Early childhood caries in relation to mode of delivery, preterm birth, tooth brushing habits, and signs of the metabolic syndrome

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Cover illustration: Josefine Roswall

To Ellen, Leo and Anders

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ABSTRACT

Background: Early childhood caries (ECC) is defined as the presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries), or filled surfaces in any primary tooth of a child under 6 years of age. ECC is highly prevalent in the world and largely untreated in children under the age of 3 years; it shares common risk factors with other non-communicable diseases such as cardiovascular disease, diabetes, and obesity. ECC can impair quality of life by causing difficulties in sleeping, eating, and attending school due to loss of tooth substance with consequent acute and chronic pain. Even though several risk factors associated with ECC are known, it is difficult to predict the condition. Variables that have been used when trying to predict ECC include oral habits, dietary habits, and socioeconomic status. It is known that children born vaginally have a gut microflora that differs from children delivered by cesarean section (C-section). Recently, the oral microbiota has also been explored in relation to mode of delivery.

Aim: The aim of this thesis was to study whether mode of delivery affects selected oral bacteria during the first 6 months of life and to explore whether mode of delivery, early oral habits, family characteristics, perinatal factors, and nutritional and nursing habits during the first 2 years of life, are associated with ECC at 5 years of age. A further aim was to explore whether there is a correlation between ECC and metabolic risk factors according to IDEFICS monitoring levels for the metabolic syndrome.

Methods: In paper I, 149 infants, delivered either vaginally or by C-section, were followed prospectively from birth to 6 months of age. Saliva samples were collected at 0, 1, 3, and 6 months of age. At 6 months a saliva sample was also taken from the mother of the child. The parents answered questionnaires on socioeconomic factors, feeding habits, prescribed antibiotics, and oral habits at baseline and at every sampling occasion. The samples were analyzed with 13 pre-determined bacterial probes using checkerboard DNA-DNA hybridization technique. In papers II–IV, 395 infants were invited to a prospective medical study at the pediatric clinic, Halland Hospital Halmstad, with a focus on growth and overweight prevention. This group was then merged with 156 infants delivered by C-section into a group that was followed for endocrine research purposes. Of the invited children, 336 were examined at the dental clinic at 2 years of age, 302 at three years of age, and 292 at 5 years of age. Two calibrated examiners at the dental clinic then assessed caries lesions (cavitated and non-cavitated) on tooth and surface levels. Background data on the mothers and perinatal data on the infants were extracted from hospital records. The parents of the child also answered sets of questionnaires about oral and feeding habits. Of the 292 children examined at the dental clinic at 5 years of age, 208 were than re-examined at the pediatric clinic at the age of 6.5 (paper IV).

Results: In paper I, the children delivered vaginally had a greater diversity of the selected oral bacteria strains, compared to children delivered by C-section. In paper II, children delivered by C-section had a significantly elevated risk of having caries at 5 years of age. The risk of ECC at 5 years of age was also increased for children born preterm. The relative risk for ECC at 5 years of age (paper III) was significantly increased for tooth brushing less than twice daily at 2 as well as 3 years of age. In paper IV, the perinatal factors preterm birth or being born small for gestational age raised the risk of ECC at 5 years of age. Preschool children with ECC had higher fasting glucose levels, but no other signs of the metabolic syndrome.

Conclusion: The findings of this thesis stress the need for a good cooperation between dental and pediatric professionals, even for infants. The health personnel should instruct and encourage parents to brush their child's teeth twice daily, as well as give appropriate advice concerning food habits. The association between fasting blood glucose and caries, the relationship between perinatal factors and caries, and the influence and evolvement of the oral microbiome are all issues to be further explored in this cohort and in future studies.

SAMMANFATTNING PÅ SVENSKA

Bakgrund: Early childhood caries (ECC) definieras som "närvaron av en eller flera karierade (intial- eller manifest karies), saknade (på grund av karies), eller fyllda ytor, i primära tänder hos barn yngre än 6 år". ECC är vanligt förekommande i världen och till stor del obehandlad när det gäller barn yngre än 3 års ålder. Karies har flera riskfaktorer gemensamt med andra kroniska sjukdomar, såsom diabetes, obesitas och kardiovaskulär sjukdom. ECC kan genom förlust av tandsubstans och akut och kronisk smärta försämra barns livskvalité på olika sätt till exempel genom att orsaka sömnsvårigheter, svårigheter att äta samt frånvaro från skolan. Trots att det finns flera kända riskfaktorer förknippade med ECC är sjukdomen svår att förutspå. Riskfaktorer som använts för att förutspå ECC är till exempel munhygienvanor, kostvanor och socioekonomisk status. Det är känt att barn födda med kejsarsnitt har en annan tarmflora än barn födda vaginalt. På senare år har även den orala floran undersökts i förhållande till förlossningssätt.

Syfte: Ett syfte med denna avhandling var att undersöka om förlossningssättet påverkade utvalda orala bakterier under ett barns första 6 levnadsmånader. Ett annat syfte var att undersöka om förlossningssättet och andra perinatala faktorer samt tidiga munhygienvanor, kostvanor och familjeförhållanden var förknippade med ECC vid 5 års ålder. Ytterligare ett syfte var att utforska om det finns ett samband mellan ECC och metabola riskfaktorer.

Material och metod: I delarbete I följdes 149 vaginal- eller kejsarsnittsförlösta spädbarn under 6 månader. Salivprov togs vid 0, 1, 3 och 6 månaders ålder. Föräldrarna besvarade frågeformulär avseende bakgrundsfaktorer samt kost- och munhygienvanor vid studiens början och därefter fortlöpande vid varje provtillfälle. Salivproverna analyserades med checkerboard DNA-DNA hybridiseringsteknik avseende 13 utvalda bakteriearter. I delarbete II-IV inbjöds 395 spädbarn att delta i en prospektiv studie på Barnkliniken, Hallands sjukhus Halmstad, med fokus på bland annat tillväxt samt övervikt- och fetmaprevention. Denna grupp med barn slogs sedan samman med 156 barn födda med kejsarsnitt, till en grupp som följdes avseende endokrina frågeställningar. Vid 2 års ålder undersöktes 336 av barnen på Specialisttandvården, Hallands sjukhus Halmstad, därefter undersöktes 302 barn vid 3 års ålder och 292 barn vid 5 års ålder. Vid undersökningen registrerades kariesförekomsten (manifest och intial karies) på tand- och ytnivå (dmft, dmfs). 208 av barnen i studien undersöktes sedan också återigen på Barnkliniken vid 6,5 års ålder. Bakgrundsdata gällande mödrar och barn utlästes ur journaler. Föräldrarna besvarade också omfattande frågeformulär

om bland annat bakgrundsfaktorer, munhygienvanor och kostvanor, vid ett stort antal tillfällen under barnens förskoleår.

Resultat: Barnen i delarbete I som var födda vaginalt visade sig ha en mer diversifierad mikroflora avseende de utvalda bakteriearterna. Barn födda med kejsarsnitt, sågs i delarbete II-IV ha en signifikant förhöjd risk för ECC vid 5 års ålder. Om tandborstning skedde mer sällan än två gånger per dag vid 2 och 3 års ålder, ökade risken för karies vid 5 års ålder (delarbete III). I delarbete IV konstaterades att de perinatala faktorerna prematur födsel (före vecka 37) och liten för gestationsåldern båda ökade kariesrisken vid 5 års ålder. Förskolebarn med ECC hade också ett högre genomsnittligt faste-glukosvärde jämfört med barn utan karies. Inga andra samband sågs mellan karies och metabola syndromet.

Slutsats: Ovanstående fynd styrker behovet av ett gott samarbete mellan tandvård och barnhälsovård redan från spädbarnsåldern. Vårdpersonal bör ge lämpliga kostråd samt instruera och uppmuntra föräldrar att borsta sina barns tänder två gånger per dag. Framtida studier bör undersöka sambandet mellan faste-glukos och karies, utvecklingen av den orala mikrofloran samt relationen mellan perinatala faktorer och ECC.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I.   Boustedt K, Roswall J, Dahlén G, Dahlgren J, Twetman S. Salivary microflora and mode of delivery: a prospective case control study. *BMC Oral Health* 2015;15:155.

II.   Boustedt K, Roswall J, Twetman S, Dahlgren J. Influence of mode of delivery, family and nursing determinants on early childhood caries development: a prospective cohort study. *Acta Odontologica Scandinavica* 2018;76:595–599.

III. Boustedt K, Dahlgren J, Twetman S, Roswall J. Tooth brushing habits and prevalence of early childhood caries: a prospective cohort study. *Eur Arch Paediatr Dent*. 2019 Jul 23. doi: 10.1007/s40368-019-00463-3. [Epub ahead of print]

IV.   Boustedt K, Roswall J, Kjellberg E, Twetman S, Dahlgren J. A prospective study of perinatal and metabolic risk factors for early childhood caries. *Submitted*.

CONTENTS

ABBREVIATIONS

BMI   Body mass index (kg/m²)

C-section   Cesarean section

dmft   decayed, missing, filled teeth

ECC   Early childhood caries

IR   Insulin resistance

LGA   Large for gestational age

RR   Relative risk

SD   Standard deviation

SGA   Small for gestational age

WHO   World Health Organization

DEFINITIONS IN SHORT

Early childhood caries The presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries), or filled surfaces in any primary tooth of a child under 6 years of age. (1)

dmft The number of decayed, missing (due to caries), and filled teeth in the primary dentition.

