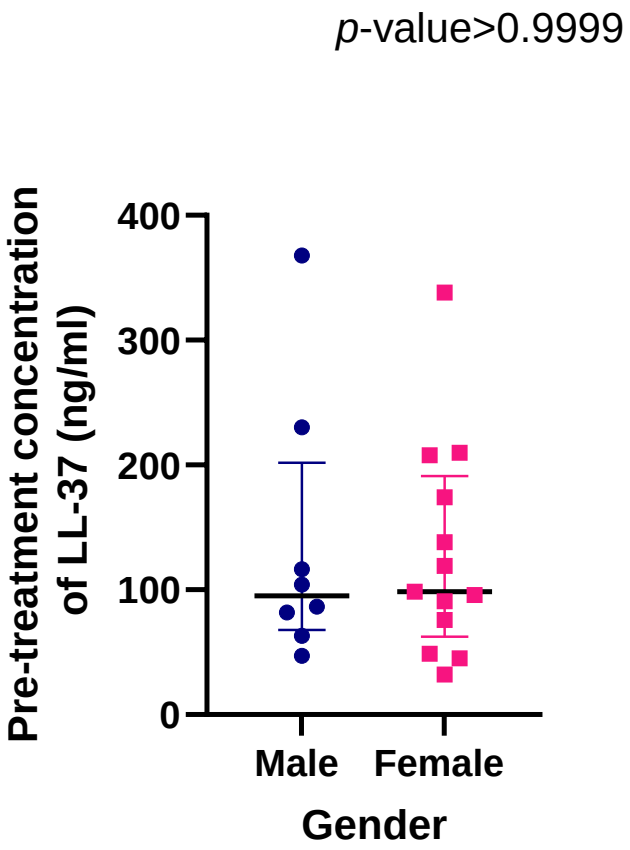
3.5.2 Relationship between the pre-treatment salivary concentration of LL-37 and gender

We next asked whether the gender of the participants influenced the salivary concentration of LL-37. To test this hypothesis, the Mann-Whitney U test was used to determine if there was a statistically significant mean difference in the pre-treatment salivary concentrations of LL-37 between males and females. The results of the test showed that there was not a statistically significant difference (U=52, *p*-value>0.9999), between the pre-treatment salivary concentration of LL-37 in males and females.

Table 3-8: Summary of Mann-Whitney U test indicates no statistically significant difference was found between males and females in the mean pre-treatment salivary concentration of LL-37.

Gender	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Sig. (2-tailed)
Male	8	11	88	52	>0.9999
Female	13	11	143		

Figure 3-10: Scatter plot for the distribution of the pre-treatment concentration of LL-37 among males and females, demonstrating the median value and interquartile range.



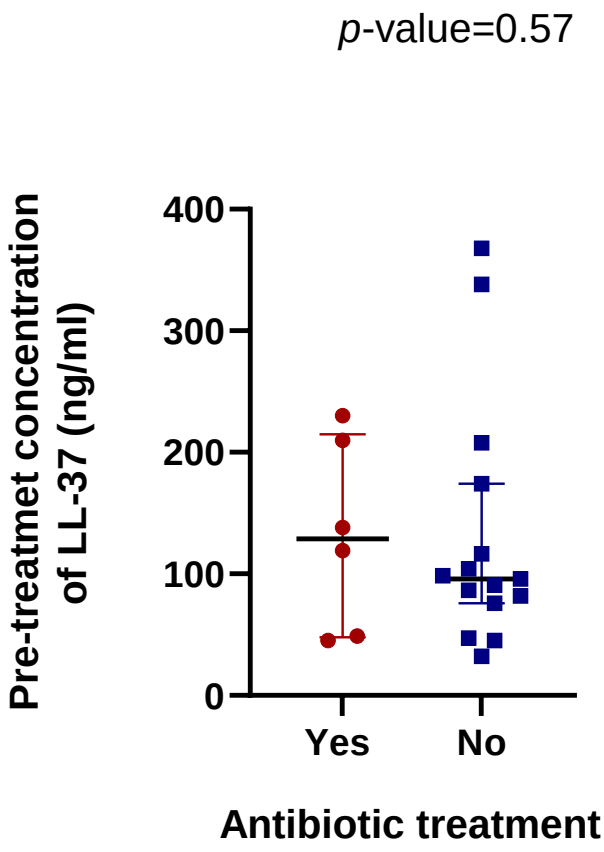
3.6 Relationship between the pre-treatment salivary concentration of LL-37 and antibiotic treatment

Of the 21 participants, six patients received antibiotic treatment (amoxicillin) 3-6 months before the day of the initial dental examination. Hence, we asked whether antibiotic treatment before dental treatment can influence the salivary concentration of LL-37 in children. To test this hypothesis, Mann-Whitney U test was performed to determine if there was a statistically significant mean difference in the pre-treatment salivary concentration of LL-37 in patients that received antibiotic treatment and those that did not receive antibiotic treatment. The difference was not statistically significant, U = 37, *p*-value= 0.57.

Table 3-9: Summary of Mann-Whitney U test indicates no statistically significant difference was found between participants that received and those that did not receive antibiotic treatment in the mean pre-treatment salivary concentration of LL-37.

Antibiotic treatment	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Sig. (2-tailed)
Yes	6	12	72	37	0.57
No	15	10.6	159		

Figure 3-11: Scatter plot for the distribution of the pre-treatment concentration of LL-37 among participants who received antibiotic treatment and those who did not receive it, demonstrating the median value and interquartile range.



