

Innate Immunity Signatures of Early Childhood Caries (ECC) And Severe Early Childhood Caries (S-ECC)

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Caries that affects children under the age of 6 is known as early childhood caries (ECC). According to the American Academy of Pediatric Dentistry (AAPD), ECC is defined as the presence of one or more teeth with caries (cavities or no cavities), missing teeth due to caries or the presence of restoration deciduous teeth in children under 71 months. If it occurred on the smooth surface of the tooth in children under 3 years of age, the disease is classified as severe early childhood caries (S-ECC). *Streptococcus mutans* plays important role in the etiology of caries of preschool children, which is a contagious pathological entity. The pathogen is an acidogenic and aciduric bacteria. The occurrence of caries is a complex process of interaction between host factors (teeth), microorganisms, substrates (carbohydrates) and the environment, including saliva. This mini review aims to summarize current findings on several markers of innate immune system related to ECC and S-ECC namely lysozyme, salivary lactoferrin, IL-8, CD36 of salivary neutrophils, and sCD14.

Keywords: ECC, S-ECC, Innate immune system, Biomarkers.

Introducing ECC & S-ECC

Caries is a chronic infectious disease that affects the hard tissues of the teeth and can occur in adults or children. Caries that affects children under the age of 6 is called early childhood caries (ECC). According to the American Academy of Pediatric Dentistry (AAPD)¹, ECC is defined as the presence of one or more teeth with caries (cavities or no cavities), missing teeth due to caries or the presence of restoration of deciduous teeth in children under 71 months or 6 years. If the children under 3 years of age are experiencing caries on the smooth surface of the teeth, it indicates severe early

childhood caries (S-ECC). The ECC starts with a white spot on the deciduous / upper maxillary incisors along the gingival margin, and if caries continues it will damage the entire crown of the tooth.²

The prevalence of ECC in developing countries reaches 85% in the weak economic group.³ The American Academy of Pediatric Dentistry (AAPD) found that 70% of children aged 2-5 years have caries. In Indonesia the prevalence of caries in 5-year-olds is 67.3%, with a caries experience rate of e^7 6, this is a severe category of ECC.⁴

The factors responsible in ECC are supportive host, fermented carbohydrate diets, dental plaques, and the number of cariogenic microbes, such as *Streptococcus mutans* and *Lactobacillus*.⁵ *S. mutans* is an essential causative agent of caries among preschoolers. This species is both acidogenic and aciduric, with the resulting caries shown to be contagious.⁶ Initial acquisition of *S. mutans* is the key to caries disease, in which acquisition can occur through vertical and horizontal transmission.⁷ The caries process occurs when the activity of bacterial (*Streptococcus mutans*) enzymes in the oral fermented carbohydrate and produce acids, it will cause demineralization of the tooth surface.⁸ If the demineralization process is more often or extensive than the remineralization process, caries occurs.³ Recent study on a geriatric population found that *S. mutans* is also intriguingly related to higher risk of suffering stroke and dementia.⁹ Whether acquisition of this bacteria is associated to increased risk of having reduced cognitive performance in children, other than causing caries, deserves further attention.

Several studies have been carried out to find out the markers related to caries. Mass *et al.* found an association between low levels of lysozyme and decreasing in the number of *S. mutans*.⁹ Klissia *et al.* found the relationship of lysozyme to dmft score, and lactoferrin versus the number of restored teeth in school children. The level of lactoferrin and lysozyme may be used to evaluate caries status.¹⁰ In view of these findings, we aimed to present current knowledge on several biomarkers associated with ECC and S-ECC, especially from the point of view of innate immune system.

Lysozyme

Lysozyme is a salivary protein that plays an important role in innate immunity. This 14 kDa basic protein is found in a vast array of body fluids such as plasma/serum, tears, amniotic fluid, saliva, urine, bile and CSF. This enzyme has the ability to act as an antibacterial agent. In the oral cavity, lysozyme is secreted from the major and minor salivary glands, gingival crevicular fluid (GCF), and salivary leukocytes. Lysozyme exhibits the activity of muramidase, which hydrolyzes β -(1-4) bonds between N-acetylmuramide acid and N-acetylglucosamine of bacterial cell wall peptidoglycan, thus degrading bacterial wall

integrity, resulting in hypo-osmolarization and bactericidal effect. As a strong cationic protein, lysozyme is a medium of aggregation and attachment of bacteria to activate the autolysin of bacteria, which also damages bacterial cell walls.^{11,12}

Octiara *et al.* found that lysozyme is a promising biomarker of ECC. Higher lysozyme level was found in caries-free controls than that of ECC groups.¹³ Rather than relying solely to salivary lysozyme level measurement, it is suggested that studying the lysozyme activity is also crucial because cariostatic activity of lysozyme is a resultant of its salivary concentration and status of activation.¹⁴ Currently, clinical study that investigates both lysozyme level and activity, and their correlations with clinical outcomes (dmft scores) in ECC and S-ECC patients is lacking.