1 INTRODUCTION

1.1 EARLY CHILDHOOD CARIES

1.1.1 DEFINITION

Early childhood caries (ECC) is defined as "the presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries), or filled surfaces in any primary tooth of a child under 6 years of age" (1). ECC differs from caries in permanent teeth, with faster progression and a special pattern, usually first affecting the maxillary primary incisors and first primary molars (2). ECC also affects smooth surfaces of the teeth, which in older children and adults are seldom affected.

ECC is associated with high costs for the family and for society in general (3). ECC shares common risk factors with other non-communicable diseases associated with excessive sugar consumption, such as cardiovascular disease, diabetes, and obesity (1). Moreover, ECC can impair quality of life (4), by causing difficulties in sleeping, eating, and attending school due to loss of tooth substance and acute and chronic pain. Severe tooth decay may also result in chronic systemic infections requiring hospitalization. There are also reports of growth delay (5-7). Untreated caries in children can in extreme cases lead to life-threating conditions (5).

Treatment of ECC includes restorative treatment and extraction of primary teeth, which may require sedation or general anesthesia (1). Caries in the primary dentition is known to increase the risk of caries in the permanent dentition (8-10). Oral health is important for children to maintain oral functions like eating and speech development, but also to maintain a positive self-image (3).

1.1.2 EPIDEMIOLOGY

Caries is the most widespread non-communicable disease, five times more common than asthma (11), affecting 560 million children worldwide (7, 12).

ECC is highly prevalent in the world and largely untreated in children under the age of 3 years (1). Sweden has for a long time had a decreasing prevalence of caries in children aged 6 years, but in recent years an increase has been seen. In 2010, 78% of 6-year-old Swedish children were caries-free (initial/non-cavitated caries not included in the report). By 2016, only 75% of 6-year-olds

in Sweden were caries-free (13). The World Health Organization (WHO) has set a goal for Europe that 80% of children aged 6 years, should be free from caries by 2020 (13).

1.1.3 ETIOLOGY

Dental caries is a complex biofilm-mediated, sugar-driven, multifactorial, dynamic disease which demineralizes the tooth (1, 12). Enamel demineralization is caused by acidogenic bacteria that ferment carbohydrates. After ingestion of carbohydrates (especially sucrose), the pH in tooth adherent biofilms falls to 5.0 or below. According to the ecological plaque hypothesis, caries development is a result of the enrichment of acid-tolerant bacteria in dental biofilms in response to prolonged periods of low pH. The beneficial organisms that preferentially grow at a neutral pH are inhibited by the prolonged lowered pH, and an increase of the dysbiotic microbiome follows, through an increase in the proportion of acidic species and changes in the composition of the biofilm matrix (1, 12, 14-16). Frequent intake of sugar leads to a sustained acid production and consequent demineralization of the tooth. Dental caries is considered a non-communicable disease, since the presence of bacteria alone is not sufficient for caries to occur (1, 17).

1.2 PREDICTORS OF EARLY CHILDHOOD CARIES

Even though several risk factors associated with ECC are known, it is difficult to predict the condition (18). The latest published review and meta-analysis found 123 different factors associated with ECC (19). In a systematic review of caries risk assessment, baseline caries prevalence was the most accurate single predictor for ECC (18); additional variables that have been used as predictors in multivariate models are, for example, oral bacteria count (*Streptococcus mutans* and *Lactobacillus*), buffering capacity of saliva and pH, visible plaque levels, dietary habits, and socioeconomic status (18).

1.2.1 SOCIOECONOMIC STATUS

Socioeconomic status is an important factor in ECC, both at the community level as well as the family level (3, 20, 21). Factors such as ethnicity and maternal educational level are associated with ECC prevalence (1, 22), as well as parents' attitudes, knowledge, and beliefs (23). Children of parents with immigrant background and/or low socioeconomic status have been identified as vulnerable for caries development in numerous studies (22, 24-26).

Maternal smoking (27) and having several siblings may also increase the prevalence of ECC (28).

1.2.2 MATERNAL FACTORS

Maternal health is important for the fetus and later for the child (29). A Norwegian longitudinal study found that, besides low maternal education and non-western origin, having an obese mother is a risk indicator for caries at 5 years of age (22). In Sweden, approximately 26% of pregnant women are overweight and 14% are obese (30), whereas the corresponding rates for Norwegian women are 21% and 9%, respectively. High BMI in pregnant women is associated with preterm birth and cesarean section (C-section) (30, 31). Maternal behavior such as oral habits, caries prevalence, and tobacco use have also been linked to the prevalence of ECC (32).

1.2.3 PERINATAL FACTORS

Perinatal factors that may influence the risk of ECC are C-section, preterm birth, birth size, and early nutrition. The mechanisms behind these factors are not completely elucidated. However, as earlier mentioned, it is established that high BMI in pregnant women is associated with preterm birth and C-section (30, 31).

Preterm birth

Moderate or late preterm children (defined as being born after gestational week 32–34 or 34–37, respectively) experience health challenges. These children more often show hyperbilirubinemia and respiratory insufficiency during perinatal life and impaired neurodevelopmental outcome when later compared with term infants (33). Preterm children are known to have an increased risk of mineralization disturbances in their primary teeth (34-36). The influence of preterm birth on ECC has been studied with conflicting results. One study showed that adolescents born before gestational week 29 had increased prevalence of dental plaque, gingivitis, and Streptococcus mutans, but no increase in the frequency of caries (34). A large Brazilian study demonstrated an increased level of ECC in children born preterm (37), as did a recent study in India (38). In contrast, other studies have not found any relationship between ECC and preterm birth (39-41).

Size at birth

Children born small for gestational age (SGA) (42) or large for gestational age (LGA) (43), are at risk of increased morbidity. True intrauterine growth retardation is most often due to insufficient maternal nutrition, low blood flow in the umbilical cord, placenta dysfunction, maternal preeclampsia, or diabetes.

But on the relationship between birth size and ECC, findings are inconsistent. A systematic review in 2001 did not find a link, but it concluded that there was a lack of studies of good quality in this area (44). Neither have some of the more recent studies found associations between low birth weight or SGA and the prevalence of caries (37, 41, 45). In contrast, O'Connell found an increased risk of ECC in children born SGA (46). A Scottish study from 2017 following more than 1,000 children from 1 to 4 years of age found that children with low birthweight had greater caries increments (27), and a recently published longitudinal study in more than 1,000 Brazilian children also supported the earlier findings of a higher risk of caries for children born with low birthweight (47).

Mode of delivery

In Sweden, 17% of all births are by C-section (31), and in the Nordic countries as a whole, the proportion varies between 16% and 22%. In comparison, over 40% of deliveries are by C-section in Cyprus, Hungary, Bulgaria, Poland, and Romania. Mode of delivery might influence the child's future health, and there is a growing understanding of the increased risk of asthma and obesity when delivered by C-section (48). Conflicting results exist regarding mode of delivery and caries. Some studies have shown elevated caries occurrence among children delivered by C-section (49, 50) while others have found more caries in vaginally delivered children (51-53).

1.2.4 ORAL HABITS

Oral habits established during infancy, such as brushing with fluoride toothpaste, are known to be maintained throughout early childhood (54, 55). Studies have shown that visible plaque (56, 57) and increased bleeding on probing (56) are associated with a higher caries experience in preschoolers. Fluoride in toothpaste influences the balance between demineralization and remineralization, but fluoride also has the capacity to inhibit acid production from saccharolytic bacteria through formation of calcium fluoride reservoirs in the biofilm matrix (12).

Systematic reviews show strong evidence that daily tooth brushing with fluoride toothpaste is effective in the prevention of ECC (58, 59). A recently published Norwegian study showed that brushing less than twice daily at 2 years of age, increases the risk of caries increment at 5 years of age (57). Infrequent tooth brushing in early preschool years also correlates with more severe caries in adolescence. (9, 60).

1.2.5 ORAL MICROBIOTA

The oral microbiome consists of approximately 700 species (61). Prior to the 1980s, it was only possible to identify bacteria in culture studies. Since then, molecular methods based on gene sequences have been developed to characterize and identify bacteria, most frequently using the 16SrRNA gene (61).

A healthy oral microbiota that lives in symbiosis with its host is beneficial for the host in several ways. For example, it helps to develop immunity in the host and prevents foreign pathogens from colonizing (16, 62). The healthy microbiome (i.e. the entire microbial community of commensal, symbiotic, and pathogenic microorganisms) is stable, but can be disrupted by changes in lifestyle or immune status, or by broad-spectrum antibiotic therapy. When it comes to lifestyle, a frequent intake of fermentable sugars can change the oral microbiota (62).

The bacteria in the oral cavity include both those associated with health and those that cause disease under special conditions. *Streptococcus mutans*, traditionally considered to be the primary ECC pathogen, is a relatively common member of the oral microbiota without causing disease (63). Furthermore, *Streptococcus mutans* can even be missing in children with ECC (64-66).

According to the ecological plaque hypothesis, it is more likely that there is not one specific pathogen that causes caries, but rather it is a shift in the environment which leads to a predominance of cariogenic bacteria (63).

Veillonella species are common in initial caries, they are thought to promote the growth of *Streptococci* by using lactic acid as energy source, and facilitating the growth of acidogenic species (61).