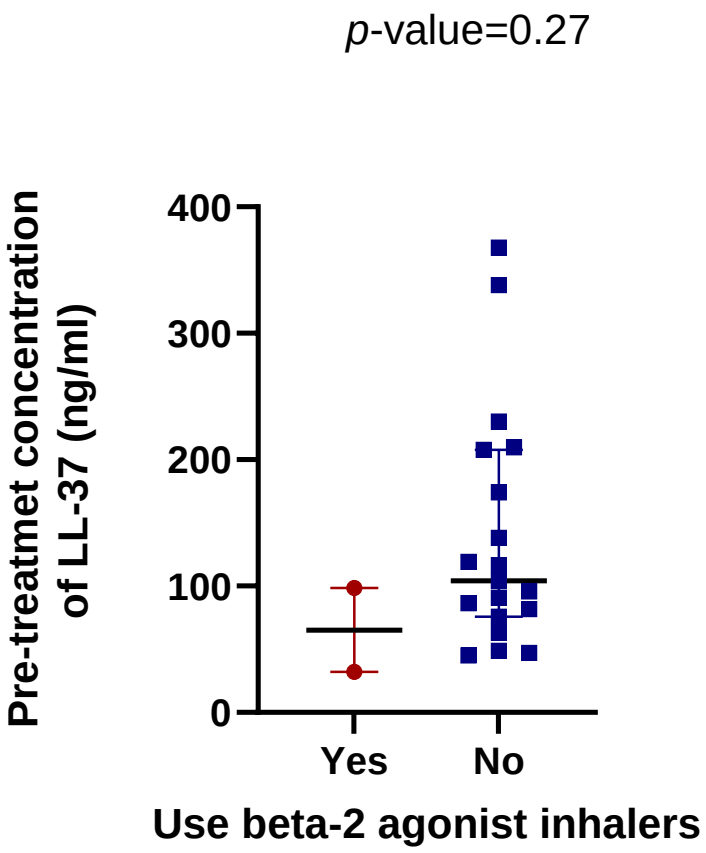
3.7 Relationship between the pre-treatment salivary concentration of LL-37 and beta-2 agonist inhalers

We then asked if the pre-treatment concentration of LL-37 differ between participants who use beta-2 agonist inhalers to manage asthma compared to healthy participants. To test this hypothesis, Mann-Whitney U test was performed, and the results showed that there was no statistically significant difference in the mean pre-treatment salivary concentration of LL-37 between the two groups, U = 9, *p*-value= 0.27.

Table 3-10: Summary of Mann-Whitney U test indicates no statistically significant difference was found between participants that use beta-2 agonist inhalers and those that do not use in the mean pre-treatment salivary concentration of LL-37.

Beta-2 agonist inhalers	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Sig. (2-tailed)
Yes	2	6	12	9	0.27
No	19	11.53	219		

Figure 3-12: Scatter plot for the distribution of the pre-treatment concentration of LL-37 among participants who use beta-2 agonist inhalers and those who do not use them, demonstrating the median value and interquartile range.



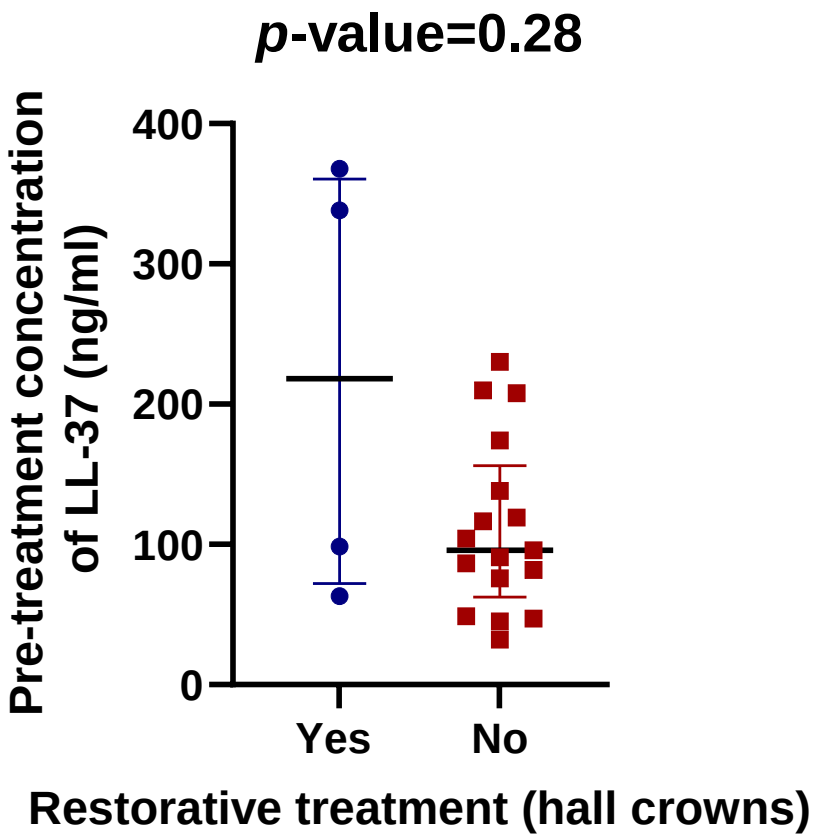
3.8 Relationship between the pre-treatment salivary concentration of LL-37 and restorative treatment (Hall technique crowns)

Of the 21 participants, four participants had received Hall technique crowns before their appointment for dental extraction under GA. Hence, we asked whether restorative treatment with Hall technique crowns that involve sealing carious lesions in primary molars could influence the salivary concentration of LL-37. To test this hypothesis, the Mann-Whitney U test was performed to determine if there was a statistically significant difference in the pre-treatment salivary concentration of LL-37 in patients that received restorative treatment and those that did not receive restorative treatment. The difference was not statistically significant, U=21, *p*-value=0.28.

Table 3-11: Summary of Mann-Whitney U test indicates no statistically significant difference was found between participants that received Hall technique crowns and those without in the mean pre-treatment salivary concentration of LL-37.

Hall crown	N	Mean Rank	Sum of Ranks	Mann-Whitney U	Sig. (2-tailed)
Yes	4	14.25	57	21	0.28
No	17	10.24	174		

Figure 3-13: Scatter plot for the distribution of the pre-treatment concentration of LL-37 in participants who received Hall technique crowns compared to those without, demonstrating the median value and interquartile range.



4 DISCUSSION

Multiple studies investigating the relationship between the salivary concentration of AMPs, including LL-37, and dental caries experience in children have been carried out. Most of these previous studies were cross-sectional where caries experience in children and the salivary concentration of AMPs were evaluated and measured at a single point in time. Thus, these studies would only provide a limited amount of information regarding the effect of dental caries progression, arrest, or regression on the salivary concentration of AMPs. Despite these previous attempts to explain the relationship between these two variables, no consensus has been reached to date.

