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New Frontiers in Conservative Dentistry: from Resin-Based Materials to Stem Cells

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“*Choose a job you love,*
and you will never have
to work a day in your life”

Confucio

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LIST OF PAPERS

This dissertation is based also on the work contained on the following papers:

Paper I:

- S. Sparabombe, M. Roncati, R. Monterubbianasi, A. Catellani, L. Manzoli, F. Bambini, M. Procaccini, A. Putignano, G Orsini, Assessment of antiplaque effectiveness of chlorhexidine-soaked gauze compared to chlorhexidine mouth rinse: Randomized clinical trial, J Investig Clin Dent. 9 (2018):e12328.

Paper II:

- M. Mascitti, G. Orsini, V. Tosco, R. Monterubbianesi, A. Balercia, A. Putignano, M. Procaccini, A. Santarelli, An overview on current non-invasive diagnostic devices in oral oncology, Front Physiol. 9 (2018): 1510.

Paper III:

- R. Monterubbianesi, G. Orsini and A. Putignano, New Trends of Colour and Background Effect in Restorative Dentistry, Global Journal of Oral Science. 4 (2018):14-17.

Paper IV:

- R. Monterubbianesi, G. Orsini, G. Tosi, C. Conti, V. Librando, M. Procaccini, A. Putignano, Spectroscopic and Mechanical Properties of a New Generation of Bulk Fill Composites, Front Physiol. 7(2016): 652.

Paper V:

R. Monterubbianesi, A. Putignano, V. Tosco, S. Sabbatini , C. Conti, G. Orilisi, G. Tosi, G. Orsini Correlation between chemical-physical properties of methacrylate and dimethacrylate resin composites after different polishing time (Paper in review).

Paper VI:

- R. Monterubbianesi, M. Bencun, P. Pagella, A. Woloszyk, G. Orsini, T.A. Mitsiadis, A comparative in vitro study of the osteogenic and adipogenic potential of human dental pulp stem cells, gingival fibroblasts and foreskin fibroblasts, Sci Rep. 9 (2019) 1761.

ABSTRACT

“Save the teeth” should be the final aim of every dentist. In the era of implantology, the attempt to maintain the tooth is critical; sometimes removing the whole tooth is easier than saving it. When the tooth is decayed, the clinician should remove the damaged tissue (enamel, dentin) and try to reconstruct it. In this phase, decision making is an important task and the knowledge of all possible treatments and materials is essential in helping the clinician to take the right decision. Dentistry has recently gone through a technological revolution, especially in dental materials science and novel instrumental platforms. Important innovations have involved all dental disciplines, including Conservative dentistry, which is devoted to the care and maximum preservation of the tooth tissues.

Preventive dentistry represents the initial and the most conservative method to accomplish the mission of preserving the teeth, therefore evaluations of new devices for dental care and oral disease were my first aim. If the tooth is decayed or traumatically injured, clinicians have a vast variety of dental materials to repair it. Nowadays, resin-based materials have become the gold standard replacements for damaged dental tissues, that can be restored using direct or indirect techniques. Recent developments and potential applications of some new resin-based materials were the second aim of the present thesis. In particular, physical and chemical properties of bulk fill composites and their behaviour after polishing procedures have been investigated. Furthermore, the cementation of indirect restoration has been evaluated by studying the kinetics of degree of conversion of luting resin agents and the effects on them of two different curing protocols.

Finally, the third aim went a bit further into the future, in order to evaluate potential approaches of regenerating dental tissues or even completely reforming the whole tooth. Every day stem cells become closer to the reality and their clinical application could be a great challenge for regenerative and conservative dentistry. Novel potential strategies for regenerating damaged tissues have been studied. Particularly, a comparative study on the differentiation potential of human gingival, foreskin fibroblasts and dental pulp stem cells was performed.

Layout

The thesis is divided in three parts; each one refers to different aspects of conservative dentistry.

The first part focuses on the basis for every treatment plan, the preventive dentistry. After a brief introduction, the findings of the research studies, in which I participated, will be presented.

The second part regards modern applications of resin-based materials. After summarizing the principal components and properties of resin-based materials, I will describe our studies on physical and chemical properties of bulk fill composites and the kinetics of resin luting agents.

The third part will discuss stem cells-based therapies and a recent study on differentiation potential of different cell types.

At the end of each chapter, I will include the papers that I authored or co-authored regarding the specific topic.

CHAPTER I:

Preventive Dentistry

Prevention is the base of all conservative treatments. It is usually said “Prevention is better than healing”. Preventive dentistry is the most low-cost treatment and the modern way to preserve a healthy mouth. Teeth, gum and oral mucosa are the main components of the mouth and they are involved in oral health. Dental caries, periodontal diseases and oral cancers are the most common pathologies. They are highly prevalent and significantly impact the society: common symptoms can be pain, discomfort, sleepless nights, limitations in eating function, and time off school or work.