Different studies show contradictory results concerning the presence of *lactobacillus* species and ECC. At the same time, some *lactobacilli* are associated with the progression of deep caries and others with reduced caries development through the process of diminishing the number *of Streptococcus mutans* in saliva (67-69).

Although there remains much to explore when it comes to the oral microbiota, a core oral microbiome in health has been proposed, which includes representatives of the following genera: *Streptococcus, Veillonella, Granulicatella, Neisseria, Haemophilus, Corynebacterium, Rothia,*

Actinomyces,   Prevotella,   Capnocytophaga,   Porfyromonas, and *Fusobacterium* (62, 70).

1.2.6 ESTABLISHMENT OF ORAL MICROBIOTA

It has been known for about a decade that mode of delivery has an impact on the infants' gut microbiota (71). Children delivered by C-section have an altered gut microbiota that seems to mainly be derived from the skin, oral microbiota, and the surroundings during delivery (72). The colonization of the oral cavity starts at birth, and the mother is traditionally considered to transmit the bacteria. In addition to getting microbiota from the mother at birth, person-to-person transmission, mode of feeding, and microbial cross-talk all appear to have a major influence on the establishment and succession of bacteria (73). The development of the oral microbiome in early childhood is not yet fully understood. One study showed that the bacterial composition and pattern changed during the first years of life, starting with early colonizers, including *Streptococcus* and *Veillonella* (74). *Lactobacilli* are also known to be early colonizers, highly abundant in vaginal microbiota and breastmilk (73). During the first 3–6 months, *Gemella, Granulicatella, Haemophilus,* and *Rothia* appear (74). A set of microorganisms that colonize after the first year of life are *Actinomyces, Porphyrmonas, Abiotrophia, and Neisseria* (74).

The oral microbiota has been studied in relation to mode of delivery, showing that infants delivered by C-section exhibit a reduced microbial diversity in saliva, in comparison with vaginally delivered children (75, 76). If, and for how long, these differences persist between children delivered by C-section and those delivered vaginally is not clear, but one study found that at 7 years of age the genus *Haemophilus* was still found to be more abundant in children delivered by C-section (74). Whether the lower oral microbial diversity in children delivered by C-section increases the risk of ECC needs to be studied further.

1.2.7 NUTRITIONAL FACTORS

Frequent sugar consumption is a well-known risk factor for developing ECC (1), which is not surprising according to the ecological plaque hypothesis, though intake of fermentable sugars can change the oral microbiota (62). A study that compared children with ECC to caries-free children, found that ECC was associated with higher food frequency and the consumption of juice and solid retentive food. *Streptococcus mutans, Streptococcus sobrinus,* and *Bifocobacteria* were also associated with ECC (66). Even though this is known, it is difficult to predict caries in preschoolers from dietary information alone. In a report from SBU (Swedish Agency for Health Technology

Assessment and Assessment of Social Services), it was concluded that intake of sugar-containing products more than once a week correlated with caries, but the predictive power of this study was limited. Dietary habits that are established as early in life as 1 year of age are known to be maintained during early childhood (54).

In systematic reviews and meta-analyses, breastfed children have been shown to be less affected by dental caries compared to bottle-fed children (77, 78). Among breastfed children, those breastfed for longer than 12 months seemed to have an increased risk of ECC compared to children breastfed for less than 12 months (78, 79). Children breastfed for longer than 12 months had a further increased risk of developing caries if they were fed more frequently or nocturnally (78). In contrast, a recent review concluded that breastfeeding up to 2 years of age does not increase the risk of ECC (21).

The oral microbial profile differs between breastfed and formula-fed infants; in particular, *Lactobacillus* species (which are associated with health, but also with caries) have been found to be more prominent in 3-month-old breastfed children (65, 73), independently of mode of delivery (73). A reason for *Lactobacilli* being favored by breastmilk, is that they ferment lactose (73). Breastfed children at 3 and 12 months of age have also been shown to have an increased prevalence of *Streptococcus* species (including both health- and disease-associated species) (73, 74). The *Lactobacilli* were also found to inhibit growth of *Streptococcus* species. Formula-fed children, on the other hand, have been found to more frequently have more anaerobic species, usually associated with gingival inflammation, but also *Neisseria*, a common inhabitant of the healthy mouth (73).

1.3 CHILDHOOD CARIES, BMI, AND SIGNS OF THE METABOLIC SYNDROME

ECC shares common risk factors with other non-communicable diseases associated with excessive sugar consumption – for example, overweight, decreased glucose tolerance, or diabetes, and ultimately cardiovascular disease (1).

Studies on the impact of BMI on ECC show contradictory results. Both dental caries and obesity are multifactorial diseases and therefore, not surprisingly, associations are complex (80). In one Swedish study, obese children had more

caries-affected teeth than non-obese children (81). The correlations may be more complex, as Swedish 5-year-old children with low BMI have been shown to have a significantly higher caries prevalence than children with normal BMI (82). A review also found signs of a U-shaped correlation between oral health and BMI (23), with studies showing that caries prevalence is higher in underweight children but also higher in overweight and obese children, although other studies do not confirm this (83, 84). These findings most likely vary according to which populations were studied and whether confounders were adjusted for. A study from the US found no significant association between childhood obesity and caries experience after controlling for age, race, and poverty–income ratio (85).

The metabolic syndrome is an aggregate of risk factors that lead to cardiovascular disease and it often includes overweight or obesity, high waist circumference, insulin resistance, low glucose tolerance, high blood pressure, and dyslipidemia (86). In adults, correlations between caries and the metabolic syndrome have been reported (87, 88), and even between caries, dyslipidemia and hyperglycemia in non-overweight/non-obese men (89); one study also found an association specifically between caries and hyperglycemia (90). In adolescents, correlations have been found between higher BMI, hypertension, and caries (91) and also between cardiometabolic risk factors and caries (92, 93). When it comes to the association between the metabolic syndrome in young children and ECC, no studies have come to light during the preparation of this thesis. A study performed in Japanese children aged 10–13 years found no associations between the metabolic syndrome and caries but an association with the prevalence of *Streptococcus mutans* (94).

Recently, IDEFICS (*Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS*), a European multicenter study, proposed a definition of metabolic syndrome in children which specifies monitoring levels that require further follow-up. Based on these cut-offs, the Halland Health and Growth study group published evidence of the metabolic syndrome in children as young as 6 years old (95). Whether metabolic risk factors could be identified at an earlier age based on coexisting childhood caries prevalence and birth data merits further evaluation.

Since the body of research on risk factors for ECC has thus far given contradictory findings, more prospective studies are needed in order to establish the impact of perinatal factors as well as other risk factors.

2 AIM

The general aim of this thesis was to study whether mode of delivery affects selected oral bacteria during the first 6 months of life and to explore early influencing factors for ECC, as well as the correlation of ECC with metabolic risk markers.

2.1 AIMS

1. To evaluate whether mode of delivery has a longitudinal impact on selected bacteria in saliva during the first 6 months of life.

2. To explore whether mode of delivery affects the prevalence of ECC in 5-year-old children.

3. To study whether early oral habits, family characteristics, and nutritional and nursing habits during the first 2 years of life are associated with ECC at 5 years of age.

4. To explore whether perinatal factors or signs of metabolic risk factors in preschool children, are associated with ECC.

2.2 HYPOTHESES

1. There is a difference in selected salivary bacteria between infants delivered vaginally and by C-section.

2. It is possible to find early risk factors for ECC by studying family characteristics, nutritional and nursing habits, and oral habits during the first 2 years of life.

3. Perinatal factors, such as mode of delivery, preterm birth, being SGA or LGA at birth, affect the prevalence of ECC in 5-year-old children.

4. There is a relationship between ECC and metabolic risk factors during preschool age.

3 PATIENTS AND METHODS

3.1 PAPER I

3.1.1 STUDY DESIGN

A prospective case-control study longitudinally following 149 infants from birth to 6 months of age.

3.1.2 STUDY PARTICIPANTS

The 149 participants were consecutively enrolled among healthy infants born at Halland Hospital, Halmstad, Sweden, between April and December 2013. During the last trimester, pregnant couples visiting the maternity clinics in southern Halland, were informed about the upcoming study. Both parents then signed a consent form for the child's participation after thorough verbal and written information at the maternity hospital. Saliva samples from the children were taken at 0 (i.e. within two days), 1, 3, and 6 months of age, and from mothers when the children were 6 months old. Ninety-six of the newborn infants were delivered vaginally and 53 by C-section. Exclusion criteria were i) preterm children, ii) neonates in need of hospital care, iii) infants with an unclear medical condition, and iv) families that planned to relocate abroad after the child's birth. The 6-month attrition rate was 20%, mainly due to lack of time, according to the parents. See figure 1.
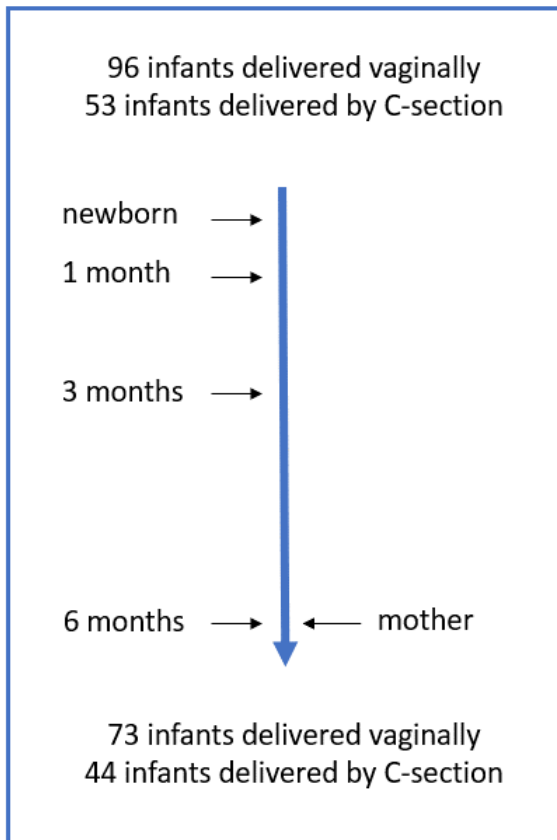
96 infants delivered vaginally
53 infants delivered by C-section

newborn ⟶

1 month ⟶

3 months ⟶

6 months ⟶   ⟵ mother

73 infants delivered vaginally
44 infants delivered by C-section

Figure 1. Saliva sampling intervals.

