Oral biofilms and secondary caries formation

Eugenio Brambilla
Andrei Ionescu

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Abstract

The presence of a huge amount of data regarding the onset of a carious lesion in close proximity with a restoration must not make ourselves less aware about the fact that, still, a lot of information is missing about secondary caries formation. Many pieces of information are highly conflicting, such as, for instance, the role that different microbial species have in the onset of the lesion, or the link between the existence – and width – of a gap between hard tissues and restoration, and the development of a secondary lesion, or, again, the clinical decisions regarding the replacement of a restoration due to secondary caries, and, if so, to what point stop excavating?
The main difficulty in this field arises from the fact that secondary caries is the result of very complex interactions taking place among already injured human tissues, overlying biofilms that often maintain the dysbiotic conditions that lead to the primitive lesion, and dental materials that may help, or even worsen that situation. Increasing our knowledge about what exactly happens after a material is placed at this three-sided interface may greatly help us in designing new dental materials able to interact in a positive way both with the host and its biofilm, ensuring longevity to restorations and helping in reducing what is nowadays their main cause of failure – secondary caries.

Secondary caries: what is it?

The answer is: we still do not know for sure. In 2009 Cenci *et al.* stated: "Up to now, there has been no conclusive evidence for the association or lack of association between gap presence and caries adjacent to restorations." After ten years, this sentence still seems to be valid. However, we must start from some definition (1).
Secondary or recurrent caries is a lesion at the marginal area of an existing restoration (2). Contrarily to residual caries, which is represented by infected tissue left behind after cavity preparation, secondary caries develop close to a restoration. Nevertheless, in clinical practice, it is most often difficult to differentiate between these situations. This is the reason why secondary caries have been receiving increasing attention over the last years. It has become a matter of concern in restorative dentistry since it has been recognized as the most common reason for premature failure of restorations, irrespective of the restorative material used (3-5).
This fact is demonstrated by a relevant amount of experimental data about the clinical performance of dental restorative materials that have been published over the last two decades (3, 6-9). In these studies, the development of a secondary caries lesion is considered one of the most important parameters to measure the performances of the restorative materials. Furthermore, recent

literature indicates that secondary caries and its prevention is one of the critical problems of the next 20 years (10).
For many reasons, epidemiological data about the prevalence and the incidence of secondary caries are far from being complete and exhaustively investigated. Firstly, there are no consensual standards to perform the detection of secondary caries. Clinicians use many empirical methods based on their field experience. This approach leads to relevant differences in decision-making regarding the need for the replacement of restorations, which might be grounded on false-positive diagnoses that may finally cause unnecessary replacement of restorations (5, 6). This considerable heterogeneity of diagnosis-subsequent treatment decisions is grounded on criteria with limited accuracy (11-14). From an operative point of view, the lack of a clear and shared strategy causes an unmotivated and increased sacrifice of sound tooth tissues and coincides with unnecessary costs and a decrease of functional shelf-life of natural teeth (15). Citing Elderton, decisions made by dentists for replacing a filling are idiosyncratic (16). This circumstance may not only explain the high variability that is reported in the literature regarding the incidence of secondary caries but to a certain extent also the higher incidence of secondary caries that has been observed in practice-based studies that included a higher number of noncalibrated operators than academic studies (17, 18). On the other hand, the prevalence and incidence of secondary caries identified in controlled clinical trials may not be representative of daily dental practice. As such, the current approach in addressing secondary caries treatment has a profound impact on health care expenses, since the replacement of existing restorations due to secondary caries takes most of the work time of the dentist (19-21). A clinical example

An estimated 60% of the replaced resin-based composite restorations, however, belong to a “redentistry cycle” which describes a continued series of repeated restoration placements, implying that the patient is supplied with more restorations depending on the existing restorations (16, Panel 1).
Moreover, subsequent replacement or repair of a restoration leads to further loss of natural tooth tissues, starting a so-called death spiral, which may eventually lead to tooth loss and reduction of the residual oral health (17, 18).
While secondary caries is primarily the same disease as primary caries and patient-related factors such as oral hygiene and dietary habits play a crucial role in its etiology, there is evidence that the problem is related to the interface between natural tooth tissues and the restorative material as well as the characteristics of the restorative material (4). Consequently, increasing efforts are made to modulate the characteristics of this interface in order to improve the ability of restorative materials to reduce the onset of secondary caries (22). However, the detection of secondary lesions has received less attention until now, even though it is essential both to estimate the incidence of the disease and to manage it effectively. In fact, this problem seems to be unsolved.
Many clinical studies showed a higher incidence of secondary caries associated with resin-based composites than with amalgam, which implies that resin-based composites could be more susceptible to secondary caries than amalgams especially in high-caries-risk subjects (20, 23, 24).
The higher susceptibility of resin-based composites to secondary caries has so far been associated with various material-inherent properties such as polymerization shrinkage and subsequent micro-leakage, higher plaque accumulation, the release of bacterial growth-stimulating compounds, the lack of antibacterial and acid-buffering effects, and changes in microbial composition (4, 25, 26).
Thus, several studies were performed, aiming to improve the behavior of resin-based composites to make the restorations more resistant to the development of secondary caries. The primary strategy is represented by changes in chemical composition produced by the addition of antimicrobial compounds, either to the formulation of the resin-based composites or the corresponding