The prevalence of dental disease is high: the most common diseases affecting humans worldwide are dental caries and periodontitis. Dental caries is defined as being the result of a localized chemical dissolution of the tooth surface caused by acid production of the dental biofilm exposed to sugars [1]; periodontal diseases (gingivitis and periodontitis) are inflammatory diseases mainly of microbial origin. According to recent global estimates, 621 million children had untreated cavities in dentin in primary teeth and 2.4 billion people had untreated cavities in dentin in permanent teeth [2]. Severe periodontitis affected 743 million people worldwide [3].

Other common oral diseases are lesions of the oral mucosa. The most common head and neck malignancy is the oral squamous cell carcinoma (OSCC) [4]. Despite advances in therapies, the overall 5-year survival rate has remained unchanged during the past decades, mainly due to delayed diagnosis [5]. Oral health is a main part of general health and wellbeing. Good oral health is important in overall quality of life, social and self confidence [6]. However, oral diseases affect a significant proportion

of the world's population and exact a heavy toll in terms of morbidity and mortality [7]. The first attempt to decrease the incidents of most oral diseases is oral home care. Nowadays, primary home care instruments are mainly the manual and electric toothbrushes [8,9]. They are used to control and remove plaque formation. Dental plaque is composed by a biofilm of bacteria which is the primary cause of tooth and gingival diseases [10]. Although the mechanical effects of toothbrushes on plaque control, caries and periodontal diseases is well known, the prevalence of gingival inflammation is still high [11]. Antimicrobial mouth rinsing is usually used together with toothbrushing in reducing the gingival inflammation and controlling the plaque formation [12]. Chlorhexidine mouth rinses have been widely used and have been proved to be potent antiseptics [13]. Chlorhexidine-based mouth rinses are recommended especially in periodontitis affected patients, following oral and periodontal surgeries [14]. Although these instruments exert a positive action, some individuals find difficult their use, as in the case of patients admitted to the intensive care unit.

New Oral Care Device

The efficacy of new oral care device, which consists in a digital brush with a chlorhexidine-soaked gauze, was tested in the Clinic of Dental School, Polytechnic University of Marche. This instrument combines the mechanical action of a rough tissue (gauze) with the antimicrobial efficacy of the chlorhexidine digluconate. Although there are few articles about such device, the results of our paper showed that

the digital brush with a chlorhexidine-soaked gauze significantly reduced the bleeding index score after two weeks of use [15]. Moreover, this method has showed a beneficial effect on plaque control as to the one performed with a 0.12 % chlorhexidine mouth rinse. Furthermore, the appreciation test VAS significantly demonstrated appreciation of gauze rather than typical mouth rinse with chlorhexidine. In patients who are aware of oral health and brush well, this additional antibacterial tool could provide benefits in terms of plaque and gingivitis control: the digital brush system can also be a useful aide to toothbrushing in improving the oral hygiene status both in young patients and in non-collaborating or poorly motivated patients.

Diagnostic tools for oral lesions

Oral cancer is another significant oral disease. Oral cancer is the eighteenth most common malignancy worldwide [16]. In 2018, cancer of the oral cavity and lip afflicted three hundred and fifty thousand patients (2.0% of the total cancer cases) of which, one hundred and seventy seven thousand patients passed away from cancer of the oral cavity and lip [16]. Oral cancer has high mortality and morbidity in the patients, especially when discovered in late stages. For this reason, clinicians should pay attention to examine the whole mouth at the first appointment. Oral mucosa, especially in high prevalence geographic area, should be carefully inspected [17]. The early detection of precancerous lesions or cancers in the early stage increases the chances of reducing mortality and morbidity [18]. OSCC is often preceded by potentially malignant disorder (PMD), a group of clinically suspicious lesions. The

PMD can be classified in high-risk PMD or in low-risk PMD for their malignity power. Although most PMDs do not progress to OSCC, identifying the grade of the malignity is not easy and is a considerable challenge of the dental practitioners [19].

Thanks to the collaboration with the Oral Pathology Unit of our Dental School, diagnostic tools for oral mucosa lesion were reviewed. In recent years, several light-based detection systems (LBDS), based on optical properties of biological tissues, have tried to make the detection of PMD and OSCC easier. Preclinical studies have showed the potential of optical diagnostic technologies (e.g., Raman spectroscopy) or imaging techniques (e.g. Magnetic resonance imaging) to enhance diagnostic accuracy [20]. However, the following factor can limit their clinical uses: 1) data does not demonstrate clear superiority of these methods compared to conventional oral examination; 2) there remains the need for well-designed multicentre prospective studies; 3) these devices exhibit a not-negligible interobserver variability, limiting their use to clinicians with significant experience in oral pathology [21]. However, current evidences suggest improving these instruments with new approaches used to analyse optical imaging data, with the aim to quantify the results obtained. Moreover, they could be improved with more accurate methods in order to better understand the biological aspects of oral carcinogenesis. Finally, the possibility of implementing LBDS through the use of tissue-marking dyes could allow for new strategies for the use of nanoparticles. For example, a promising nanotechnology in oral diagnostic research is the quantum dots, consisting in nanometre-sized semiconductor crystals [22]. Compared with traditional immunohistochemistry (IHC), the quantum dots with

IHC is more accurate and precise at low protein expression levels and can achieve quantitative detection which could provide much more information for personalized treatment or for cancer identification [23,24].

