Faculty of Health Sciences

Department of Clinical Medicine

Oral health in pregnancy: changes in oral bacterial milieu related to cariogenic bacterial load, oxidative stress and nitric oxide levels in the saliva and their effect on pregnancy outcome

Madhu Wagle Parajuli

A dissertation for the degree of Philosophiae Doctor [September 2022]

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Abbreviations

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt

CFU – colony forming unit

DMFS – decayed, missed, filled surface

DMFT – decayed, missed, filled tooth

IQR – interquartile range

LB – lactobacillus

LBW – low birth weight

MDA - malondialdehyde

NO – nitric oxide

OD – optical density

OS – oxidative stress

PTB – pre-term birth

ROS – reactive oxygen species

SM – streptococcus mutans

WHO – World Health Organization

List of papers

Paper I

Wagle, M., Basnet, P., Vårtun, Å., Trovik, T. A., & Acharya, G. (2020). Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. non-pregnant women. *BMC oral health*, *20*(1), 245. https://doi.org/10.1186/s12903-020-01230-3

Paper II

Wagle, M., Basnet, P., Vårtun, Å., & Acharya, G. (2021). Nitric Oxide, Oxidative Stress and *Streptococcus mutans* and *Lactobacillus* Bacterial Loads in Saliva during the Different Stages of Pregnancy: A Longitudinal Study. *International journal of environmental research and public health*, *18*(17), 9330. https://doi.org/10.3390/ijerph18179330

Paper III

Wagle, M., D'Antonio, F., Reierth, E., Basnet, P., Trovik, T. A., Orsini, G., Manzoli, L., & Acharya, G. (2018). Dental caries and preterm birth: a systematic review and meta-analysis. *BMJ open*, *8*(3), e018556. https://doi.org/10.1136/bmjopen-2017-018556

Abstract

The physiological changes that occur during the pregnancy are known to affect women´s oral health. Pregnant women are more vulnerable to oral diseases, such as gingivitis, periodontal disease and dental caries, compared to non-pregnant. Better understanding of the relation between changing oral microbial milieu and risk of pregnancy complications as well as the associated mechanisms could help to improve maternal and perinatal outcomes. Thus, the aim of this thesis was to investigate the pregnancy associated changes in oral bacterial milieu with a focus on cariogenic bacterial load, oxidative stress and nitric oxide levels in the saliva, and their effect on pregnancy outcome.

The study included a total of 146 participants, with a cohort of 96 healthy pregnant women recruited from University Hospital of North Norway (UNN) at 18-20 weeks of gestation and 50 age-matched non-pregnant women that were recruited from the University campus of Tromsø or the UNN for comparison. The saliva samples from both groups were collected under similar conditions using the same technique to investigate oral bacterial milieu represented by the load of dental caries related bacteria, Streptococcus mutans (SM) and Lactobacillus (LB), and for determining antioxidant capacity, oxidative stress and nitric oxide (NO) levels in the saliva. In addition, we summarized the evidence from published literature to investigate the association between dental caries and preterm birth.

We found that the salivary oxidative stress level increases and the antioxidant capacity of saliva decreases in pregnant women together with an increase in colonization by SM compared to non-pregnant women. In addition, the salivary NO levels were also higher among the pregnant women compared to nonpregnant and levels increased with advancing gestational age. However, an association between dental caries and risk of preterm birth was not observed based on data obtained from the published literature.

In conclusion, pregnant women had increased load of caries related bacteria, especially SM, in the saliva with higher oxidative stress levels and decreased antioxidant capacity compared to non-pregnant women. Likewise, there was an increase in salivary NO levels with advancing gestational age, and an increase in bacterial colonization by SM and LB was found among the pregnant women in their second and third trimesters. However, no significant association was observed between dental caries and increased risk of preterm birth.

1 INTRODUCTION

Oral health as per the FDI World Dental Federation "is multifaceted and includes the ability to speak, smile, smell, taste, touch, chew, swallow, and convey a range of emotions through facial expressions with confidence and without pain, discomfort, and disease of the craniofacial complex." As such, it attributes to fundamental components of health and physical and mental wellbeing. It exists along a continuum influenced by the values and attitudes of individuals and communities, reflecting the physiological, social, and psychological attributes that are essential to quality of life; it is influenced by the individual's changing experiences, perceptions, expectations, and ability to adapt to circumstances." (Glick *et al*., 2016). Essential elements describing the various components of oral health are schematically presented in figure 1.
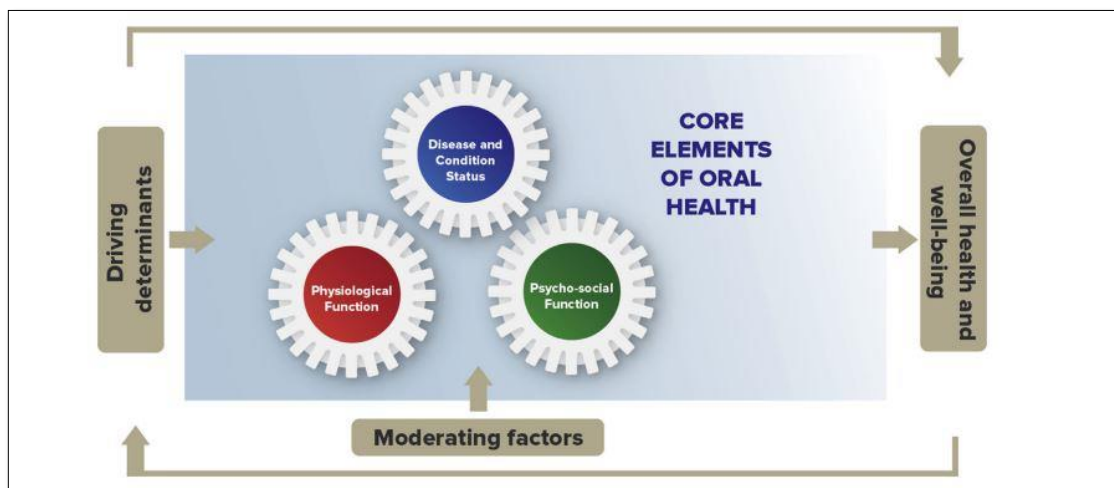
Figure 1: Essential elements describing the main components of oral health. (Reprinted with the permission from the Elsevier publisher group)

Good oral health is essential for overall general health and is particularly essential for a sense of wellbeing and being free of any oral diseases (Petersen, 2003). An individual's oral health depends on various factors, such as their knowledge and attitude towards taking good care of hard and soft tissues of the oral cavity, their dietary habits, and personal oral hygiene. Oral disease affects all human beings regardless of their age, sex, ethnicity, or socio-economic status, but risk may vary. It is considered the fourth most expensive disease requiring treatment

worldwide. Although preventable, dental caries and periodontal diseases are most prevalent among all the oral diseases due to the fact that they are asymptomatic over longer time-periods with sporadic painful exacerbations and are considered a major disease burden globally (Petersen, 2003; Watt, 2005; Boggess & Edelstein, 2006; Frencken *et al.*, 2017).

1.1 Oral health: oral hygiene and risk factors

Human oral cavity harbours about 770 different taxa of microorganisms also known as oral microflora or oral microbiome (Dewhirst, *et al.,* 2010; Ye & Kapila, 2021). These microorganisms are both commensals and pathogenic types. Commensals are microflora that are living in the host but do not cause disease under normal circumstances; however, they can turn pathogenic with alteration in microflora (Avila, Ojcius & Yilmaz, 2009; Cugini *et al*., 2021). Moreover, an oral cavity constitutes an entry port into the body and taking good care of cavities is important for optimal health and wellbeing.

In recent years, it has been established that the oral diseases also share common risk factors with other chronic diseases such as cardiovascular diseases, cancer, chronic respiratory diseases and diabetes, for example unhealthy diet, tobacco and alcohol use (Sheiham & Watt, 2000; Petersen *et al*., 2005; Peres *et al.,* 2019; WHO, 2020). However, low importance and priority is given to oral health compared to general physical health due to lack of knowledge, interest, awareness, and forgetfulness leading to postponement of dental appointments/treatments (Rocha *et al*., 2018) and high cost as well as low priority in resource allocation (Peres *et al.,* 2019). In addition to all these factors, poor oral hygiene practice also causes and/or deteriorates oral diseases (WHO, 2020).

Poor oral hygiene leads to the formation of a thin biofilm on the tooth surface known as dental plaque that harbours many microbiomes and sometimes desquamated epithelial cells (Abebe, 2021). This biofilm or plaque containing bacterial colonies in turn leads to oral disease, such as dental caries, gingival and periodontal disease. Dental caries and periodontal disease are one of

the major causes for tooth loss (Frencken *et al.,* 2017). Even though oral diseases are prevalent, they are preventable. The high treatment cost of oral diseases could be minimized by utilizing early preventive as well as health promotion measures (Suga *et al*., 2014).

1.2 Oral health in pregnancy

During puberty, the female body undergoes various changes including physical, hormonal, psychological and so forth. Similarly, during pregnancy, many physiological changes are observed in women, such as endocrine, cardiovascular, respiratory, digestive, metabolic, immunological, psychological etc., and their oral health is also affected (Silva de *et al.*, 2017). "A tooth for a child" during pregnancy is a common proverb and belief in many parts of the world (Christensen *et al*., 1998). Improving the oral health of women during pregnancy may avert dental/periodontal diseases and their complications, thereby decreasing the possibility of preterm birth and low birthweight (Khanna & Malhotra, 2010). Optimal oral health is equally essential during pregnancy to optimal general health (Achtari *et al*., 2012). Pregnant women are more susceptible to dental caries and gingivitis/periodontal disease than non-pregnant women (Martinez-Beneyto *et al.*, 2011; Kamate, Vibhute & Baad, 2017). A possible explanation for this could be nausea or vomiting due to morning sickness, leading to erosion of the enamel layer, changes in dietary patterns including food cravings, intolerance to foreign objects in mouth such as toothpaste/toothbrush, lack of adequate knowledge, awareness about appropriate oral health care and oral hygiene, and not least the hormonal and immunological changes that occur during pregnancy (Ye and Kapila, 2021). Most of the above-mentioned factors could lead to changes in oral microflora in pregnancy. Studies have also shown that the oral flora from the mother can easily be transmitted to their infants and cariogenic oral flora can predispose the infant to early childhood dental caries (Boggess & Edelstein, 2006).

1.3 Attitude, knowledge, and awareness

Attitude in general can be perceived as one's view or opinion towards something specific. Attitude can be defined through three components: cognitive (thoughts, beliefs, and knowledge), affective (strength of belief, emotions and feelings) and behavioural (readiness to act on certain situation/objective or action) (Eagly & Chaiken, 1993; Stenberg, Håkansson & Åkermann, 2000). Attitude with reference to dental care can be defined as self-assessment of one's dental health (cognitive), concerns about one's dental health (affective) and the inclination to attend for regular oral/dental examination (behavioural) (Stenberg, Håkansson & Åkerman, 2000).

Knowledge is expertise and skills acquired by a person through experience or education and the theoretical or practical understanding of a subject with the ability to use it. Knowledge can be defined as comprising three forms: declarative or knowing what, procedural or knowing how, and conditional or knowing when and why (Schrader & Lawless, 2004). Knowledge acquisition involves complex cognitive processes: perception, learning, communication, association, and reasoning (Sharda & Shetty, 2008). Previous studies have shown that a low level of education and knowledge in pregnant women has some impact on oral hygiene care beliefs and practices resulting in avoidance of visiting their dentists during pregnancy (Lydon-Rochelle *et al*., 2004; Boggess *et al*., 2011). In addition, providing valuable information, awareness, and services to pregnant women about basic and preventive oral health care procedures could benefit both the mother and the child (Steinberg *et al*., 2013).

1.4 Saliva

Saliva is an extremely important oral fluid that is crucial for the maintenance and preservation of oral tissues. Besides this, saliva also performs other important functions, such as helping in

speech, taste perception, bolus formation, swallowing and digestion (Humphrey & Williamson, 2001; Amerongen & Veerman, 2002; Pedersen *et al*., 2018). Saliva acts as an important link between different soft tissues and structures that are present in the oral cavity maintaining natural homeostasis between the intra-oral tissues and oral microflora (Hofer *et al*., 2004; Pedersen *et al*., 2018). Previous studies have reported that changes in oral microflora and increases in dental caries and periodontal disease activity occur with the alteration in the flow of the saliva (Hofer *et.al*, 2004; Novakovic *et al*., 2014; Marsh *et al*., 2016).

In recent years, saliva is being widely used in the screening and diagnosis of several conditions and diseases, for monitoring disease progression, and detection of drugs due to ease in its collection, the non-invasiveness of the procedure, the abundant presence of biomarkers, its durability and repeatability (Chiappin *et al*., 2007; Navazesh & Kumar, 2008; Lee, Garon & Wong, 2009; Chojnowska *et al*., 2018). Saliva is also considered a "mirror of the body" and plays an important role in immunological as well as enzymatic defence mechanisms against certain microbes' antioxidant systems. In recent years, many studies have indicated that the body's overall oxidative stress is also expressed in saliva to a greater extent that also accounts for the increased popularity of saliva as a diagnostic tool (Lee, Garon & Wong, 2009; Ahmadi-Motamayel *et al*., 2013; Javaid *et al*., 2016).

1.5 Gingivitis

The hormonal and physiological changes in pregnancy can affect oral tissues and the marked soft tissue change can be observed most commonly in the gingival tissues (Laine, 2002). Gingivitis or inflammation of gums/gingiva is very common during pregnancy and is experienced by almost 30-100% women wherein the gums may become red, swollen, enlarged and bleed easily during brushing or even during chewing (Löe & Silness, 1963; Laine, 2002). Even though pregnancy itself does not cause gingivitis, it may be intensified by a pre-existing

gingival condition (Laine, 2002). Pregnancy gingivitis and pregnancy epulis (also called as pregnancy granuloma or epulis gravidarum) is the most common gingival disease observed during pregnancy and is a reversible condition (Khanna & Malhotra, 2010). The changes in gingiva are observed as early as the 2nd month of pregnancy until the 8th month and the condition reverses after delivery.

1.6 Periodontitis / periodontal disease

Periodontal disease can be defined as any inherited or acquired disorder of the tissues surrounding the teeth. According to the American Academy of Periodontology, periodontal disease is a chronic inflammatory disease usually of bacterial origin affecting the surrounding and supporting structures of teeth (American Academy of periodontology, 2001). Periodontal disease could, in its mildest form, is also known as gingivitis (inflammation of gums, a reversible condition) or periodontitis (inflammation extending to underlying deep connective tissues and the surrounding alveolar bone, an irreversible condition). The destruction of the teeth surrounding tissues is instigated by the pathogenic microflora present in microbial biofilms or dental plaque formed on the tooth surface, which can be a result of poor oral hygiene (Pihlstrom, Michalowicz & Johnson, 2005; Krejci & Bissada, 2012). Many studies have investigated an association between periodontal disease with other systemic diseases such as cardiovascular disease, stroke, Alzheimer's disease, diabetes complications and adverse pregnancy outcomes (Borgnakke *et al*., 2013; Lafon *et al*., 2014; Gurav 2014; Martende *et al*., 2014; Cassini *et al*., 2013; Sgolastra *et al*., 2013; Takeuchi *et al*., 2013). Periodontal disease has been a focus in studies in pregnant women populations after it was reported to have an association with risk of preterm birth (PTB), pre-eclampsia, and low birthweight (LBW) (Offenbacher *et al*., 2001; Jeffcoat *et al*., 2003; Huck, Tenenbaum & Davideau 2011; Horton & Boggess, 2012; Takeuchi *et al*., 2013; Sgolastra *et al*., 2013; Teshome & Yitayeh, 2016).

1.7 Dental caries

Dental caries is the localized destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates. It is the result of the metabolic activities of bacteria in microbial communities of dental biofilm or dental plaque. Dental caries initiation and progression depends upon the imbalance between pathologic (bacteria) and protective factors (Pitts *et al.,* 2017). Bacteria like mutans streptococci [Streptococcus mutans (SM) and Streptococcus sobrinus] and Lactobacillus (LB) present in the dental plaque produce organic acids resulting in demineralization of teeth which in the long term leads to the formation of cavities on the tooth surface also called dental decay (Selwitz, Ismail & Pitts, 2007). SM plays an important role in the initiation and progression of dental caries whereas a high LB count indicates a high content and high frequency of intake of carbohydrates in diet, which is also a risk factor for the development of dental caries. The process of dental caries formation is presented in figure 2.
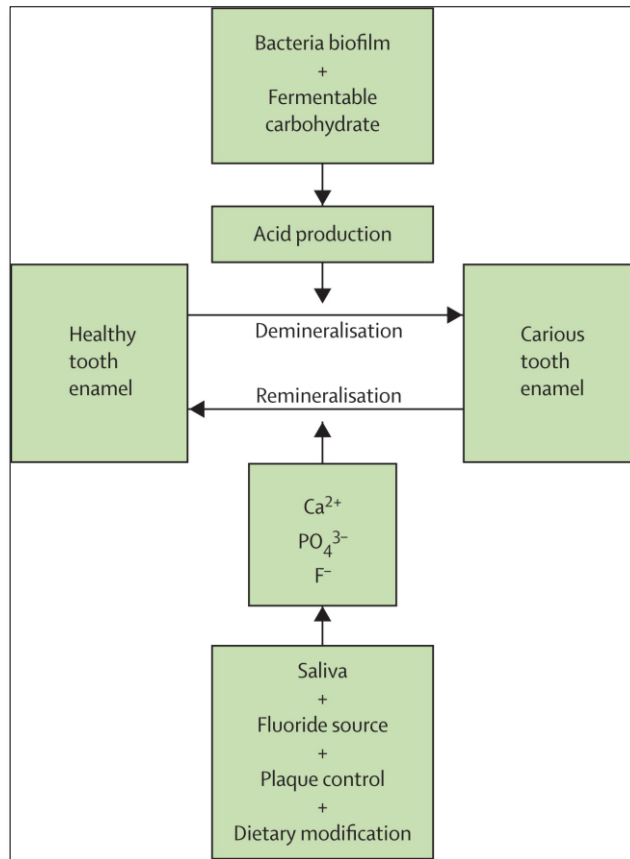
Figure 2: Schematic diagram showing dental caries formation pathways. (The figure was adapted from Essentials of dental caries: The disease and its management 2nd ed. by Kidd & Joystone-Bechyl, 1997 with permission from the authors and publisher)

The formation of dental caries depends upon several risk factors, such as altered salivary composition, inadequate salivary flow, increased load of cariogenic bacteria, poor oral hygiene, poor dietary habits, inadequate fluoride exposure, gingival recession, inappropriate methods of feeding, and poverty. Essential requirements for the development of dental caries are cariogenic bacteria, bacterial plaque, stagnation or plaque retaining areas, fermentable bacterial substrate or sugar, including sweetened baked food, susceptible tooth surface, and time period (Selwitz *et al.,* 2007; Cawson & Odell, 2017).