adhesive, providing generally satisfying results *in vitro* on the short-middle term (27-30). Clinical data on the performance of such materials are generally lacking. It is challenging to design studies that produce relevant and compelling data about the progress of initial secondary caries, which is due to ethical reasons and the intrinsic difficulties associated with clinical trials, such as, for instance, their duration. Therefore, the identification of potential prognostic factors is difficult, mainly because they are associated with the individual caries risk of the patient, the operator's skills, and the performance of the restorative material applied.
Clinical trials are, however, the only current way to convey reliable indications to clinicians. Problems associated with this kind of study occur for two main reasons, including (1) the high number as well as different types of restorative materials available on the market, and (2) the specific application techniques. Moreover, restorative materials have one of the shortest turnover times. In many cases, restorative materials investigated in a clinical study have already been replaced by the next-generation materials when the study is finished, meaning that the results are outdated before the completion of the study. Moreover, results and conclusions drawn by analyzing a particular type of restorative material can hardly be transferred to the behavior of other restorative materials from the same class (31).
From this point of view, the duration of clinical studies produces other critical limitations. Planning, performing, and reporting the outcomes of a clinical trial on secondary caries is challenging as there may be high dropout rates of the participants, and manufacturers often ask for experimental designs involving observation periods that are less than three years. However, the favorable clinical performance of a restorative material over short periods is no predictor for favorable long-term behavior. Thus, we need alternative strategies to improve the performance of new generations of restorative materials in order to protect the marginal area of surrounding tissues by secondary caries.

Microbiology of secondary caries

The development of a caries lesion is caused by an imbalance between pathological factors that lead to a loss of minerals and protective factors that cause an uptake of ions by the tooth tissues (32). Cariogenic bacterial species represent the leading etiologic agent for this process. Their fermentative metabolism produces organic acids, such as carbonic, lactic, and propionic acid. It has been known for years that those acidogenic bacterial species are also aciduric and can live under acid conditions (33). The microbiology of secondary caries is even more complicated due to the presence of the restorative material that unevenly interacts with the biofilm colonizing both the surface and the interface.
Although the histopathology of secondary caries is described as similar to that of primary caries, its etiology is not as clear. Beginning in the 1990s, research efforts in this field have until now failed to produce consistent data. Kidd et al. showed no significant differences between the microflora in samples from cavity walls involving primary caries and secondary caries in the proximity of amalgam restorations (34). Thomas et al., using an *in situ* model to investigate the composition of biofilms colonizing the surfaces in primary and secondary caries lesions identified a higher proportion of cariogenic bacteria on restorations fabricated from resin-based composite. These data show that the composition of biofilms in primary caries lesions differs from that of secondary lesions developing in the proximity of resin-based composite restorations (35).
Another complex topic is the colonization of tissues under the restorations. Mejàre et al. found that the bacterial flora below resin-based composite restorations is similar to the flora observed in dental plaque, mainly including *Streptococcus* and *Actinomyces* spp. (36). Splieth et al. (37)