Therefore, this was a pioneering approach to investigate and quantify the salivary concentration of LL-37 in 6- to 10- year-old children with diagnosed dental caries, and to evaluate the differences in the concentration of this AMP after extraction of carious teeth. Our results showed no correlation between the salivary concentration of LL-37 and the caries experience in children before dental treatment. However, the results after dental treatment were interesting where we detected a significant reduction in the salivary concentration of LL-37 in the saliva samples of all participants. Hence, this finding raises the hypothesis that salivary LL-37 has a fundamental role in innate immunity against inflammation and microbial infection.

4.1 Salivary LL-37 quantitative ELISA

We found that both the pre-and post-dental treatment salivary concentrations of LL-37 were highly variable among the participants. However, the concentrations were not associated with the participants' level of caries experience in this study population. This inter-subject variation in the peptide concentration is in agreement with several previous studies that found variable salivary peptide concentrations in children and adults in their study populations (Colombo et al., 2016, Davidopoulou et al., 2014, Malcolm et al., 2014, Davidopoulou et al., 2012, Phattarataratip, 2010, Tao et al., 2005). Moreover, the pre-treatment salivary concentration of LL-37 in this present study falls within the ranges of the reported concentration of the peptide in the previous studies in children aged 36-60 months, 2-18 years, and 11-15 years old, respectively (Colombo et al., 2016, Davidopoulou et al., 2012, Tao et al., 2005). Tao et al. (2005) utilised the slot blot technique for the detection of salivary LL-37 and reported the concentration of the peptide in the range of 0.12-12 µg/ml. Other studies that utilised the ELISA technique reported the salivary concentration of the peptide in the ng/ml range (Stojkovic et al., 2020, Colombo et al., 2016, Davidopoulou et al., 2014, Davidopoulou et al., 2012, Phattarataratip, 2010). Davidopoulou et al. (2012) detected the peptide in the range of 0.22-275 ng/ml when investigating the salivary concentration of LL-37 in children with and without caries. The detection level of salivary concentration of LL-37 in children in the ng/ml range by utilising the ELISA technique resembles those detected in other biological fluids in children, such as plasma or bronchoalveolar lavage fluid (Stukes et al., 2016, Cakir et al., 2014). Despite using a similar methodology to detect the salivary concentration of LL-37 in children 12- to -24 months of age, Malcolm et al. (2014)

found that 25.5% of children displayed undetectable concentrations of the peptide.

Differences in the methodology, such as experimental design, peptide detection method, population, participant's age, sample size as well as dental caries levels should all be considered in attempting to explain the discrepancies in the peptide concentration reported in the literature. Regarding the peptide detection method, the slot blot technique produces qualitative and semi-quantitative data regarding the molecule of interest. In contrast, the ELISA technique detects and quantifies molecules by first immobilising them on a solid surface and then by interacting with a highly specific antibody. Although ELISA is a highly sensitive and specific technique, it cannot assess LL-37 peptide quality and activity.

4.2 Reduction in the salivary concentration of LL-37 after dental treatment

The main interesting finding of the present study is that the salivary concentration of LL-37 was reduced after extraction of carious teeth and the reduction was statistically significant. It should be emphasized that information regarding LL-37 expression in the course of dental caries is scarce. Also, the association between caries experience and the salivary concentration of LL-37 in children has been controversial and inconclusive in literature. Moreover, it was not possible, in the present study, to determine precisely why the salivary concentration of LL-37 significantly decreased following the extraction of carious teeth. However, several complex oral events described in the literature should be considered in attempting to explain our study findings.

4.2.1 Reduction of pro-inflammatory cytokines

Firstly, a complex network of inflammatory and immune responses is known to occur during the initiation and progression of dental caries, and dental treatment can significantly alter these responses. Several studies have investigated the association between pro-inflammatory cytokines and dental caries and many suggested that a strong correlation exists between them. It was shown previously that adolescents with dental caries exhibited significantly elevated salivary levels of IL-6, IL-8, and TNF-α compared to caries-free controls (Gornowicz et al., 2012). A more recent study further validated this hypothesis where the researchers demonstrated that the salivary concentration of IL-6 is significantly higher in children with caries compared with caries-free children (Lo Giudice et al., 2020). Also, Menon et al. (2016) reported that the salivary concentration of IL-6 was significantly reduced three months after dental treatment in 3- to 6- year old children with ECC.

Although information regarding the regulation of cathelicidin gene expression is sparse, it was previously suggested that cathelicidin gene expression may be actively regulated during infection and inflammation. Several *in vitro* and *in vivo* studies have reported that human odontoblast and pulp tissue cells increase the expression and production of pro-inflammatory cytokines in response to microbial invasion and dental hard tissue damage. These pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-8, subsequently stimulate leukocyte migration and infiltration (Malekafzali et al., 2014, Horst et al., 2011). Also, it has previously been shown that in response to bacterial LPS, odontoblasts produce IL-8 which stimulates neutrophil migration to the site of inflammation. Moreover, the results of an *in vivo* study have also hypothesized that a significantly elevated concentration of the AMP LL-37 occurs in response to pulp tissue inflammation

compared to that in normal pulp and that the increased expression of the peptide is derived from increased neutrophil infiltration (Sarmiento et al., 2016). A recent *in vivo* study has also demonstrated that odontoblast-like MDPC-23 cells obtained from mouse pulp tissue produce IL-6 when stimulated with lipoteichoic acid from Gram-positive bacteria and express LL-37 constitutively (Odlén et al., 2020). Interestingly, it was reported that odontoblast-like cells in rats that are in contact with reparative dentine express CRAMP, confirming the role of cathelicidin in dentine formation during dental hard tissue damage (Horibe et al., 2018). Taken together, these previous findings indicate that odontoblasts, pulp tissue cells, and infiltrating immune cells secrete inflammatory cytokines and produce AMPs in response to infection from dental caries, dental hard tissue damage, and pulp tissue inflammation. In addition, these locally produced inflammatory mediators can further up-regulate the expression of the AMPs, including LL-37, to restore and re-establish the ecological balance in the oral microbiome. Hence, the reduction of the pro-inflammatory cytokines after dental treatment might subsequently reduce the expression of cathelicidin in odontoblasts, pulp tissue cells, and leukocytes and thus reduce the concentration of LL-37.

