Red fluorescent dental plaque: An indicator of oral disease?
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General discussion
In the previous chapters, associations were described between red fluorescent dental plaque (RFP) and the age and thickness of a biofilm. The results indicate that the composition of plaque affects the development of red fluorescence. A novel aspect of RFP in this thesis is its association with the gingival inflammatory response of the subject.

Reliable screening for developing oral diseases of a population is desirable to disclose which patients are at high risk, subsequently allowing for more effective prevention. The use of a fluorescence tool that visualizes pathogenic plaque, may be an effective method to perform this screening. To use RFP for oral health assessments in daily practice, thorough validation of a fluorescence tool is needed on both the possibility to use RFP for screening of caries risk and resilience to gingival inflammation.

The serendipity finding of RFP on teeth was at first related to caries lesions (König et al., 1993), while a possible relationship between RFP and gingival inflammation was later on also patented though without comprehensive scientific data (Kanbara et al., 2006). Promising results have been reported using fluorescence when excavating caries, called Fluorescence Aided Caries Excavation (FACE; Lennon et al., 2006a). The latter results point towards a relationship between the presence of a caries-associated microbiota and red fluorescence, indicating that community composition is related to its red fluorescence. If this fluorescence is purely related to the presence of a large amount of biofilm, a correlation between RFP and gingiva inflammation is to be expected. However, this hypothesis cannot explain the observation of the presence of specific fluorescent spots within fluorescent biofilms in chapter 4, which suggests that some specific microorganisms or microbial communities contribute to this red fluorescence. In chapter 8, the microbial composition of RFP reflects the composition of a mature biofilm, which is more related to gingivitis with respect to the composition of the plaque. This possible relationship between RFP and gingivitis is also described in the chapters 4 and 6. New questions are raised from the results in this thesis, such as whether specific groups of microorganisms cause biofilm red fluorescence, what characterizes this group of microorganisms and what is the effect of the individual inflammatory response on RFP. In the future, novel methods will enable the study of red fluorescence within the microbial community and its functions in a more detailed way (Franzosa et al., 2015).

Methodological considerations

In vitro studies

The present techniques in the field of microbiology are enabling us to study the microbiome of a biofilm in more detail, within its current limitations (Goodrich et
The composition of the oral biofilm turned out to be far more diverse than expected from culturing methods (Aas et al., 2005). Microbes interact with each other and influence each other’s functioning (Kuramitsu et al., 2007), which makes the results on solitary microorganisms, as described in chapter 2, less representative for the translation towards the clinical situation of a biofilm.

All existing in vitro biofilm models have limitations (Sissons, 1997). However, the use of models enables us to control specific circumstances of biofilm growth, e.g., the thickness of a biofilm as presented in chapter 3. In this study, the effect of biofilm age, thickness and cariogenicity on red fluorescence was assessed in two separate experiments. In these experiments biofilms were assessed at different time points and using different substrates (Teflon vs dentin). In this model, the pH of the biofilms was not assessed, while it is known that pH influences fluorescence (Polo et al., 1988; DaCosta et al., 2003). Since the pH of a biofilm is a key parameter in caries commencement and development (Stephan, 1944), it can be of interest to study the effect of pH on RFP to obtain more insights in the effect of caries activity on plaque fluorescence.

When designing an in vitro study, a biofilm model is chosen that controls the parameters of interest and that is feasible to perform. In some models the intensity of red fluorescence appeared to be difficult to assess in a reliable way, e.g., due to reflections of the excitation light on the glass substrate. In some models, like the CDFF (Pratten, 2007) and the ACTA active attachment model (Exterkate et al., 2010), samples had to be sacrificed to assess their fluorescence. The CDFF enabled RF measurements, while allowing to harvest the biofilm for composition analysis without contamination. The microfluidic BioFlux flow system, on the other hand, allowed real-time fluorescence measurements, while the biofilms could not be harvested. Therefore, both types of studies contribute to different insights in the mechanism of red fluorescence. Photobleaching of the red fluorescence is a parameter to be considered as well (Hope et al., 2011), although we did not experience this during the experiments described in chapters 2 to 4. Another possible influence to take into account in all studies is the detection threshold for red fluorescence.