compared the microbial flora under resin-based composite and amalgam restorations focusing on the anaerobic species, identifying a similar bacterial composition under both materials. In particular, the latter study indicated that inadequate resin-based composite restorations might stimulate the growth of cariogenic as well as obligate anaerobic bacteria potentially pathogenic to the pulp. There are many possible explanations for these observations. Firstly, the micro space between the restoration and the cavity floor favors the ecological niche of obligately anaerobic bacteria. In fact, it is not surprising to discover many obligate anaerobic bacteria commonly colonizing the oral environment, even in subjects without clinically detectable endodontic or periodontal lesions. In addition to that, it does not necessarily mean that subjects without clinical symptoms such as toothache or pulpitis do not have chronic or arrested caries lesions, which implies that it is still possible to detect such anaerobic bacteria. However, it must be highlighted that co-existence does not mean involvement. Therefore, it is still necessary to demonstrate the participation of those obligate anaerobic bacteria in the progress of secondary caries. These considerations suggest that the restorative material plays a crucial role in the composition of the biofilms colonizing surfaces and interfaces of dental restorations and surrounding tooth tissues.
According to the viewpoint of Philipp Marsh, any species with an acidogenic ability that is able to tolerate the cariogenic environment can contribute to the progress of dental caries (38). For a long time, mutans streptococci (*S. mutans*), lactobacilli, and *Actinomyces naeslundii* have been used in several in vitro models to study secondary caries. *S. mutans* and lactobacilli can produce a variety of organic acids and can withstand a low pH environment for a long time, thus leading to the demineralization of dental tissues and the onset of caries. It has been shown that these bacterial species are widely present and might play a crucial role in the development of secondary caries around amalgam restorations (39). However, in a recent in situ study, *S. mutans* was not detected in any sample, contrarily to lactobacilli spp. Furthermore, *Actinomyces odontolyticus* and *Candida* spp. were also found in most samples (35). In fact, David Beighton recently put forward a distinct point of view, insisting that *S. mutans* might be a useful marker of secondary caries without necessarily being its etiological agent (40). These assumptions are supported by the previously described experiments performed by Renske Thomas's research group.
As a consequence, scientists speculated that there might exist unknown caries-associated bacteria that cannot be isolated on selective agar plates (35). In the past decade, the detection of *A. odontolyticus* and *Candida* spp. associated with caries development has caused reactions of surprise in the scientific community. It has been shown that *Candida albicans,* despite its lower growth rate (41), can dissolve hydroxyapatite at a much higher rate than *S. mutans*.
Klinke et al. assumed that *C. albicans* might make a significant contribution to caries pathogenesis in caries-active children, and it could be responsible for an increase in caries pathogenicity (42). Some experiments performed by the Authors of this chapter show signs of increased virulence (enhanced adherence to the pellicle-treated enamel surfaces, increased replication rate and extracellular matrix production) by *S. mutans* when co-cultured with the yeast, while none of these microorganisms seemed to suffer from the presence of their "partner", possibly meaning they express synergistic relationships (Panel 2).
Apart from that, it should be noted that many subjects may have high caries activities without having a prevalence of *S. mutans* in the composition of oral biofilms. Therefore, further research is needed to define better the microbiological features of secondary caries, such as the role of the different pathogenic species and inter-species relationships.

Carious tissues features and removal strategies of secondary caries

In a secondary lesion, we can identify two distinct regions: the surface lesion, which develops perpendicular to the tooth surface and can be considered as a primary lesion developing next to a restoration, and the wall lesion, which develops in-depth, perpendicular to the tooth/restoration interface (Figure 3, 43).
Histological analysis of artificial, caries-like lesions and natural lesions around restorations may yield lines of demineralized tissue running along the cavity wall. These are called wall lesions, are considered to be the result of microleakage, and can be identified in natural teeth with occlusal amalgam restorations. In this case, the wall lesions are probably the consequence of an initial leakage that occurred just after the placement of the restoration and before the filling of the marginal gap with corrosion products.
Particular attention must be paid not to mix up secondary caries with histological signs of microleakage. The difficulty to clinically distinguish between these situations is a crucial factor in making a clinical decision for the placement of restorations, which often causes overtreatment.
Furthermore, it is also important not to mix up secondary caries with residual caries, which is to be regarded as residual demineralized tissues that are more or less intentionally left during cavity preparation. This distinction is a very difficult matter for both clinicians and researchers because the clinician needs to know how much carious dentin should be removed and how deep the excavation has to be extended (44, 45). The criteria defining the amount of excavation and caries removal are influenced both by the interaction of the surgical procedure itself with pulp tissues and with the management and application of the adhesive procedures. Clinical studies of Mertz-Fairhurst have questioned the consolidated approach to how much demineralized tissue may be left during cavity preparation (46). In their clinical studies, the enamel lid from large occlusal lesions was removed, leaving extensively demineralized dentin. The cavities were then restored with adhesive material techniques. After ten years, data very surprisingly showed that these restorations were still satisfactory, with no need for replacement. These results put into question the conventional teaching in restorative dentistry. In the latter case, we currently assume that the infected, demineralized dentin that is part of the carious lesion must be removed entirely in order to arrest the progression of a carious lesion.
Nevertheless, if no negative effect happened after leaving the infected tissue on the bottom of the cavity, the endpoint that we must consider for the surgical excavation procedures has mainly to do with the requirements and characteristics of the adhesive procedures that are used to restore the function and esthetics of the tooth. We may better explain these results after accepting that the bacterial metabolism of biofilms is the driver for caries development and progression at all stages of this pathology. In this sense, if the process is arrested by removing the biofilm inside the lesion, or, better said, by tuning down its metabolic activity, the entity of the remaining carious tissue removal is merely a function of the tissue's ability to support the overlying restoration reliably.
In this sense, we need to define criteria to address restorative interventions rationally (44). The first step is the choice of a threshold for carious tissue removal. This choice is still a matter of huge debate in the dental field. In general terms, clinicians and researchers agree that the operator should retain sound, remineralizable tissues and attempt to seal residual carious dentin beneath the restoration by creating a durable bond with sound tooth tissues surrounding the lesion (Panel 1). Another crucial target is to save the vitality of the pulpal tissues by avoiding as much as possible accidental exposures.
Schwendicke et al. summarized five main strategies to maximize the success of the removal of carious tooth tissues. Following these indications, our operative intervention can span from the drastic removal of all the softened dentin to completely avoiding this step. The real focus of the discussion is, regardless of the excavation strategy, that the remaining tissues still harbor vital