The published review [25] analysed the advantages and limits of LBDS, and possible future applications of nanotechnologies. In our review, we concluded that, although the LBDS showed great potential for screening and monitoring oral lesions, there are several factors that hinder an extensive use of these devices. These devices seem to be useful in the identification of lesion margins which must be biopsied, thus assisting the surgical management. Moreover, we suggest, as others authors, that lowering the cost of these devices could indirectly lead to greater attention for oral lesions among both patients and general dental practitioners, allowing in turn to promote a culture of oral cancer prevention [21,26]. However, conventional oral examination and tissue biopsy remain the gold standard for OSCC diagnosis. The use of nanotechnologies could be the next step in the evolution of LBDS, thus providing devices that can help clinicians to better detect and monitor oral lesions because prevention and their early diagnosis improve their survival rate.

PAPERS REFERRING TO CHAPTER I

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ORIGINAL ARTICLE

Periodontics

WILEY

Assessment of antiplaque effectiveness of chlorhexidine-soaked gauze compared to chlorhexidine mouth rinse: Randomized clinical trial

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Abstract

Aim: The aim of the present study was to compare the efficacy of a disposable gauze soaked with chlorhexidine and rolled up on the finger vs chlorhexidine mouth rinse (CM) to maintain oral hygiene.

Methods: In this single-blind, randomized trial, both groups were instructed to brush their teeth and use the 0.12% chlorhexidine intervention twice per day (experimental group: toothbrushing + chlorhexidine-soaked gauze; control group: toothbrushing + mouth rinse). The main outcome was the change in full mouth plaque index scores from baseline to 2 weeks. Secondary outcomes were the patient's appreciation (visual analog scale) and the bleeding index.

Results: The final sample consisted of 60 young patients: 31 in the experimental group and 29 in the control group. After 2 weeks, both interventions were effective in reducing plaque index. The percentage of score reduction was 33% in the experimental group and 30% for the controls, with no significant difference between groups. Both interventions reduced the bleeding index, but this reduction was significant only in the experimental group ($P < .001$).

Conclusions: There is no difference between chlorhexidine-soaked gauze and CM regarding plaque control. In addition, gauze soaked with chlorhexidine was significantly more effective in reducing the bleeding index score and was appreciated by the patients.

KEYWORDS

chlorhexidine, mouth rinse, oral hygiene, plaque index score, prevention

1 | INTRODUCTION

Manual and electric toothbrushes are the primary tools for plaque control.[1,2] Despite the emphasis on mechanical methods of plaque control, the prevalence of gingival inflammation is high.[3,4] Interdentally cleaning is generally advised, but is not always effective. A number of studies have shown a reduction of gingival inflammation and better bacterial biofilm control if antimicrobial mouth rinsing is associated with toothbrushing.[5-7]

Chlorhexidine-based mouth rinses (CM) are widely used and have proved to be potent antiseptics.[8] In combination with brushing, CM are effective in controlling plaque and gingivitis.[8-13] Moreover,

the use of CM is recommended, especially in periodontitis-affected patients and after oral and periodontal surgeries.[14]

Oral hygiene using toothbrushes or mouth rinses alone can be difficult to perform in patients admitted to the intensive care unit. Nurses usually prefer to clean patients' teeth, gums, and tongue using cotton swabs or gauzes. Two recent clinical trials proposed to combine the use of such cotton tissues with .12% CM in mechanically-ventilated patients.[12,14] In addition, a recently-commercialized device, Digital Brush (DB), was introduced, which combines the mechanical action of a rough tissue (gauze) to the antimicrobial efficacy of the chlorhexidine digluconate.[15] Therefore, the primary aim of the present study was to control dental plaque in healthy young patients by comparing the efficacy of a gauze soaked with chlorhexidine digluconate and rolled up on a finger to the use of a commercially-available 0.12% chlorhexidine digluconate mouth rinse. Secondary aims were to assess antigingivitis action (by measuring the bleeding score) and patients' level of appreciation of the tested methods.

2 | MATERIAL AND METHODS

The study was carried out at the Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Ancona, Italy, between May and October 2013. The study protocol was approved by the local ethics committee (no.: 212497), and was registered in the Australian New Zealand Clinical Trials Registry (no.: 365043).