The bacteria present in the bacterial biofilm results in the formation of weak acid during the fermentation or metabolism of the carbohydrates. This weak acid produced during the process will cause a lowering of local pH value resulting in the demineralization (dissolution of calcium, phosphate and carbonate from the enamel) of the teeth. Demineralization process at an early

stage is reversible due to uptake of calcium, phosphate, and fluoride from the saliva. Further continuation of the dissolution of tooth structure results in the formation of dental cavitation. This cavitation process leads to a breakdown of dental hard tissues, e.g., enamel and dentine, and at the later stage involves the dental soft tissue (pulp) causing severe pain and irreversible tooth damage (Selwitz *et al.,* 2007).

1.7.1 Streptococcus mutans

Streptococcus mutans (SM) are the facultative gram-positive cocci (stained dark blue or violet by gram staining) that are the major culprit in the commencement and development of dental caries and are used as a marker in risk assessment and monitoring the outcomes of dental caries prevention programs (Rabe, Winterscheid & Hillier, 1988). SM is especially seen in people with high caries activity and high dietary sucrose intake. It is transmitted to newborns/infants via vertical transmission from the mother as well as horizontal transmission (Berkowitz, 2003; Köhler & Andreen, 2012).

The cariogenic property of SM is that it produces lactic acid from sucrose, it can survive at a low pH, as low as 4.2, it forms in large amounts of extracellular, sticky and insoluble glucan plaque matrix adhering to pellicle and contributing to plaque formation, and it produces intracellular polysaccharides reserves for future survival when substrate is scant (Karpinski & Szkaradkiewicz, 2013; Cawson & Odell, 2017; Al-Shahrani, 2019).

1.7.2 Lactobacillus

Lactobacillus (LB) is also a gram-positive bacterium. The presence of LB in plaques is low but has been reported to inhabit in high levels in the caries lesions of people with high-caries activity (Van Houte, 1993). Previous studies have reported LB counts to be related to caries activity, and the strongest correlation was observed between low LB counts and low caries

activity (Birkhed, Adwardsson & Andersson, 1981). High LB count indicates a high content and high frequency of intake of carbohydrates in the diet which is a risk factor for the development of dental caries. Usually, LB are found in deep carious lesions and root caries (Bardow, Nyvad & Nauntofte, 2001; Karpinski & Szkaradkiewicz, 2013; Al-Shahrani, 2019). They are responsible for dentin caries lesions and are rarely isolated from enamel caries or early caries lesions contributing to the progression of the carious lesion rather than the initiation (Loesche, 1986; Karpinski & Szkaradkiewicz, 2013; Klinke *et al*., 2014).

1.7.3 Decayed, missing, filled tooth (DMFT) and decayed, missing, filled surface (DMFS) indices

For the numerical expression of caries prevalence two popular indices are used: decayed, missing, filled teeth (DMFT) and decayed, missing, filled surfaces (DMFS) indices. Both indices are used for individuals' or groups' caries assessment as well as commonly used in oral health epidemiological surveys. DMFT/DMFS score is calculated by adding up permanent teeth that are caries affected. If a tooth has both a filling and caries lesion, then it is counted as D for DMFT index. Whereas filling (F) + caries surface is counted as D, if there is F on one and D on another surface, then they are counted differently for DMFS index. In brief, each tooth or surface is counted only once. The anterior teeth up to canine have four (mesial, distal, buccal and lingual/palatal) surfaces while pre-molars and molars have five (mesial, distal, buccal, lingual/palatal and occlusal) surfaces respectively, in DMFS index. D+M+F = caries prevalence or caries score of an individual wherein the maximum score for DMFT and DMFS index is 28 and 128, respectively. All 28 permanent teeth are included in both indices whereas all 4 wisdom molar teeth are excluded (Shulman & Cappelli, 2008; WHO, 2021).

1.8 Oxidative stress

Oxidative stress (OS) is the state of disproportion between oxidants and antioxidants with the former leading to and causing potential damage of the living cells (Sies, 1997). Oxidative reaction is a constant process that occurs within the living body with constant forming and neutralizing of the free radicals and reactive oxygen species (ROS). ROS has the potential to generate new free radicals (Duhig, Chappell & Shennan, 2016), and if the concentration of free radicals and ROS increases, it may lead to the permanent damage of tissue cells. Therefore, it is very important to maintain oxidative equilibrium. OS has been considered to be the main contributor to the development of several systemic diseases such as diabetes, obesity, Sjörgen's syndrome and also several other oral conditions, such as salivary gland dysfunctions, xerostomia, periodontitis, precancerous lesions, and oral carcinogenesis. Oral cavity and/or saliva acts as the first line of defense against oxygen free radicals (Battino *et al*., 2002; Zukowski, Maciejczyk & Waszkiel, 2018) and OS biomarkers are evident in saliva also (Araujo et al., 2020).

The OS levels in oral cavity can be expressed as Malondialdehyde (MDA) content present in the saliva. MDA acts as an indicator of OS as it is one of the final products of lipid peroxidation. The increase in MDA level signifies increased OS level. Canakci *et al*. in 2009 reported higher MDA levels and lower salivary antioxidant activity in periodontitis (Canakci *et al*., 2009) whereas in 2006, Rai *et al*. reported increased salivary MDA level in several other oral diseases including dental caries (Rai *et al*., 2006). Similarly, previous studies have also indicated the inverse relationship between serum antioxidants and periodontitis (Chapple, Milward & Dietrich, 2007).

In recent years, studies have highlighted that OS may have influences on the reproductive system of humans and can be associated with endometriosis, polycystic ovary syndrome (POS) amongst other conditions. Pregnancy may express increased susceptibility to OS that may result

in spontaneous abortion, recurrent pregnancy loss, pre-eclampsia, and gestational diabetes. In addition, OS is also one of the causes of sub/infertility (Poston *et al*., 2011; Giebultowicz *et al*., 2013).

1.9 Antioxidant capacity of saliva

In general, oxidation is defined as a loss of electrons of molecules and the oxidant/oxidizing agent is the substance that accepts electrons causing another reactant to be oxidized (Prior & Cao, 1999). An antioxidant is a substance that prevents or delays free radicals and ROS initiated oxidation when present at low concentrations compared with an oxidant (Halliwell & Gutteridge, 1990). Antioxidants' defense systems could be divided into three variations according to their mode of action: 1. preventive antioxidants (subpresses free radical formation as superoxide dismutase, catalase, albumin, transferrin etc), 2. radical scavenging antioxidants (scavenges radicals inhibiting chain initiation and breaking chain propagation as carotenoids, bilirubin, uric acid, vit A., vit C., vit D. etc) and 3. repair and "de novo" enzymes (repairs and reconstructs the damage and membranes as DNA repair enzymes, lipase, protease, transferase etc) (Niki, 1996). Due to the complexity of different antioxidants' activities and economic circumstances, researchers are focused on assays that assess the "antioxidant capacity " of biological fluids including saliva (Battino *et al*., 2002). As mentioned earlier, saliva could be the first line of defense for the oral entry due to its antioxidant properties. There are two types of salivary antioxidant based on functional and structural characteristics: enzymatic and non-enzymatic, wherein the former is involved in neutralization of free radicals and the latter neutralizes secondary oxidative products (Novakovic *et al*., 2014). The antioxidant capacity of saliva can be measured by three methods: 1. spectrophotometric assay 2. enhanced chemiluminescence assay, and 3. cyclic voltammetry assay (Prior & Cao, 1999). Previous studies have reported an increase in the antioxidant capacity of saliva with increased caries activity (Tulunoglu, Demirtas & Tulunoglu, 2006; Hegde, Rai &Padmanabhan, 2009).

1.10 Inflammation and Nitric oxide

Nitric oxide (NO) is also a free radical that reacts constantly with several molecules to form Nitrite (NO_2^-) and Nitrate (NO_3^-). NO is synthesized from L-arginine and is involved in physiological and pathological processes such as metabolism and inflammation (Yang *et al.*, 1997). The overall hormonal, cardiovascular, immunological, metabolic changes in pregnancy affect the oral condition leading to changes in oral milieu that is favorable for pathogenic bacterial growth, leading to inflammation and OS in oral cavity. NO is considered one of the indicators that expresses the level of inflammation.

NO plays an important role during pregnancy and is regarded as a key regulator of both maternal and fetal homeostasis for cardiovascular functions. It enables the maternal cardio-vascular changes, the growth and development of the fetus and adaption to afterbirth (Sutton, Gemmel & Powers, 2020). NO levels in serum have been observed to increase along with gestational age in normal pregnancy (Shaamash *et al.*, 2000; Choi, Im & Pai, 2002; Hodzic *et al.*, 2017). Inorganic nitrate is found profoundly in saliva, almost 10 times higher in concentration compared to its serum concentration (Duncan *et al.*, 1995; Bayindir, Polat & Seven, 2005) and it has been reported to have an antimicrobial effect in different systems of the human body such as oral cavities, the gastrointestinal tract and skin (Duncan *et al.*, 1995, Duncan *et al.*, 1997; Weller *et al.*, 2001). Increased salivary NO levels have been reported in patients with dental caries and poor oral hygiene status, and higher NO concentration has been reported more in plaque than in saliva (Bayindir *et al.*, 2005) suggesting a probable host defense mechanism by the body to counteract oral inflammation.

1.11 Pregnancy complications associated with poor oral health

Taking good care of oral health during pregnancy is very important. Poor oral health in pregnancy may lead to further complications regarding general health. Nausea and vomiting during pregnancy may lead to the erosion of the enamel layer of the teeth, predisposing them

for caries. Periodontal disease has been linked to adverse pregnancy outcome as threatened preterm labor (Ye *et al*., 2020; Pokpa *et al*., 2021). A recent review on periodontal disease and adverse pregnancy outcome has reported periodontal disease's link with PTB, LBW, and pre-eclampsia (Choi *et al*., 2021; Pokpa *et al*., 2021).

2 AIMS AND OBJECTIVES

The main objective of this thesis was to understand the relations among pregnancy associated changes in oral bacterial milieu with a focus on cariogenic bacterial load, oxidative stress and nitric oxide levels in the saliva, and their effects on pregnancy outcome.

The specific aims were:

1. To measure and compare the dental caries-related bacterial load, level of oxidative stress and antioxidative capacity in the saliva of pregnant women in reference to non-pregnant women.

2. To investigate the association of oral bacterial load, represented by Streptococcus mutans (SM) and Lactobacillus (LB), with nitric oxide (NO) and oxidative stress (OS) levels longitudinally during the development of pregnancy.

3. To synthesize the scientific evidence on the association between dental caries and the risk of preterm birth based on a systematic review of published literature.

3 METHODOLOGY

3.1 Ethical approval

For paper I and II, the study protocol was approved by the Regional Committee for Medical Research ethics – North Norway (Project number 19353- Ref no:2012/633/REK Nord). The research was conducted in accordance with the Helsinki Declaration. Informed written consent was obtained from all the study participants.

For Paper III, a formal ethical approval was not required as it was a systemic review and meta-analysis of already published data. It followed a priori designed protocol that was prospectively registered in PROSPERO database (registration number: CRD42017062573) and reported according to Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (Moher *et al*., 2009).

3.2 Study design

3.2.1. Prospective observational cohort

We investigated the pregnancy-related changes in cariogenic bacterial (SM and LB) loads, OS and NO levels in the saliva from pregnant women. One of the studies has a cross-sectional design (paper I) that included a cohort of pregnant women at 18-20 weeks of gestation and a comparison group of non-pregnant women matched for age. Another study (paper II) has a longitudinal design. The saliva samples from pregnant women were collected four times during pregnancy, from 18 weeks of gestation until term, and compared with age matching non-pregnant women.

Paper III is a systematic review and meta-analysis aiming to investigate association between dental caries and preterm birth.

3.3 Study setting and study population

This study was conducted at the Department of Obstetrics and Gynaecology, University Hospital of Northern Norway and Institute of Clinical Medicine, UiT the Arctic University of Norway, Tromsø, Norway. The study comprised a total of 146 participants, of which 96 healthy pregnant women and a comparison group of 50 healthy non-pregnant women of reproductive age were included. Low-risk pregnant women attending the hospital for their routine second trimester ultrasound screening at 18-20 weeks were recruited. The inclusion criteria were: age > 18 years, low risk singleton pregnancy, no previous history of any pregnancy-associated complications (e.g preeclampsia, preterm birth or gestational diabetes), and absence of any preexisting medical condition that may have an impact on the course and outcome of pregnancy. Pregnant women not willing to participate, who could not communicate in Norwegian or English, and those who have been diagnosed to have a fetus with a chromosomal or structural fetal anomaly and did not plan to continue their pregnancy, were excluded. The non-pregnant healthy volunteers of reproductive age were recruited among women working at the University of Tromsø or University Hospital of North Norway, Tromsø, Norway. Women with a history of any acute or chronic illness requiring regular medical treatment were excluded.

3.4 Study material

Saliva samples were collected from all pregnant and non-pregnant women participants (using identical methods) for investigating oral bacterial milieu specially for dental caries related bacteria SM and LB; and for determining antioxidant capacity, OS and NO levels in the saliva. Saliva samples for both groups were collected during day-time between 9:00 and 15:00. The paraffin wax provided by the manufacturer in the supplier kit was chewed by the participants for about 5 mins to stimulate saliva secretion. The saliva sample was then obtained by expectorating in disposable cups. For antioxidative capacity, OS and NO measurements, 1.8 ml

of saliva was collected in cryo-tubes vials and stored at -70ºC until samples were analyzed. Samples were transferred to a refrigerator at 4 °C for one day before the analyses and all samples were left for 2h on the working table to bring it to room temperature and centrifuged at 10000 x g for 10 min to remove cell debris and supernatant that was separately collected for further analysis. For all of the sample collection procedures, the commercial kit manufacturer's instructions were strictly followed.

3.5   Study methods

3.5.1. Measurement of SM and LB loads

Commercial kits, Dentocult® LB (kit for LB) Dentocult® SM Strip mutans (kit for SM) provided by Orion Diagnostica Oy, Espoo, Finland were used to cultivate SM and LB samples until development of bacterial colony forming units (CFU). Both pregnant and non-pregnant women were requested to chew a paraffin pellet to stimulate the secretion of saliva and promote transfer of SM from tooth surfaces into the saliva. The round-tipped test strip supplied in the kits was pressed against the saliva on the women's tongue. The strip was then placed in the cap of the vail containing culture broth and recapped. The vial cap was loosened according to the instruction and incubated at 37 °C and 5% CO_2 for 48 h. Results were interpreted by scoring as 0, 1, 2, and 3 for 0, $<10^5$, $10^5 - 10^6$ and $>10^6$ CFU/mL, respectively, by comparing the samples to the template reader provided in the kits. In case of LB culture, both sides of the modified Rogosa agar test surface (that are fixed along with the cap) were thoroughly made wet by saliva, excess saliva was drained, and the cap was refitted. It was then incubated for 4 days at 37 °C and 5% CO_2. Results were interpreted scoring as 0, 1, 2, 3 and 4 for 0, 10^3, 10^4, 10^5 and 10^6 CFU/mL, respectively, by comparing to the template reader provided by the manufacture (figure 3 & figure 4).
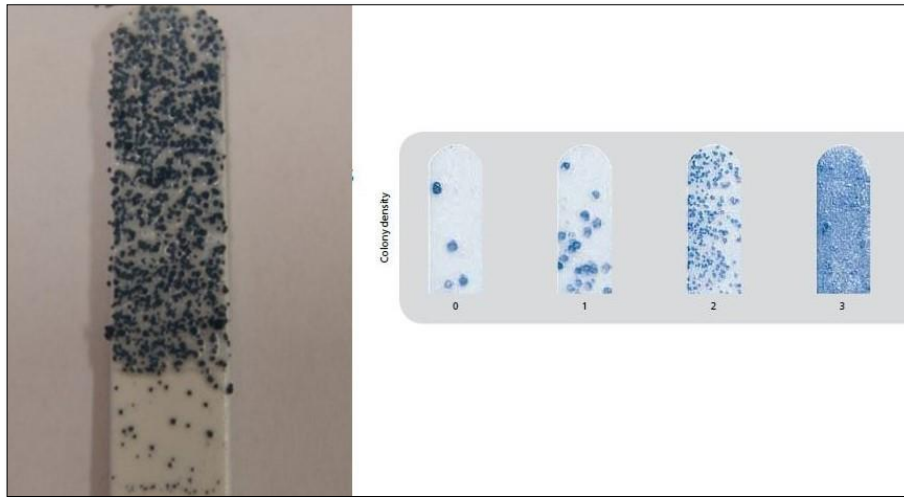
Figure 3: Representative picture of Streptococcus mutans (SM) colony formation on left after 48 hours incubation showing score 3 (>106 CFU/mL) compared to the manufacturer template on the right side of the figure.
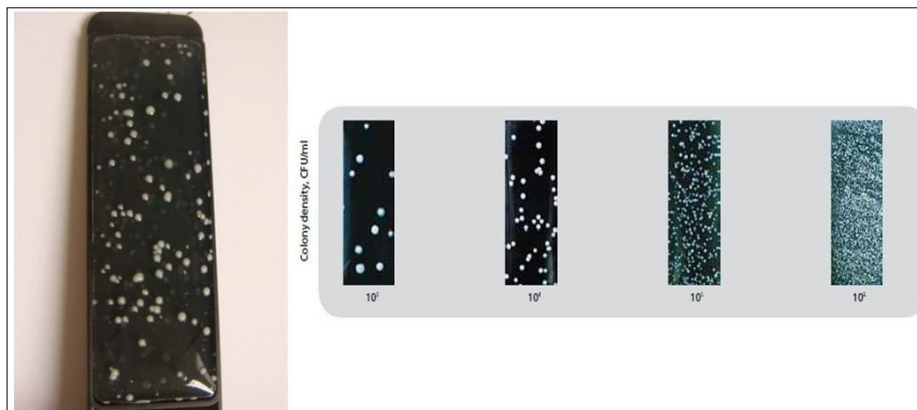
Figure 4: Representative picture of Lactobacillus (LB) colony formation on the left after 4 days incubation showing score 2 (104 CFU/mL) compared to the manufacturer template on right side of the figure.