bacteria. The new approach is based on the idea that the removal of all microorganisms in the tissue is not quite necessary (44).
From this point of view, the strategy involving nonselective removal to hard dentine (formerly complete excavation or complete caries removal) is now considered overtreatment and no longer recommended. Less invasive, and consequently more selective removal to firm dentine leaving a bottom layer of leathery tissue and sound margins, is the treatment of choice in carious lesions that are not in close proximity with the pulp. Deeper lesions produce a significant risk of pulpal exposure during selective removal procedures to sound dentin. Therefore, an option is to leave soft carious tissue to avoid exposure and stress to the pulp.
Another option is represented by stepwise removal, that is two-step carious tissue removal: a first intervention involves leaving the soft carious tissue close to the pulp, while time is allowed for remineralization and production of secondary dentin under a provisional restoration, possibly showing bioactivity/remineralization capabilities. In a second stage, additional excavation is performed to reach firm dentin and, at the same time, reducing the risk of pulpal exposure. However, there is still a consistent debate regarding the application of this technique. Indeed, the main disadvantages are related to extra treatment costs due to additional clinical sessions required by this procedure and to the increased risk of pulpal exposure.
A last and most controversial strategy may be to leave all carious tissues. The options include either to seal the carious lesion completely or to open the lesion to expose it to the oral environment, and to manage it without further restorative procedures. Sealing of non-cavitated or minimally cavitated lesions in areas not subjected to occlusal load was demonstrated to be a highly effective option. The second possibility is based on the induction of massive changes in the composition of the biofilm that colonizes the superficial layers of the carious tissues and its pathogenicity. Once a lesion is exposed to the oral environment, the increased interaction with saliva and its components dramatically reduces the lesion's activity. This possibility, however, requires very high compliance from both the patient and the operator, involving frequent recalls and follow-up and, at the bottom line, was not found to be more effective than its alternatives.

Secondary caries and microleakage

The presence of a not perfectly sealed interface between the restorative material and the surrounding hard tissues has been regarded as the main reason for the development of secondary caries for many years. The reason was assumed to be microbial penetration into a gap between the restorative material and the surrounding sound dental tissues and the production of acidic metabolites in this new microenvironment (43, 47, 48).
One of the possible situations leading to the development of both outer and wall lesions is the existence of the gap itself. The size of this gap can vary from a few microns to some hundred microns (49-51). The presence of a clinically visible gap is regarded as a sign of the presence of defective restoration margins and often leads to the decision to replace the restoration (52). Therefore, the role of this structure seems to be crucial for clinical decision-making and has to be carefully considered to avoid overtreatment.
Two explanations attempt to put into relation gaps with the development of a cavity wall lesion. The first one postulates the penetration of bacterial cells and, especially, their metabolites through the gap (microleakage), thus initiating the demineralization process. According to this "microleakage theory," bacterial colonization increases along with the size of the gap (26, 53, 54). Nevertheless, this microleakage-based theory has been questioned recently by research data indicating that secondary caries is but a primary lesion that develops in the marginal area of a restoration. The evolution of a lesion at the restoration margin is determined by the activity of the