Lactoferrin

Lactoferrin is a salivary protein that plays an important role in innate immunity (non-specific body defense system). Lactoferrin is a 75 kDa salivary protein that has a high affinity for the iron binding glycoprotein. The cariopreventive role of this molecule is due to its effect in attracting iron (iron chelation) from bacteria.¹¹ Epithelial cells in the mucosa and neutrophils are known as the endogenous producers of this protein.¹⁵ Lactoferrin is present in saliva, tears and secondary granule of leukocytes. Intriguingly, iron-free lactoferrin (apolactoferrin) has also antibacterial ability through direct binding of lactoferrin to bacteria and agglutination of *S. mutans*, thereby facilitating removal of agglutinated bacteria through mechanical action of salivary ingestion. *In vitro*, lactoferrin has been shown to exhibit anti-inflammatory and antimicrobial effects.^{15,16}

Studies on the association of lactoferrin and salivary lysozyme that are not stimulated on ECC showed that the levels of lactoferrin and lysozyme are higher in caries-free individuals than ECC-positive individuals.¹⁶ The levels of lactoferrin and lysozyme decrease significantly after 3 months of comprehensive dental treatment of ECC patients.¹⁷ However, other study have shown the opposite, i.e., the upregulation of salivary lysozyme levels along with the increased lysozyme activity in preschool children with S-ECC compared with caries-free groups. The inconsistency between studies may be the resultant

of a number of factors such as different ways applied for lysozyme quantification, dentition status (deciduous versus permanent), and the temporal regulation of lysozyme level (i.e., age-related changes).¹⁸

Interleukin 8 (IL-8)

Neutrophils in saliva is the first defense of the immune cell against microbial pathogens, it can recognize bound surfaces or free molecules secreted by bacteria including *S. mutans* such as peptidoglycan, lipoprotein, lipoteichoic acid (LTA), lipopolysaccharide (LPS), CpG-containing DNA, and flagelin. These pathogenic molecules, collectively known as pathogen associated molecular patterns (PAMPs), interact directly with a number of pathogen recognition receptors (PRRs) expressed on the cell surface, including toll-like receptors (TLRs).¹⁹

Interleukin-8 (IL-8) is a member of CXC family chemokine. IL-8 is a key mediator in the migration of neutrophils to areas of inflammation and infection.^{20,21} Interleukin-8 (IL-8) is synthesized by polymorphonuclear leukocytes (PMNs), with lactoferrin as an endogenous stimulator for the secretion.²² If there is stimulation of microbial groups, G-CSF or GM-CSF, tumor necrosis factor- α (TNF- α) or type I and II interferon in the inflamed tissue, activated neutrophils will produce IL-8.²³ IL-8 is secreted by macrophages and endothelial cells as neutrophil attracting chemicals, lead the infiltration of neutrophils. *In vitro* IL-8 results in the recruitment of neutrophils

and increases the release of lysosomal enzymes against bacteria.^{24,25}

Luthfi *et al.* showed that IL-8 expression was lower in S-ECC (early childhood caries) than caries-free early childhood. The lower expression of IL-8 in S-ECC salivary neutrophils causes the killing of *S. mutans* to be less effective because the oxidative activity of NADPH was low. The degranulation process is also becoming less active so that the killing in the phagosome decreased, thus the cariogenesis is continued. The reduced expression of IL-8 in salivary neutrophils may be one of the causes of the increasing number of *S. mutans* in S-ECC.²⁶

AZurophilic Granules (CD63) In Salivary neutrophil

Neutrophils are phagocytic cells in the first line of defense against pathogenic bacteria. Primary granules (azurophilic granules) in salivary neutrophils contain antimicrobial molecules, such as defensin like human neutrophil peptide 1-3 (HNP1-3), elastase, cathepsin G and proteinase 3, and CD63. These granules are related to the phagocytosis tool which releases its contents in the phagosome which causes microbes are phagocytosed. Gram-positive and gram-negative microbes, including *S. mutans*, are the main bacteria that cause dental caries.²⁷ Lutfi's study showed that CD63 neutrophil salivary expression decreased in S-ECC.²⁵

The low neutrophil salivary CD63 expression on S-ECC is caused by *S. mutans* which