Participants were fully informed of study risks and procedures at the first visit, and were asked to sign informed consent according to the policies of the Marche Polytechnic University. The ethics statement was conducted in full accordance with the World Medical Association Declaration of Helsinki.

The participants were mostly medical, dental, and engineering students from Marche Polytechnic University. Each patient had to meet the following inclusion criteria: (a) age 18-35 years; (b) good general health; and (c) healthy periodontium or with slight gingival inflammation (evaluated by full mouth bleeding score [FMBS]: cut-off of 15%). The exclusion criteria were as follows: (a) diseases or medications that might interfere with the oral hygiene procedures or with oral tissues healing (e.g. diabetes, neurological diseases); (b) allergies; in particular, patients sensitive to tested products commonly used as dental materials; (c) antibiotic and anti-inflammatory therapy in the previous 6 months; (d) pregnancy and lactation; (e) patients with chronic (moderate or severe) gingival/periodontal disease; (f) people taking medications regularly (except contraceptives); and (g) individuals with severe dental disease (abscesses, tooth fractures, large cavities with compromised crown integrity).

Eligible participants were divided into two groups (Figure 1): the experimental DB group and the control CM group. The groups were randomly created by the statistical unit using a computer-generated random table,[16] and investigators were neither involved in the randomization process nor were they aware of the assigned group in all outcome evaluations. Randomly-assigned identification codes for each patient were printed on sealed boxes containing one of the two treatments: DB (Enacare, Micerium S.p.A., Avegno, Genoa, Italy) and 0.12% CM (Pharmatek, PMC S.R.L., Cremosano, Italy). In the pre-experimental phase, all

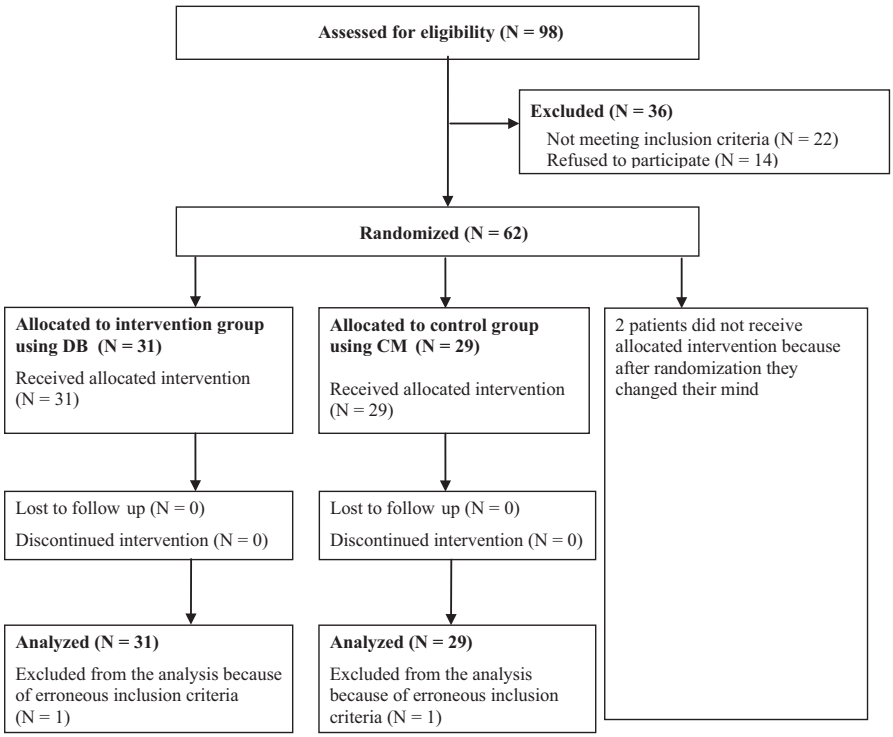

FIGURE 1 Flowchart of study participants. CM, chlorhexidine mouth rinse; DB, Digital Brush

eligible patients underwent a session of professional oral hygiene (mean duration: 20 minutes) using ultrasonics and polishing using rubber cups with prophy paste. They were instructed to perform normal, at-home toothbrushing for the next 7 days. Each patient was also recommended to discontinue any oral hygiene at home 24 hours prior to the next appointment (T_0). At baseline (T_0), 7 days after professional oral hygiene, all enrolled patients were evaluated for the full mouth plaque score (FMPS)[17] and the FMBS.[18]

All assessments were made by the same dental hygienist (SS), who has >10 years of experience, and who was unaware of each patient's group. The same examiner evaluated each patient throughout the trial. During each visit, the occurrence of potential adverse effects was assessed by investigators through both clinical survey and participant enquiry.