3.1.3 SALIVA SAMPLING

Samples of saliva were collected with aid of sterile cotton buds. At the first occasion at the maternity hospital, the parents were carefully instructed and shown how to collect the sample and transfer it to a marked plastic tube. The parents also received an illustrated instruction sheet. The parents were requested to collect the sample in the morning by soaking the cotton bud in saliva and immediately return it to the laboratory. When a sample was not received, the parents were reminded through a telephone call within 5 days. In a similar way, a saliva sample from the mothers was obtained 6 months after delivery. All samples were stored frozen at -20 C°.

Figure 2. Saliva sampling in an infant.

3.1.4 MICROBIAL METHOD

The samples were processed with the checkerboard DNA-DNA hybridization technology according to Wall-Manning et al. (96). Whole genomic DNA probes were prepared from a panel of 13 bacterial species of potential interest for oral disease and health. The bacterial counts were obtained by the percent method, in which the intensity of the signal (biomedical light units) was expressed as a fraction of a high standard sample (106 cells) using a LumiImager Workstation (Boehringer Mannheim, Mannheim, Germany). The detection level was >104 cells per mL sample. Table 1 shows the 13 bacteria species with strain designation according to the culture collection Oral Microbiology, Gothenburg, Sweden.

Table 1. Bacteria species with strain designation according to the culture collection Oral Microbiology, Gothenburg, Sweden.

Bacteria species
Actinomyces naeslundii 2466*
Actinomyces odontolyticus G67
Bifidobacterium dentium G174
Fusobacterium nucleatum 2865
Lactobacillus casei 3184
Lactobacillus salivarius 3830
Rothia dentocariosa 1956
Streptococcus gordonii 2471
Streptococcus mitis 1770
Streptococcus mutans 2482
Streptococcus salivarius 2473
Streptococcus sanguinis 2478
Veillonella parvula G186

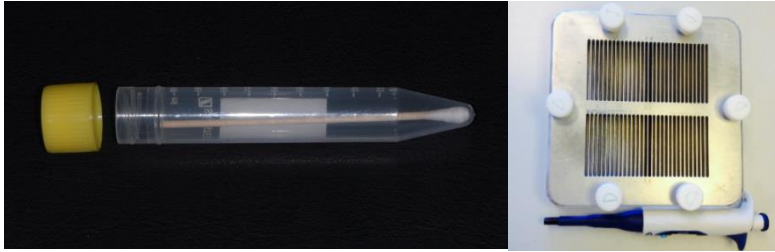**A.naeslundii* is now designated *A. Oris*.

Figure 3. Cotton swab in plastic tube, MiniSlot and pipette, and the resulting patterned membrane of the Checkerboard DNA-DNA analysis.

3.1.5 QUESTIONNAIRE

The parents were asked to complete a 20-item questionnaire on family characteristics, socioeconomic status, lifestyle factors, and hygiene habits at the inclusion of the study. A further questionnaire on nursing and feeding habits, oral habits, and prescribed antibiotics was attached to the follow-up mailings and returned together with the samples.

3.2 PAPER II–IV

3.2.1 STUDY DESIGN

A prospective cohort study longitudinally following children from birth to 6.5 years of age.

3.2.2 STUDY PARTICIPANTS

This study is part of a larger ongoing population-based birth cohort of children born between 2007 and 2009 in the county of Halland, in the western part of Sweden (n=2,666), with a focus on health and growth – the Halland Health and Growth study (H2GS) (97). Infants born in Halmstad at gestational age 32–43 weeks were then included in a sub-study evaluating endocrine health. To increase the power of the study with respect to the research question, this group of 395 infants was later merged with 156 infants delivered by C-section (but otherwise following the same inclusion criteria as the 395 infants) between May 2010 and February 2012 into what was termed the endocrine cohort (n=551). These children were followed by two nurses at regular visits to the pediatric clinic at Halland Hospital Halmstad. Anthropometric measurements and extensive endocrine follow-up were conducted at birth and at 4, 12, 36, 60, and 78 months.

At 2 years of age, the 551 children in the endocrine cohort were invited to the dental part of the study. The parents of 346 of these (179 boys, 52%, and 167 girls, 48%) consented to their participation, whereupon they were examined at 2 (n=336), 3 (n=302), and 5 (n=292) years of age at the dental clinic at Halland Hospital Halmstad. The attrition rate from inclusion at the age of 2 years was 10.1% at 3 years of age and 13.1% at 5 years. These children are included in papers II and III. See figure 5.

In paper IV, a total of 208 of the original endocrine cohort had both a dental examination at 5 years of age, and an extensive endocrine examination with successful blood sampling at 6.5 years of age. The examination at 6.5 years of age had a special focus on signs of the metabolic syndrome.
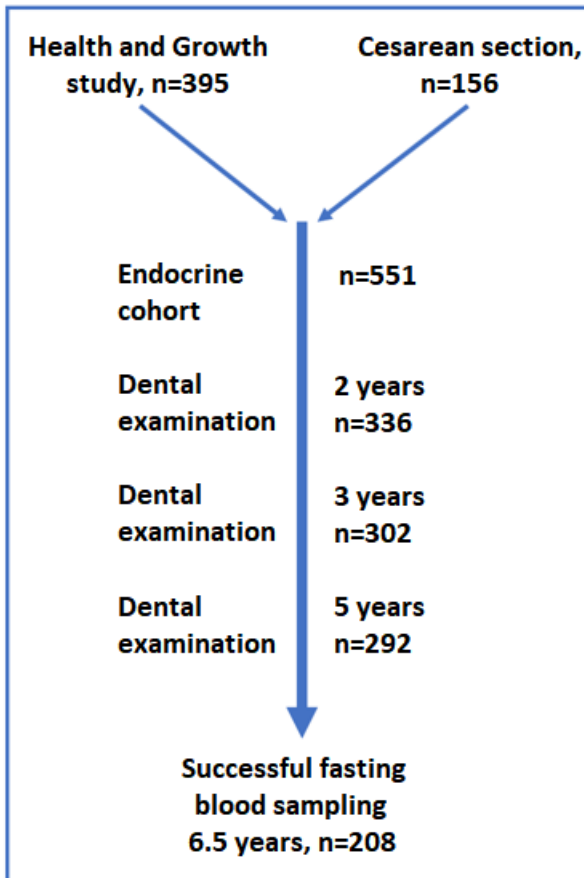
Figure 4. Flow chart of participants in papers II–IV.

At inclusion in the Halland Health and Growth study, pregnant mothers received written information about the study when visiting their local maternal health care unit during the last trimester; they then gave consent to participation in the study when arriving at the delivery unit at Halland Hospital Halmstad. Children delivered by C-section were recruited separately to ensure recruitment of elective C-section for other analyses in this study. In papers II and III, 35.5% (n=123) of the children were delivered by C-section, and in paper IV, 37.5% (n=78) children were delivered by C-section.

The main reason for attrition in papers II and III was family relocation, although a few families reported lack of interest or time. In paper IV, unsuccessful blood sampling was another reason for exclusion.

3.2.3 QUESTIONNAIRES

A set of comprehensive questionnaires covering maternal and birth-related factors was distributed at the baseline of this project and additional medical data, such as history of neonatal hospital care and prescription of antibiotics, were extracted from the hospital records. The questionnaires covered family characteristics (such as parents born outside Sweden, parental education level, parental smoking, siblings), nursing factors (such as being breastfed or bottle-fed, use of formula milk and cereal drink, night meals), and other food habits (such as drinks with meals and intake and frequency of different types of food, including sugary snacks). Age-adapted subsets of these questionnaires were administered continuously by the personnel at the pediatric clinic until the children reached. 6.5 years of age. In addition, information on sucking habits (pacifier, finger, comfort blankets, etc.) and dental variables (tooth brushing routines and the use of toothpaste, etc.) was gathered through questionnaires and personal interviews at the dental clinic.

Figure 5. A subset of questionnaires used in papers II–IV.

3.2.4 MATERNAL FACTORS

Maternal factors such as age at parturition, weight, height, smoking habits, educational level (university, high school, or elementary school), and employment (yes/no), were extracted from medical records and through questionnaires.