3.5.2. Measurement of antioxidative capacity in saliva

Antioxidative capacity in the saliva was expressed by measuring 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS) free radical scavenging activity. Dark green colored ABTS free radicals were generated by mixing 2 mL each of the solutions of ABTS (7.4 mM) and potassium peroxodisulfate (2.6 mM) for 24 hours. Both ABTS and potassium peroxodisulfate were purchased from Sigma-Aldrich, Oslo. The reaction mixture was then diluted to 100 mL with distilled water as a working solution of ABTS free radical with

the optical density (OD) of the approx. 0.5 to 0.6. Supernatant of centrifuged saliva samples were used in a similar way for both pregnant and non-pregnant participants. Reactions were carried out by mixing 450 µL of working solution of ABTS radical and 50 µL supernatant saliva following by incubating the samples for 30 min in darkness. The change in green color of ABTS free radicals scavenged by the antioxidants present in saliva samples was measured for its OD using spectrophotometric methods (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) at 731 nm. Vitamin C (a water soluble one) (Sigma-Aldrich) was used as standard and quantified as µg/mL vitamin C equivalent levels, representing the antioxidant capacity with the help of standard curve and regression equation ($R^2 = 0.9331$ and $y = - 0937x + 0.7357$).

3.5.3. Salivary OS levels malondialdehyde (MDA) assay

Salivary malondialdehyde (MDA) levels were measured using the MDA Assay Kit (Sigma-Aldrich, Lipid Peroxidation MDA Assay Kit). MDA levels are expressed as OS levels. A mixture of 100 µL saliva fluid is diluted with 200 µL buffer provided in the kit. Saliva sample in buffer and TBA solution each of 600 µL were mixed thoroughly and incubated at 95°C for 60 minutes. Of the reaction mixture after cooling in ice, 150 µL was transferred to a 96 well microplate in duplicates and absorbance measured flurometrically (Epoch Microplate, BioTek Instrument, Vermont, USA) by measuring fluorescence intensity ($\lambda_{ex} = 532/ \lambda_{em} = 553$). The MDA levels in the saliva were calculated by the MDA standard provided in the kit with the help of standard curve and regression equation ($R^2 = 0.9903$ and $y = 728.95x + 111.6$).

3.5.4. Measurement of NO Levels

The levels of NO in saliva were expressed by measuring nitrite quantitatively using the Griess method spectrophotometrically with modification. Griess reagent was prepared as 1% sulfanilamide and 0.1% N-naphthylethylene diamine dichloride in 5% orthophosphoric acid (v/v). This reagent reacts with nitrite and produces a purple azo dye end-product, which is

measured spectrophotometrically with a maximum absorbance at 546 nm. Triplicate samples of saliva (10 μL) were transferred to a tube containing 290 μL of distilled water, and a 300 μL of Griess reagent was added to each tube. After mixing thoroughly and allowing to react in the dark for 30 min, the changed colour was measured. The quantitative expression of NO in saliva is taken from the analysis of triplicates of sodium nitrite ($NaNO_2$) at concentrations of 25, 20, 15, 10, 5, 2, 1, and 0 μM as final concentrations and with the help of standard curve and regression equation ($R^2 = 0.9995$ and $y = 0369x - 0.0035$).

3.5.5. Socio-demographic characteristic and information on oral health status

The pregnant women participants were requested to fill in a set of close-ended self-administered questionnaires containing questions on background information about their age, civil status, highest education level, family (number of children), habits related to general health, oral health habits in general and during pregnancy, knowledge and attitude towards oral health and about their current oral health.

3.5.6. Data on general health and outcome of pregnancy

The rest of the information regarding general health such as weight, height, medical history current/previous if any, about previous pregnancies if any and current pregnancy outcome was extracted from the health-card for pregnant women and electronic patient journal. All of the study participants were designated with a code number. Further investigation and data handling was done using the provided code number to maintain their anonymity.
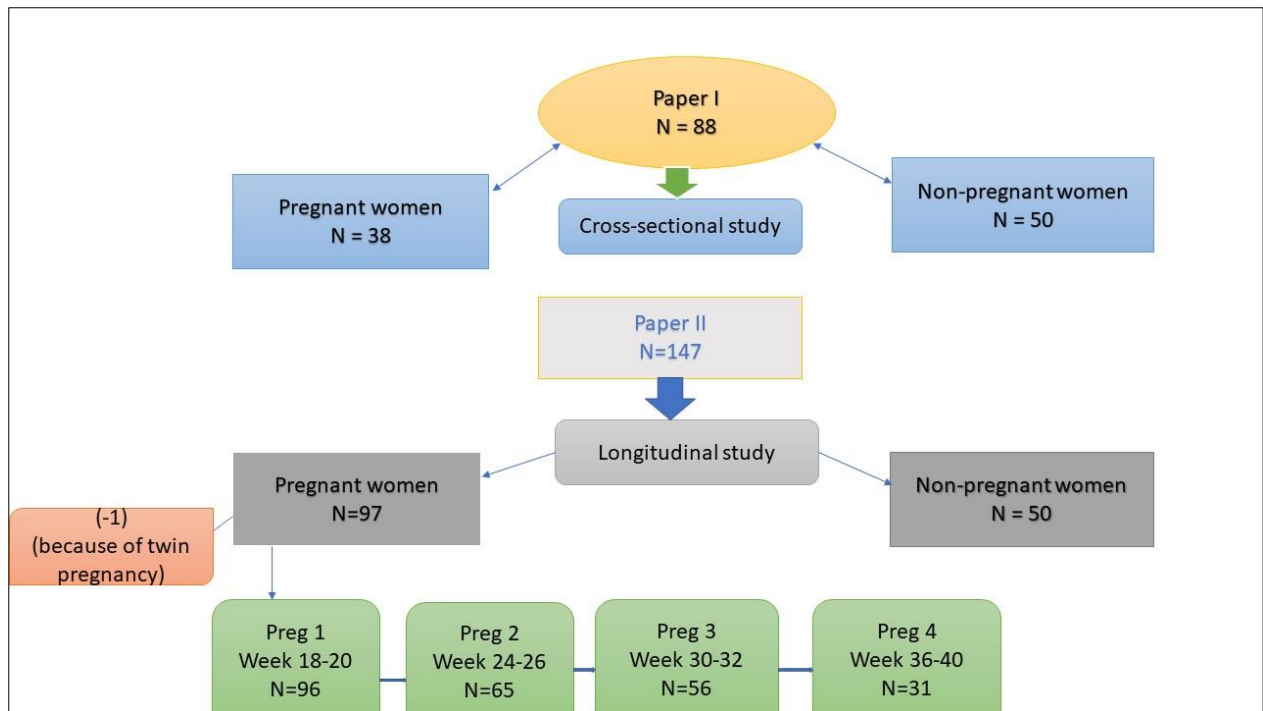
Figure 5: Flow chart of study participants.

3.5.7. Systematic review and meta-analysis

In this part of the study (paper III), we synthesized the evidence-based knowledge on association between dental caries and the risk of preterm birth among pregnant women using systematic review and meta-analysis of available published literature.

Study population

Pregnant women with preterm birth with dental caries and pregnant women with preterm birth without dental caries.

Study selection

Studies were assessed according to the following criteria: population, outcome, gestational age at birth and clinical characteristics of the caries during pregnancy.

A systematic literature search was performed in the following databases: Ovid MEDLINE(R) (In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R), Daily, Ovid MEDLINE(R) and Ovid OLDMEDLINE(R), Embase Classic + (Ovid), The Web of Science® (Thomson

Reuters) and The Cochrane Library (Wiley) and CINAHL Plus (EBSCOhost) after developing search strategy and full search was performed twice, once in November 2015 and repeated in December 2016. All references were exported to EndnoteTM (x7.4 – Thompson Reuters) and the duplicates were removed accordingly. There were no restrictions regarding languages or publication year for the searches. Only full text articles were considered eligible and included. Case reports, conference abstracts, and case series with fewer than three cases were excluded.

3.6 Statistical analyses

Paper I and II

The data collected from the study participants were entered and analyzed using IBM SPSS software package for Windows, Version 25.0. (IBM Corp, Armonk, NY). Descriptive data were presented as mean (SD) or median (IQR) as appropriate. The comparison between the pregnant and non-pregnant groups was carried out by conducting χ2 (chi-squared) test for categorical variables with Bonferroni adjustment and an independent sample t-test was used for parametric continuous variables. Comparisons between different stages of pregnancy were made using analysis of variance (ANOVA), and Turkey's posthoc test was used to find out specific differences between multiple groups when the ANOVA was significant. Associations of laboratory measured saliva parameters (i.e. NO, antioxidant capacity, MDA, and SM and LB colony forming units) with pregnancy outcomes were analyzed using linear regression. The strength of association between two continuous variables was assessed by Pearson's correlation coefficient. A *p*-value of <0.05 was considered statistically significant.

Paper III

Random-effects meta-analysis of binary outcomes was used to compute the summary OR (and relative 95% CI) of PTB among women with caries versus women without caries. Continuous outcome measures (mean DMFT and DMFS scores) were summarized using a random-effects

model with restricted maximum likelihood estimation of between study variance (Van Houwelingen, Zwinderman & Stignen, 1993). The degree of heterogeneity across studies was quantified using the I^2 statistic (Higgins & Thompson, 2002).

All computations were made using Review Manager (RevMan), V.5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). Meta-analysis of observational studies in epidemiology (MOOSE) guidelines were followed (Stroup *et al*., 2000).

4 RESULTS – SUMMARY OF THE PAPERS

4.1 Paper I

Oxidative stress levels and oral bacterial milieu comparison in the saliva from pregnant vs. non-pregnant women

In this cross-sectional study, we measured and compared the bacterial milieu of Streptococcus mutans (SM) and Lactobacillus (LB), oxidative stress (OS), and antioxidative capacity in the saliva of pregnant and non-pregnant women. A total of 88 study participants were included in the analysis, 38 pregnant women and 50 age-matched non-pregnant women.

We found that the bacterial milieu of SM colonies were more abundant and significantly higher in the saliva of pregnant compared to non-pregnant women ($p= 0.003$) and almost 74% of the pregnant women participants had developed higher SM colonies ($\geq 10^5$ CFU/mL) after 48 hours of culture.

Although statistically non-significant ($p = 0.266$), among the pregnant women, the LB bacterial colonies were found to be more abundant in the saliva of pregnant women compared to non-pregnant. Almost 15 % of the pregnant women had developed higher LB colonies ($\geq 10^6$ CFU/mL) after 4 days of culture in comparison to 4 % among the non-pregnant women. The majority of the non-pregnant women had $\leq 10^3$ CFU/mL LB colonies after 4 days of culture.

The antioxidative capacity that was calculated from the average ABTS free radical scavenging capacity in the saliva was found to be 46 % lower in pregnant women's saliva compared to non-pregnant women's saliva ($p<0.001$). We found that the pregnant women had a 16 % higher level of OS in their saliva compared to non-pregnant women. The average OS levels in the saliva as derived from the MDA measurement was found to be significantly higher in pregnant women's saliva compared to non-pregnant women's saliva ($p = 0.023$).

4.2   Paper II

Nitric oxide, oxidative stress and Streptococcus mutans and Lactobacillus bacterial loads in saliva during different stages of pregnancy: a longitudinal study

In this longitudinal study, we investigated the changes in salivary NO, OS, and antioxidant capacity levels during the development of pregnancy and correlated it with caries-related bacterial loads as illustrated by SM and LB. A total of 146 participants were included in the analysis with 96 pregnant women in one group and 50 non-pregnant women of reproductive age in the comparison (reference) group.

We found out that the NO level of saliva was higher in pregnant women in second trimester by 30% compared with the non-pregnant group and it continued to increase as the pregnancy advanced, reaching its maximum level by the end of third trimester. Similarly, our study has shown increased NO and OS with a decrease in antioxidant capacity of saliva compared to non-pregnant women and there was increase in colonization of SM and LB during the second and third trimester.

4.3   Paper III

Dental caries and pre-term birth: a systemic review and meta-analysis

In this study, we evaluated the evidence regarding the association between dental caries and preterm birth (PTB) and determined the differences in dental caries characteristics (DMFT and DMFS scores) between women who deliver preterm and those who do not deliver preterm. This systemic review and meta-analysis included observational cohort, case-control studies after performing MEDLINE, Embase, Pubmed, CINAHL and Cochrane databases searches and studies reporting the risk of PTB in women affected by and those not affected by dental caries.

A total of 1786 articles were identified during the search, 20 full text articles were accessed for eligibility for inclusion, of which nine observational studies with a total of 4826 pregnancies were included in the final analysis.

Five studies exploring the risk of PTB in pregnant women affected by dental caries versus those not affected by dental caries, did not show significant increased risk of PTB, i.e. delivering <37 weeks of gestation (OR: 1.16, 95%CI: 0.90 to 1.49, p= 0.25, $I^2 = 35\%$).

Among all the included studies, five stratified women by caries characteristics using DMFT and three studies using DFMS, respectively. Meta-analyses showed no significant differences in the mean DMFT (p = 0.10) and DMFS PTB (p = 0.9) scores between women who delivered preterm and those who did not.

5 DISCUSSION

5.1 Main findings

In our study, we found that the salivary OS level increases, and the antioxidant capacity of saliva in pregnant women decreases with an increase in colonization by SM compared to the non-pregnant women. Similarly, the salivary NO levels were also higher among the pregnant women with its level increasing with advancement in gestational period. However, we did not find any association between dental caries and risk of pregnancy of PTB.

5.2 Interpretation of results

In our study, we found an increased level of cariogenic SM bacteria in the saliva of pregnant women as represented by the abundance of colonization of the saliva by SM (paper I and II), which is in concordance with previously published reports (Molnar-Varlam *et al.*, 2011; Martinez-Pabon *et al.*, 2014; Kamate *et al.*, 2017; Xiao *et al.*, 2019; Sparvoli *et al.*, 2020). In addition, increased level of SM counts in the saliva of pregnant women has also been observed among women with untreated carious lesions (Xiao *et al.*, 2019) and those with high-risk pregnancies (Merglova *et al.*, 2012). High levels of SM indicate high caries risk (Köhler, Pettersson & Bratthall, 1981; Singh & Shah, 2017) and increases the risk of developing caries post pregnancy as well (Yousefi *et al.*, 2020).

A significant increase in LB count was reported during the 2nd trimester of pregnancy (Molnar-Valam *et al.*, 2011), which is also in line with the findings of our study, with some differences in LB colonization pattern found with advancing gestational age (paper II). A non-significant increase in LB colonization with the advancement of pregnancy has been reported by a study conducted in Columbia (Martinez-Pabon *et al.*, 2014). An increase in carious bacterial activity during pregnancy could be due to many factors, such as a drop in saliva pH and decrease in buffering capacity during pregnancy (Lopez *et al.*, 2011; Yousefi *et al.*, 2020).

Our study demonstrated a significantly higher level of OS and lower antioxidant capacity in saliva of pregnant women compared to non-pregnant (paper I and II), whereas OS and antioxidant capacity levels remained almost constant throughout the pregnancy period (paper II). Similarly, increased OS and decreased antioxidant capacity have also been observed in the serum of pregnant women compared with non-pregnant groups (Kareem, Hassan & Laylani, 2021). Evidence from the review of published literature suggests that certain changes in oral microbiome occur in pregnancy when compared to a non-pregnant stage, however, the microflora remains comparatively stable otherwise throughout the gestation (Jang H *et al.*, 2021). This could be one of the reasons why salivary OS and antioxidant capacity levels were relatively constant during the second half of pregnancy in our study.

There have been reports suggesting an increase in salivary antioxidant capacity level with increase in caries activity (Ahmadi-Motamayel *et al.*, 2013; Pani, 2018; Araujo *et al.*, 2020). In contrast, a significant increase in MDA (a surrogate marker for OS) and decrease in antioxidant activity level observed in active caries groups further creates the dilemma of whether the increase in OS is the aggravating factor or the effect of dental caries (Ahmadi-Motamayel *et al.*, 2018).

We found that salivary NO production is significantly increased during pregnancy compared to the non-pregnancy state and continues to increase as pregnancy advances. This was the first time that NO levels in saliva were reported. Several studies have reported an increase in serum NO during pregnancy reaching its peak in the third trimester (Jo *et al.*, 1998; Shamaash *et al.*, 2000; Choi *et al.*, 2002), while others have reported no differences in serum nitric oxide level during the course of pregnancy (Brown *et al.*, 1995; Smarason *et al.*,1997). In contrast to these studies, one previously published study reported a decrease in serum NO level during normal pregnancy (Hata *et al.*, 1999). However, these studies have measured the NO levels in blood or

serum samples, and whether a correlation exists between serum and salivary levels has not been checked.

Chaudhary *et al*. have reported that due to its capability of generating NO from nitrite, SM can sustain itself in the oral cavity milieu (Chaudhary *et al*., 2007). This could explain our findings of increasing salivary NO and SM colonization with advancement in gestation (paper II). High level of NO produced in saliva in high caries risk group may be a defense response to suppress bacterial growth due to antibacterial property of NO (Hegde *et al*., 2012). Use for serum NO as the biomarker of caries risk has been suggested (Surdilovic *et al*., 2008; Sayed Sachdev & Chopra, 2016). Although a recent study has reported that no significant association exists between salivary NO level and dental caries (Gorji *et al*., 2021), our findings support that salivary NO could potentially be used as a biomarker for the assessment of dental caries risk in pregnant women.

In paper III, we investigated the possible association between dental caries and risk of PTB by performing a systemic review of published literature. Our study concluded that dental caries does not significantly increase the risk for PTB.