At T_0, right after the clinical examinations, the experimental DB group was instructed to use DB in addition to toothbrushing for 14 days, whereas the control CM group was instructed to use CM in addition to toothbrushing for 14 days. The experimental group had to perform at-home oral hygiene twice per day using a toothbrush (without toothpaste) associated with DB, by rolling it on the index finger, using circular motions from the gums to teeth, in each sextant (lasting approximately 30 seconds); the gauze has to be used only once. The control group received instructions to perform at-home oral hygiene twice per day with toothbrushing (without toothpaste) plus rinsing for 30 seconds using 10 mL of .12% CM. Group adherence was improved with: (a) instructions made to the participants; (b) explanatory demonstration of its use during the first appointment; and (c) daily patient diary. At this time (T_0), right after the procedure demonstration, patients' appreciation indexes were recorded by means of the visual analogue scale.[19,20]

At T_1, FMPS, FMBS, and appreciation indexes were re-evaluated.

The primary outcome was the difference across groups between the mean changes of plaque index scores (FMPS) from baseline to the end of follow up (T_1). According to a previous study,[21] we expected a mean reduction of 0.15 ± 0.15 in the control CM group, and 0.45 ± 0.45 in the experimental DB group. Assuming a 0.05 alpha error and an expected withdrawal/dropout rate of 5%, a minimum of 29 patients per group were needed to achieve 80% statistical power.

Wilcoxon matched-pairs signed ranks test was used to compare the mean plaque index (and other scores) before and after the intervention within each group. The differences between groups in plaque index or other scores at any time point were evaluated using Kruskal-Wallis test. The differences between groups in the rate of responses (reduction from baseline to the end of follow up of ≥.1 in the plaque index score, ≥1 in patients' appreciation score, ≥0.05 in bleeding index score) were evaluated using χ^2-test.

Statistical significance was defined as a two-sided *P*-value of <.05, and all analyses were carried out using Stata version 13.1 (Stata, College Station, TX, USA).[16]

3 | RESULTS

Of a total of 98 eligible patients, 62 agreed to participate, and were thus randomized, as shown in the detailed flow of the trial participants (Figure 1).

In the control CM group, two patients refused to participate immediately after randomization because they changed their minds. All other patients attended all examinations. The final sample consisted of 60 patients: 31 in the experimental DB group and 29 in the control CM group. The mean age was 22 years, and sex was equally distributed, with no differences in demographic variables or baseline plaque score between the groups (Table 1).

At T_0, both DB and CM significantly reduced the mean plaque index, which decreased by 33% in the DB group, and 30% in the control group (both $P < .001$) (Table 2). Such a reduction was not significantly different between the groups ($P = .7$).

In contrast, the participants' appreciation score increased significantly in the intervention group ($P = .028$), where the baseline mean value of 3.14 ± 2.08 increased to 4.45 ± 1.96 at the end of follow up ($P < .001$) (Table 2). Notably, the patients' appreciation score did not increase in the control group.

Regarding the bleeding score, 11 patients scored 0 at baseline in both groups and were not considered in the analysis. Within this limited individual sample, both interventions reduced the bleeding index during the 2 weeks of follow up, but this reduction was only significant in the experimental group ($P < .001$) (Table 2). The comparison between groups was not significant.

TABLE 1 Demographic characteristics of the sample by group

Sample characteristics1	Intervention[a](N = 31)	Control[b] (N = 29)	*P*-value[c]
Mean age in years (SD)	22.4 (4.0)	22.1 (3.3)	.8
Male sex, %	51.6	41.4	.4
Current smoker, %	38.7	34.5	.7
Mean no. daily coffees (SD)	2.0 (1.2)	1.8 (1.4)	.5
Oral contraceptives, %	12.9	13.8	.9

SD, standard deviation.
[a]Toothbrushing + Digital Brush.
[b]toothbrushing + .12% chlorhexidine digluconate mouth rinse.
[c]χ^2-test for categorical variables, t-test for continuous variables.

TABLE 2 Comparison between groups of the mean plaque index and other scores at baseline and end of follow-up