3.2.5 PERINATAL FACTORS

Perinatal factors such as gestational age (days), weight, length, and head circumference were extracted from the medical records. We defined moderate to late preterm birth as a gestational age of 32+0 to 36+6 weeks. Infants were classified as appropriate for gestational age (AGA) if birth weight and/or birth length was between +/-2 standard deviation scores (SDS); below -2 SDS was classified as small for gestational age (SGA), and above +2 SDS as large for gestational age (LGA), according to Niklasson and Albertsson-Wikland (Niklasson et al. 2008).

3.2.6 DENTAL FACTORS

Dental habits, such as tooth brushing frequency and use of fluoride toothpaste, as well as eruption of the first tooth, sucking habits, and whether siblings suffered from caries, were recorded through questionnaires and personal interviews. The fluoride level in the piped drinking water was <0.3 ppm in the part of the region from which the infants were recruited.

3.2.7 NUTRITIONAL AND NURSING FACTORS

Nutritional and nursing factors, such as being breastfed or bottle-fed, use of formula milk and cereal drink, night meals, and juice with meals, as well as other food habits, were recorded by questionnaires at the clinical visits.

3.3 CLINICAL EXAMINATIONS

3.3.1 DENTAL EXAMINATION

The children were examined in a fully equipped pediatric dental clinic by one of two experienced and calibrated examiners. The occurrence of manifest caries (cavitated lesions) was scored on tooth and surface level according to the WHO criteria (98). In addition, initial non-cavitated early lesions were defined as any visual "whitish" change of the enamel, with a rough surface but without enamel breakdown. The caries prevalence was expressed as the

proportion of children with a sum of initial and cavitated, missing, or filled teeth (dmft >0). The 5-year-olds had radiographs taken based on individual need. At each visit, the parents were given the opportunity to discuss lifestyle issues related to oral health, and the importance of using fluoride toothpaste twice daily was reinforced. The families were also encouraged to limit the intake of free sugars and to avoid soft drinks. At the implementation of the project, the two examiners scored ten preschool children independently, and any discrepancies were discussed and resolved through consensus. The inter-examiner agreement was 90%.

3.3.2 MEDICAL EXAMINATION

The children were examined by one of two trained research nurses. At 6.5 years of age, height was measured with an electronic stadiometer, weight was measured with calibrated scales, and waist circumference was measured after exhaling according to WHO (99). On the morning of the examination, children rested at least 10 minutes before blood pressure was recorded with a Welsh-Allyn Spot Vital Signs digital monitor (Welsh, New York). Fasting blood sampling (cholesterol, triglycerides, high-density lipoprotein, glucose, insulin, HbA1C, and homeostatic model assessment for IR) was performed under local anesthesia supplied by a patch. BMI was calculated using the algorithm weight in kilograms divided by height in meters squared. Overweight and obesity were defined according to Cole and Lobstein (100).

Cut-offs for the metabolic syndrome, namely insulin resistance, high blood lipids, and high blood pressure, were chosen in the same way, based on IDEFICS publications (101-105). Several of these papers redefine metabolic syndrome in children with reference intervals as well as cut-offs for monitoring and action levels for metabolic risk factors.
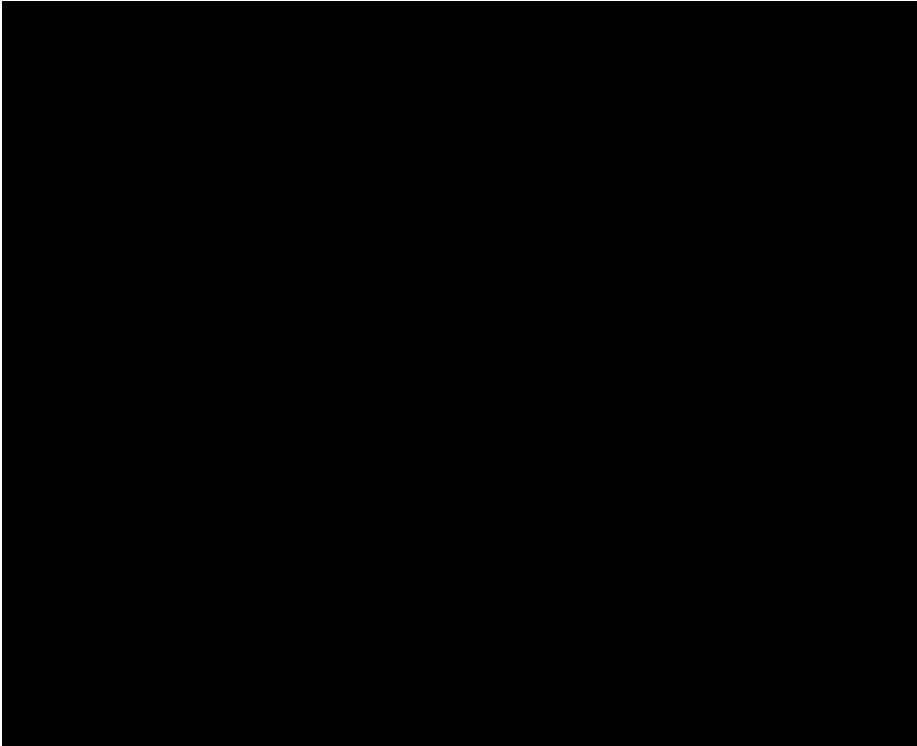
Figure 6. Measuring waist circumference.

3.3.3 STATISTICAL METHODS

All data were processed with the IBM SPSS software (version 22.0, 23.0 and 25.0; IBM Corp., Armonk, NY, USA). Normally distributed measurements are presented as means and standard deviation (SD). In paper I, differences in percentage distributions between the groups were analyzed with chi-square tests, and correlations between the bacterial counts in mothers and their offspring were analyzed with Spearman's rank correlation coefficient.

In paper II and III, the influence of the background factors and tooth brushing on the presence of caries (non cavitated + cavitated lesions) at 5 years of age was analyzed with chi-square tests and expressed as relative risk (RR) with 95% confidence intervals. Chi-square tests were used for the comparison of two proportions expressed as a percentage. A multivariate logistic regression was then performed in paper II on categorical data, taking into account the confounding effects of individual-level variables. Adjusted odds ratios (aORs) were thereby estimated, reflecting the effects of each explanatory variable. The

relationship between continuous variables was analyzed by correlation tests. Finally, a multivariate logistic regression was performed on data in paper II and III that were shown to be statistically associated with ECC at 5 years of age.

In paper IV, the Student's t-test and chi square test, complemented by Fisher's exact test, were used for comparisons between the groups, according to whether the data were normally distributed or not. Spearman's rank correlation coefficient was used for correlation analyses.

All tests were two-tailed, and p-values of <0.05 were considered statistically significant.

3.3.4 ETHICAL CONSIDERATIONS

All studies were approved by the Regional Ethical Review Board in Lund (approval number 44/2008, 2010/362, 2012/483). No examination was performed without written consent from the parents or guardians, and verbal assent was obtained from the children who were still in the study at 6.5 years of age. All the examinations were made with special care taken regarding the needs of the children. The examiners were all used to working with children, including children with special needs. Except for saliva sampling and measurement of waist circumference, there was no other examinations or measurements that were new routines to the personnel at the dental and pediatric clinics. All the children had parallel access to regular preventive dental care during the entire project, provided free of charge by the regional public dental service.

4 RESULTS

4.1 PAPER I – MODE OF DELIVERY AND SELECTED STRAINS OF BACTERIA

There was no statistical difference between the two delivery groups at baseline concerning background factors such as gender, socioeconomic factors, antibiotics prescribed to the mothers, and having more than two siblings.

At 1, 3, and 6 months of age, there were no significant differences between delivery groups concerning feeding habits. Neither were there significant differences in the use of pacifiers, teething, or oral cleaning, and there were no differences between the groups concerning prescribed antibiotics to the children or mothers.

Analysis of the saliva samples detected on average a higher number of cells in the children delivered vaginally compared to those delivered by C-section, at all sampling occasions.

Of the selected bacteria, gram-positive streptococci (*S. mitis, S. mutans,* and *S. salivarius*) displayed the highest counts in both delivery groups. The vaginally delivered infants showed a greater diversity among the species, since *A. naeslundii, A. odontolyticus, F. nucleatum*, and *L. salivarius* were detected in this group only. The prevalence of *B. dentium*, *R. denticariosa, S. gordonii, S. mutans*, and *S. sanguinis* increased with age in both groups, but the prevalence was significantly higher in the vaginally delivered group. Concerning the correlation of bacteria strains in mother and child, at 6 months of age there was a statistically significant correlation between five of the strains in the C-section group and eight in the vaginally delivered group. There was a correlation for *B. dentium, S. gordonii, S. mitis, S. mutans*, and *S. salivarius* in both groups, but no correlation between mother and child in the C-section group for *A. odontolyticus, R. denticariosa*, and *S. sanguinis*. See table 2.

Table 2. Prevalence (≥10⁴cells) of the selected bacteria strains in the two delivery groups at birth and at designated follow-up, and the correlation between mother and child at the 6-month follow-up for each bacteria strain.