Several studies as well as systemic reviews have highlighted the association between periodontal diseases and adverse pregnancy outcomes (Daalderop *et al*., 2018: Pokpa *et al*., 2021; Choi *et al*., 2021), such as PTB, low birth weight (LBW), preterm LBW, preeclampsia and other pregnancy complications. A recent study from the Republic of Korea reported no association between dental caries and adverse pregnancy outcomes such as PTB and preeclampsia, but pregnant women with untreated dental caries had a risk of delivering large-for-gestational-age infants (Cho *et al*., 2020). However, a recent systematic review has reported a significant association between apical periodontitis and adverse pregnancy outcome (Jakovljevic *et al*., 2021). As apical periodontitis is a chronic inflammatory condition at the periapical tissues of the root of the teeth with different etiologies (Jakovljevic *et al*., 2021), and

one of the reasons for apical periodontitis is untreated dental caries (Larsen & Fiehn, 2017), further investigation in this area is warranted.

5.3   Strengths and limitations

One of the main strengths of our study is that we have validated pregnancy associated change in oral bacterial milieu not only by the assessment of colonization of saliva with SM and LB, but also by the measuring the salivary antioxidant capacity and OS levels, thus by demonstrating the direct consequences of altered milieu. Furthermore, the NO levels in saliva of the pregnant women was measured longitudinally to demonstrate increase in salivary NO level with advancing gestational age. We had adequate statistical power to demonstrate statistically significant differences in salivary OS and NO levels between pregnant and non-pregnant women. Similarly, for the systematic review, we used a robust methodology and comprehensive literature search to capture all available data from the published studies, used appropriate methods to assess data quality and synthesized the evidence.

Our study is not without limitations. We have restricted our investigation to SM and LB, bacteria that are responsible for caries initiation and progression, and no other oral microbiota were assessed. Another limitation is that the intra-oral examination was not performed before or after saliva sampling. The pregnant participants were recruited among those who were attending their routine second trimester prenatal ultrasound check-up that and our non-pregnant participants those working in the university or hospital who might have good knowledge and practice of oral hygiene. Therefore, some risk of selection bias can be expected. We only collected saliva samples and no other biological samples for accessing the OS and NO. This may limit the generalizability of our findings. Similarly, other lifestyle factors such as physical activity and dietary habits which might have influence on the OS and antioxidant capacity were not investigated.

The limitations our systematic review are related to the limitations of the original research articles on which it was based. A small sample size in some of the included studies, differences in their design, different follow-up periods, and dissimilarity of the populations studied were the main limitations. In addition, the lack of description of caries classification limited our stratification for sub-group analysis according to the severity of dental caries.

5.4   Future Perspective

From a research perspective, further large-scale clinical and epidemiological studies need to be conducted in order to investigate the association between dental caries and adverse pregnancy outcomes. A detailed intra-oral examination should be conducted along with the salivary sample collection, and biomarkers measured in saliva should be correlated with similar markers in other samples (such as serum, urine, genital secretion etc). Oral microbiota other than caries related bacteria that have potential to alter oral health during pregnancy should be taken into consideration.

From a clinical perspective, just like general health, good oral health is important in pregnancy. Therefore, awareness about good oral healthcare and oral hygiene should be given high priority during pregnancy. It is important to inform pregnant women that most of routine oral healthcare, pain management, management of periodontal diseases, and dental caries treatment can be performed with certain precautions during pregnancy and need not be postponed until after delivery (Hummel *et al*., 2015; Kessler, 2017; Silva de Araujo *et al*., 2017). In fact, appropriate management of oral health problems during pregnancy may improve pregnancy outcomes. It is well documented that oral microflora of the mother can be vertically transmitted to the offspring, thereby increasing the risk of early onset of oral diseases in childhood. Therefore, policymakers should consider providing free oral health consultations (and treatment when indicated) for pregnant women and simultaneously educating them to take good care of their oral health.

6 CONCLUSIONS

1. Abundant bacterial colonization of oral cavity with caries related bacteria (SM and LB), was observed among pregnant women during mid-pregnancy compared to non-pregnant women. Pregnancy appears to have an adverse impact on oral bacterial milieu as demonstrated by increased colonization with SM together with higher OS levels and decreased antioxidant capacity in the saliva.

2. The salivary NO levels increased with advancing gestational age in pregnant women. Profuse bacterial colonization with SM and LB was observed among the pregnant women during the second and third trimester of pregnancy. Likewise, an increase in the levels of NO and OS and decrease in antioxidant capacity was observed in pregnant women when compared with the non-pregnant women.

3. Evidence synthesis based on published literature demonstrated that dental caries does not significantly increase the risk of preterm birth.

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Paper I

BMC Oral Health

RESEARCH ARTICLE

Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. non-pregnant women

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Abstract

Background: Saliva plays a significant role in maintaining oral health and oral bacterial milieu. Difference in oxidative stress (OS) levels in saliva in conjunction with bacterial load between pregnant and non-pregnant women has not been studied previously. We hypothesized that the physiological changes in pregnancy alter oral bacterial milieu by promoting growth of *Streptococcus mutans* (SM) and *Lactobacillus* (LB), and increase OS in saliva. The aim of this study was to measure and compare the oral bacterial milieu, OS and total anti-oxidative capacity (TAC) in the saliva of pregnant and non-pregnant women.

Method: In this cross-sectional study, we assessed oral bacterial milieu by culturing the SM and LB by using commercial kits, TAC by measuring 2, 2′-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) free radical scavenging activity spectrophotometrically and OS levels by measuring malondialdehyde (MDA) levels with commercial kits in the saliva of pregnant women ($n = 38$) at 18–20 weeks of gestation, who were compared with age-matching healthy non-pregnant women ($n = 50$).

Results: *Streptococcus mutans* were found to be more abundant in the saliva of pregnant women compared with non-pregnant women ($p = 0.003$) but the difference was not significant for the LB ($p = 0.267$). TAC was found to be 46% lower in pregnant women's saliva compared to non-pregnant women [optical density (OD) measured at 731 nm as 0.118 ± 0.01 vs. 0.063 ± 0.02; $p < 0.001$]. OS, expressed as saliva MDA levels, was found to be 16% higher in pregnant women compared to non-pregnant women (1.07 nM MDA vs. 0.92 nM MDA; $p = 0.023$).

Conclusion: Pregnancy has an adverse impact on oral bacterial milieu as demonstrated by increased colonization with *Streptococcus mutans* together with higher OS levels and decreased TAC levels in saliva. This emphasizes the importance of improved oral hygiene and provision of oral healthcare services during pregnancy care.

Keywords: Oral health, Bacterial milieu, Oxidative stress (OS), Total anti-oxidant capacity (TAC), Malondialdehyde (MDA), Saliva, Pregnancy

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Background

Saliva is an important aqueous oral fluid that contributes to the maintenance, preservation, protection and healing of oral tissues along with other functions such as helping in speech, lubrication, taste perception and digestion. Saliva is also considered the "mirror of body", and in recent years is being widely used as a tool to screen and diagnose diseases, monitor disease progression, measure drug levels etc. due to its ease of collection and abundance of biomarkers present [1–8]. In addition to this, saliva also has a role in immunological and enzymatic defence mechanisms against certain microorganisms´ antioxidant system and the body's overall oxidative stress (OS) is expressed in saliva [8, 9].

Oxidative stress is the state of an imbalance between oxidants and anti-oxidant systems leading to and causing potential damage of cellular physiology [10]. OS is recognized as a major contributor to several oral conditions, such as salivary gland dysfunctions, xerostomia, periodontitis, precancerous lesions and oral carcinogenesis. Malondialdehyde (MDA) is an indicator of OS as it is one of the final products of lipid peroxidation reaction resulting from increased levels of reactive oxygen species (ROS). Higher MDA levels and lower salivary antioxidant activity have been reported in patients suffering from periodontitis [11]. In recent years, studies have highlighted that OS may have an influence on the human reproductive system [12–14]. Increased vulnerability to OS during pregnancy may predispose to spontaneous abortion, recurrent pregnancy loss, pre-eclampsia, and gestational diabetes [15, 16]. Offenbacher et al. were the first, in 1996, to point out that periodontal disease is a potential risk factor for preterm birth [17]. Since then, the link between periodontal infections and preterm birth has been one of the frontiers in dental research. However, recent epidemiological studies largely support a strong association between poor oral health and adverse pregnancy outcomes, while some controversy still remains [18].

Hormonal fluctuation and immunological changes are physiological phenomena during pregnancy that may predispose to poor oral health. Although poor oral health has been shown to be associated with adverse pregnancy outcomes, preventive dentistry and oral health care is neither the focus nor a part of routine prenatal care in most countries, including Norway. OS in the blood samples of pregnant women was found to be higher than that of healthy non-pregnant women [14]. Therefore, OS measurement in the saliva of pregnant women, together with the assessment of oral bacterial milieu, could be important to understand the cross-link between OS, oral health, and pregnancy outcome.

Streptococcus mutans (SM) and *Lactobacillus* (LB) are reported as the major culprit causing dental caries in humans [19–22]. Therefore our main focus in this study is on these bacterial species. We hypothesized that the physiological changes in pregnancy alter oral bacterial milieu by promoting growth of SM and LB, and increase OS in saliva. The objective of this study was to measure and compare the bacterial milieu, OS and total antioxidative capacity (TAC) in the saliva of pregnant and non-pregnant women.

Methods

This cross-sectional study was part of an ongoing prospective study on oral health in pregnancy conducted at the University Hospital of North Norway, Tromsø, Norway. Saliva samples collected consecutive from 38 healthy pregnant women and 50 healthy non-pregnant women were used for determining the bacterial milieu and OS levels. Women were recruited to the study when they attend the hospital for routine second trimester ultrasound screening at 18–20 weeks. Inclusion criteria were: age > 18 years, low risk singleton pregnancy, no previous history of any pregnancy-associated complications such as preeclampsia, preterm birth or gestational diabetes, and absence of any preexisting medical condition that may have an impact on the course and outcome of pregnancy. Pregnant women who were not willing to participate, could not communicate in Norwegian or English, and those who have been diagnosed to have a fetus with a chromosomal or structural fetal anomaly and did not plan to continue their pregnancy, were excluded. Age matched non-pregnant healthy women of reproductive age were recruited among women working at the University of Tromsø or the University Hospital of North Norway, Tromsø. A history of any acute or chronic illness requiring regular medical treatment excluded participation. All participants were informed about the study in advance and a written consent was obtained from all participants. The study was approved by the Regional Committee for Medical and Health Research Ethics - North Norway (Ref no: 2012/633/REK nord).

Collection of saliva samples

Saliva samples for both groups were collected using identical methods. In brief, paraffin wax stimulated saliva samples were obtained by expectorating in disposable cups. For oral bacterial milieu, two main bacteria, SM and LB, were tested. For OS study, 1.8 ml of saliva was collected in cryo-tubes vials and stored at -70 °C until samples were analyzed. For the TAC and OS analysis, samples were stored in a refrigerator at 4 °C for 1 day before analysis was performed. On the day of analysis, samples were kept at room temperature for 2 h and centrifuged at 10000 x g for 10 min to remove cell debris and supernatant that was collected for further analysis. Storing-procedures and laboratory analyses were processed according to the kit manufacturer's instructions.

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Bacterial milieu assessment in saliva

Oral bacterial milieu was assessed by the cultivation and development of bacterial colony forming units (CFU) of two main bacteria, SM and LB, using commercial kits Dentocult® LB (kit for LB), and Dentocult® SM Strip mutans (kit for SM) (Orion Diagnostica Oy, Espoo, Finland). Women were requested to chew a paraffin pellet to stimulate the secretion of saliva and promote transfer of SM from tooth surfaces into the saliva. A round-tipped test strip supplied in the kits was pressed against the saliva on the woman's tongue. The strip was placed in the cap of the vail containing culture broth and was recapped in the vail. The vial was loosely capped and incubated at 37 °C and 5% CO_2 for 48 h. Results were interpreted by scoring as 0, 1, 2, and 3 for 0, $< 10^5$, 10^5–10^6 and $> 10^6$ CFU/mL, respectively, by comparing to the template reader provided in the kits. In case of LB culture, the test strip was thoroughly made wet by saliva, fixed in the cap and fitted in the vials containing culture broth. It was then incubated for 4 days at 37 °C and 5% CO_2. Results were interpreted scoring as 0, 1, 2, 3 and 4 for 0, 10^3, 10^4, 10^5 and 10^6 CFU/mL, respectively, by comparing to the template reader provided in the kits. Results are expressed as the percentage among pregnant and non-pregnant women based on the development of bacterial CFU.

Measurement of Total Antioxidant Capacity (TAC) in saliva

Total antioxidant capacity (TAC) in the saliva was expressed by measuring 2,2′-azino-bis(3-ethylbenzo-thiazoline)-6-sulfonic acid diammonium salt (ABTS) free radical scavenging activity [23]. In brief, a dark green color of ABTS free radicals was generated by mixing 2 mL of each of the solutions of ABTS (7.4 mM) and potassium peroxodisulfate (2.6 mM) for 24 h. Both chemicals, ABTS and potassium peroxodisulfate were purchased from Sigma-Aldrich, Oslo. The reaction mixture was diluted to 100 mL with distilled water as a working solution ABTS free radical. Optical density (OD) of the working ABTS radical solution was approx. 0.5 to 0.6. Supernatant of saliva samples were used for both groups. Reactions were carried out by mixing 450 μL of working solution of ABTS radical and 50 μL supernatant part of saliva followed by incubating for 30 min in darkness. The change in the green color of ABTS free radicals scavenged by the antioxidants present in saliva fluid was measured for OD using spectrophotometric methods (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) at 731 nm. Higher OD_{731} value represents lower level of TAC. Water soluble vitamin C (Sigma-Aldrich) was used as a standard and TAC was quantified as μg/mL vitamin C equivalent level, representing the total antioxidant capacity (TAC) with the help of standard curve and regression equation ($R^2 = 0.9331$ and $y = - 0937x + 0.7357$).

Oxidative stress levels in saliva by malondialdehyde (MDA) assay

We measured saliva MDA content using a commercially available MDA Assay Kit (Sigma-Aldrich, Lipid Peroxidation MDA Assay Kit) following the instructions provided by the supplier; MDA levels are expressed as OS levels [24]. In brief, a mixture of 100 μL saliva fluid is diluted with 200 μL buffer provided in the kit. The saliva sample in buffer and Thiobarbituric acid (TBA) solution, 600 μL each, were mixed thoroughly and incubated at 95 °C for 60 min. Of the reaction mixture, after cooling in ice, 150 μL was transferred to a 96 well microplate in duplicates and absorbance was measured flurometrically (Epoch Microplate, BioTek Instrument, Vermont, USA) by measuring fluorescence intensity ($\lambda_{ex} = 532/\lambda_{em} = 553$). The MDA levels in the saliva were calculated by the MDA standard provided in the kit with the help of standard curve and regression equation ($R^2 = 0.9903$ and $y = 728.95x + 111.6$).

Statistical analysis

The sample size required a detection of 15% difference in the OS level between pregnant and non-pregnant women, with 80% power at an alpha of 0.05, calculated to be at least 38 individuals per group on the basis of mean MDA level and standard deviation reported in the saliva of 25 healthy female controls in a previous report [25] using an online sample size calculator [26].

Data analysis was performed using IBM SPSS Statistics for Windows, Version 25.0. (IBM Corp, Armonk, NY). Data are presented as mean (SD) or median (IQR) as appropriate. Frequency tables were made and comparison between the pregnant and non-pregnant groups was carried out by conducting χ^2 (chi-squared) test for categorical variables with Bonferroni adjustment when appropriate, and an independent sample t-test for parametric continuous variables. The strength of correlation between two continuous variables was assessed by Pearson's correlation coefficient. A *p*-value of < 0.05 was considered statistically significant.

Results

Data from a total of 38 pregnant and 50 non-pregnant women were included in the analysis. The median (IQR) age of the pregnant and non-pregnant groups were 31.5 (5.8) and 30 (8) years, respectively.

Oral bacterial milieu

Figures 1 and 2 show the distribution of salivary levels of SM and LB in pregnant and non-pregnant women.

The SM bacterial milieu profiles as compared between the groups of pregnant and non-pregnant women are shown in Fig. 1. SM colonies were found to be more abundant and significantly higher in the saliva of
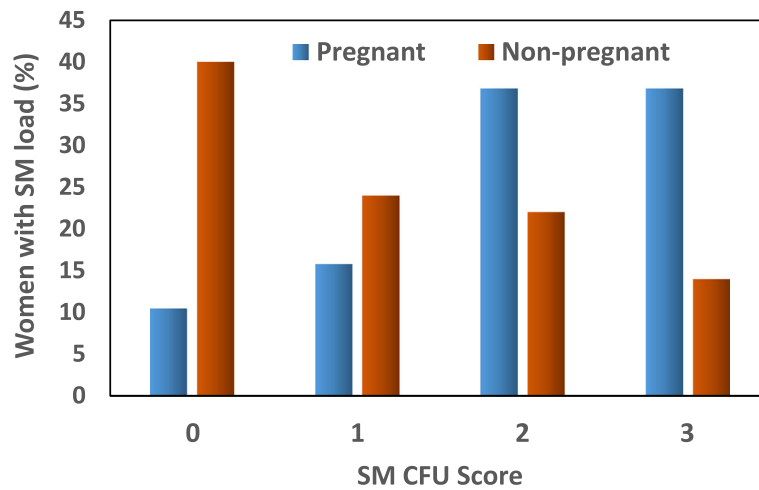
Fig. 1 Comparison of *Streptococcus mutans (SM)* bacterial milieu in the saliva of pregnant and non-pregnant women. The bars in the diagram represent the percentage of women scoring 0, 1, 2, and 3 based on the number of colony-forming units (CFU) of bacteria identified after culture, i.e. 0, 10^5, 10^5–10^6 and > 10^6 CFU/mL, respectively. *Pregnant vs. non-pregnant group; $p < 0.05$ (chi-squared test). **Pregnant vs. non-pregnant group (difference was only significant ($p < 0.05$.) between subgroups with SM score 0 and 3 (chi-squared test with Bonferroni adjustment)

pregnant compared to non-pregnant woman (χ^2 statistic = 13.984; p = 0.003). The majority of pregnant women were highly colonized with SM compared to non-pregnant women. In the group of pregnant women, 73.6% were found to have developed 10^5 or more CFU/mL in the culture.