biofilm colonizing the outer surface - regardless, within reasonable limits, of the presence of microleakage. The cavity wall lesion is, therefore, a consequence of the extension of the process already taking place on the external surface. Furthermore, the microleakage-based theory does not take into account that biofilm development is dependent on environmental parameters such as the oxygen diffusion gradient or chemical compounds leaching from the material's surface. In a tiny gap, these variables may not necessarily be more favorable than in a more substantial gap.
The second explanation is based on the recent advance of knowledge regarding the microbiology of cariogenic biofilms and their role in secondary caries development. Both *in vitro* and clinical experimental data showed that microleakage alone does not necessarily promote an active demineralization process on the cavity wall of a restoration (55). Bacterial colonization of the tooth-restoration interface is a mandatory prerequisite for the development of a secondary carious lesion, just as in primary caries.
Nevertheless, there are relatively few experimental data in the literature dealing with the relationships between gap size and wall lesion development. Indeed, we still do not know if a minimum gap size can be determined to cause the lesion development. Moreover, even if this information were available, its use by the clinician in the decision-making process on the replacement of a restoration or parts of it would be tough. Data about the relationship between gap presence and secondary caries development is still controversial (1). Some studies identified a lack of correlation between these factors (56-58), while other researchers have found a positive relationship based on gap size (54, 59).
In particular, regarding the results gathered by the most recent *in vitro* studies, Totiam et al. and Nassar and González-Cabezas used different sucrose-cycling *S. mutans* models, showing that in experimental gaps the size of the gap is positively correlated with the size of dentinal wall lesions (53, 54). Only Diercke et al. (60) demonstrated a statistically significant increase in lesion depth in enamel (50-µm to 250-µm gap) and dentin (50-µm to 100-µm gap). It must be pointed out that in this study, as in the one by Nassar and González-Cabezas, no adhesive system was used, so probably the microenvironment was very different from the clinical situation. Furthermore, the experimental design of these studies shows other bias sources related to a relatively short incubation period, the use of a static setup (no bioreactor to simulate biofilm formation under shear stresses), and the use of monospecies *S.mutans* biofilm. For these reasons, the results above have to be interpreted with caution.
In recent years, the improvement of microbiological techniques and the diffusion of the use of bioreactors seem to be improving the reliability and translational value of results obtained by *in vitro* studies. The correlation between a gap and secondary caries is confirmed by the results of Hayati et al. (61), that - using a bioreactor – studied the gap colonization by a multispecies cariogenic biofilm. The influence of an adhesive system was also evaluated. The results showed that the presence of an adhesive system significantly reduced the progression of the secondary lesion. These findings show that the results of the previously described studies might be overestimated due to the absence of an adhesive system. Finally, Maske et al. (62) investigated the development of dentin wall lesions next to resin composite using an in vitro microcosm model. Their aim was to evaluate the influence of the gap size on the wall lesion development. They found that wall lesions in dentin developed even in tiny gaps, and the threshold for secondary wall lesion development was around 30 µm.
Regarding *in situ* studies, Thomas et al. (51) indicated that an average gap size of 225 µm was necessary for the development of a wall lesion, even if their results had a broad data range (80-560 µm). In 2009, Lima et al. demonstrated a significant impact of biofilm control in the prevention of enamel lesion demineralization. Their results suggest that microleakage and surface roughness do not influence the formation of secondary caries lesions. In 2015, Montagner et al. demonstrated

that the presence of a bonding agent on the composite side of a restoration–dentin gap increases wall lesion development (63). One year later, the same group demonstrated that composite-dentin interfaces that failed after aging showed different demineralization patterns depending on the presence of an adhesive system (64). These data showed that the restorative procedure and the application of the adhesive system deeply influence the response of the tissue structure to secondary caries challenge (Panel 4).
In conclusion, it is interesting to note that the integrity of the composite-tissue interface may be of critical importance for the development of secondary caries in adhesive restorations.

Is secondary caries a material-related problem?