Strain	Birth CS/V	1 month CS/V	3 months CS/V	6 months CS/V	Mother–child CS/V
A. naeslundii	0/5	0/0	0/2	0/2	NS/NS
A. odontolyticus	0/12*	0/32*	0/30*	0/32*	NS/0.43*
B. dentium	5/32*	13/61*	8/49*	16/63*	0.54*/0.28*
F. nucleatum	0/2	0/11	0/10	0/14*	NS/NS
L. casei	0/2	0/0	0/0	0/0	NS/NS
L. salivarius	0/0	0/0	0/0	0/3	NS/NS
R. dentocariosa	0/12*	3/22*	10/21	8/39*	NS/0.45*
S. gordonii	3/15*	10/19	13/32*	13/38*	0.48*/0.36*
S. mitis	73/84	85/94	90/94	93/96	0.59*/0.32*
S. mutans	53/76*	65/88*	58/88*	68/92*	0.85*/0.36*
S. salivarius	28/36	45/67*	53/71	48/70	0.71*/0.48*
S. sanguinis	0/35*	5/44*	10/47*	13/53*	NS/0.56*
V. parvula	0/0	3/6	5/6	8/24	NS/NS

*Statistically significant differences between the groups (p<0.05, chi-square test). Prevalence is expressed as %. The correlation between mother and child at 6 months was measured with a two-tailed Spearman correlation coefficient. CS = cesarean section; V = vaginally delivered; NS = not statistically significant.

4.2 PAPER II AND III – EARLY RISK FACTORS FOR ECC

According to the socioeconomic measures, more than 92% of the parents of the participating children were born in Sweden. Less than 5% of the parents were smokers, and 64% of the mothers and 45% of the fathers reported education to university level at inclusion in the endocrine study. The proportion of obese mothers was higher in the C-section group (14.4 versus 5.9%, p<0.05). Among children delivered by C-section, a significant higher proportion were preterm births or required neonatal hospital care than among vaginally delivered children (16.5 versus 1.1%, p<0.05). At 3 months of age, breastfeeding was more common in the vaginally delivered group (p<0.05), whereas the C-section infants were more commonly fed with formula (p<0.05).

4.2.1 MODE OF DELIVERY

The caries prevalence (cavitated and non-cavitated lesions) was in total 5.6% at 3 years of age and 18.9% at 5 years. Of the children delivered by C-section, 29% had caries at 5 years of age, compared to 13% of the children delivered vaginally. See table 3. The 5-year-old children delivered by C-section displayed a significantly elevated risk of having caries (relative risk (RR) 2.2; 95% confidence interval (CI) 1.4–3.6; p<0.05). Multivariate logistic regression of the factors included in paper II showed that parental smoking and siblings with caries were the most influential family determinants for having ECC at 5 years of age (p<0.05), while drinking juice with meals at 2 years of age was most influential among the nutritional and nursing factors (p<0.05). Being delivered by C-section showed an adjusted odds ratio of 2.5 (1.7–4.0) for having ECC at 5 years of age.

Table 3. Dental caries prevalence and frequency at 3 and 5 years according to mode of delivery.

Variable	Vaginal	C-section	Total
At 3 years			
Caries prevalence (dmft >0)	6.6%	3,8%	5.6%
Number of decayed and filled teeth, mean dmft (SD)	0.06 (0.47)	0.02 (0.19)	0.04 (0.39)
Number of teeth with initial lesions, mean (SD)	0.14 (0.72)	0.06 (0.34)	0.11 (0.61)
At 5 years			
Caries prevalence (dmft >0)	13.0%	29.0%	18.9%
Number of decayed and filled teeth, mean dmft (SD)	0.17 (0.76)	0.31 (1.09)	0.22 (0.90)
Number of teeth with initial lesions, mean (SD)	0.31 (1.0)	0.52 (1.01)	0.39 (1.01)

C-section = cesarean section; dmft = decayed, missing, filled teeth; SD = standard deviation.

4.2.2 ORAL HABITS

The majority of the parents assisted their child with tooth brushing (88.4% at 2 years of age, 87.9% at 3 years of age, and 83,7% at 5 years of age), and more than 98% were using fluoride toothpaste at all three assessment points. At 5 years of age, 90% brushed their teeth twice daily, compared to 77.3% at 2 years of age, and 86.7% at 3 years of age. Only 2.1% reported that they brushed non-

daily at 5 years of age (compared to 4.8% at 2 years of age, 1.7% at 3 years). Difficulties (minor or major problems) with performing tooth brushing decreased from 30.5% at 2 years to 5.1% at 5 years of age. The relative risk of having ECC at 5 years of age was statistically significant for the variables (at 2 years of age) "tooth brushing less than twice daily" (RR 2.1 (95% CI 1.3–3.3)) and "problems with brushing (major and minor)" (RR 1.5 (95% CI 1.0–2.4)).

A logistic regression of the variables that were significantly associated with caries at 5 years of age in paper II and paper III revealed that the variables "preterm birth", "mode of delivery (C-section)", "juice with meals", and "tooth brushing less than twice daily" significantly increased the risk of ECC at 5 years of age, also in a multivariate logistic regression. See table 4.

*Table 4. Multivariate logistic regression of influencing variables for early childhood caries at 5 years of age. ***

Variable	Bivariate			Multivariate		
	p-value	OR	95% CI	p-value	OR	95% CI
Preterm birth (32–36 weeks)	0.001	5.0	1.9–12.6	0.015	4.4	1.3–14.3
Cesarean section	0.001	2.7	1.5–4.9	0.047	2.2	1.0–5.0
Juice with meals (at 2 years)	0.031	2.2	1.1–4.5	0.006	3.0	1.4–6.6
Tooth brushing < twice daily (at 2 years)	0.001	2.7	1.5–5.1	0.067	2.1	1.0–4.7

OR = odds ratio; CI = confidence interval.

*= NGA was tested against SGA and LGA, respectively, which gave a p-value of 0.099 in the bivariate analysis. However, in the multivariate analysis, this variable (3 categories: NGA, SGA and LGA) did not contribute significantly (p-value 0.82)

4.3 PAPER IV – PERINATAL FACTORS, BMI, AND METABOLIC RISK FACTORS

The 208 children who participated in both the dental part of the study at 5 years of age and the endocrine study at 6.5 years of age did not differ significantly from the endocrine cohort (n=551) regarding gestational age, head circumference, weight, and length at birth. Of these 208 children, 6.3% were born moderately to late preterm (defined as gestational age 32–36 weeks), 4.8% were born LGA, and 11.6% were born SGA. Among their mothers, 24.4% were overweight and 11.4% were obese. What significantly differed between the groups was that 5.4% of the included mothers were born outside Sweden, compared to 10.8% in the endocrine group. The mothers in the dental group were also significantly older, smoked less, and had a higher educational level compared to the endocrine cohort.
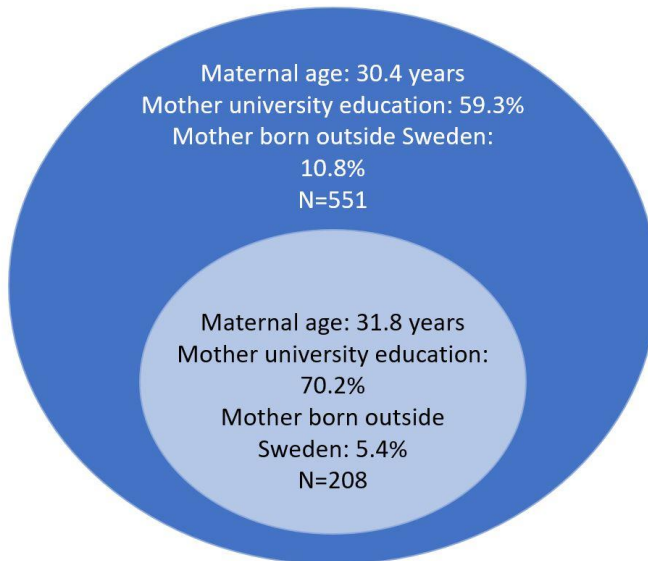

Maternal age: 30.4 years
Mother university education: 59.3%
Mother born outside Sweden:
10.8%
N=551

Maternal age: 31.8 years
Mother university education:
70.2%
Mother born outside
Sweden: 5.4%
N=208

Figure 7. Significantly different family characteristics between the endocrine cohort (n=551) and the dental subgroup in the study (n=208).

4.3.1 ASSOCIATIONS BETWEEN ECC, GESTATIONAL AGE, AND BIRTH WEIGHT

Of the 208 children, 17.8% of the children had caries in at least one tooth at the age of 5 years. Moderate to late preterm children displayed at 5 years of age a significantly higher caries prevalence than the children born full-term (61.5% versus 14.9%, p=0.000). Children born SGA had a significantly higher prevalence of caries than children born AGA (33% versus 14.5%, p=0.036), whereas the difference for children born LGA did not reach significance (40%, p=0.054). Being born preterm or SGA significantly increased the relative risk of having caries at 5 years of age. Table 5 shows the relative risk (RR) for caries in relation to perinatal data.

Table 5. Prevalence of ECC at 5 years of age in preterm children and children born SGA or LGA. Differences compared with children born full-term or AGA are expressed as relative risk (RR) with 95% confidence interval (CI).

Variable	n	ECC (%)	RR	95% CI	p-value
Preterm birth (32–36 weeks)	13	61.5	4.1	2.4–7.1	<0.001
Small for gestational age	24	33.3	2.3	1.2–4.5	0.036
Large for gestational age	10	40.0	2.8	1.2–6.4	0.054 (NS)

ECC = early childhood caries; RR=relative risk; CI = confidence interval; NS = Not significant.

4.3.2 ASSOCIATIONS BETWEEN ECC AND BMI

At 6.5 years of age, 5.8% of the children were underweight, 12.0% overweight, and 3.4% obese according to Cole and Lobstein (100). No correlation was found between BMI at 6.5 years of age and ECC at 5 years of age.