The LB bacterial milieu profiles as compared between the groups of pregnant and non-pregnant women are shown in the Fig. 2. The LB bacterial colonies were abundant in the saliva of pregnant women, but not significantly higher compared to non-pregnant women (χ^2 statistic = 5.208; p = 0.266). Among pregnant women, 15% developed 10^6 CFU/mL after the culture compared to 4% among the non-pregnant women. The majority of

the non-pregnant women showed 10^3 or less CFU/mL in their saliva. Approximately 45% of pregnant women showed 10^4 or more CFU/mL of LB bacterial colonies compared to 40% in non-pregnant women.

Total Anti-oxidative Capacity (TAC) in saliva

The results of TAC in the saliva of pregnant and non-pregnant women are shown in Fig. 3. The average ABTS radical scavenging capacity in the saliva of pregnant women were 46% lower compared to that of non-pregnant women (OD_{731}: 0.118 ± 0.01 vs. 0.063 ± 0.02; $p < 0.001$). TAC levels in the saliva of pregnant women (n = 38) and non-pregnant women (n = 50) were calculated as 6.59 μg/mL and 7.17 μg/mL vitamin C equivalent, respectively.

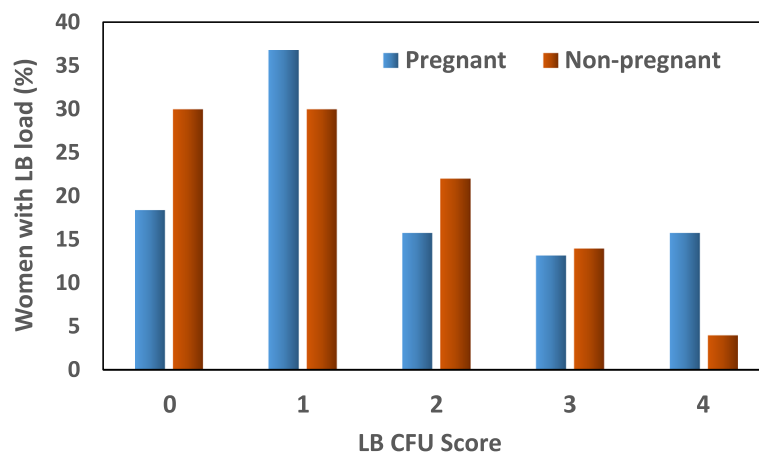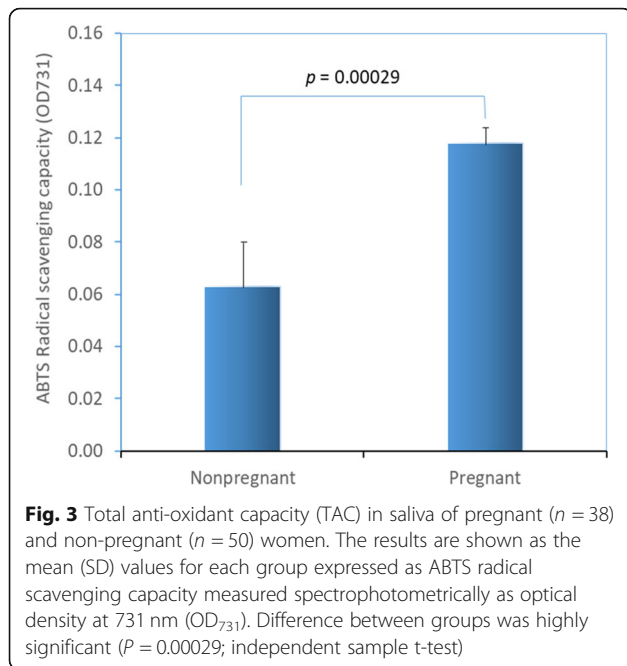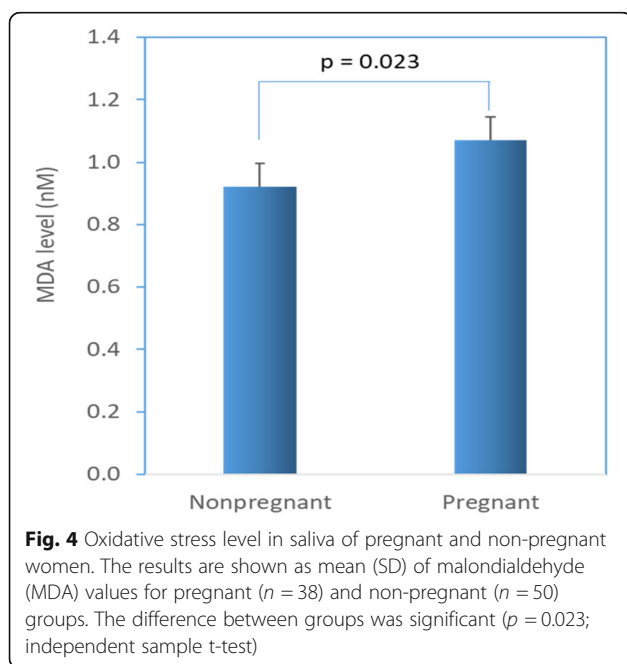**Fig. 2** Comparison of *Lactobacillus (LB)* bacterial milieu in the saliva of pregnant and non-pregnant women. The bars in the diagram represent percentage of women scoring 0, 1, 2, 3 and 4 based on the number of colony-forming units (CFU) of bacteria identified after culture, i.e. 0, 10^3, 10^4, 10^5 and 10^6 CFU/mL, respectively. There were no significant differences between pregnant and non-pregnant groups

Fig. 3 Total anti-oxidant capacity (TAC) in saliva of pregnant ($n = 38$) and non-pregnant ($n = 50$) women. The results are shown as the mean (SD) values for each group expressed as ABTS radical scavenging capacity measured spectrophotometrically as optical density at 731 nm (OD_{731}). Difference between groups was highly significant ($P = 0.00029$; independent sample t-test)

Oxidative Stress (OS) levels in saliva

The results of MDA contents in the group of pregnant and non-pregnant women are shown in Fig. 4. The OS levels are expressed as the MDA content present in the saliva. The pregnant women had a 16% higher level of OS in their saliva compared to the non-pregnant women. The average OS levels, expressed as MDA levels in the saliva of pregnant women ($n = 38$) and non-pregnant women ($n = 50$) were 1.07 nM and 0.92 nM; $p = 0.023$), respectively.

Fig. 4 Oxidative stress level in saliva of pregnant and non-pregnant women. The results are shown as mean (SD) of malondialdehyde (MDA) values for pregnant ($n = 38$) and non-pregnant ($n = 50$) groups. The difference between groups was significant ($p = 0.023$; independent sample t-test)

Oxidative Stress (OS) and Total Antioxidant Capacity (TAC) in saliva in relation to oral bacterial load

The results of correlation analysis between OS or TAC in the saliva and oral bacterial load expressed as colonization by SM and LB after 48 h or 96 h of culture for both pregnant and non-pregnant group are presented in Table 1. No statistically significant correlations were found. (Table 1).

Discussion

Saliva, in addition to minerals, mucus, antibacterial compounds and enzymes [27], also carries a portion of antioxidants, such as vitamin C and vitamin E. Saliva has a pivotal role in maintaining the microbial taxa in the oral cavity, as well as oral health. However, there are limited studies on the effect of pregnancy on oral bacterial milieu and OS. In this study, we explored the differences in the oral bacterial milieu and OS levels in saliva between pregnant and non-pregnant women, demonstrating that pregnancy may adversely affect oral health by promoting abnormal bacterial growth and increasing OS levels in the saliva.

More than 700 microbial taxa are found in the oral cavity [28, 29]. Several microbial species reported in the oral cavity are known to cause intrauterine infection without being found in the urogenital tract [30–32]. Surprisingly, a study on microbiomes has demonstrated that microbes found in term placenta are similar to oral rather than vaginal microbes [33]. There are two main hypothetical routes for oral microbes to cause intrauterine infection: either hematogenous dissemination, particularly with periodontal disease [34], or colonization of the vaginal tract with microbes from the oral cavity during receptive oral sex [35]. Periodontal disease is associated with a two to seven-fold increase in preterm birth [36, 37] and a link between maternal periodontal disease

Table 1 Correlation between oxidative stress (OS) or total antioxidant capacity (TAC) with bacterial load (SM or LB) among the groups of pregnant and non-pregnant women

Variables	Groups	Pearson r	p-value
ABTS			
SM	Pregnant	0.20764	0.2386
LB		0.10593	0.5509
SM	Non-pregnant	0.10082	0.4860
LB		0.11193	0.4389
MDA			
SM	Pregnant	0.11009	0.5105
LB		0.29464	0.0725
SM	Non-pregnant	0.17653	0.2200
LB		0.01280	0.9296

2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), *Lactobacillus* (LB), malondialdehyde (MDA), *Streptococcus mutans* (SM)

and preeclampsia has been suggested [38]. A large multi-center trial comparing women treated for periodontal disease at < 21 weeks vs. post-delivery found a trend for reduced early preterm birth < 32 weeks [39]. These scientific findings demonstrate that oral microbiota are associated with pregnancy outcome. Furthermore, studies have demonstrated that pregnant women are at high risk of caries development [21]. Among oral microbes, SM and LB are most strongly associated with the dental caries [21, 22]. SM is not found anywhere else except in human oral cavity [40]. Based on this background, we measured oral bacterial milieu of SM and LB in the saliva of pregnant women and compared with non-pregnant women. Among pregnant women, only four (10.5%) women out of 38 did not show the presence of SM in the saliva, whereas among non-pregnant women, 20 (40%) women out of 50 did not have SM in their oral cavity. A significantly higher percent of pregnant women participating in our study were colonized with SM (almost 89%), which is in line with previous studies conducted on pregnant populations where 100% of women were found to be infected by SM [21, 41]. Both SM and LB were found to be abundant in pregnant women's saliva although the difference between pregnant and non-pregnant groups was not statistically significant for LB.

Increased bacterial colonization of oral cavity could be connected with TAC and OS in saliva. Therefore, we measured TAC and OS in the saliva of the pregnant women and compared these with non-pregnant women. We found lower TAC and higher OS in pregnant women compared to non-pregnant women. However, the oral bacterial load of SM and LB did not significantly correlate with TAC or OS in either group. It is not clear to us whether the decreased levels of TAC and increased levels of OS makes a favorable condition for bacterial milieu in the pregnant women or whether increased bacterial growth leads to decreased TAC and increased OS. In general, for the purpose of counteracting and minimizing the damage produced by the ROS, living cells operate antioxidant systems such as enzymes, macromolecules and an array of small molecules. Low levels of TAC could be a sign of increased OS and increased potential for oxidative damage [42, 43].

We measured a 46% lower value of anti-oxidant capacity in the saliva of pregnant women compared to that of non-pregnant women. Saliva contains vitamin C and vitamin E which enhance the total anti-oxidative system of the oral cavity. Vitamin C concentration in saliva has been reported to be 6 to 10 μg/mL [44]. In our study, TAC levels in the saliva of pregnant women and non-pregnant women were calculated to be 6.59 μg/mL and 7.17 μg/mL of vitamin C equivalent, respectively. Expression of total antioxidants in the saliva correlates with the vitamin C level in saliva. We did not measure vitamin C

concentration directly in the saliva. However, we measured ABTS radical scavenging activity of saliva, and the majority of the TAC effect is due to the vitamin C reflecting the range of saliva vitamin C concentration. Vitamin C plays an important role in maintaining the integrity of teeth and also contributes to non-enzymatic anti-oxidant defense. Decreased serum and/or salivary vitamin C levels correlate with dental caries [44]. Therefore, decreased TAC may predispose women to poor oral health during pregnancy.

OS occurs when the production of reactive oxygen species (ROS) overwhelms the anti-oxidants that conquer them [45–47]; the net result is damage to cellular structures such as DNA, protein and lipids. ROS are constantly formed within the cells as a by-product of metabolic processes, and a low to moderate level of ROS is physiological and serves as signaling molecules [45, 46]. The levels of ROS and OS directly relate to the corresponding metabolites. MDA is one of the cellular lipids metabolites generated by the ROS reaction. Hence, increased levels of MDA indicate higher levels of OS. We measured MDA contents in the saliva of pregnant and non-pregnant women in order to determine the level of OS. In this study, OS was found to be 16% higher in the saliva of pregnant women compared to non-pregnant women ($p = 0.023$).

Previous studies have described the association between poor periodontal health and risk of preterm birth and low birth weight [36, 37, 48]. However, in a recent systematic review on dental caries and preterm birth, we found that dental caries was not significantly associated with preterm birth [49]. Whether the level of OS in the oral cavity rather than specific disease categories would be more predictive of adverse pregnancy outcomes needs further investigation.

One major strength of our study is that the pregnancy associated change in oral bacterial milieu was validated not only by the assessment of colonization of oral cavity by SM and LB, but also by the measurement of TAC and OS levels in the saliva demonstrating direct consequences of altered milieu. However, pathophysiological mechanisms related to poor oral health leading to adverse pregnancy outcomes need to be further elucidated.

Our study has some limitations. Firstly, the non-pregnant group consisted of a selected population of women working in the hospital and university who could have better oral knowledge of oral hygiene, and thus the results may not be generalizable to other populations. Secondly, we only investigated colonization of oral cavity by SM and LB rather than investigating the whole oral microbiome. Although these are the most important pathogens, the possible role of other microbes in causing pregnancy associated changes in oral cavity cannot be ignored. Furthermore, we did not perform clinical oral

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examination before saliva sampling. However, our study participants were healthy and none of them reported having any significant medical illness or oral health problems. Additionally, as our study had a cross-sectional design, the question of whether there are gestational-age-related serial changes in the oral bacterial milieu from beginning to the end of pregnancy remains unknown.

Conclusion

Abundant bacterial colonization of oral cavity by both SM and LB was observed among healthy pregnant women during mid-pregnancy. Pregnancy appears to have an adverse impact on oral bacterial milieu as demonstrated by significantly increased colonization with SM together with higher OS levels and decreased TAC levels in the saliva. This emphasizes the importance of improved oral hygiene and provision of oral healthcare services during pregnancy.

Abbreviations
ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt; CFU: Colony forming unit; LB: *Lactobacillus*; MDA: Malondialdehyde; OD: Optic density; OS: Oxidative stress; ROS: Reactive oxygen species; SM: *Streptococcus mutans*; TBA: Thiobarbituric acid; TAC: Total anti-oxidative capacity

Authors' contributions
Study concept, design and methodology were carried out by MW, PB, GA. Data collection and data entry by MW and AV. Supervision by TAT, PB and GA. Analysis and interpretation of data by MW, AV, TAT, PB, GA. Writing, review, critique, comments and revision of manuscript by MW, AV, TAT, PB, GA. All authors read and approved the manuscript.

Availability of data and materials
The dataset generated and/or analyzed during the current study are not publicly available due to concerns over participant confidentiality but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Ethical approval for conducting the study was obtained from the Regional Committee for Medical and Health Research Ethics - North Norway (Ref no: 2012/633/REK nord). The research was conducted in accordance with the Helsinki Declaration and informed written consent was obtained from all the study participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Paper II

Nitric Oxide, Oxidative Stress and Streptococcus mutans and Lactobacillus Bacterial Loads in Saliva during the Different Stages of Pregnancy: A Longitudinal Study.

Article

Nitric Oxide, Oxidative Stress and *Streptococcus mutans* and *Lactobacillus* Bacterial Loads in Saliva during the Different Stages of Pregnancy: A Longitudinal Study

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Abstract: Hormonal changes associated with pregnancy promote oral bacterial growth, which may affect salivary nitric oxide (NO) levels, oxidative stress (OS), and antioxidant capacity (AC). We hypothesized that caries-related bacterial load, NO level, and OS in the saliva change with advancing gestation. The aim of this study was to investigate longitudinal changes in salivary NO, OS, and AC during pregnancy and correlate them with *Streptococcus mutans* (*SM*) and *Lactobacillus* (*LB*) colonization at different stages of pregnancy. We assessed NO level by Griess method, OS by measuring malondialdehyde (MDA), AC by ABTS radicals and bacterial load by culturing *SM* and *LB* in the saliva of pregnant women ($n = 96$) and compared with non-pregnant women ($n = 50$) as well as between different stages of pregnancy. Compared with non-pregnant women, NO was 77% higher (4.73 ± 2.87 vs. 2.67 ± 1.55 µM; $p < 0.001$), MDA was 13% higher (0.96 ± 0.27 vs. 0.85 ± 0.22 nM; $p = 0.0055$), and AC was 34% lower (60.35 ± 14.33 vs. $80.82 \pm 11.60\%$; $p < 0.001$) in the late third trimester. NO increased with advancing gestation, but AC and OS did not change significantly during pregnancy. *SM* were more abundant in pregnant women compared with non-pregnant ($p = 0.0012$). Pregnancy appears to have an adverse impact on oral health emphasizing the importance optimal oral healthcare during pregnancy.