	Intervention (N = 31)	Control (N = 29)	Intervention vs control *P*-value[b]
Plaque index score (primary outcome)			
Score at T_0	0.56 ± 0.13	0.50 ± 0.16	.10
Score at T_1	0.38 ± 0.15	0.33 ± 0.12	.15
Comparison T_1 vs T_0 within groups (*P*-value[a])	<0.001	<0.001	
Difference between T_1 and T_0 score	−0.18 ± 0.12	−0.17 ± 0.15	.7
Reduction in means from T_1 to T_0 (%)	−32.7 ± 22.0	−30.4 ± 26.1	.7
Patients' appreciation score (secondary outcomes)			
Score at T_0	3.14 ± 2.08	4.10 ± 2.40	.10
Score at T_1	4.45 ± 1.96	4.00 ± 2.71	.5
Comparison T_1 vs T_0 within groups (*P*-value[a])	<0.001	0.8	
Difference between T_1 and T_0 score	1.37 ± 1.47	−0.10 ± 2.44	.007
Reduction in means from T_1 to T_0 (%)	102 ± 165	−19.2 ± 111	.028
Bleeding index score (secondary outcomes)[c]	(N = 20)[c]	(n = 18)[c]	
Score at T_0	0.12 ± 0.08	0.10 ± 0.15	.7
Score at T_1	0.06 ± 0.05	0.06 ± 0.06	.9
Comparison T_1 vs T_0 within groups (*P*-value[a])	<0.001	0.15	
Difference between T_1 and T_0 score	−0.06 ± 0.06	−0.04 ± 0.10	.5
Reduction in means from T_1 to T_0 (%)	−38.9 ± 47.6	−20.3 ± 63.0	.3

All values are expressed as means and standard deviations.
[a]Wilcoxon matched-pairs signed ranks test.
[b]Kruskal-Wallis test.
[c]only patients with bleeding scores >0 at baseline were included. T_0, baseline; T_1, after 2 weeks.

TABLE 3 Rate of responses (reduction from baseline to the end of follow up of ≥.1 in the plaque index score, ≥1 in patients' appreciation score, ≥.05 in bleeding index score) in each group

Test	Intervention (N = 31)	Control (N = 29)	*P*-value[a]
Plaque index score	77.4	65.5	.3
Patients' appreciation score	54.8	24.1	.015
	(N = 20)[b]	(N = 18)[b]	
Bleeding index score[b]	55.0	27.8	.09

[a]χ^2-test.
[b]11 patients had a bleeding index score of 0 in both groups at baseline, thus caution is required to interpret the results of this comparison.

When the rates of responses in the two groups were compared, we found similar results (Table 3). While no differences between the groups were observed in plaque and bleeding index scores, the patients' appreciation score increased by ≥1 point in significantly more patients receiving the compared with the controls (54.8% vs 24.1%, respectively; *P* = .015).

4 | DISCUSSION

Brushing daily is the primary method through which plaque can be removed. Indeed, several clinical studies have shown that an individual's ability to brush properly is an important factor, as is the type of toothbrush used, in terms of effectiveness.[22,23] Therefore,

a clinician's choice of the most suitable oral care method should be driven in part by a patient's motivation and dexterity.[24] Bacterial plaque control is still a problem, and some of the following issues should be considered: the need for methods that maximize comfort, degree of compliance of the consumer, lack of time, reduced dexterity, disinterest in own health, or lack of correct information of the patients.[24,25] Therefore, as highlighted by Watt and Marinho's, the promotion of oral health and interventions therein bring short-term reductions in plaque and gingival bleeding.[26] In addition, improving compliance to oral hygiene instructions with the use of psychological interventions has often proven to be weak and limited.[27] An individual's characteristics and behavior, as well as the environment in which this behavior is performed, all interact with each other.[11] As such, keeping patients motivated is very important; however, it can create frustrations if the technique is excessively complicated or boring, or if the objectives proposed appear unachievable.[18,19] The literature highlights the need for educational strategies,[24,25] and alternative oral care devices,[28,29] that can, at least partially, compensate for patient with a low grade of compliance.

The DB method investigated in the present study can be considered an auxiliary oral care device, which might be preferred by patients based on the following characteristics: it is easy and fast to use and can be used anywhere, it has been demonstrated to have antibacterial actions, and the mechanical action of the gauze rolled up on the finger might favor plaque removal from dental surfaces.[15] The chlorhexidine concentration used in the present study was 0.12% because, as highlighted in several studies, there is no evidence that one concentration of chlorhexidine rinse is more effective than another.[8,28,29] The present study confirms that, after 14 days of use (T_0- T_1), the added benefits of the DB method in reducing plaque and bleeding indices is consistent with the results reported by Lucchese et al. in 2012.[15] Although the latter article was not a randomized, clinical trial, the study involved a total of 120 patients, showing a significant reduction of dental plaque, evaluated by means of an erythrosine-containing detector, after 2 minutes of DB use, which is in agreement with the data of the present trial.

It is important to highlight that statistical analyses of the present study were obtained from a very homogeneous sample in terms of sex, age, and smoking habits. In the pre-experimental phase, all patients underwent a session of professional oral hygiene and used both devices (DB and MC) in addition to normal toothbrushing, as suggested by a similar previously-performed clinical trial,[30] thus undoubtedly obtaining a uniform, standardized sample.

To summarize, our findings demonstrated significantly reduced mean plaque indices in both the DB and CM groups, whereas better control of gingival inflammation was obtained in patients using DB compared to those using CM. It is also important to note the enthusiasm demonstrated by patients during the DB adjunctive regimen, supporting its utility in daily practice, especially for increasing compliance in patients struggling with traditional oral hygiene devices.