4.3.3 ASSOCIATIONS BETWEEN ECC AND METABOLIC RISK FACTORS

At 6.5 years of age, 19.7% (n=41) of the children had one, 13.9% (n=29) had two, and 13.4% (n=28) had three or more risk factors for the metabolic syndrome according the IDEFICS monitoring level.

Using the IDEFICS cut off, corresponding to the monitoring level of risk factors of the metabolic syndrome, none of the metabolic risk factors differed significantly between children with or without caries at 5 years of age. However, a difference was found between the mean fasting glucose levels (4.6 (SD 0.4) versus 4.8 (SD 0.4) mmol/L; p=0.05), and a correlation was found between glucose as a continuous variable and caries frequency (dmft) (r=0.15; p<0.05).

5 DISCUSSION

The main findings of this thesis are that perinatal factors beyond oral habits, feeding habits, and socioeconomics, have an impact on the risk of developing ECC. Being delivered by C-section and being born preterm or SGA seem to increase the risk. Mode of delivery affects the oral microbiota, since children delivered by C-section display a different oral flora compared to vaginally delivered children.

When studying mode of delivery and prevalence of caries at 5 years of age, we found a significantly raised risk of having caries in children delivered by C-section. This is in line with our finding that, during infancy, children who had been delivered by C-section displayed a different oral flora of selected bacteria compared to children delivered vaginally. The mode of delivery affected the composition of the oral microbiota, which is in accordance with lower diversity in gut microbiota if delivered by C-section (106, 107). During vaginal delivery, the mothers' vaginal microbiota is transmitted to the child, whereas children delivered by C-section get their microbiota mainly from the skin of their mother (108). We found that children delivered by C-section displayed a lower diversity among selected oral bacteria. A highly diversified microbiota is considered beneficial, since caries, according to the ecological plaque hypothesis, is associated with a less diversified, more acidogenic oral microbial flora (109). The group delivered vaginally also displayed a higher count of cells at every interval during infancy. Four of the selected species (*A. naeslundii, A. odontolyticus, F. nucleatum* and *L. salivarius)* were only detected in the vaginally delivered group. The prevalence of *B. dentium*, *R. denticariosa, S. gordonii, S. mutans* and *S. sanguinis* increased with age in both groups, but the prevalence was significantly higher in the vaginally delivered group. We also found a clear mother-to-child correlation for 8 of the 13 strains in the vaginally delivered group.

The early composition of the oral microbiota is also affected by several other factors apart from mode of delivery, such as nursing factors (breastfeeding, for example, favors acquisition of *Lactobaccilli* strains), antibiotic treatment (which might affect the balance of the normal oral flora), and use of a pacifier or finger sucking (73, 110, 111). In the present study, such early factors affecting the composition of the early salivary flora were well balanced between the group of infants delivered by C-section compared to the group delivered vaginally, which can be considered as a strength of the study. The finding that the majority of all children in the study harbored *S. mutans* at 6 months of age, is in accordance with a recent study (74) and supports earlier

findings that *S. mutans* can also be found in caries-free children (112). Since the microbiome has evolved together with humans over a long time and evolution has been driving adaptation and selection, it can be hypothesized that vaginal delivery favours healthy colonization. It is known that the endogenous human microbial communities contribute to the differentiation and maturation of the host immune system, food digestion and nutrition, energy generation, metabolic regulation, processing and detoxification of environmental chemicals, maintenance of skin and mucosa barrier function, as well as prevention of pathogen invasion (109).

The finding that children delivered by C-section have an increased risk of caries at 5 years of age could be due to a less diversified oral flora but currently we lack data to confirm this. The elevated risk of caries for children delivered by C-section is in accordance with a recent study (38). Another study found a tendency towards more severe caries in children delivered by C-section (50). Others have found the opposite: a Polish study in a water-fluoridated area found more caries in the first permanent molars in vaginally delivered children aged 5–13 years (52) and a study of Thai children aged 3–5 years found a relation between vaginal delivery and elevated caries prevalence (51). A register-based retrospective study of 7-year-old children found a tendency toward more caries in vaginally delivered children (53). The reason for these conflicting findings could be that different populations were studied, with different preconditions for caries prevalence, proportion of C-section deliveries and other variables, such as socioeconomics. The decision to perform C-section differs widely between different nations. In Europe, the median rate is 27%, but in four European countries the rate is above 40%. Poland is one of these. In Sweden, the main reason to perform C-section is medical, whereas European countries that have a high rate of C-section probably have other criteria. If the decision to deliver by C-section or not is influenced by the economic resources of the parents, this might be a confounder when analyzing the outcome of delivery in relation to caries prevalence.

In addition to our finding that children delivered by C-section have a less diverse oral microflora, which can explain the elevated risk of caries, it is also known that children delivered by C-section display an elevated risk of childhood asthma (113). Asthma can increase the risk of ECC because the medication inhaled contains lactose and causes a reduction of salivary flow as well as reduced pH (114). We did not investigate this relationship, but future evaluation is planned. Antibiotic treatment in either the mother or the child might have an effect on the establishment of the developing oral microbiota and by extension on the prevalence of caries. Mothers giving birth by C-section

are often given antibiotics either as a preventive measure or due to actual infection. We could however not find any correlations between the prevalence of caries and the antibiotics prescribed to the mother or the child.

In the cohort of children followed to 5 years of age, breastfeeding was more common in the first 3 months of life in children delivered vaginally, compared to children delivered by C-section, for whom formula was more common. We do not know whether the early nursing factors and/or mode of delivery have affected the oral microflora in this cohort, but since we collected saliva samples at all three dental examinations, we will have an answer to this in the future. Apart from having an elevated risk of ECC at 5 years of age, the children delivered by C-section also differed significantly from the vaginally delivered children concerning gestational age. This is not surprising, since children delivered by C-section are more often preterm and SGA. (115, 116).

In this sample of preschool children, we found moderately to late preterm children to have a significantly increased risk of developing caries, thereby confirming several previous studies (37, 38, 117). In Brazil 2006, Gravina et al. found a higher dmft in full term children aged 0–3 years, which was hypothesized to be due to better follow-up in preterm children, but the significance was lost by the age of 4–6 years (39). In a study from London, Davenport et al. found that children born preterm and with low birth weight had significantly increased dmft compared to normal birth weight children. In this study, there was also a clear relationship between poor diet (frequency of sugar consumption and snacking between meals) in the preterm low-birth-weight group (118). In a study from Thailand an inverse relation between preterm birth and dental caries was found, but in that group (3–4 years), the caries prevalence was high (88.2%). The author suggested that it was possible that the high caries prevalence masked a true correlation (41). It is interesting that studies of extremely preterm children (34, 40), did not find a correlation between gestational age and caries, in contrast to the above-mentioned studies.

Several studies have also found that children born preterm have an increased risk of enamel hypoplasia (36, 117). One explanation for this, might be that children born extremely preterm are at risk of enamel hypoplasia, but they still might get a better overall quality of the enamel compared to children born more moderately preterm, as children born extremely preterm (children weighing less than 1500 g) have in the last decade received mineral supplements, and moderately preterm children do not receive these supplements.

This thesis has shown that children born SGA seem to have higher risk of ECC. The correlation between birth weight and caries has previously been unclear.

Some earlier studies have found no difference in ECC when comparing children born with low birth weight or SGA (45, 119) , while others found that children born SGA more often had caries when compared to children born AGA (46) The latter is interesting and in accordance with our findings. Whether children born LGA have increased risk of caries is currently not fully understood and not supported by our findings, although a non-significant tendency was found. Hisano et al., on the other hand, found in 3-year-old children that high birth weight was associated with a higher prevalence of caries, but they did not find an association between low birth weight and caries (120). It is important to note that few studies have evaluated caries risk according to size at birth after adjusting for gestational age; since low or high birth weight per se is not equal to being born SGA or LGA, birth size should be adjusted for gestational age, to be regarded as a better marker of below-normal or above-normal intrauterine growth.

Additionally, both children born preterm and those born SGA are more often given formula as a supplement in the neonatal period because of difficulties with breastfeeding and to avoid hypoglycemia. This extra supplementation may be prolonged throughout infancy, with extra meals becoming a habit. Furthermore, in the cohort of children followed to 5 years of age, formula feeding was more common in children delivered by C-section. The lower incidence of breastfeeding in infants delivered by C-section probably raises the risk of a less diverse oral microbiota, which can explain the elevated risk of caries at 5 years of age in children born preterm or SGA. The multivariate regression analysis showed a persistent increased risk of ECC if delivered preterm.

Nutrition is important for health, and breastfeeding, even as little as once a day, is known to promote higher diversity of the gut microbiota (72). The protective role of breastfeeding in caries development has been debated (77). The studies in this thesis did not provide any evidence that breastfeeding was protective against ECC in our study group, probably because the majority of the children were breastfed (completely or partly) during the first 6 months of life. Intake of cereal drink at 2 years of age, as well as juice with meals, increased the risk of ECC at 5 years of age. There was also a positive correlation between the intake frequency of candy, cookies, and ice cream at 2 years of age and caries prevalence at 5 years.