Table 1. Summary on Biomarkers Related to ECC and S-ECC

Markers	Conditions	Related Molecular/ Cellular Events	Outcomes	References
Lysozyme	High	High sIgA level	Associated to ECC-free phenotype and lower dmft score	13
Lactoferrin	High	Anti-inflammatory action (<i>in vitro</i>)	Associated to caries-free phenotype	12, 15
IL-8	Low	Reduced neutrophil recruitment	Associated to S-ECC severity or progression	20, 23, 24,
CD63 in salivary neutrophils	Low	Higher level of <i>S. mutans</i> ; Elastase and cathepsin G deficiency	Associated to S-ECC severity	25, 26, 28
sCD14	Undetectable or Low	Formation of stable complex with bacterial LPS	Associated to ECC phenotype	31, 32, 34

Table 2. Genes, Chromosomal Loci, and Local Sources of ECC- and S-ECC Potential Biomarkers

Markers	Encoding-genes	Cytogenetic Loci	Oral Sources	References
Lysozyme	<i>LYZ (LYZF1, LZM)</i>	12q15	Salivary glands, salivary neutrophils	11, 12, 36
Lactoferrin	<i>LTF (LF)</i>	3p21.3	Mucosal epithelial cells and neutrophils	15, 37, 38
IL-8	<i>CXCL8</i>	4q13.3	PMN leukocytes	22, 39
CD63	<i>CD63 (MLA1)</i>	12q13.2	PMN leukocytes	40, 41
CD14	<i>CD14</i>	5q31.3	Monocytes (secreted upon activation)	42, 43

has been internalized by neutrophils through the process of phagocytosis mediated by Fc α R (CD89) or CR1 (CD35) which can develop into three defense system strategies to avoid intracellular killing, first, leaving the phagosome, second, the combined blocking of phagosome-lysosomes, and third, using a mechanism that allows survival in phagolysosomes.²⁸ Low neutrophil salivary CD63 on S-ECC is also caused by protein elastase and cathepsin G deficiency.²⁹

Lack of active neutrophils will produce less extracellular traps (NETs) that work to kill extracellular microbes because NETs contain lactoferrin, cathepsin and enzymes which are highly toxic for microbes, in addition to facilitating the phagocytosis process.³⁰ Decreased expression of salivary neutrophil CD63 is found to be associated with higher *S. mutans* levels in S-ECC cases.²⁶

Soluble CD14

Other potentially useful salivary biomarker of early carious lesions in children is soluble or saliva CD14 (sCD14). One study finds that this molecule is undetectable through Western blotting in the saliva of early childhood caries patients, meanwhile the opposite state does exist in normal control. Following dental treatment against the lesions, sCD14 is detectable in the saliva.³¹ Similar pattern of relationship between sCD14 and ECC is recently revealed by Biria *et al.*³² Therefore, sCD14 can be viewed as a potentially promising biomarker of ECC. The level of sCD14 can be used as a molecular cue to differentiate carious from noncarious lesions in clinical setting, and in monitoring or evaluating the effectiveness of cariostatic treatment.

Soluble or salivary CD14 is a bacterial pattern recognition receptor (PRR) known to be secreted into the saliva by the salivary glands

in a constitutive manner.³³ Other study suggests that sCD14 act as immunological component against bacterial LPS, by forming CD14-LPS complex. This complex can be generated under LPS-binding protein (LBP)-independent mechanism, with LBP acting as a catalysator of CD14-LPS complex genesis.³⁴ Soluble CD14 is also known to facilitate internalization of *Actinobacillus actinomycetemcomitans* (a periodontopathic species) by human oral epithelial cells *in vitro*, using actin cytoskeleton-dependent mechanism and up-regulate IL-8 synthesis.³⁵ Although these later findings further underline the role of sCD14 as an important player of innate immune response “symphony” in oral region, up to date, the occurrence of similar cellular event directed against *S. mutans* is unknown. Overall, this body of evidences put sCD14 in the limelight as an essential molecule in oral homeostasis, with further investigations being awaited to reveal its exact immunological role in caries pathobiology.

CONCLUSION

Some promising innate immunity markers associated with ECC / Early Childhood Caries or S-ECC / Severe-Early Childhood Caries are lysozyme, lactoferrin, IL-8, CD36 of salivary neutrophil, and sCD14. Low levels of these biomarkers are associated with carious phenotype in children (as summarized in table 1, and with the oral sources and genetic aspects being summarized in table 2). Further studies are warranted in revealing the complete picture (complex “spider web” of interactomes) of these molecules in the pathogenesis of ECC and S-ECC, and how the profiles relate to the clinical outcomes.