4.2.2 Reduction of short-chain fatty acids

Secondly, it is well known from the literature that oral cariogenic and aciduric bacteria initiate the process of dental caries by anaerobic fermentation of carbohydrates leading to the production of a spectrum of organic acids. Butyrate, acetate, and propionate are some of the major short-chain fatty acid (SCFAs) end-products of the fermentation process. These organic acid compounds decrease the dental plaque pH below the critical pH value which can initiate the demineralisation of dental hard tissue.

Several *in vivo* and *in vitro* studies have demonstrated that butyrate induces the expression of the cathelicidin gene in colonic, intestinal, and lung epithelial cells (Zhao et al., 2018, Jiang et al., 2013, Schwab et al., 2007, Kida et al., 2006, Raqib et al., 2006, Schauber et al., 2003). However, there is no published research investigating the effect of this short-chain fatty acid on oral epithelial cells. The effect of butyrate therapy has been evaluated by a randomised, double-blinded clinical trial on humans with shigellosis which is an intestinal bacterial infection caused by *Shigella*. The study demonstrated that patients who received butyrate therapy had a significantly higher expression of cathelicidin LL-37 in rectal epithelia compared with the patients who received placebo treatment (Raqib et al., 2012).

Concerning dental caries, Fidalgo et al. (2013) examined salivary metabolite markers in children with and without caries in the primary, mixed and permanent dentition stages. In this study, the salivary concentrations of several organic acids, including acetate, *n*-butyrate, and lactate, were higher in children with carious lesions compared to children without caries (Fidalgo et al., 2013). The same authors reported that the salivary concentrations of acetate, saccharides, n-butyrate, and propionate were reduced after dental treatment together with the reduction of salivary *Streptococcus mutans* and *Lactobacillus* species counts in children with dental caries (Fidalgo et al., 2015). While a direct relationship between the salivary concentration of butyrate and the expression of LL-37 in oral epithelial cells and immune cells has not been investigated yet, the above-mentioned findings might suggest an interplay between butyrate and the expression of LL-37 in children with dental caries.

4.2.3 Reduction of circulating neutrophils and oral cariogenic bacteria

Thirdly, as stated earlier in the introduction, LL-37 is expressed by the junctional epithelium which serves as the main route for the migration of local neutrophils into the GCF and saliva from the connective tissue (Puklo et al., 2008, Dale et al., 2001). Verifying the results from a later study suggested that LL-37 is mainly released by the circulating neutrophils in the oral cavity, indicating that most salivary LL-37 is produced and supplied by the neutrophils (Türkoğlu et al., 2009). This finding thus confirms that the expression of LL-37 in a proximal location to the tooth surfaces provides a significant role in defending against microbial infection. Interestingly, when the salivary concentration of LL-37 was investigated in edentulous adults using the ELISA technique, the authors found that the levels of the free LL-37 peptide were extremely lower than in dentate adults (Davidopoulou et al., 2013). These levels were irrespective of the inflammatory condition of the gingival tissue in either the edentulous or dentate participants. A similar observation has been reported in edentulous subjects who exhibited almost absent salivary α-defensins (Fanali et al., 2008). The previous two studies hypothesized that the extreme reduction in the salivary concentration of the AMPs can be explained by the absence of natural teeth. Therefore, the presence of natural teeth implies the presence of gingival tissues and sulcus which serve as the main route for the migration of neutrophils from the junctional epithelium into the GCF and saliva.

Moreover, it is known that dental treatment can alter the microbial community in the oral environment. It has been shown previously that full-mouth dental extraction can initiate the elimination or decrease the prevalence of periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Danser et al., 1994). Similar results have been reported in a more recent cohort study where they evaluated the changes in oral microflora after full-mouth extraction using both culture and quantitative real-time PCR (qPCR) techniques (de Waal et al., 2014). A similar trend was observed in studies where they investigated the oral counts of cariogenic bacteria, specifically *Streptococcus mutans*, after dental treatment in children diagnosed with ECC. Hughes et al. (2012) reported that *Streptococcus mutans* counts were significantly reduced following restorative and preventive dental treatment in children with s-ECC. Moreover, further studies have demonstrated that in children with dental caries presenting with higher counts of *Streptococcus mutans,* dental treatment significantly reduced their counts (Fidalgo et al., 2015, Tanner et al., 2011). Hence, the reduction of the circulating neutrophils together with the reduction of the microbial by-products and stimuli as a result of extraction of carious teeth in the present study might provide another explanation for the reduction of the salivary concentration of LL-37 after dental treatment.

4.2.4 Inflammation up-regulates LL-37 expression

Lastly, enhanced LL-37 expression has also been demonstrated in response to several other oral and systemic inflammatory disorders. It has previously shown that the extent of erosive lesions in patients diagnosed with oral lichen planus correlated with the increased salivary concentration of LL-37 (Davidopoulou et al., 2014). In that study, systemic treatment with corticosteroids improved the clinical appearance of the erosive lesions as well as significantly reduced the salivary concentration of LL-37 with a -34.5 ng/ml median value of concentration change after a 7-day treatment. The previous findings are consistent with another study that showed a positive correlation between the salivary concentration of LL-37 and the number of monthly oral ulcers in patients diagnosed with Behcet's disease (Mumcu et al., 2012). The authors suggested that poor dental and periodontal health in patients with Behcet's disease triggered an inflammatory immune response that stimulated oral epithelium and enhanced neutrophilic activity and function. Elevated levels of LL-37 in GCF and saliva in patients with periodontitis have also been reported in the literature, and it has been suggested that this increase is due to enhanced migration of neutrophils into the gingival crevice (Turkoglu et al., 2017, Takeuchi et al., 2012, Türkoğlu et al., 2009).

In terms of systemic inflammatory disorders, researchers have also reported significantly increased serum concentration of LL-37 in adults with bacterial pneumonia and pulmonary tuberculosis as well as in neonates with congenital pneumonia compared to healthy controls (Majewski et al., 2018, Majewski et al., 2017, Gad et al., 2015). Additionally, patients diagnosed with infective cellulitis showed significantly increased levels of cathelicidin and HBD-1 mRNA expression in the involved skin compared with their expression in the skin of the healthy controls (Stryjewski et al., 2007). This increase in expression has also

been detected in normal-appearing skin distal to the site of active infection suggesting that these AMPs further act to limit the spread of the infection to distal uninfected areas. Therefore, these previous findings present sufficient evidence to support the important role of AMPs, including LL-37, in providing immunity against various inflammatory conditions. Thus, it seems reasonable to assume similar immune responses may occur in relation to dental caries and oral inflammation in general.