Both dental caries and obesity are multifactorial diseases and therefore, not surprisingly, associations between dental caries and BMI are complex. In Sweden, approximately 26% of pregnant mothers are overweight and 14% obese (31). The percentage of pregnant women with obesity in papers II and

III was lower than the Swedish average (27% overweight, 9% obese), as well as in paper IV (24% overweight, 11% obese). Obesity is also linked to socioeconomic status, and Swedish pregnant women with lower educational level are more often obese (121). High BMI in pregnant women is associated with preterm birth and C-section (30). A Norwegian study found that having an obese mother is a risk indicator for caries at 5 years of age (22), but we did not find a relationship between BMI of the mothers and risk of caries at 5 years of age (papers II–IV). Nonetheless, we did find that a higher proportion (14%–16%) of the mothers who gave birth by C-section were obese, compared to 6%–9% of the mothers delivering vaginally, and our finding that being delivered by C-section increased the risk of caries at 5 years of age might be indirectly connected to the fact that others found a relationship between mothers' BMI and ECC. Maternal BMI and education, parental ethnicity, parental smoking, and siblings with caries can all be a proxy for socioeconomic status. The latter two were the most influential family factors in paper II and III, but the significance was lost in the multivariable logistic regression, which could be due to the population being too homogeneous, with few smokers.

The reasons for several studies finding maternal smoking to be a risk factor for ECC, can go beyond socioeconomics. It may also be due to the fact that maternal smoking during pregnancy is known to elevate the risk of fetal growth restriction as well as preterm birth (122-124). The mothers and children included in both paper I and papers II–IV are assumed to have good socioeconomic status based on the fact that the mothers were relatively well-educated, few had an immigrant background, and a large majority did not smoke. Several earlier studies show that poor socioeconomic conditions are risk factors for developing ECC (3, 22, 24, 125, 126). The details of how socioeconomic factors affect the risk of ECC are not fully understood, but the parents' knowledge about, and attitudes toward, health-promoting activities including oral habits and food habits are probably of importance. In contrast to maternal health and socioeconomic status, tooth brushing is probably a factor that is easier for the individual to modify. This can only be answered by interventional studies. A Scottish study presented evidence that a tooth brushing intervention in preschoolers (who were provided with a toothbrush and at tube of fluoride toothpaste on at least six occasions by the age of 5), saved more than two and a half times the cost of the program implementation through avoided dental extractions, fillings and potential treatments for decay (127).

In paper III, tooth brushing less than twice daily increased the risk of ECC at 5 years of age. The majority of the parents reported at all three measurement points (2, 3 and 5 years of age) that they assisted their children with tooth

brushing, and more than 98% also used fluoride toothpaste. The fluoride content should be stressed, since some years ago in Sweden there was a debate among parents about whether children should avoid fluoride in toothpaste. It is known that a regular disruption of the oral biofilm is important for oral health (17), but it is also known that brushing teeth with toothpaste that does not contain fluoride, has failed to show a benefit in terms of reducing the incidence of dental caries (58, 128, 129). Our study is in accordance with previous studies in Scandinavia (57, 130), which showed that establishing early (at 2 years of age) brushing habits twice daily is beneficial. In paper III, the variables "brushing with fluoride toothpaste" and "teeth always brushed by an adult" failed to reach statistical significance. This might be due to the fact that the vast majority (98%) used fluoride toothpaste, and the majority of children had their teeth brushed by an adult.

Oral health behavior established early in life has been shown to be stable through the preschool years (55). Recently, recall intervals to the dentist seem to have been extended in Swedish children. Longer recall intervals might lead to less well-informed parents, who brush their children's teeth more seldom. It is important to emphasize to the parents that they should brush twice daily, right from the start of tooth brushing, to establish the habit and maximize the effect of the fluoride ions in the oral biofilm. Parents struggling with brushing their children's teeth should be offered hands-on training by oral health personnel, as it prevents caries and lowers the cost for the individual as well as for the society.

In the present cohort, no correlation was found between ECC and BMI of the child. This is in accordance with a study from US that found no association between childhood obesity and caries experience after controlling for age, race, and poverty–income ratio (85). This stands in contrast to other studies, where obese children are shown to have more caries-affected teeth than overweight or non-obese children (81). However, the correlations could be more complex, as preschool children with low BMI seem to have a significantly higher caries rate than children with normal BMI (82). Interestingly, other researchers have also found a U-shaped correlation between oral health and BMI, with a higher prevalence of caries in underweight children and in overweight and obese children (23, 84, 131). Different findings might depend on which populations are studied and whether confounders are adjusted for.

In adults, correlations between caries and the metabolic syndrome have been described (87, 89). Previously, studies in adolescents have found associations between caries and signs of the metabolic syndrome (91, 92). The European multicenter network IDEFICS has recently developed references and

evidence-based cut-offs for metabolic risk factors in preschool children, as described in the Introduction. Not only did we lack correlations with obesity, but we did not find any differences between the prevalence of caries and the presence of metabolic risk factors according to the monitoring levels proposed by the IDEFICS consortium, which may be due to a lack of power in the study. In a recent publication from the same cohort, we identified preschool children with increased metabolic risk based on these cut-offs, the majority of these being either overweight or obese (95). Therefore, it was surprising that the only significant correlation was found between the fasting blood glucose values and the number of dmft. On the other hand, either higher fasting blood glucose or higher fasting insulin is the first sign to emerge in the conglomerate of the classic metabolic syndrome (132). In paper IV, the mean fasting glucose value was 4.8 mmol/L in children with caries at 5 years of age and 4.6 mmol/L for children without caries; this may appear to be only a minimal difference, but it is in fact of clinical relevance. It should be emphasized that in healthy individuals, serum glucose is strictly regulated and clamped in a narrow interval by diurnal insulin secretion to avoid vascular damage (133). In the Bogalusa Heart study, 10-year-old non-obese children with fasting glucose in the third and fourth quartile (with a fasting glucose of ≥ 5.0 mol/L) had a three times higher risk of type 2 diabetes in adulthood without any dose–response effect observed (134).

The finding of higher blood glucose at 6.5 years of age in children with ECC is consistent with results reported in adults (89, 90). A study performed in Japanese children aged 10–13 years found no associations between the metabolic syndrome and caries but an association with the prevalence of *Streptococcus mutans (94)*.

5.1 STRENGTHS AND LIMITATIONS

An overall strength of this thesis is the longitudinal prospective design, although this design results ultimately in a smaller cohort due to dropouts. Since the participants of papers II–IV were already a part of a larger ongoing study, it was not possible to increase the power by including more children at 2 years of age. The restricted number of examiners, both at the dental clinic and the pediatric clinic, is considered a strength, although the calibration of the two dental examiners in papers II–IV could have been recorded more often. Nevertheless, throughout the study the examiners continuously discussed potential diagnoses when difficulties appeared.

The analysis method in paper I has both strengths and limitations. The checkerboard DNA–DNA hybridization technique can analyze many species

in a large number of samples (135), although the risk of cross-reactions and varying reproducibility for different strains have been discussed (136). It is also important to note that the detection level of the method is approximately 10^4 cells, which means that a smaller amount of the selected strains could be present in the samples but not in enough quantities to be detected.

The study was conducted in a relatively homogeneous society, with relatively small socioeconomic segregation (high level of education, low proportion of parents who smoked, and a low proportion of parents born abroad), an overall high rate of breastfeeding and, in relation to other countries, a low prevalence of caries. The homogenous society can be considered a limitation, since the conclusions can be difficult to transfer to other populations (low external validity), but also a strength since confounders are limited.

6 CONCLUSION

1. Mode of delivery had a longitudinal impact on oral bacteria during the first 6 months of life. Children delivered vaginally had more of the selected salivary bacteria in common with their mothers. They also had higher counts of bacteria, as well as more of the selected bacteria strains compared to children delivered by C-section.

2. Mode of delivery affected the prevalence of ECC in 5-year-old children. Children delivered by C-section had an increased risk of ECC.

3. Oral as well as nutritional habits had an influence on ECC. Children who did not have their teeth brushed twice daily at the age of 2 years had an elevated risk of ECC at 5 years of age. We did not find a protective role of being breastfed on the risk of ECC at 5 years of age, but intake of cereal drink as well as juice with meals at 2 years of age increased the risk.

4. The perinatal factors being born preterm or SGA, increased the risk of ECC at 5 years of age. No differences between the prevalence of caries at 5 years of age and the presence of risk factors for the metabolic syndrome at 6.5 years of age could be found, but a significant correlation was found between dmft and fasting blood glucose.

7 FUTURE PERSPECTIVES

The study of selected bacteria strains, presented in this thesis revealed differences in oral bacteria diversity caused by different modes of delivery, but how long these differences persist remains to be seen. Additional studies following children longitudinally, and following the whole oral microbiota (for example with 16s rRNA amplification and sequencing), are needed. In papers II–IV, saliva samples were taken at the dental clinic at every visit (at 2, 3, and 5 years of age); the future plan is to analyze these samples to get a better understanding of the early and evolving oral microbiome in preschool children. Since children delivered by C-section or born moderately preterm or SGA seem to have an elevated risk of ECC, this makes it important to maintain and reestablish good cooperation between the staff at childcare centers and dental practitioners, from infancy onwards. Both the childcare centers and dental practitioners should also make efforts to encourage parents to start brushing their children's teeth with fluoride toothpaste at an early age. Future studies are needed to explore why perinatal factors, such as being born moderately preterm among other perinatal factors, is associated with an elevated risk of ECC. The association between fasting blood glucose and caries should also be further explored in this cohort as well as in future studies.

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