Keywords: antioxidant capacity (AC); *Lactobacillus*; malondialdehyde (MDA); nitric oxide (NO); oral health; oxidative stress (OS); pregnancy; saliva; *Streptococcus mutans*

1. Introduction

Saliva plays a vital role in the digestive tract contributing to the maintenance, preservation, lubrication, protection, and healing of intra-oral hard and soft tissues. Saliva is widely used as a screening and diagnostic tool because of its simple non-invasive collection process and the presence of several biomarkers [1–3]. Normal pregnancy is associated with altered flow, composition, and pH of saliva, which promotes bacterial growth and predisposes women to poor oral health [4]. Furthermore, physiological endocrine, cardiovascular, immunological, and metabolic adaptations of pregnancy may also contribute to increased colonization of oral cavity by pathogenic microbes leading to inflammatory response and oxidative stress. Nitric oxide (NO) has a key role in pregnancy as a regulator of both maternal and fetal homeostasis [5], and its levels in the saliva are likely to reflect local conditions of the oral cavity. However, although there are a limited number of cross-sectional studies measuring NO levels in maternal serum during pregnancy [6,7], no study to our knowledge has performed NO measurements in the saliva of pregnant women longitudinally. Saliva also plays an important role in the immunological and enzymatic

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defense mechanisms against certain microorganisms' antioxidant systems, and human body's oxidative stress (OS) levels are expressed in saliva [8]. A recent systematic review on maternal oral microflora has reported that the pregnancy is associated with abundance of micro-organisms compared to non-pregnant state [9]. In a cross-sectional study, we have previously demonstrated an increased level of OS in the saliva of pregnant women compared to non-pregnant in association with increased colonization of oral cavity by dental caries-related pathogen, *Streptococcus mutans* (*SM*) [10]. Increased levels of NO and OS have been measured in the blood samples of pregnant women compared to healthy non-pregnant women [6,11]. Therefore, longitudinal measurements of NO and OS levels in the saliva of pregnant women, together with the assessment of pathogenic bacterial load could help to elucidate the link between OS and oral health during the course of normal pregnancy. We hypothesized that the colonization by caries-related bacteria, OS level, and antioxidant capacity (AC) change during pregnancy with advancing gestational age. We aimed to investigate associations of oral pathogenic bacterial load represented by *SM* and *Lactobacillus* (*LB*), with salivary NO and OS levels during the progression of pregnancy.

2. Materials and Methods

2.1. Study Design

This single center observational cohort with comparison group was part of a longitudinal study on oral health in pregnancy conducted at UiT-The Arctic University of Norway and University Hospital of North Norway, Tromsø, Norway. Saliva samples were collected consecutively from healthy pregnant women four times during gestation starting at 18–20 weeks to term. The samples were divided into 4 groups by gestational age; early second trimester (18–20 weeks), late second trimester (24–26 weeks), early third trimester (30–32 weeks), and late third trimester (36–40 weeks). The comparison group comprised of healthy non-pregnant women of reproductive age without history of any chronic illness or recent disease. These were recruited among women working at the UiT-The Arctic University of Norway or the University Hospital of North Norway, Tromsø. Pregnant women were approached and recruited to the study when they attended the hospital for routine ultrasound screening at 18–20 weeks of gestation. General inclusion criteria were: (1) age over 18 years, (2) low risk singleton pregnancy, (3) women without any previous history of pregnancy-associated complications, such as preeclampsia, preterm birth, or gestational diabetes, and (4) absence of any pre-existing medical condition that may have an impact on the course and outcome of pregnancy. Those pregnant women who were unable to communicate in Norwegian or English, and those who have been diagnosed with a fetal chromosomal or structural anomaly and did not plan to continue their pregnancy, were excluded. A history of any acute or chronic illness requiring regular medical treatment excluded participation for the non-pregnant women. Pregnant study participants were followed from mid-gestation until childbirth, and the outcome of pregnancy was recorded.

All participants were informed about the study in advance and a written consent was obtained from all the participants. The study was approved by the Regional Committee for Medical and Health Research Ethics-North Norway (Ref No: 2012/633/REK nord).

2.2. Collection of Samples

For both pregnant and non-pregnant groups, saliva samples were collected during daytime between 9:00 and 15:00 h with the same method as described previously [10]. Women were not eating or brushing their teeth for approximately 1.0 to 1.5 h before sampling. In brief, the participants were asked chew paraffin wax pellets for 5 min to stimulate saliva production which was then collected by expectorating in sterile disposable cups.

For the assessment of caries related bacterial load in the saliva, *SM* and *LB* were cultured and colony forming units (CFU) were evaluated by a single investigator (MW). For the measurement of NO and OS levels, 1.8 mL of saliva was collected in two separate cryo-tube vials and stored at $-70\,^{\circ}$C until samples were analyzed by a single investigator (PB). Samples were put in a refrigerator at $4\,^{\circ}$C for one day before the analyses were

performed. All samples were left for 2 h on the working table before the analysis to defrost and bring to room temperature. Sample containing tubes were centrifuged at $10,000 \times g$ for 10 min to remove cell debris and supernatant was collected for further analysis. Storing-procedures and laboratory analyses were followed according to the instructions by the kit supplier.

2.3. Chemicals

All chemical used were of analytical grade. 2,2′-azino bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), vitamin C (ascorbic acid), acetic acid, and sodium nitrite were purchased from Sigma-Aldrich, Chemie GmbH, Steinheim, Germany. Potassium peroxodisulphate was a product from Merck KGaA, Darmstadt, Germany. Griess reagent was prepared from 1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid, all products were from Sigma-Aldrich Norway AS, Oslo.

2.4. Measurement of NO Levels

Because NO is highly labile, measurement of the relatively stable metabolite, nitrate and nitrite (NO_x), is employed as an index of NO production and, also, as a marker of NOS enzyme activity [12]. The levels of NO in saliva were expressed by measuring nitrite quantitatively using the Griess method spectrophotometrically with modification [13]. Griess reagent was prepared as 1% sulfanilamide and 0.1% N-naphthylethylene diamine dichloride in 5% orthophosphoric acid (v/v). This reagent reacts with nitrite and produces a purple azo dye end-product, which is measured spectrophotometrically with a maximum absorbance at 546 nm. Triplicate samples of saliva (10 μL) were transferred to a tube containing 290 μL of distilled water, and a 300 μL of Griess reagent was added to each tube. After mixing thoroughly and allowing to react at dark for 30 min, the changed color was measured. The quantitative expression of NO in saliva is taken from the analysis of triplicates of sodium nitrite ($NaNO_2$) at concentrations of 25, 20, 15, 10, 5, 2, 1, and 0 μM as final concentrations and with the help of standard curve and regression equation ($R^2 = 0.9995$ and y = 0369x − 0.0035). The intra-assay coefficient of variation was 4.89%

2.5. Measurement of Antioxidant Capacity (AC)

Antioxidant capacity (AC) in the saliva was assessed by measuring free radical scavenging activity by 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS) methods with appropriate modifications [10,13]. In brief, ABTS free radicals ($ABTS^{\bullet+}$) having dark green color was generated by allowing the reaction equal volume (each 2 mL) of the solutions of ABTS (7.4 mM) and potassium peroxodisulfate (2.6 mM) for 24 h. The reaction mixture was diluted to 100 mL with distilled water as a working solution $ABTS^{\bullet+}$. Optical density (OD) of the working $ABTS^{\bullet+}$ solution was approximately 0.5 to 0.6. Supernatant of saliva samples were used for both groups. Reactions were carried out by mixing 450 μL of working solution of ABTS radical and 50 μL supernatant part of saliva followed by incubating for 30 min in darkness. The changes in the green color of ABTS free radicals scavenged by the antioxidants present in saliva fluid was measured for OD using spectrophotometric methods (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) at 731 nm. Higher OD_{731} value represents lower level of TAC. Water soluble vitamin C (Sigma-Aldrich) was used as a standard and AC was quantified as μg/mL vitamin C equivalent level, representing AC with the help of standard curve and regression equation ($R^2 = 0.931$ and y = −0.0304x + 0.6134). The intra-assay coefficient of variation was 0.73%.

2.6. Measurement of Oxidative Stress

We evaluated OS levels in saliva by measuring MDA content using a commercially available MDA Assay Kit (Sigma-Aldrich, Lipid Peroxidation MDA Assay Kit (MAK085), Darmstadt, Germany) [10]. In brief, a mixture of 100 μL saliva supernatant, 100 μL buffer provided in the kit, and 600 μL thiobarbituric acid (TBA) solution were mixed thoroughly

and incubated at 95 °C for 60 min. Of the reaction mixture, after cooling in ice, 150 μL was transferred to a 96 well microplate in duplicates and absorbance was measured fluorometrically (Epoch Microplate, BioTek Instrument, Winooski, VT, USA) by measuring fluorescence intensity ($\lambda_{ex} = 532/\lambda_{em} = 553$). The MDA levels in the saliva were calculated by the MDA standard provided in the kit with the help of standard curve and regression equation ($R^2 = 0.9951$ and $y = 783.07x + 12.095$). The intra-assay coefficient of variation was 6.91%.

2.7. Assessment of SM and LB in Saliva

Oral bacterial culture was assessed by a single investigator (MW) by saliva culture and development of bacterial colony forming units (CFU) of two main bacteria, *SM* and *LB*, using commercial kits Dentocult® *LB* (kit for *LB*), and Dentocult® *SM* Strip mutans (kit for *SM*) (Orion Diagnostica Oy, Espoo, Finland) as described previously [10]. Women were requested to chew a paraffin pellet to stimulate the secretion of saliva and promote transfer of *SM* from tooth surfaces into the saliva. A round-tipped test strip supplied in the kits was pressed against the saliva on the woman's tongue. The strip was placed in the cap of the vail containing culture broth and was recapped in the vail. The vial was loosely capped and incubated at 37 °C and 5% CO_2 for 48 h. Results were interpreted by scoring as Category 0, 1, 2, and 3 for 0, $<10^5$, 10^5–10^6, and $>10^6$ CFU/mL, respectively, by comparing to the template reader provided in the kits. In case of *LB* culture, the test strip was thoroughly made wet by saliva, fixed in the cap and fitted in the vials containing culture broth. It was then incubated for 4 days at 37 °C and 5% CO_2. Results were interpreted scoring as Category 0, 1, 2, 3, and 4 for 0, 10^3, 10^4, 10^5, and 10^6 CFU/mL, respectively, by comparing to the template reader provided in the kits. Results are expressed as the percentage of women in each category based on the development of bacterial CFU. Both Dentocult® *SM* and Dentocult® *LB* have been shown to have good reliability in determining different categories of CFU counts and have a good correlation with standard culture techniques using Agar plates [14,15].

2.8. Statistical Analysis

The sample size required for the detection of 15% difference in the OS level between pregnant and non-pregnant women, with 80% power at an alpha of 0.05, was calculated to be at least 38 individuals per group based on mean MDA level and standard deviation (SD) reported in the saliva of 25 healthy female controls in a previous report [16] using an online sample size calculator [17]. As our study on the pregnant women was longitudinal, we recruited more participants to compensate for possible non-attendance or drop-outs at follow-up visits, inadequate sample collection or analysis failure, to ensure that there would be adequate number of samples/measurements for each stage of pregnancy.

Data analysis was performed using IBM SPSS Statistics for Windows, Version 25.0. (IBM Corp, Armonk, NY, USA). Data are presented as mean (SD) as appropriate. Frequency tables were made and comparison between the pregnant and non-pregnant groups was carried out by conducting χ^2 (chi-squared) test for categorical variables an independent sample t-test for parametric continuous variables. Bonferroni correction was used when multiple comparisons were performed. Comparisons between different stages of pregnancy were made using analysis of variance (ANOVA) and Turkey's posthoc test was used to find out specific differences between multiple groups when the ANOVA was significant. Associations of laboratory measured saliva parameters (i.e., NO, AC, MDA, and *SM* and *LB* colony forming units) with pregnancy outcomes were analyzed using linear regression. The strength of association between two continuous variables was assessed by Pearson's correlation coefficient. A *p*-value of <0.05 was considered statistically significant.

3. Results

Flow of study participants is shown in Figure 1. Data from a total of 96 pregnant and 50 non-pregnant women were included in the final analysis. The mean ages of the pregnant

and non-pregnant groups were 30.81 ± 4.32 and 30.32 ± 6.09 years, respectively. The mean gestational age at delivery was 39.51 ± 1.91 weeks with 80.2% ($n = 77$) women delivering vaginally, whereas 19.8% ($n = 19$) had a caesarean section. Six women delivered preterm (<37 weeks). Mean estimated blood loss during delivery was 498.06 ± 627.11 mL. Similarly, 53.3% of the neonates born were male and 46.7% females, and the mean weight and length of the neonates were 3507.32 ± 631.67 gm and 48.87 ± 7.87 cm, respectively. The median one- and five-minute Apgar scores of the neonates were 9 (range 1 to 10) and 10 (range 3 to 10), respectively.
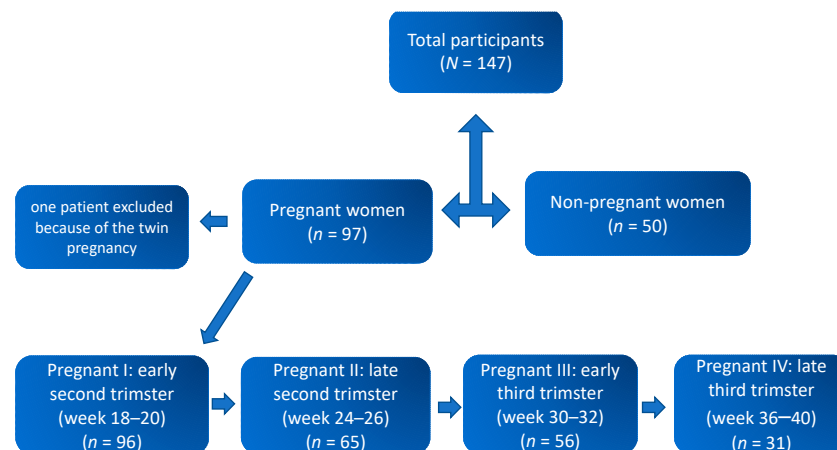

Figure 1. Chart illustrating the flow of study participants.

For the longitudinal evaluation, saliva samples were collected consecutively from 96 healthy singleton pregnant women four times starting at 18–20 weeks to term pregnancy and from 50 healthy non-pregnant women. The samples were divided into four groups by gestational age: early second trimester (18–20 weeks, $n = 96$), late second trimester (24–26 weeks, $n = 65$), early third trimester (30–32.0 weeks, $n = 56$), and late third trimester (36–40 weeks, $n = 31$). The comparison group comprised of healthy non-pregnant women of reproductive age ($n = 50$) without history of any chronic illness or recent disease.

3.1. Nitric Oxide (NO) Levels in Saliva

Results for saliva nitrite concentrations of the subjects are shown in Table 1. The average saliva NO equivalent nitrite concentration in the 96 pregnant women measured in the early second trimester was 3.47 ± 2.48 μM, which was 30% higher compared to the group of non-pregnant women (2.67 ± 1.55 μM; $p = 0.00029$). Results showed that NO level continue to increase throughout the pregnancy, attaining peak levels in the end of third trimester (4.73 ± 2.87 μM). The levels of NO equivalent nitrite levels in late third trimester were 36% and 77% higher than in early second trimester of pregnancy (4.73 ± 2.87 μM) and in non-pregnant women (2.67 ± 1.55 μM), respectively. We found no statistically significant association between salivary NO level measured with pregnancy outcomes, such as gestational age at delivery, birthweight, or Apgar score.

3.2. Antioxidant capacity (AC) in Saliva

The results of average AC in the saliva of pregnant and non-pregnant women are shown in Table 2. The AC is expressed as the measure of free radical scavenging capacity of the saliva. Higher level of AC shows the saliva containing strong antioxidants. It can be generalized that higher level of AC indicates low level of OS. The average saliva AC levels of pregnant women ($n = 96$) measured at the early second trimester were $60.35 \pm 14.33\%$ which is a 25% decrease in free radical scavenging capacity compared to non-pregnant women ($80.82 \pm 11.60\%$; $p < 0.00001$). The AC levels did not change significantly throughout pregnancy. AC levels in the saliva of pregnant women ($n = 96$) and non-pregnant women ($n = 50$) were calculated as 16.26 μM and 11.89 μM vitamin C equivalent, respectively, with

the regression equation: y = −0.0304x + 0.6134. No statistically significant association was found between AC and pregnancy outcomes.

Table 1. Nitric oxide (NO) equivalent nitrite levels (μM) in the saliva of pregnant women at different gestational ages and non-pregnant women.

Nitric Oxide (μM)	NP	Preg. I	Preg. II	Preg. III	Preg. IV
Mean	2.67	3.47	3.67	4.56	4.73
SD	1.55	2.49	3.04	4.23	2.87
MAX	7.58	13.49	16.89	22.69	13.97
MIN	0.52	0.20	0.19	0.43	1.41
p-value vs. NP *		0.0004	0.0002	<0.0001	<0.0001
p-value vs. Preg. *			0.4279	0.0005	<0.0001

Results are expressed as mean ± SD. * Bonferroni correction and Turkey's posthoc test was used when multiple comparisons were performed. Preg. I: the early second trimester (18–20 weeks, *n* = 96), Preg. II: the late second trimester (24–26 weeks, *n* = 65), Preg. III: early third trimester (30–32 weeks, *n* = 56) and Preg. IV: late third trimester (36–40 weeks, *n* = 31), and NP: non-pregnant (*n* = 50).

Table 2. Antioxidant capacity (AC) in the saliva of pregnant women at different gestational ages and non-pregnant women.

AC (%)	NP	Preg. I	Preg. II	Preg. III	Preg. IV
Mean (%)	80.82	60.35	60.80	58.40	60.50
SD	11.60	14.33	15.18	15.18	17.58
p-value vs. NP *	-	<0.0001	<0.0001	<0.0001	<0.0001
p-value vs. Preg. I *	-	-	0.8745	0.1972	0.8745

The results are shown as the percentage mean and SD values for each group expressed as ABTS radical scavenging capacity measured spectrophotometrically as optical density at 731 nm (OD$_{731}$). * Bonferroni correction and Turkey's posthoc test was used when multiple comparisons were performed Preg. I: the early second trimester (18–20 weeks, *n* = 96), Preg. II: the late second trimester (24–26 weeks, *n* = 65), Preg. III: early third trimester (30–32 weeks, *n* = 56) and Preg. IV: late third trimester (36–40 weeks, *n* = 31), and NP: non-pregnant (*n* = 50).

3.3. Oxidative Stress (OS) Levels in Saliva

The results of MDA contents in the group of pregnant and non-pregnant women are shown in Table 3. The OS levels are expressed as the MDA content present in the saliva. The pregnant women had higher levels of MDA in their saliva compared to the non-pregnant women reflecting higher level of OS in pregnant women. The average OS levels, expressed as MDA levels in the saliva of pregnant women in early second trimester gestation (*n* = 96) and non-pregnant women (*n* = 50) were 0.93 nM and 0.85 nM; *p* = 0.008), respectively. OS levels did not change significantly throughout second half of pregnancy. Association between salivary OS level and pregnancy outcomes was not statistically significant.

Table 3. Oxidative stress level measured as MDA level in the saliva of pregnant women at different gestational ages and non-pregnant women.