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REFERENCES

- American Academy of Pediatric Dentistry. Reference manual 2002-2003. Policies on early childhood caries; Unique Challenges and Treatment Options. *Pediatric Dent.* **23**:24-5 (2003).
- Pinkham JR. Pediatric Dentistry: Infancy through Adolescence, 3rd ed. WB Saunders Co., London, UK. (1999).
- Kawashita Y, Kitamura M, Saito T. Early Childhood Caries. *Int J Dent.* **2011**:1-7(2011).
- Risikesdas. Prevalensi Karies dan Rata-rata Karies Anak-anak. Kementerian Kesehatan RI (2018). (Article in Indonesian Language or Bahasa Indonesia).
- Ripa LW. Nursing caries: a comprehensive review. *Pediatric Dentistry.* **10**(4):268-282 (1988).
- Banas JA. Virulence properties of *Streptococcus mutans*. *Front Biosci.* **9**(1-3):1267-77 (2004).
- Berkowitz RJ. Causes, Treatment and Prevention of Early Childhood Caries: A Microbiologic Perspective. *J Can Dent Assoc.* **69**(5):304-7(2003).
- Finn SB. Clinical Pedodontics, 4th ed. W.B. Saunders. Co., Philadelphia. 2003.
- Watanabe I, Kuriyama N, Miyatani F, Nomura R, Naka S, Nakano K, *et al.* Oral Cnm-positive *Streptococcus mutans* Expressing Collagen Binding Activity is a Risk Factor for Cerebral Microbleeds and Cognitive Impairment. *Sci Rep.* **6**:1-10 (2016).
- Mass E, Gadoth N, Harell D, Wolff A. Can salivary composition and high flow rate explain the low caries rate in children with familial dysautonomia? *Pediatr Dent.* **24**:581-6(2002).
- Felizardo KR, Gonçalves RB, Schwarcz WD, Poli-Frederico RC, Maciel SM, de Andrade FB, An evaluation of the expression profiles of salivary proteins lactoferrin and lysozyme and their association with caries experience and activity, *Rev Odonto Ciênc.* **25**(4):344-349 (2010).
- Marsh P, Martin M.V. *Oral Microbiology.* 4th ed. Reed Educational and Profesional Publishing Ltd. Oxford (2000).
- Octiara E, Sutadi H, Siregar Y, Primasari A. sIgA and Lisozim as Biomarkers of Early Childhood Caries Risk. *Advances in Health Science Research.* **8**:96-101 (2018).
- Jenzano JW, Hogan SL, Lundblad RL. Factors influencing measurement of human salivary lysozyme in lysoplate and turbidimetric assays. *J Clin Microbiol.* **24**:963-967 (1986).
- Giansanti F, Panella G, Leboffe L, Antonini G. Lactoferrin from Milk: Nutraceutical and Pharmacological Properties. *Pharmaceuticals (Basel).* **9**(4):1-15 (2016).
- Moslemi M, Sattari M, Kooshki F, Fotuhi F, Modarresi N, Sadrabad ZK, *et al.* Relationship of Salivary Lactoferrin and Lysozyme Concentrations with Early Childhood Caries. *J Dent Res Dent Clin Dent Prospects.* **9**(2): 109-14 (2015).
- Sharma A, Subramaniam P, Moiden S. Analysis of Salivary IgA, Amylase, Lactoferrin, and Lysozyme Before and After Comprehensive Dental Treatment in Children: A Prospective Study. *Contemp Clin Dent.* **8**(4):526-530(2017).
- Lertsirivorakul J, Petsongkram B, Chaiyarit P, Klaynongsruang S, Pitiphat W. Salivary Lysozyme in Relation to Dental Caries among Thai Preschoolers. *J Clin Pediatr Dent.* **39**(4):343-7 (2015).
- Lehner T. *Imunologi Pada Penyakit Mulut.* Edisi 3. Penerbit Buku Kedokteran, EGC, Jakarta. (1995) (Book in Indonesian Language or Bahasa Indonesia).
- Baratawidjaya KG, Rengganis I. *Imunologi Dasar.* Edisi IX, cetakan ke-2. Badan Penerbit Fakultas Kedokteran UI, Jakarta: 217-256; 257-83. (2014) (Book in Indonesian Language or Bahasa Indonesia).
- Luthfi, M., Indrawati, R., Arundina, I., Dachlan, Y.P., Korelasi Jumlah Streptococcus mutans (*S. mutans*) dan Level Ekspresi Interleukin 8 (IL-8) pada Severe Early Childhood Caries. *Maj Kedokt Gigi Indon.* **1**(2):142 – 8 (2015) (article in Indonesian Language or Bahasa Indonesia).
- Shinoda I, Takase M, Fukuwatari Y, Shimamura S, Köller M, König W. Effects of Lactoferrin and Lactoferricin® on the Release of Interleukin 8 from Human Polymorphonuclear Leukocytes. *Biosci Biotechnol Biochem.* **60**(3):521-3 (1996).
- Baggiolini M, Alfred WA, Kunkel SL. Neutrophil-activating Peptide-1/Interleukin 8, a Novel Cytokine That Activates Neutrophils. *J Clin Invest.* **84**:1045-49 (1989).
- Abul KA, Andrew HL, Shiv P. Cellular and Molecular Immunology. 6th ed. Elsevier-Saunders: 245-269 (2007).
- Male D, Brostoff J, Roth B, Roitt I. *Immunology.*