4.3 The salivary concentration of LL-37 and caries experience in children

In the current pilot study, we did not find an association between the salivary concentration of LL-37 and caries experience in this population. In addition, the salivary level of this AMP did not correlate with the severity of caries as measured by the number of DT/dt. Similarly, a recent exploratory study found that the salivary levels of HNP-1, hBD-2, and LL-37 in children aged 11-13 years old did not correlate with the incidence of caries and therefore, concluded that these salivary peptides cannot be used as potential caries risk predictors in children (Stojkovic et al., 2020). In addition, Tao et al. (2005) reported that the salivary concentration of LL-37 varied between participants and concluded that the salivary LL-37 does not associate with caries experience in children. These findings are in contrast to the results from a previous study by Davidopoulou et al. (2012) which showed that caries-free children and children with low to moderate caries activity exhibited higher levels of salivary LL-37 than children with high caries activity. Caries severity in the previous study was determined by the number of the DT/dt score which is in accordance with the method in which we measured caries experience in our population. However, caries status in the three groups, classified as mild, moderate, or severe, were not defined by the same number of DT/dt in both studies. A weak positive association between caries experience and salivary LL-37 in children was found in a study by Colombo et al. (2016) where caries extent was determined by the number of decayed missed filled surfaces (dmfs). The different criteria utilised for identifying and recording dental caries might help in understanding the differences in the results generated by the current study and the previous studies.

4.4 Participant demographics and the salivary concentrations of LL-37

Regarding participant demographics, the salivary concentration of LL-37 did not correlate with age in the present study. A positive correlation between cathelicidin and age was shown when examining LL-37 concentrations in plasma in healthy children aged two to seven years (Stukes et al., 2016). Moreover, Davidopoulou et al. (2012) reported a significant positive correlation between the salivary concentration of LL-37 and participant age. Participants recruited into the latter study were between two and 18 years of age and the authors suggested that this correlation is influenced by the type of dentition (primary vs permanent) rather than age itself. Furthermore, they concluded that the salivary concentration of LL-37 significantly increases during the mixed dentition stage and tends to plateau in late adolescence when the permanent dentition is almost fully present. Malcolm et al. (2014) observed that the concentration of the AMPs studied, including LL-37 and bacteria-specific sIgA, increased in children who cultured positive for *Streptococcus mutans* compared to those who cultured negative when the children were followed from 12 to 24 months of age to three years of age. The previous findings can be attributed to the introduction of the primary dentition which initiates a dynamic change in oral microbial colonisation and therefore changes the expression of salivary AMPs. Hence, age-related variations would not have been expected to play a role in the present study since all children recruited were in their mixed dentition stage in the age range of 6 to 10 years.

In addition to age, we found that the pre-treatment salivary concentrations of LL-37 were not statistically significantly different between males and females. These results are in agreement with a previous study that found no correlation between the salivary concentrations of the AMPs investigated, including LL-37, and the participant's age and gender (Tao et al., 2005). Despite demonstrating a higher salivary concentration of LL-37 in girls compared to boys, Davidopoulou et al. (2012) reported that the difference did not reach a statistically significant level. When the salivary concentration of the peptide was investigated by the same group of researchers in adults aged 18 to 83 years, they found that the concentration of LL-37 was higher in females compared to males and the difference was statistically significant. However, no correlation was found between the salivary concentration of LL-37 and the age of the participants (Davidopoulou et al., 2013).

4.5 Study limitations and strengths

This study has some limitations. Information regarding ethnicity, socioeconomic and nutritional status, such as vitamin D status, of the participants, was not collected in the present study. In addition, information regarding dental caries status and severity were collected from the participants' dental files. Collecting retrospective data from previously examined participants performed by multiple uncalibrated examiners would introduce information bias leading to inaccurate estimates of the association between variables. Also, loss-to-follow-up was high after dental treatment, mainly due to the COVID-19 pandemic. However, as there are no similar studies to date, our results have led to a hypothesis that should be confirmed with larger and more representative numbers of participants to allow greater statistical power in the future.

Conducting clinical studies presents some difficulties, including the recruitment process and the occurrence of dropouts during the follow-up visits. Despite these limitations and difficulties, as far as we know, this is the first prospective pilot study that has evaluated changes in salivary concentration of LL-37 after dental treatment involving extraction of carious teeth in children with dental caries. Also, another strength presented in this study is in terms of information regarding the caries status of the participants. The numbers of DT/dt and DS/ds were collected from the participants' clinical dental notes and confirmed by their radiographic examination records. In addition, saliva samples from all participants were collected by a non-invasive standardised method for saliva collection. Finally, the salivary concentration of LL-37 was detected by performing the ELISA technique which is a highly sensitive and specific method for peptide detection.

4.6 Suggestions for future research

The results of the current pilot study have been promising in terms of providing some evidence to support the important role of salivary AMPs, particularly LL-37, in providing innate immunity and defence against microbial invasion during the carious process, although limited. Hence, future well-designed studies might help in predicting the status of dental caries over time and their association with the salivary biomarkers and biomolecules. We suggest conducting a dental examination on the research participants by the chief examiner or by a group of calibrated examiners using a standardised epidemiological method for caries assessment, such as the International Caries Detection and Assessment System (ICDAS). Utilising a standardised method for caries assessment would increase the level of agreement among the examiners and would facilitate the comparison of the results from different studies. In addition, it would be more valuable to include a control group to minimise the effect of potential confounders, such as gingivitis and fasting, and allow comparing the difference in the salivary concentration of LL-37 between the groups. The control group of caries-free children would receive no dental intervention; however, it would be used as a baseline to compare and assess the effect of dental extraction on the salivary concentration of LL-37.

4.7 Null hypothesis outcome

The null hypothesis ‘’There are no differences in salivary concentration of the AMP LL-37 in children before and after dental treatment to remove their carious teeth’’ can be rejected as a significant difference was detected between pre-and post-treatment salivary concentrations of LL-37.