In vitro studies, when designed and performed correctly, provide indications what to expect in the clinical setting. These in vitro results enable the design of clinical studies with clear hypotheses. Moreover, the origin of red fluorescence has been suggested to be from porphyrin derivatives (König, 1994b; Buchalla et al., 2008), but more fundamental studies are required to determine the exact mechanism responsible for red fluorescence from bacteria. It is known that porphyrins are important in many enzymatic reactions in the cell, especially where electrons are transported (Beale, 1995). When a porphyrin is bound to an enzyme, it loses
its fluorescence. Therefore it can be speculated that red fluorescence is related to intracellular energy transport and thereby to cell growth. This hypothesis applies only to cells dependent on enzymes in need of porphyrins, which could be subject for further research.

In *in vitro* biofilm studies it is feasible to study factors influencing red fluorescence without burdening volunteers with participation in clinical research. Even when red fluorescence proves to indicate pathogenic dental plaque, biofilm models are useful to easily assess the response of red fluorescence to, e.g., recent food intake or the use of oral rinses or simulated biofilm ‘alterations’ (pre- or probiotics).

A striking example of how *in vitro* research complements *in vivo* research is described in chapter 4. The results from this biofilm study appeared to be in line with the results from the related clinical study in chapter 6, where RFP after a few days without oral hygiene correlates to the clinical bleeding on marginal probing after a challenge of 14 days without hygiene. The gingival inflammation after this challenge was more determining for the *in vitro* red fluorescence than the clinically determined percentage of tooth surface covered with RFP after the challenge. These results provide opportunities for new experimental studies in the laboratory.

**In vivo studies**

Up to now, no gold standard is available predicting high risk individuals or high risk oral niches. Risk assessment based on pathogenic plaque is of great importance, since plaque is the main cause of most oral diseases. This enables the user to look at direct disease-influencing factors before symptoms of disease are present. The absence of a gold standard for caries risk makes it difficult to validate the usefulness of RFP for risk assessments. Longitudinal clinical studies should be performed to investigate the applicability of RFP for risk assessment regarding caries and gingivitis.

Most of the clinical studies in this thesis were not performed with studying RFP as the main aim of the trial. The opportunity to collaborate with colleagues to include RFP as a variable enabled us to obtain important directions for future research. Based on these results effect size calculations may be performed to do power analysis for future studies. The performance of a clinical study with a sample size that is too small, may lead to overlooking clinically significant outcomes. An over-estimation of the sample size of a clinical study, however, leads to an unnecessary burden for participants (Hochster, 2008).

The study described in chapter 6 follows participants before, during and after a period of 14 days without performing any oral hygiene. Part of the participants showed a strong RFP response during a 14 day period of refraining from oral hygiene. These participants did already have RFP before suspending their oral
hygiene measures. Additional insight in red fluorescence may be obtained from the ecology of the plaque and the caries activity of the participants to determine how these participants differ from those without RFP at the start of the experimental period. For this, a recall appointment after a longer period of time can be performed to record possible new caries lesions that have developed in time.

Assessment of caries activity starts with a well-performed scoring of caries at the start of a study. This includes professional cleaning of the teeth and using a suitable caries scoring system. Due to ethical principles, it is often not possible to take radiographs as part of a clinical study. All these arguments interfere with an ideal caries assessment. And even when performing the most thorough caries assessment, we do not observe the sub-clinical initial lesions, which are in a dynamic state of progression and regression.

The main cause of dental caries is plaque. To visualize this plaque, a disclosing solution is frequently used in the dental practice. The strong correlation between disclosed plaque scored clinically or on photographs in chapter 5, together with high inter-rater reliability, confirms the validity and reliability of the use of intra-oral photographs when scoring plaque on the buccal surfaces of teeth. Only little time is needed to obtain a set of full-mouth fluorescence photographs. However, it is hard to obtain photographs from sites that are difficult to access in the oral cavity, while these are at the same time most prone to the development of disease. Chapter 5 describes that the amount of RFP of the anterior teeth correlates moderate to strong with the total amount of plaque present. Only a weak to moderate correlation was found between RFP and blue disclosed plaque, raising questions on how to relate RFP to conventional plaque visualization methods and the hypothesis of RFP being old plaque.

As presented in this thesis, both in vitro and in vivo studies are required to answer the questions concerning the applicability of RFP to the daily dental practice. In vitro studies can focus on the pathways used by microorganisms to generate red fluorescence as well as on further biofilm studies, e.g., to determine what the high intensity red fluorescent biofilm spots (chapter 4) consist of. For solid oral health risk assessments, prognostic studies are required with a longitudinal design and within a general population. In this thesis, associations were found between anaerobic biofilm and RFP, with clear indications that the composition of plaque affects the development of red fluorescence. In addition, the association between RFP and gingival inflammation provides new directions for future research.