- 7th ed. Mosby, New York. 147-167 (2006).
26. Luthfi, M. Expression Analysis of CD63 in Salivary Neutrophils and The Increased Level of *Streptococcus mutans* in Severe Early Childhood Caries. *Dental J (MajKedok Gigi)*. **48**(2):89-93 (2015).
 27. Skubitz KM. *Neutrophilic leukocytes*. In: Greer JP, eds. *Wintrobe's clinical hematology*. 12th ed. Philadelphia: Lippincott Williams and Wilkins: 170-213 (2009).
 28. Rosenberger, CM, Finlay, BB, 2003. Phagocyte sabotage: disruption of macrophage signaling by bacterial pathogens. *Nat Rev Mol Cell Biol*. **4**(5):385-96 (2003).
 29. Urban CF, Lourido S, Zychlinsky A. How do microbes evade neutrophil killing? *Cell Microbiol*. **8**(11):1687-96 (2006).
 30. Phillipson M, Kubes P. The neutrophil in vascular inflammation. *Nat Med*. **17**(11):1381-90. (2011).
 31. Bergandi L, Defabianis P, Re F, Pretti G, Aldieri E, Garetto S. *et al.* Absence of soluble CD14 in saliva of young patients with dental caries. *Eur J Oral Sci*. **115**:93-6 (2007).
 32. Biria M, Sattari M, Golpayegani MV, Kooshki F. Association of Salivary sCD14 Concentration Levels with Early Childhood Caries. *Iran J Immunol*. **7**(3):193-7 (2010).
 33. Uehara A, Sugawara S, Watanabe K, Echigo S, Sato M, Yamaguchi T, Takada H. Constitutive expression of a bacterial pattern recognition receptor, CD14, in human salivary glands and secretion as a soluble form in saliva. *ClinDiagn Lab Immunol*. **10**(2):286-92(2003).
 34. Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, *et al.* Lipopolysaccharide (LPS)-binding Protein Accelerates the Binding of LPS to CD14. *J Exp Med*. **179**:269-77 (1994).
 35. Takayama A, Satoh A, Ngai T, Nishimura T, Ikawa K, Matsuyama T, *et al.* Augmentation of *Actinobacillusactinomycetemcomitans* invasion of human oral epithelial cells and up-regulation of interleukin-8 production by saliva CD14. *Infect Immun*. **71**(10):5598-604 (2003).
 36. Genetics Home Reference – NIH U.S. National Library of Medicine. LYZ gene. Available at:<https://ghr.nlm.nih.gov/gene/LYZ#location>(published June 11, 2019).
 37. Kim SJ, Yu D, Pak K, Jeong S, Kim S, Lee K. Structure of the Human Lactoferrin Gene and its Chromosomal Localization. *Mol Cells*. **8**(6):663-8 (1998).
 38. NCBI Gene Resources. LTF lactotransferrin [*Homo sapiens* (human)]. Available at:<https://www.ncbi.nlm.nih.gov/gene/4057>(updated June 5, 2019).
 39. NCBI Gene Resources. CXCL8 C-X-C motif chemokine ligand 8 [*Homo sapiens* (human)]. Available at:<https://www.ncbi.nlm.nih.gov/gene/3576> (updated June 11, 2019).
 40. Rijkschroeff P, Jansen IDC, van der Weijden FA, Keijser BJB, Loos BG, Nicu EA. Oral polymorphonuclear neutrophil characteristics in relation to oral health: a cross-sectional, observational clinical study. *Int J Oral Sci*. **8**:191-8 (2016).
 41. NCBI Gene Resources. CD63 CD63 molecule [*Homo sapiens* (human)]. Available at:<https://www.ncbi.nlm.nih.gov/gene/967> (updated May 12, 2019).
 42. CD14 molecule [*Homo sapiens* (human)]. Available at:<https://www.ncbi.nlm.nih.gov>