MDA (nM)	NP	Preg. I	Preg. II	Preg. III	Preg. IV
Mean	0.85	0.93	0.95	0.97	0.96
SD	0.22	0.25	0.24	0.26	0.27
p-value vs. NP *	-	0.0083	0.0018	0.0005	0.0055
p-value vs. Preg. I *	-	-	0.4901	0.2017	0.4673

The results are shown as mean (SD) of malondialdehyde (MDA) nM values for pregnant and non-pregnant groups. * Bonferroni correction and Turkey's posthoc test was used when multiple comparisons were performed. Preg. I: the early second trimester (18–20 weeks, *n* = 96), Preg. II: the late second trimester (24–26 weeks, *n* = 65), Preg. III: early third trimester (30–32.0 weeks, *n* = 56) and Preg. IV: late third trimester (36–40 weeks, *n* = 31), and NP: non-pregnant (*n* = 50).

3.4. Oral Bacterial Loads

3.4.1. *Streptococcus mutans* (SM)

The *SM* bacterial loads as compared between the groups of pregnant and non-pregnant women are shown in Table 4. Almost 40% of pregnant women in their early and late third trimester groups developed more than 10^5.CFU/mL *SM* colonies compared to 22% of non-pregnant women. SM colonies were abundant and significantly higher in saliva of pregnant compared to non-pregnant women (χ^2 statistic = 5.375; $p = 0.001$). The colony formation was significantly different at different stages of pregnancy ($p = 0.0024$) with the highest proportion (38%) of women being colonized in the early third trimester. However, the degree of SM colonization in the early second trimester was not significantly associated with pregnancy outcomes.

Table 4. *Streptococcus mutans* (*SM*) counts in the saliva of pregnant women at different gestational ages compared with non-pregnant women.

Group	Category 0 (%)	Category 1 (%)	Category 2 (%)	Category 3 (%)
Preg. I	11 (11.5)	25 (26.0)	35 (36.5)	25 (26.0)
Preg. II	6 (9.2)	22 (33.9)	24 (36.9)	13 (20.0)
Preg. III	6 (10.7)	13 (23.2)	22 (39.3)	15 (26.8)
Preg. IV	3 (10.0)	8 (26.7)	12 (40.0)	7 (23.3)
NP	20 (40.0)	12 (24.0)	11 (22.0)	7 (14.0)

Results are shown as number of subjects (%) in the categories 0 to 3. Preg. I: the early second trimester (18–20weeks, $n = 96$), Preg. II: the late second trimester (24–26 weeks, $n = 65$), Preg. III: early third trimester (30–32 weeks, $n = 56$), Preg. IV: late third trimester (36–40 weeks, $n = 31$), and NP: non-pregnant ($n = 50$). Categories 0, 1, 2, and 3 represent the scoring for 0, $<10^5$, 10^5–10^6, and $>10^6$ CFU/mL SM bacterial colony forming unit (CFU) counts, respectively.

3.4.2. *Lactobacillus* (LB)

The *LB* bacterial loads as compared between the groups of pregnant and non-pregnant women are shown in the Table 5. The *LB* bacterial colonies formation were high and significant during the different stages of pregnancy ($p = 0.0027$). Even though the *LB* bacterial colonies were abundant in the saliva of pregnant women, it was not significantly higher compared to non-pregnant women (χ^2 statistic = 1.165; $p = 0.489$). Among pregnant population almost 17% had developed 10^6 CFU/mL *LB* colonies in their late pregnancy stage compared to 4% of non-pregnant women. Majority of non-pregnant showed 10^3 or less CFU/mL *LB* colonies in their saliva. The degree of *SM* colonization in the early second trimester was not significantly associated with pregnancy outcomes.

Table 5. *Lactobacillus* (*LB*) counts in the saliva of pregnant women at different gestational ages compared with non-pregnant women.

Group	Category 0 (%)	Category 1 (%)	Category 2 (%)	Category 3 (%)	Category 4 (%)
Preg. I	23 (24.0)	41 (42.7)	16 (16.7)	9 (9.4)	7 (7.3)
Preg. II	11 (16.9)	21 (32.3)	20 (30.8)	4 (6.2)	9 (13.8)
Preg. III	5 (8.9)	24 (42.9)	13 (23.2)	5 (8.9)	9 (16.1)
Preg. IV	2 (6.7)	16 (53.3)	4 (13.3)	3 (10.0)	5 (16.7)
NP	15 (30.0)	15 (30.0)	11 (22.0)	7 (14.0)	2 (4.0)

Results are shown as number of subjects (%) in categories 0 to 4. Preg. I: the early second trimester (18–20 weeks, $n = 96$), Preg. II: the late second trimester (24–26 weeks, $n = 65$), Preg. III: early third trimester (30–32 weeks, $n = 56$), Preg. IV: late third trimester (36–40 weeks, $n = 31$), and NP: non-pregnant ($n = 50$). Categories 0, 1, 2, 3, and 4 represent the scoring for 0, 10^3, 10^4, 10^5, and 10^6 CFU/mL *LB* bacterial colony forming unit (CFU) counts, respectively.

4. Discussion

In this study, we investigated the changes in NO production in saliva during pregnancy and the levels of NO production were compared with non-pregnant healthy women of reproductive age. We found a significantly higher NO production in the pregnant women compared to the non-pregnant women and NO production also increased with gestational age during normal pregnancy. It is, to our knowledge, the first report of the NO measurements is saliva of pregnant women, however NO production in the blood of pregnant women has been previously reported [6]. Based on some reports evaluating blood samples, NO production is increased in the second trimester and peaks in the third trimester [6,18,19]. In contrast to this, Hata et al. has reported that maternal circulating nitrite level decrease with advancing gestation [20]. Brown et al. [21] and Smarason et al. [22] found no changes in NO production during normal pregnancy compared to non-pregnant women. These studies suggest that the status of NO production in women during normal pregnancy is still controversial and needs further investigation. These discrepancies may derive from methodological variations. Many of the previous studies have relied on the measurement of NO_x in the plasma; however, the plasma level is influenced by the clearance, as well as the production of NO metabolites [23].

Saliva also has another very important function in maintaining oral health by regulating the microbial taxa in the oral cavity. Altered oral bacterial milieu and chemical components of saliva have been associated with several diseases [1–3,8]. However, only a few studies have investigated the effects of advancing gestational age on oral bacterial load together with OS. In this study, we have measured NO levels in the saliva at different gestational stages together with the OS and oral bacterial load of cries-related pathogens. It is remarkable to find that pregnancy adversely affects oral health by promoting abnormal bacterial growth and increasing NO and OS levels which are expressed in the saliva. It could be that the NO production is a defense mechanism of the body against deteriorating oral hygiene [24]. In 1999, Silva Mendez et al. reported that the nitrite present in saliva could influence growth and survival of cariogenic bacteria [25].

Oral cavity harbors over 700 microbial taxa [26,27] and some microbial species are shown to cause intrauterine infection without being present in the urogenital tract [28–30]. A study by Aagaard et al. has shown that microbes found in the term placenta are more similar to those found in the oral cavity rather than vaginal microbes [31]. Pregnancy is known to increase the risk for the development of dental caries compared to the non-pregnant women [32]. *SM* and *LB* are considered as the causative bacteria for developing dental caries [32,33]. Among these, *SM* is reported to be found only in human oral cavity [34]. With this background, we measured *SM* and *LB* load in the saliva of pregnant women at the different stages of physiological pregnancy and compared this with that of the non-pregnant healthy women of similar age. Among healthy non-pregnant women, 20 out of 50 (40%) did not show the presence of *SM* in their saliva. Whereas in the pregnant population, only 27 (10.5%) out of 248 total tested cases at various stages of pregnancies, did not demonstrate the presence of *SM* in the saliva. These results clearly show that a significantly higher number of pregnant women carry *SM* bacterial load in their oral cavity (almost 89.5%) compared to non-pregnant women (60%). Our results partly support the finding of previous studies reporting all pregnant women (100%) to have abundant *SM* colonies in their saliva [32,35]. *SM* bacterial counts at different stages of pregnancy were similar to each other. In general, both *SM* and *LB* bacterial loads were found higher in pregnant women's saliva compared to non-pregnant women, however, the difference was statistically significant only for *SM*.

It is commonly accepted that OS has a part in the initiation and progression of most oral diseases [36]. However, how the pregnancy associated changes in oral microbial load (especially that of pathogenic bacteria) influence salivary OS and AC has not been elucidated. We found lower levels of AC and higher levels of OS in pregnant women. Nevertheless, the oral bacterial counts of *SM* and *LB* did not significantly correlate with AC or OS neither in pregnant nor in non-pregnant groups. Therefore, it still remains unclear if

it is the decreased levels of AC and increased levels of OS that creates a favorable condition for oral bacterial growth in pregnant women or is it the increased bacterial growth that leads to decreased AC and increased OS. Low levels of AC could be suggestive of increased OS and increased potential for oxidative damage [37].

Antioxidants, such as vitamin C and vitamin E, are found in the saliva and play an important role in the total antioxidative system of the oral cavity [38]. Salivary vitamin C concentration has been reported to be 6 to 10 µg/mL by Hegde et al. [39]. In concordance with this, in our study, mean AC levels in the saliva of pregnant and non-pregnant women were 6.59 µg/mL and 7.17 µg/mL of vitamin C equivalent, respectively. We did not measure vitamin C concentration directly in the saliva. However, on measuring ABTS radical scavenging activity vitamin C equivalent of saliva provides a good estimate, as the main AC effect is due to the vitamin C. Vitamin C helps to maintain the integrity of teeth and overall oral health by contributing to non-enzymatic antioxidant defense. Decreased serum and/or salivary vitamin C levels have been considered as one of the factors associated with dental caries [39]. We measured a 34% lower value of AC in the saliva of pregnant women compared to that of non-pregnant women. Therefore, decreased AC may predispose women to poor oral health and increase the risk of dental caries during pregnancy.

Excessive and uncontrolled production of ROS leads to OS, that in turn damages cellular structures and alters functions of DNA, protein, and lipids. Antioxidants and antioxidative system counteracts the ROS and/or prevent ROS formation [40–42]. Various kinds of ROS are regularly generated during cellular metabolic processes, and a low to moderate levels of ROS are physiological acting as signaling molecules [40,41]. MDA is one of the cellular lipid metabolites generated by the ROS reaction, and therefore, OS levels are generally expressed by the corresponding MDA metabolites concentration. Hence the increased level of OS is indicated by higher MDA levels. In this study, OS was found to be 16% higher in the saliva of pregnant women compared to non-pregnant women ($p = 0.023$).

Our study has some limitations. We did not measure NO in the serum samples parallelly with the saliva samples to avoid repeated invasive blood sampling from the pregnant study participants. However, salivary and serum NO levels are shown to have a positive correlation [43]. We compared healthy pregnancies from a general population with a selected group of non-pregnant women which mainly consisted of women working in the hospital and university and may possess better knowledge of oral health and oral hygiene. Additionally, our study population mainly consisted of White European women, and therefore the findings may not be directly applicable to other multi-ethnic populations. The oxidative stress and antioxidative capacity may depend on life-style factors including food intake and physical activity, which were not taken into account in this study. Similarly, salivary components might have changed due to some variation in its collection timing. Our study had adequate statistical power to demonstrate statistically significant differences in salivary OS levels between pregnant and non-pregnant women as well as differences between early second, late second and early third trimesters, among pregnant women. However, the desired sample size was not reached for the late third trimester due to increasing dropout of study participants with advancing gestational age. Although ABTS free radical assay and MDA measurement are well documented reliable assays, we have not performed other types of assays or measurements in serum or other body fluids from the same women to evaluate OS. This may limit the generalizability of our findings. Our study focused only on two caries-related bacteria, i.e., *SM* and *LB*, rather than investigating the whole oral microbiome. Although these are the most important pathogens, the possible role of other microbes in causing pregnancy associated changes in oral cavity cannot be ignored. Furthermore, we did not perform any clinical oral examination before saliva sampling. However, our study participants were healthy and none of them reported having any significant medical illness or oral health problems.

5. Conclusions

We report, for the first time, NO levels measured longitudinally in the saliva of pregnant women and demonstrate that salivary NO increases with advancing gestational age. OS and AC levels were stable during the second half of pregnancy. Normal pregnancy was associated with increased levels of NO and OS, and decreased antioxidant capacity compared with the non-pregnant state, and an abundant bacterial colonization of oral cavity by both *SM* and *LB* was observed among healthy pregnant women during second and third trimester of pregnancy indicating that pregnancy may have an adverse impact on oral health. Therefore, it is important to provide essential awareness and provision of optimal oral healthcare during pregnancy.

Author Contributions: Study concept, design and methodology were developed together by M.W., P.B. and G.A. Data collection and data entry by M.W., P.B. and Å.V. Supervision by P.B. and G.A. Analysis and interpretation of data by M.W., Å.V., P.B. and G.A. All authors contributed for the study and on preparation of manuscript. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The research followed with the Helsinki Declaration. All participants are informed the study and written consent was obtained. The study was conducted only after ethical approval obtained from the Regional Committee for Medical and Health Research Ethics-North Norway (Ref No: 2012/633/REK nord).

Informed Consent Statement: Written informed consent has been obtained from the study participants to publish this paper.

Data Availability Statement: The data presented in this study are available, in anonymized form, on request from the corresponding author. The data are not publicly available due to privacy rules.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviation

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt
CFU	Colony forming unit
LB	*Lactobacillus*
MDA	Malondialdehyde
NO	Nitric oxide
OD	Optical density
OS	Oxidative stress
ROS	Reactive oxygen species
SM	*Streptococcus mutans*
TBA	Thiobarbituric acid
AC	Anti-oxidant capacity

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Paper III

BMJ Open

Dental caries and preterm birth: a systematic review and meta-analysis

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ABSTRACT

Objectives The primary objective of this systematic review was to evaluate the association between dental caries and preterm birth (PTB). The secondary objective was ascertaining the difference between women with dental caries who experienced PTB and those who did not with regard to decayed, missing and filled teeth (DMFT), and decayed, missing and filled surfaces (DMFS) indices.

Methods MEDLINE, Embase, CINAHL and Cochrane databases were searched initially in November 2015 and repeated in December 2016. We included observational cohort and case–control studies. Only studies reporting the risk of PTB in women affected compared with those not affected by dental caries in pregnancy were included. Random-effect meta-analyses were used to compute the summary OR of PTB among women with caries versus women without caries, and the mean difference in either DMFT or DMFS indices between women experiencing PTB and those without PTB.

Results Nine observational studies (4826 pregnancies) were included. Women affected by dental caries during pregnancy did not show a significantly higher risk of PTB (OR: 1.16, 95% CI 0.90 to 1.49, P=0.25, I^2=35%). Also, the women with PTB did not show significantly higher DMFT or DMFS indices (summary mean differences: 1.56, P=0.10; I^2=92% and −0.15, P=0.9, I^2=89%, respectively).

Conclusion Dental caries does not appear to be a substantial risk factor for PTB.

Trial registration number NCT01675180; Pre-results.

Strengths and limitations of this study

► Strength of the study is its robust methodology. We tried to cover all available studies, access data quality and synthesise suitable data.
► Small number of cases in some of the included studies, their design, different follow-up periods and dissimilarity of the population studies are the limitations.
► Similarly, the lack of description or classification of dental caries stage is another limitation due to which the stratification of analysis according to the disease severity could not be performed.

INTRODUCTION

Preterm birth (PTB) is the major cause of perinatal mortality and morbidity in the developed countries, with an estimated incidence of 5%–13%.[1–4] Although advances in neonatal care have led to a reduction in the neonatal mortality rate, infants born prematurely remain at a risk of developing a wide array of short-term and long-term complications such as respiratory, gastrointestinal and neurodevelopmental disabilities.[4]

Several risk factors have been associated with PTB[1 5]; among these, intrauterine infection has emerged as one of the most important factors. Despite this, PTB cannot be considered a unique disease but rather a syndrome characterised by multiple aetiology and in which different factors may play a peculiar role.[5]

Periodontal disease has been shown to carry an increased risk for PTB; the rationale for this association is based on the suggestion that periodontitis may lead to maternal and fetal inflammation, thus triggering the common pathway of preterm parturition syndrome including increased uterine contractility, cervical ripening and decidua/membrane activation.[6–11] Although dental caries, defined as a localised destruction of the tooth and its structure by the acidic by-product produced by the bacteria during the dietary carbohydrate fermentation,[12] is one of the major oral health problems in developed countries, the effects of dental caries on pregnancy outcome have not been consistently explored. Pregnant women are more susceptible to dental caries and gingivitis compared with their non-pregnant counterparts[13] because of the change in their diet, frequent snacking due to food craving and oral health negligence.[14] If left untreated, dental caries may result in further inflammatory complications,[15] which could influence pregnancy outcomes. Several studies reported that dental caries causing bacteria may have some influence on the pregnancy outcome as PTB and/or low birth weight, while in contrary, the other showed no association between these two factors.[16–27]

The primary aim of this systematic review was to explore the association between dental caries and PTB; the secondary aim was to

ascertain the differences in dental caries characteristics between women who deliver preterm and those who do not deliver preterm.

METHODOLOGY

Protocol, eligibility criteria, information sources and search

This review was performed according to an a priori designed protocol and recommended for systematic reviews and meta-analysis.[28 29]

We developed a search strategy, and a systematic literature search was performed in the following databases: Ovid MEDLINE (In-Process and Other Non-Indexed Citations, Ovid MEDLINE, Daily, Ovid MEDLINE and Ovid OLDMEDLINE, Embase Classic + EMBASE (Ovid), The Web of Science (Thomson Reuters), The Cochrane Library (Wiley) and CINAHL Plus (EBSCOhost).

The full search was performed in November 2015 and repeated in December 2016. The online supplementary material 1 shows the complete search string as it was performed in MEDLINE. The controlled vocabulary of Medical Subject Headings (MeSH) from MEDLINE and the Emtree thesaurus from Embase, including subheadings, were used when applicable. In addition, the search fields, title, abstract and keywords, were searched when applicable. In The Web of Science, the search fields, title and topic were used. All references were exported to Endnote (X7.4, Thompson Reuters), where duplicates were removed. There were no restrictions regarding languages or publication year for the searches.