There is currently a broad spectrum of restorative materials available from which clinicians can choose. Each type has its own physical and chemical characteristics that influence its field of application. As a consequence, we have an equally broad spectrum of interfaces between dental tissues and restorative materials. It is, therefore, challenging to obtain univocal results in the study of secondary caries development. During the past decade, restorative materials underwent a paradigm shift, changing from amalgam to adhesive materials. This development caused significant improvements in the esthetic performance and posed the basis to propagate minimally invasive surgical techniques. Resin-based composites have become the most commonly used restorative material (65, 66), and they have gradually replaced the amalgam since the latter has been associated with mercury toxicity (not yet demonstrated) and environmental problems (67, 68). Furthermore, their bonding ability to tooth tissues allows a wide variety of potential applications. For instance, direct treatment of clinical situations that had previously to be treated with indirect techniques is now possible. Composites also represent the ideal interface for bonding ceramic materials to tooth tissues, allowing a level of performance unknown until recently. One of the fields in which these materials have allowed for most significant developments is the minimally invasive approach. Adhesive techniques made it possible to remove only the necessary amount of dental tissues, thus minimizing the sound tissue sacrifice (69, 70).
Speaking about interfaces from a material's point of view, the terms used for any type of restorative material are "margin," "adaptation," or "gap." Other terms, for example, "marginal seal or sealing," are primarily associated with the adhesive interface of a restorative material. "Ditch" or "flowing" are mainly used for amalgams. The differences among the terms used represent the existence of a broad spectrum of situations that are related to different chemical and physical properties of the restorative materials. Moreover, the interfaces are dynamic microenvironments that show a complex balance based on the exchange of chemical compounds leaching from the restorative material and ions and other compounds from the external environment (saliva) and the dental tissues.
From a clinical point of view, different materials differ in the behavior of their interfaces. Amalgams produce a greyish halo in the tooth tissues due to the depositions of corrosion products of the material (Figure 5). Glass-ionomer cements, resin-modified glass ionomers, and resin-based composites may exhibit visible gaps and/or marginal staining related to the interface colonization, and biofilm overgrowth in the marginal area (6, 71).
The adhesive restorative materials show a significant influence on the microenvironment of the marginal area and play a crucial role in the determination of the composition and characteristics of oral biofilms in different ways (72-76). As previously mentioned, the chemical and structural characteristics of the material itself, as well as the application technique and the ability of the operator, contribute to this behavior. The latter two variables deeply influence the biological

behavior of restorative materials by multiplying the possible types of interfaces that can be obtained. Indeed, the adhesion process is very susceptible to a moist environment. Therefore, controlling humidity in the operating field is fundamental to improve the longevity of adhesive restorations. The best way to achieve this objective is considered to be the use of the dental dam (77). Nevertheless, in many countries, a relatively low percentage of dental practitioners use dental dams in adhesive procedures regularly.

The other variable that influences the characteristics of the interface is represented by the ability of the operator. The production of a new dental surface anatomy using the restorative material is often far from the natural one, showing gaps and grooves in the marginal area. The roughness of the material and the tissues surrounding the restoration that are prepared for the adhesive process are frequently much higher than that of the original natural tissues (73, 78). This problem reduces mechanical biofilm removal both by natural mechanisms and by oral hygiene procedures (79). Finally, restorative materials differ from natural tissues in several surface parameters such as surface free energy and chemical composition.

In fact, clinical studies reported shorter longevity and higher failure rates for direct adhesive restorations in comparison to amalgam, and secondary caries is the main reason for failure. These data are strictly related to the diagnostic problems posed by secondary caries, as previously explained (71).

A very different way to build an interface with dental hard tissues is represented by materials such as glass ionomers and resin-modified glass ionomers that are designed to release compounds, mainly ions. These materials can bond to tooth surfaces by chemical interaction. They are usually composed of alumino-fluorosilicate glass powder that reacts with an aqueous solution of polyacrylic acid (80). Glass ionomers can release several ions, including fluoride, already starting from the setting reaction. There is a first quick release, the so-called “burst effect,” where most of the ions are released in the first two days after placement. According to several studies, the amount of fluoride released varies from 5 to 155ppm. After that, there is a relatively long period of slow but constant release that can last up to three years. The release has an effect on the surrounding tissues and the interface and has been related to a protective action of the material against secondary caries occurrence. Furthermore, this kind of restorative material can be recharged by re-uptake of fluoride ions from the surrounding environment when the concentration of these ions is increased, for instance, after toothbrushing or the application of fluoride-containing mouthrinses (74, 81). Nevertheless, from a mechanical and esthetical point of view, glassionomers are outperformed by composites, and cannot be used in load-bearing restoration. They showed a higher risk of fracture in extended cavities and cannot adequately support the requested performances in highly aesthetic areas.