5 CONCLUSION

From the results of this study, it can be concluded that salivary concentration of LL-37 did not correlate with the level of caries experience in this pilot study, and hence it is not a salivary biomarker that can predict caries experience in children. However, in a larger study, the possibility of LL-37 levels predicting caries experience and severity in children could be investigated further.

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APPENDICES

Appendix A : REC approval letter

NHS
Health Research Authority

Yorkshire & The Humber - Leeds West Research Ethics Committee
NHSBT Newcastle Blood Donor Centre
Holland Drive
Newcastle upon Tyne
NE2 4NQ

Telephone: 0207 1048 088

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

23 April 2019

Mrs Fatemah Almusaileekh
Professional Doctorate in Paediatric Dentistry
Postgraduate room, Level 6, Worsely building
Clarendon Way
Leeds
LS2 9LU

Dear Mrs Almusaileekh

Study title:	**Dental Treatment and the Antimicrobial Peptide LL-37 in Children with Caries.**
REC reference:	**19/YH/0119**
Protocol number:	**N/A**
IRAS project ID:	**255435**

The Research Ethics Committee reviewed the above application at the meeting held on 12 April 2019. Thank you for attending to discuss the application.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

The members of the Committee present gave a **favourable** ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Recommendations:

Number	Recommendation
1.	The Committee recommended that hard copies of the forms were kept with a permanent member of staff, e.g. academic supervisor, as this would be more secure than a locked cupboard in the Postgraduate area.
2.	Include the following sentence in the adult PIS, at the end of part 4: 'Your child will receive standard dental treatment. The collection of the saliva samples is the extra part, for the research.'
3.	Proof read all participant facing documents

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System, at www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be

permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non-NHS Sponsors only) [indemnity]	1	17 September 2018
IRAS Application Form [IRAS_Form_07032019]		07 March 2019
Letter from sponsor [indemnity]	1	17 September 2018
Other [Patient summary sheet]	0.1	06 March 2019
Other [Code link]	0.1	06 March 2019
Participant consent form [Parent_consent]	0.3	27 February 2019
Participant consent form [Child_assent]	0.4	27 February 2019
Participant information sheet (PIS) [Child_IS]	0.4	27 February 2019
Participant information sheet (PIS) [PIS]	0.6	27 February 2019
Research protocol or project proposal [Protocol]	0.7	27 February 2019
Summary CV for Chief Investigator (CI) [Fatemah_CV]	6.1	04 March 2019
Summary CV for supervisor (student research) [Prof_Devine_CV]	0	04 March 2019
Summary CV for supervisor (student research) [Dr_Jinous_CV]	0	04 March 2019
Summary CV for supervisor (student research) [JM_CV]		05 March 2019

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at: https://www.hra.nhs.uk/planning-and-improving-research/learning/

19/YH/0119	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely
pp

Sarah Prothero

Dr Rhona Bratt
Chair

E-mail: nrescommittee.yorkandhumber-leedswest@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Copy to: *Faculty NHS Research Ethics Officer*

Lead Nation - England: HRA.Approval@nhs.net

Appendix B : HRA approval letter

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Mrs Fatemah Almusaileekh
Professional Doctorate in Paediatric Dentistry
Postgraduate room, Level 6, Worsely building
Clarendon Way
Leeds
LS2 9LU

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

23 April 2019

Dear Mrs Almusaileekh,

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Dental Treatment and the Antimicrobial Peptide LL-37 in Children with Caries.**
IRAS project ID:	**255435**
Protocol number:	**N/A**
REC reference:	**19/YH/0119**
Sponsor	**University of Leeds**

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Please now work with participating NHS organisations to confirm capacity and capability, in line with the instructions provided in the "Information to support study set up" section towards the end of this letter.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) have been sent to the coordinating centre of each participating nation. The relevant national coordinating function/s will contact you as appropriate.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?

The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **255435**. Please quote this on all correspondence.

Yours sincerely,
Alex Thorpe

Approvals Manager

Email: hra.approval@nhs.net

List of Documents

The final document set assessed and approved by HRA and HCRW Approval is listed below.

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [indemnity]	1	17 September 2018
IRAS Application Form [IRAS_Form_07032019]		07 March 2019
IRAS Application Form XML file [IRAS_Form_07032019]		07 March 2019
IRAS Checklist XML [Checklist_26032019]		26 March 2019
Other [Patient summary sheet]	0.1	06 March 2019
Other [Code link]	0.1	06 March 2019
Participant consent form [Parent_consent]	0.3	27 February 2019
Participant consent form [Child_assent]	0.4	27 February 2019
Participant information sheet (PIS) [Child_IS]	0.4	27 February 2019
Participant information sheet (PIS) [PIS]	0.6	27 February 2019
Research protocol or project proposal [Protocol]	0.7	27 February 2019
Summary CV for Chief Investigator (CI) [Fatemah_CV]	6.1	04 March 2019
Summary CV for supervisor (student research) [Prof_Devine_CV]	0	04 March 2019
Summary CV for supervisor (student research) [Dr_Jinous_CV]	0	04 March 2019
Summary CV for supervisor (student research) [JM_CV]		05 March 2019

IRAS project ID	255435

Information to support study set up

The below provides all parties with information to support the arranging and confirming of capacity and capability with participating NHS organisations in England and Wales. This is intended to be an accurate reflection of the study at the time of issue of this letter.

Types of participating NHS organisation	Expectations related to confirmation of capacity and capability	Agreement to be used	Funding arrangements	Oversight expectations	HR Good Practice Resource Pack expectations
All participating organisations will undertake the same activities, as detailed in the protocol and supporting documents.	Research activities should not commence at participating NHS organisations in England or Wales prior to their formal confirmation of capacity and capability to deliver the study.	A statement of activities has been submitted and the sponsor intends to use this with participating sites.	No funding.	The Chief Investigator will act as the Principal Investigator at the single participating site.	The applicant is undertaking a healthcare placement and is directly supervised by NHS staff. She has routine patient contact and all HR Good Practice, Occupational Health and DBS clearances are expected to be in place already.

Other information to aid study set-up and delivery

This details any other information that may be helpful to sponsors and participating NHS organisations in England and Wales in study set-up.
This is a non-portfolio study.