Although the differences in antiplaque efficacy between the DB and the CM groups were not statistically significant in the present study, the fact that the DB group had elevated patient compliance and appreciation scores, and exhibited the same efficacy in plaque control as that observed in the CM group, is very encouraging. Regarding the positive results obtained by DB in reducing bleeding scores, caution is required in interpreting these results, considering that 11 patients in each group were excluded from the statistical analysis because of bleeding scores at baseline being 0.

The present study has some limitations. First, the follow up was short, and this prevented evaluation of some side-effects of these materials, such as pigmentation and chlorhexidine staining.[8] Second, the patients were young, within the range 18-35 years, and had a good level of oral hygiene. The patients were mostly medical and dental students, who would be more aware of oral health care.[10] Therefore, generalizing the results is difficult, and it would be mandatory to plan further studies enrolling patients from the general population.

Regarding future trials, it would be interesting to compare the use of the DB and other digital tools as a substitute for toothbrushing. Indeed, it would be noteworthy to evaluate the effects of these tools, initially in periodontally-healthy patients, and then in patients with diverse systemic chronic diseases, elderly patients with several pathologies, and patients with physical or mental disabilities, who have difficulties in managing motor skills and find home dental care complicated.[13] Some of these individuals are not self-sufficient with oral hygiene procedures and often do not collaborate with caregivers, which create barriers in providing consistent and thorough oral hygiene instructions to these patients. Caregivers often resort to the use of cotton swabs and gauzes for cleaning patients' teeth and gums.[13,14]. However, presently, there are no other randomized, clinical trials to support or discourage the use of disposable gauze as a potential alternative to traditional mouth rinsing. Indeed, DB could represent a possible alternative to toothbrushing when there is low or no patient collaboration.

4.1 | Conclusion

The results of the present study showed that DB has a beneficial effect in plaque control, equivalent to a mouth rinse based on .12% chlorhexidine. Moreover, DB seems to reduce the bleeding index scores, after 2 weeks of utilization. Furthermore, the use of DB is significantly preferred compared to the CM. Introducing an additional antibacterial tool for patients who are aware of oral health and can brush well can provide additional benefits in terms of plaque and gingivitis control. The DB device can be suggested in association to toothbrushing for improving oral hygiene status in young patients, and could be beneficial when there is low or no patients collaboration.

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MINI REVIEW
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An Overview on Current Non-invasive Diagnostic Devices in Oral Oncology

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Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy, and despite advances in cancer therapies, the overall 5-year survival rate has remained below 50% over the past decades. OSCC is typically preceded by potentially malignant disorders (PMD), but distinguishing high-risk from low-risk PMD is challenging. In the last years, several diagnostic methods as light-based detection systems (LBDS) have been proposed to facilitate the detection of OSCC and PMD. Furthermore, the recent evolution of nanotechnology may provide new opportunities to detect PMD and OSCC at an early stage. Indeed, several preclinical studies showed the potential of nanotechnology to enhance diagnostic accuracy. For these reasons, it is fundamental to conduct studies to evaluate the efficacy of nanotechnology implementation in LBDS. The aim of this article is to review the current literature on LBDS and to provide a summary of the sensitivity and specificity of each technique, and possible future applications of nanotechnologies. The LBDS showed great potential for screening and monitoring oral lesions, but there are several factors that hinder an extensive use of these devices. These devices seem to be useful in assessing lesion margins that must be biopsied. However, to date, conventional oral examination, and tissue biopsy remain the gold standard for OSCC diagnosis. The use of nanotechnologies could be the next step in the evolution of LBDS, thus providing devices that can help clinicians to detect and better monitor oral lesions.

Keywords: light-based detection system, early diagnosis, OSCC, chemiluminescence, autofluorescence, nanotechnology

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy and the sixth most common tumour worldwide (Warnakulasuriya, 2009). Despite advances in therapies, the overall 5-year survival rate has remained unchanged during the past decades, mainly due to delayed diagnosis (Gomez et al., 2009). OSCC is typically preceded by potentially malignant disorders (PMD), a group of clinically suspicious lesions. Although the majority of PMD do not progress to OSCC, distinguishing high-risk PMD from low-risk PMD is challenging for dental practitioners (Yang et al., 2018). Furthermore, patients treated for OSCC are at risk of developing recurrences and secondary primary tumours, due to field cancerization and/or incomplete surgery (Day and Blot, 1992). Currently, conventional oral examination (COE), consisting in visual and tactile assessment of accessible oral structures, followed by tissue biopsy still constitutes the gold

standard for diagnosis of PMD and OSCC. However, there are some limitations of this procedure, such as sampling bias that can lead to underdiagnosis or misdiagnosis, particularly in multifocal lesions (Yang et al., 2018).