Resin-modified glass ionomer cements have been introduced to provide the best of both worlds: the resin components add strength and aesthetics, while the material maintains its ion releasing capabilities. These materials feature a lower release of fluoride than conventional glass ionomers. Their mechanical and esthetic properties are nonetheless inferior to those of composites.

Polyacid modified composites (compomers) were another try in this sense. They do not show an initial fluoride release burst effect (82) as glass ionomers and RMGICs, yet the levels of released fluoride seem to remain much more constant over time, probably due to the characteristics of the composite resin matrix (83). A drawback of these materials is that fluoride ions hamper the polymerization processes of the resin matrix, producing materials that reach a sub-optimal polymerization degree. This phenomenon, affecting the mechanical characteristics, leads to surface degradation, and has been related to increased microbial colonization. In fact, compomers seem to elicit the highest biofilm formation when compared to the surfaces of the previously mentioned materials (84).

Giomers are a new kind of ion-releasing material, consisting of a composite where the filler particles are made of pre-reacted glass ionomers. Similar to compomers, they do not show an initial 'burst' effect regarding the release of fluoride, and the amount of fluoride released is considered to be somewhere halfway between conventional glass ionomers and compomers (85). These materials have shown notable performances in terms of a reduction of biofilm formation *in vitro* and *in situ* (86, 87). Nevertheless, clinical data supporting the protective action against secondary caries by these materials are still controversial (88). From the point of view of secondary caries occurrence, glass ionomer restorations unexpectedly showed similar performance when compared with composites. In spite of generally very promising in vitro results, it was not possible to demonstrate for this class of restorative materials a clear protection *in vivo* against secondary caries occurrence (26, 89). Furthermore, the last generation of glass ionomer materials, featuring improved performances due to the application of nanotechnologies, has not been sufficiently tested clinically quite yet. Nevertheless, several countries have adopted glass ionomers as the standard amalgam alternative as a result of their easy applicability and low placement costs (71).
New generations of restorative materials based on bioactive glasses, calcium silicates, and calcium orthophosphates showing active interaction both with the surrounding tissues (remineralization) and with biofilms to prevent secondary caries occurrence are furtherly discussed in Chapter 9.

Artificial biofilm-induced secondary caries models

The occurrence of secondary caries is related to a plurality of factors, and an increasing amount of data suggests that interactions of these materials with the oral ecosystem and in particular with biofilms play a crucial role. As previously described, these interactions can compromise the integrity of a restoration by modifying the structure and the characteristics of materials, mainly at the surface and interface level. In this perspective, in recent years, the research field of biomaterial-biofilm interactions and of the experimental models to study them have raised increasing interest. While the methods to analyze the physical properties and biocompatibility of restorative materials are relatively standardized, as we are going to see in the next Chapter, methods to investigate their microbiological behavior are far from reaching similar levels of standardization (90-93). Studying biofilms is challenging because of their intrinsic characteristics: they are highly heterogeneous and complex. In addition to that, biofilms represent a living community in rapid evolution and are extremely sensitive to even small changes in the surrounding microenvironment (Chapter 1). For these reasons, high intra-sample and sample-to-sample variabilities are most often found in results. This situation has led to considerable heterogeneity in experimental setups regarding biofilm growth conditions and quantitative determination methods. A wide range of choices is also available for the microorganisms that should be tested in these models; for example, monospecific vs. microcosms models – which produces a severe problem when comparing the outcomes of studies with different experimental designs. Difficulties trying to summarize and to interpret the results obtained by such different methodologies can lead to misleading conclusions.
Another factor to consider is that few data are available in the literature regarding the influence of restorative materials on the biophysical properties, structure, and composition of biofilms *in vivo*, yet these are also potentially useful predictors of clinical efficacy.
Currently, the research community is discussing if single- or multispecies biofilm models are more appropriate to study recurrent caries. The experimental model to be used should ultimately depend on the specific aim of a study (94). A typical example involves the use of single-strain biofilms of *S. mutans*. While it is recognized that monospecies biofilms cannot adequately mimic the complexity of *in vivo* multispecies ones, they are still a useful simplification of a highly cariogenic biofilm that

may provide valuable information that requires, however, interpretation within the limitations of the model. In fact, it should be noted that the single cariogenic species can express a surprisingly wide diversity of phenotypes that can bring significant differences in growth characteristics and metabolism such as, for instance, acidogenicity/aciduricity and tolerance to oxidative stress (94). Studies on biomaterial-biofilm interactions can take advantage of multispecies models since they allow them to provide a more accurate approximation of clinical conditions. In this way, results can reach a better translational value, since they can reduce the variability that is a peculiar characteristic of *in situ* or *in vivo* studies.
Concluding, the importance of *in vitro* experimental setups is increasing due to the several advantages that they deliver. Current research in this field is gradually progressing towards a better ability to simulate oral conditions in order to provide faster, more economical, and accurate reproductions of the clinical environment.