Appendix C : LTHT R&I approval

Re. Salivary Antimicrobial Molecule in Tooth Decay (Caries) in Children , R&I No: DT19/124003

This email confirms that the Leeds Teaching Hospitals NHS Trust has the capacity and capability to deliver the above research study, based upon **Protocol version 0.7 dated 27/02/2019**. You may now begin the study at this organisation.

Please find attached:

- agreed statement of activities
- agreed schedule of events

It is the responsibility of the principal investigator to ensure that the study is conducted in accordance with the terms of the Health Research Authority approval and Leeds Teaching Hospitals NHS Trust policies and procedures including the requirements for research governance and clinical trials performance management. These are available at https://www.leedsth.nhs.uk/assets/Research/636ce652fc/PI-responsibilities-v2.0-27072018.pdf

Please note: If your study will involve the testing or use of an **interventional procedure which is new to LTHT** you must obtain the approval of the New Interventional Procedures Group (NIPG). Details and application form are available from Jason Dunne, secretary to NIPG, telephone 0113 - 206 6951 or email jason.dunne@nhs.net If your study will involve an interventional procedure which is new to you as an individual (but not to LTHT) you must ensure you have agreement from your clinical director, clinical lead and general manager

If you have any queries please do not hesitate to contact the R&I team at ltht.researchoffice@nhs.net.

Best Wishes

Donna Johnstone
Research and Innovation Manager

Appendix D : Child information sheet

Version 0.4 IRAS Project ID: 255435

School of Dentistry UNIVERSITY OF LEEDS

Why some children get more holes in their teeth than others?

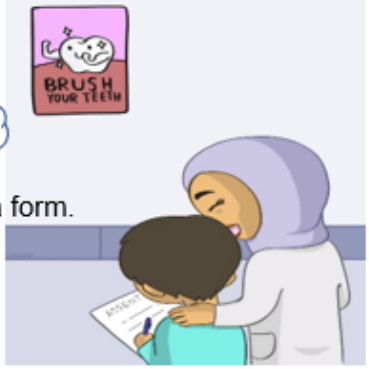
Child information sheet (6-10 years)

1- Hi
My name is Fatemah.
I am inviting you to take part in my project.

2- **Why you?**
We want to talk to you because your dentist has sent you here to fix your poorly teeth.

3- What's going to happen?

- I will tell you about the project.
- Decide if you want to take part Yes No
- If yes, we will ask you to write your name on a form.
- You will help me collect some of your spit.



4- How much do I need to spit?

About a teaspoon that might take a few minutes and I will also ask you to provide some spit again when you come back to the next visit to see me.

5- Can I change my mind?
Of course, you can change your mind at anytime. You do not need to tell us why.

6- Will you still fix my teeth if I choose not to join the project?
Yes, of course.

Appendix E : Parent/guardian information sheet

Version 0.6

IRAS Project ID: 255435

School of Dentistry

UNIVERSITY OF LEEDS

Parental/guardian Information Sheet

Salivary antimicrobial molecule and tooth decay (caries) in children (SAMCARE)

Name of researcher: Fatemah Almusaileekh.

Your child is being invited to take part in a research study. Before you decide whether your child should take part, it is important you understand why the research is being undertaken and what will be involved. Please take some time to read the following information carefully, discuss it with others if you wish and decide whether you wish to take part. Please don't hesitate to ask us if there is anything is unclear, or if you want more information.

1. **What is the purpose of the study?**

We aim to investigate the relationship between immune defences in saliva and tooth decay in children's mouths.

2. **Who is conducting this study?**

A postgraduate student is carrying out this study under the supervision of their supervisors, as part of a doctorate degree at the University of Leeds.

3. **Who is being asked to participate?**

We are inviting children who are due to have decayed teeth removed under general anaesthesia to take part.

4. **What will taking part involve?**

If you are happy for your child to participate, we will ask them to dribble in a pot until approximately one teaspoon of saliva has been collected on the day of the dental treatment under general anaesthesia. Also, we would like to invite you to come back in 1 month as part of your child routine follow up appointment to provide him/her with the proper prevention care (i.e. topical fluoride application) and to collect approximately one teaspoon of saliva again. The saliva samples will be stored for a short period in freezers in Leeds Dental Hospital prior to transfer to the Oral Biology laboratories at St James's Hospital where they will be analysed. At the end of the research the saliva samples will be disposed in accordance with the Human Tissue Authority's Code of Practice.

5. **What are the Advantages and Disadvantages of taking part in this study?**

Taking part will take up a little of your time, adding approximately 15 minutes to each visit. Some children might feel a little embarrassed about spitting into a collection tube. While there may be no personal benefits to your child through participation in this study, we hope the information provided by this study will increase our understanding of the relationship between our natural immune defences and tooth decay. All the information you provide will be kept secure, the analysis of your child's saliva will be anonymised, and the results can only be accessible by members of the research team.

6. **Does my child have to take part & what will happen if my child withdraws from the study?**

Your child participation is entirely voluntary. Please be assured that if you, or your child decline to participate it will not affect the treatment provided. If you or your child decide to withdraw from the study this will not affect the treatment provided to your child, but the collected data may still be used for this research.

7. **What will happen to the results of the research?**

The information will be stored safely and securely in accordance with the Data Protection Act 2018. All the data collected will be kept confidential and anonymised so that those reading reports from the research will not know

who has contributed to it. All research data are intended to be used for the Professional Doctorate research project by Fatemah Almusaileekh and will be published in her thesis.

The University of Leeds is the sponsor for this study based in the United Kingdom. We will be using information from your child and their medical records to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. The University of Leeds will keep identifiable information about your child for 2 years after the study has finished.

Your rights to access, change or move your child's information are limited, as we need to manage your information in specific ways for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about your child that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

Leeds teaching Hospital NHS Trust will use your child's name, NHS number and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from The University of Leeds and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Leeds teaching Hospital NHS Trust will pass these details to University of Leeds along with the information collected from your child and their medical records. The only people in University of Leeds who will have access to information that identifies you will be people who need to contact you to the research or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details. University of Leeds/LTHT will keep identifiable information about your child from this study for 2 years after the study has finished. You can find out more about how we use your information by contacting the University of Leeds Data Protection Officer on DOP@leeds.ac.uk

8. What if I need to complain?

The normal complaints process will apply through the NHS complaints procedure. You can also contact the Patient Advice and Liaison Service (PALS) office in Leeds.