Reference lists of relevant articles and reviews were hand searched for additional reports. Meta-analysis of observational studies in epidemiology (MOOSE) guidelines were followed.[30]

The study was registered with the PROSPERO database (registration number: CRD42017062573).

Study selection, data collection and data items

We aimed to compare the incidence of PTB among the pregnant women population with dental caries with those who do not have dental caries.

The primary outcome was the occurrence of PTB, defined as birth <37 weeks of gestation. We aimed to categorise the analysis according to the type of PTB (spontaneous vs iatrogenic vs term) and according to the gestational age at birth moderate to late preterm (32 to <37 weeks), very preterm (28 to <32 weeks) and extremely preterm <28 weeks[31].

The secondary objective was to ascertain the difference between women with dental caries who experienced PTB and those who did not experience PTB in either decayed, missing and filled teeth (DMFT) or decayed, missing and filled surfaces (DMFS) indices.[32]

DMFT and DMFS indices are numerical expressions of the caries prevalence of an individual or groups and are widely used in epidemiological surveys of oral health. DMFT/DMFS is calculated by adding up permanent teeth that are caries affected wherein D is for decay, M is missing due to caries and F is filled teeth (T) or surfaces (S). If one tooth has filling as well as a caries lesion, then it is counted as D for the DMFT index, whereas the filling+caries surface is counted as D but if there is F on one and D in other surface, then they are counted differently for the DMFS index. The anterior teeth up to canine have four and premolars and molars teeth have five surfaces, respectively, in the DMFS index. D+M+F=caries prevalence of an individual [maximum of 28 for DMFT and 128 for DMFS, if 28 permanent teeth are included (excluding 4 wisdom molar teeth)].[32 33]

Studies were assessed according to the following criteria: population, outcome, gestational age at birth and clinical characteristics of the caries during pregnancy. Observational cohort and case–control studies were included. Similarly, studies reporting the occurrence of PTB in women affected compared with those not affected by dental caries in pregnancies and the full-text articles were considered suitable for the inclusion in the present systematic review. Case reports, conference abstracts and case series with fewer than three cases were also excluded to avoid publication bias.

Two authors (MW and FD) reviewed all abstracts independently. Agreement regarding potential relevance was reached by consensus; full-text copies of those papers were obtained and the same two reviewers independently extracted relevant data regarding study characteristics and pregnancy outcome. Inconsistencies were discussed among the reviewers and consensus reached. Any dispute was resolved by discussion with a third author. If more than one study was published for the same cohort with identical endpoints, the report containing the most comprehensive information on the population was included to avoid overlapping populations. For those articles in which information was not reported but the methodology was such that this information would have been recorded initially, the authors were contacted.

Quality assessment of the included studies was performed using the Newcastle-Ottawa Scale (NOS)[34]; according to NOS, each study is judged on three broad perspectives: the selection of the study groups, the comparability of the groups and ascertainment outcome of interest. An assessment of the selection of a study includes the evaluation of the representativeness of the exposed cohort, selection of the non-exposed cohort, ascertainment of exposure and the demonstration that outcome of interest was not present at the start of study. The NOS tool for the quality assessment of the studies is provided in the online supplementary material 2. According to the tool, a study can be awarded a maximum of one star for each numbered item within the selection and outcome categories. A maximum of two stars can be given for comparability.[34]

Statistical analysis

A first random-effect meta-analysis of binary outcomes was used to compute the summary OR (and relative 95% CI) of PTB among women with caries versus women without caries (controls).

Table 1 General characteristics of the included studies

Author	Year	Country	Period analysed (year)	Study design	Gestational age at dental examination	Number of subject (n)	Definition of PTB
Martinez-Martinez et al[35]	2016	Mexico	2013–2014	Retrospective	From the first trimester of pregnancy until 8 weeks postpartum	70	<37 weeks
Harjunmaa et al[24]	2015	Malawi	2011–2013	Prospective	Within 6 weeks after delivery	1024	<37 weeks
Acharya et al[23]	2013	India	2009	Retrospective	Within 1 day after delivery	316	<37 weeks
Vergnes et al[22]	2011	France	2003–2006	Retrospective	Within 2–4 days post partum	2201	<37 weeks
Ryalat et al[21]	2011	Jordan	2009	Prospective	Within 1 week post partum	200	<37 weeks
Durand et al[17]	2009	France	2005–2006	Prospective	Within 8 weeks after delivery	107	<37 weeks
Heimonen et al[20]	2008	Finland	2002–2004	Retrospective	Within 2 days post partum	328	<37 weeks
Mumghamba and Manji[19]	2007	Tanzania	NS	Retrospective	Within 40 days from delivery	373	<37 weeks
Meurman et al[18]	2006	Finland	1998–2000	Retrospective	From the first trimester of pregnancy	207	<37 weeks

PTB, preterm birth.

Other two meta-analyses evaluated continuous outcomes: DMFT and DMFS. As the included studies did not differ in their outcome definitions, we used a random-effect approach to compute the mean difference in either DMFT or DMFS between PTB and non-PTB. In one study by Martinez-Martinez et al,[35] the SD were not available, and we thus conservatively used the largest values recorded in the other included studies.

For all meta-analyses, the heterogeneity across studies was quantified using I^2 statistic, and all computations were made using Review Manager (RevMan), V.5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

RESULTS
General characteristics
A total of 1786 articles were identified, 20 were assessed with respect to their eligibility for inclusion (online supplementary material 3) and 9 studies were included in the systematic review (table 1, figure 1). These nine studies included 4826 pregnancies.

Results of quality assessment of the included studies using NOS for cohort studies are presented in table 2. Most of the included studies scored at least one star in each of the three categories: the selection and comparability of the study groups, and ascertainment of the outcome of interest. The main weaknesses of these studies were their retrospective design, small sample size with even smaller number of events (PTB) and different gestational ages at assessment.

Synthesis of the results
Five studies explored the risk of PTB in women who had caries compared with those who did not have caries during pregnancy and reported that women affected by caries in pregnancy did not have an increased risk of delivering <37 weeks of gestation (OR: 1.16, 95% CI 0.90 to 1.49, P=0.25; I^2: 35%) (figure 2).

Stratification according to DMFT and DMFS indices to evaluate the association between caries and PTB was performed only by five and three studies, respectively. There was no difference in either DMFT (1.56, 95% CI −0.28 to 3.41, P=0.10) and DMFS (−0.15, 95% CI −3.40 to 3.09, P=0.9) (table 3 and figure 3).

Due to very small number of included cases and lack of information from the original study, it was not possible to perform any subanalysis according to different gestational age at birth and type of PTB (spontaneous vs iatrogenic vs term).

DISCUSSIONS
Summary of evidence
The findings from this systematic review showed that pregnant women with dental caries are not at increased risk for PTB. Furthermore, there was no difference in the mean DMFT and DMFS indices between women with dental caries who experienced PTB and those who did not.

Strength and limitations
This is, to our knowledge, the first systematic review exploring the strength of association between dental caries and PTB. The strength of this meta-analysis is its robust methodology. We tried to cover all available studies, access the quality of the data and synthesise all suitable data.

The small number of cases in some of the included studies, their retrospective non-randomised design, different periods of follow-up, dissimilarity of the populations studies (due to various inclusion criteria) and lack of standardised criteria for the antenatal management of pregnancies with dental caries represent the major limitations of this systematic review. Lack of data on early PTB, which is typically associated with infection and inflammation, was another major limitation of the present systematic review. Furthermore, we could not stratify the analysis according to maternal characteristics and caries stage at diagnosis in view of the lack of such
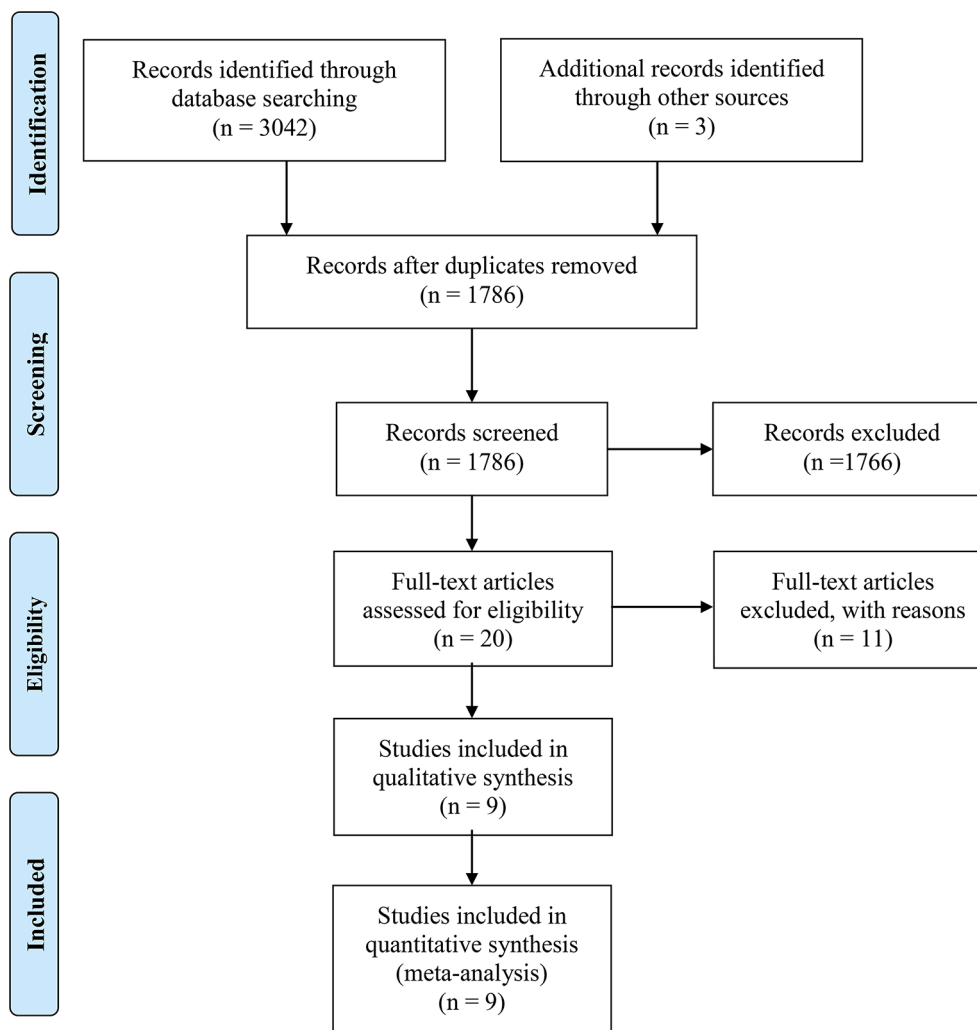
Figure 1 Systematic review flow chart.

information in the large majority of included studies. Assessment of the potential publication bias was also problematic because of the nature of the outcome evaluated (outcome rates with the left side limited to a value of zero), which limits the reliability of funnel plots, and because of the small number of individual studies, which strongly limits the reliability of formal tests. Finally, statistical heterogeneity among the included studies was another major limitation of the present review which may potentially bias the study findings. In view of these limitations, the findings from this systematic review should be interpreted with cautions.

Table 2 Quality assessment of the included studies according to Newcastle-Ottawa Scale, a study can be awarded a maximum of one star for each numbered item within the selection and outcome categories

Author	Year	Selection	Comparability	Outcome
Martinez-Martinez et al[35]	2016	★★	★	★
Harjunmaa et al[24]	2015	★★	★	★
Acharya et al[23]	2013	★★	★	★★
Vergnes et al[22]	2011	★★★	★★	★
Ryalat et al[21]	2011	★★★	★	★★
Durand et al[17]	2009	★★★	★★	★★
Heimonen et al[20]	2008	★★	★	★
Mumghamba and Manji[19]	2007	★★	★	★
Meurman et al[18]	2006	★★	★	★

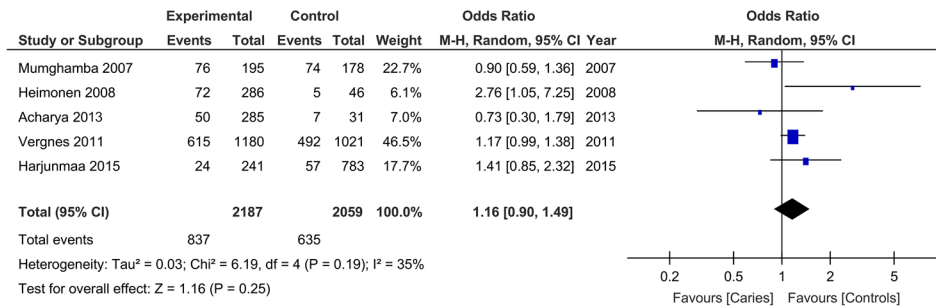A maximum of two stars can be given for comparability.

Figure 2 Pooled OR for the risk of preterm birth in women compared with those without dental caries.

Implication for clinical practice

The consequences of overall oral health including the oral health in pregnant women is of a great concern.[36] Dental caries and periodontal disease are the most common oral diseases worldwide. The higher prevalence of gingival alterations during pregnancy, especially bleeding during brushing, is a problem that is commonly encountered by pregnant women. Properly maintained oral hygiene care is known to have an impact on the oral health of pregnant women[37 38] and availability of free dental care also appears to influence this.[39] Whereas in contrast, if proper oral hygiene is not maintained during pregnancy, the chances to develop oral health problems such as enamel erosions, dental caries[40] and gingivitis increase.

There are no reports indicating that the incidence of dental caries increases during pregnancy, but the chances of getting dental caries could increase[14] and the prevalence of dental caries seemed to be higher in older pregnant women.[41] Despite the high dental caries prevalence in most developed countries, very few studies have explored the potential association between oral health and adverse pregnancy outcome.

Identification of women at higher risk of PTB is fundamental to prevent the likelihood of delivering preterm. Several risk factors have been associated with PTB, such as prior history of PTB, cervical disease and infection. Despite this, finding an association between a given risk factor and the occurrence of PTB is challenging.

Table 3	Selected outcomes evaluating the association between dental caries and PTB			
Outcomes	**N studies (n/N)**	**OR (95% CI)**	**P**	**I² %***
PTB, women with dental caries versus controls	5 (1472/4246)	1.16 (0.90 to 1.49)	0.25	35
DMFT (PTB vs non-PTB)	5 (2963)	1.56 (−0.28 to 3.41)	0.10	92
DMFS (PTB vs non-PTB)	3 (2594)	−0.15 (−3.40 to 3.09)	0.9	89

*I² is a measure of the heterogeneity among the included studies. a value ≥50% indicates high while <50% low heterogeneity.
DMFT, decayed, missed and filled teeth; DMFS, decayed, missed and filled surface; n, number of events; N, total number of participants; PTB, preterm birth.
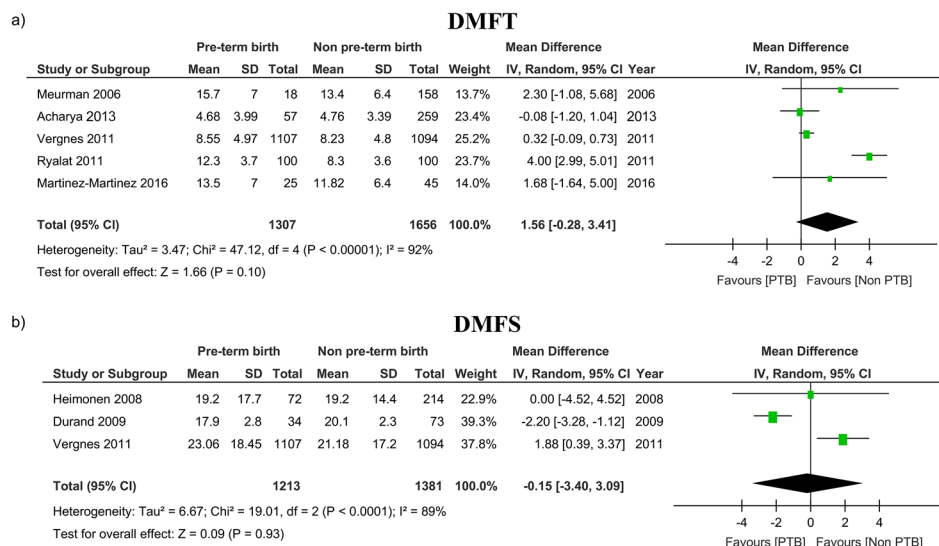

Figure 3 Mean differences in DMFT and DMFS indices in women with dental caries compared with those who did not experience PTB. DMFS, decayed, missing and filled surface; DMFT, decayed, missing and filled teeth; PTB, preterm birth.

Dental caries is a frequently encountered oral health problem in pregnancy as pregnant women are more susceptible to caries compared with non-pregnant women.[13] Being caused by an infectious process, dental caries can theoretically lead to inflammation and thus increase the risk of PTB.[12] Despite this, we could not find any significant association between dental caries and PTB; furthermore, we did not find any significant difference in the severity of caries assessed by DMFT and DMFS indices between women who experienced PTB compared with those who did not. In addition to this, since most of these studies have evaluated women after delivery, this may also have influenced the results.

The lack of association between dental caries and PTB is difficult to explain. The initiation and progression of the caries lesion is very slow and the destruction caused by caries in initial stage can be reversible.[12] In addition to this, pregnancy itself does not cause dental caries but it may exacerbate the existing condition. Dental caries is symptomless until there is severe and irreversible destruction of teeth.[42] It might be possible that bacterial spreading during caries formation and the subsequent production of proinflammatory mediators induced by oral pathogens may not be of the magnitude to cause production of proinflammatory mediators enough to initiate PTB.

Even though we found no significant relationship between the dental caries and PTB, it is still important for the health professionals to promote oral health among the pregnant women. This is because pregnant women are susceptible to dental problems and have very limited knowledge and awareness about the importance of oral health and its potential impact on pregnancy outcomes.[39 43] Furthermore, the risk of transmitting the oral cariogenic flora from the mother to her infant through feeding practices and predisposing the infant to early childhood caries in the future should not be neglected.[44–47] Therefore, large prospective studies aiming at ascertaining the association between dental caries and spontaneous PTB, according to the gestational age at occurrence, severity of the disease and presence of other co-morbidities are needed in order to elucidate the role, if any, of dental caries in increasing the risk of PTB.

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