Figure legends

All pictures were acquired during experimental or clinical activity performed by the Authors of the present chapter.

Panel 1. A clinical case is shown where restorations were performed or replaced due to primary and secondary caries. The diagnosis step is crucial and can never be underestimated. It can be seen that the lesion considered as secondary caries on the lower first molar was, in fact, a superficial stain (3d). The latter could have easily been removed by finishing procedures applied to the marginal area of the restoration. The choice of replacing the restoration of the lower first molar was, in this case, only motivated by occlusal reasons (3b). Nevertheless, it must be highlighted that a similar result could have probably been achieved in a more conservative way by a reshaping of the restoration performed with a finishing bur. On the contrary, relatively minor signs on the marginal area of the lower second molar restoration (3c) hide the presence of deep secondary caries extended beyond the floor of the original restoration (3e). The lower second premolar also showed that deep caries originated both from the occlusal and interproximal surfaces (3c, 3e). Re-performing restorations always leads to bigger cavities and, consequently, to reduced residual health of the tooth. The excavation was limited to reaching firm tissue with a leathery consistency after checking that restorations' margins were made of sound tissues ensuring a perfect seal.

Panel 2. A series of SEM micrographs showing a co-culture of *S. mutans* and *C. albicans* at increasing magnifications. The microorganisms were cultivated, starting from a 1:1 inoculum using a sucrose-enriched salivary mucin medium over enamel surfaces for 24h. The adherence surfaces were pre-treated with sterile human saliva for 24 h before inoculation to allow the formation of a salivary pellicle. Signs of active growth can be seen for both microorganisms, such as the formation of streptococcal chains showing several microorganisms replicating at the same time, and budding of a high number of yeast blastospores. At this timepoint, *S. mutans* is outcompeting *C. albicans* in terms of cell numbers, and the pH near the surface is less than 4.5. Despite that, neither microorganism shows signs of suffering. More interestingly, *S. mutans* preferentially adheres to *C. albicans* cells and more promptly produces extracellular matrix than when directly adhering to enamel surfaces, suggesting that the yeast improves the virulence of the bacterium. At the same time, the latter's activity is not detrimental to the yeast, and the extracellular matrix produced by the coccus may serve as protection for both microorganisms.

Figure 3. In a secondary lesion, two distinct regions exist, the outer lesion, and the wall lesion. The first one can be considered as a primary lesion developing next to a restoration, while the wall lesion develops perpendicular to the tooth/restoration interface and is the result of microleakage.

Panel 4. A series of SEM micrographs are shown of an aerial view of an enamel-composite interface. In all micrographs, the enamel is on the right, and the composite restoration surface is on the left, while the interface is displayed vertically.
4a. A composite restoration was performed on an enamel slab, and *in vitro, S. mutans* biofilm formation was obtained using a bioreactor for 48 h. The microorganism's colonization can be predominantly seen on the composite surface and in the microgap between the materials. (2000x magnification).
4b and 4c. The enamel-composite interfaces are shown where different adhesive systems were used to bond composite restorations to enamel slabs (600x). In 2b, a conventional self-etch adhesive system was applied, while in 2c, a self-etch adhesive featuring an antibacterial monomer (MDPB) was used. In the first case, after *in vitro S. mutans* biofilm formation, intense colonization of the composite surface and the adhesive interface can be seen. On the contrary, the antibacterial adhesive system was able to reduce bacterial colonization not only at the interface but also on the surfaces in close proximity. No evidence of gap can be seen, suggesting that both adhesives performed optimal sealing of the interface and that microbial degree of colonization of the interface was not dependent on the presence of a gap.

Figure 5. Section of an extracted lower molar with an amalgam restoration. Clinical appearance does not reflect the condition of the underlying tissues, not affected by a secondary lesion. This typical appearance is due to the ability of the material to seal the marginal gap with greyish corrosion products and has to be carefully considered by the clinician during the decisional process. In the right part of the section, a primary lesion can be seen, developing independently from the existence of a restoration on the occlusal side of the tooth

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