PALS contact details:

T: 0800 052 5790
E: pals.lypft@nhs.net

The PALS Office
Leeds and York Partnership NHS Foundation Trust
The Becklin Centre
Alma Street
Leeds
West Yorkshire
LS9 7BE

The University has in force a Public and Products Liability policy which provides cover for claims for "negligent harm" and the activities of this study are included within that coverage subject to the terms, conditions and exceptions of the policy.

9. Who is organising and funding the research?

This research is funded by the Faculty of Medicine and Health, University of Leeds.

10. Who has reviewed this study?

This Study has been reviewed and approved by the Health Research Authority (HRA) and a local Research Ethics Committee (REC). These bodies have responsibility for scrutinising all proposals for medical research on NHS patients. This research will also be monitored by The University of Leeds and the Leeds Teaching Hospitals Trust (LTHT).

If you agree for your child to take part and would like more information or have any questions, concerns or complaints about the study please contact:

Name	Telephone number	Email address
Mrs Fatemah Almusaileekh	**07568917277**	**dnfra@leeds.ac.uk**
Dr Josephine Meade	+44 (0)113 343 7561	j.l.meade@leeds.ac.uk
Professor Deirdre Devine	+44 (0)113 343 6116	d.a.devine@leeds.ac.uk
Dr Jinous Tahmassebi	01133436138	j.tahmassebi@leeds.ac.uk

Thank you for taking the time to read this information sheet.

Appendix F : Child assent form

Version 0.4 IRAS Project ID: 255435

School of Dentistry
FACULTY OF MEDICINE & HEALTH

UNIVERSITY OF LEEDS

Participant Code: ☐

Assent Form for child aged 6-10 years

(To be completed once parents/guardian has consented)

Project Name: Why some children get more holes in their teeth than others?
Chief investigator: Fatemah Almusaileekh

Tick the boxes if you agree

- I have read (have read to me) and understand the information about this study. ☐
- I have asked all the questions about the study that I want to and my questions have been answered. ☐
- I know I can stop being in the study whenever I want, for any reason and I will still be looked after the same. ☐
- I understand that all my information will be kept safe. ☐
- I understand that Fatemah will collect some of my dribbles/Spits for the project. ☐
- I agree to be in this study. ☐

Participant's name: **Date:**
Signature:
Name of the researcher: **Date:**
Signature:

Appendix G : Parent/guardian consent form

Version 0.3 IRAS Project ID: 255435

School of Dentistry
FACULTY OF MEDICINE & HEALTH

UNIVERSITY OF LEEDS

Participant code: ☐

Parental/guardian consent form

Salivary antimicrobial molecule and tooth decay (caries) in children (SAMCARE)

1	I confirm that I have read and understand the information sheet explaining the above research project and I have had the opportunity to ask questions about the project.	☐
2	I understand that my child's participation is voluntary and that my child is free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should my child not wish to answer any particular question or questions, he/she are free to decline.	☐
3	I understand that my child's name will not be linked with the research materials, and we will not be identified or identifiable in the report or reports that result from the research.	☐
4	I agree my child's notes can be looked at by the researchers/dental team for the purpose of this research.	☐
5	I agree for my child's data collected from our participation can be used anonymously in this research.	☐
6	I agree for my child saliva to be collected and to be used for this dental research.	☐
7	I agree for my child to take part in the above research project.	☐

_____	_____	_____
Initials of the Parent	Date	Signature
(*Or legal representative*)		
_____	_____	_____
Dental Operator	Date	Signature

To be signed and dated in presence of the participant

Copies:
Once this has been signed by all parties the participant should receive a copy of the signed and dated participant consent form, the letter/pre-written script/information sheet and any other written information provided to the participants. A copy of the signed and dated consent form should be kept with the project's main documents which must be kept in a secure location.

Appendix H : Summary of raw data of experiment 1 (1st technical repeat).

	Pre-treatment		Post-treatment	
Participant's code	Corrected average OD	salivary concentration of LL-37 (ng/ml)	Corrected average OD	salivary concentration of LL-37 (ng/ml)
1	0.33	84.25	0.13	32.87
2	0.92	238.27	0.48	125.38
3	0.91	235.54	0.25	63.69
4	1.47	379.77	0.18	46.21
5	0.22	57.90	0.12	30.17
6	0.40	104.18	-	-
7	0.64	165.36	0.16	40.22
8	0.44	114.39	-	-
9	0.99	256.40	0.46	120.38
10	0.22	56.87	0.02	5.99
11	0.45	116.76	0.03	7.22
12	0.55	143.06	0.16	41.11
13	0.37	96.96	0.00	0.21
15	1.41	366.49	-	-
16	0.54	140.92	-	-
17	0.14	36.33	-	-
18	0.19	48.22	-	-
22	0.25	65.09	-	-
24	0.44	112.86	-	-
26	0.38	99.12	-	-
29	0.81	209.49	-	-

Appendix I : Summary of raw data of experiment 2 (2nd technical repeat).

	Pre-treatment		Post-treatment	
Participant's code	Corrected average OD	Salivary concentration of LL-37 (ng/ml)	Corrected average OD	Salivary concentration of LL-37 (ng/ml)
1	0.18	67.5	0.08	31.88
2	0.58	222.01	0.23	87.83
3	0.48	184.24	0.15	56.61
4	0.93	356.23	0.06	24.05
5	0.10	39.83	0.05	17.37
6	0.27	103.91	-	-
7	0.29	111.19	0.09	36.09
8	0.13	49.25	-	-
9	0.42	159.46	0.21	79.35
10	0.10	37.33	0.02	8.90
11	0.20	75.24	0.01	5.17
12	0.25	95.08	0.11	42.42
13	0.20	75.9	0.00	0.25
15	0.81	309.6	-	-
16	0.15	55.87	-	-
17	0.07	27.87	-	-
18	0.11	42.2	-	-
22	0.16	61.46	-	-
24	0.31	120.26	-	-
26	0.22	82.75	-	-
29	0.36	138.66	-	-