The possibility of making an early diagnosis is crucial for reducing high mortality rate and morbidity of OSCC patients. In the last years, several light-based detection systems (LBDS), based on optical properties of biological tissues, have emerged with claims of enhancing oral mucosal examinations and facilitating the detection of PMD and OSCC.

Furthermore, the recent evolution of nanotechnology may provide new opportunities to detect PMD and OSCC at an early stage (El-Sayed et al., 2005). Several preclinical studies showed the potential to enhance diagnostic accuracy of optical diagnostic technologies (e.g., Raman spectroscopy) or imaging techniques (e.g., Magnetic resonance imaging) (Chen et al., 2018). Among the latter techniques, reflectance confocal microscopy seems to improve the evaluation of oral lesions, by detecting backscattered light from illuminated tissue, producing high resolution tissue map. However, several technological limitations need to be resolved to validate diagnostic accuracy (Lucchese et al., 2016). LBDS showed several advantages compared to the aforementioned approaches, such as low cost and ease of use. For these reasons, it is fundamental to conduct studies to evaluate the efficacy of nanotechnology implementation in LBDS.

The aim of this article is to review the literature on LBDS currently on the market (**Tables 1**, 2), providing clinicians with a better understanding of their advantages and limits, and possible future applications of nanotechnologies.

ViziLite®

ViziLite® (Zila Pharmaceuticals, Phoenix, AZ, United States) is a chemiluminescence-based detection device designed to facilitate the early identification of PMD and OSCC. In 2002 ViziLite® became the first device approved by FDA for this purpose (Oh and Laskin, 2007). This is a disposable capsule formed by an outer shell of flexible plastic containing acetyl salicylic acid and an inner glass vial containing hydrogen peroxide. To activate it, the capsule is bent to break the inner glass vial, triggering the reaction of the chemicals contained in the two compartments. Consequently, a bluish-white light (430–580 nm) is produced,

TABLE 1 | Published studies on VELscope® for clinical detection of oral lesions.

Author and year	Patients	Type of lesion	Sens	Spec	PPV	NPV
Poh et al., 2006	20	OSCC	95%	–	100%	–
Lane et al., 2006	44	PMD, OSCC	98%	100%	100%	86%
Roblyer et al., 2009	65	OL	95.9%	96.2%	–	–
Jayaprakash et al., 2009	60	OL	72%	50%	76%	46%
Mehrotra et al., 2010	156	OL	50%	38.9%	6.4%	90.3%
Koch et al., 2011	78	PMD, OSCC	94%	16%	45%	77%
Paderni et al., 2011	175	PMD, OSCC	80.7%	97.5%	93.9%	91.3%
Awan et al., 2011a	126	PMD	84.1%	15.3%	37.8%	61.1%
Scheer et al., 2011	64	OSCC	100%	80.8%	54.5%	100%
Marzouki et al., 2012	85	OL	92%	77%	–	–
McNamara et al., 2012	130	OL	66.7%	6.0%	4.1%	75%
Farah et al., 2012	112	PMD	30%	63%	19%	75%
Rana et al., 2012	123	PMD	100%	74%	16.7%	100%
Hanken et al., 2013	60	PMD	97.9%	33.3%	85.5%	80%
Sawan and Mashlah, 2015	71	OL	100%	74.1%	46.4%	100%
Kaur and Jacobs, 2015	130	OL	67%	62%	29.8%	89%
Jane-Salas et al., 2015	60	OL	40%	80%	62.5%	66.7%
Elvers et al., 2015	20	PMD	–	–	–	–
Ayoub et al., 2015	30	Screening	–	–	–	–
Ohnishi et al., 2016	20	OSCC	91%	100%	100%	58.3%
Scheer et al., 2016	41	OSCC	33.3%	88.6%	33.3%	88.6%
Yamamoto et al., 2017	62	PMD, OSCC	85.9%	26.7%	83.3%	30.8%
Ganga et al., 2017	200	OL	76%	66.3%	24.4%	95.1%
Burian et al., 2017	90	OSCC	–	–	–	–
Huang et al., 2017	140	PMD, OSCC	98.3%	77.6%	91.7%	93.8%
Amirchaghmaghi et al., 2018	21	PMD, OSCC	90%	15%	40%	71%
Farah et al., 2018	11	PMD	–	–	–	–
Canjau et al., 2018	18	OL	94.4%	100%	100%	50%

OL, oral lesions; PMD, potentially malignant disorders; OSCC, oral squamous cell carcinoma; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value. Where possible, missing data were recalculated.

TABLE 2 | Published studies on light-based detection systems other than VELscope® for clinical detection of oral lesions.

Author and year	Device	Patients	Type of lesion	Sens	Spec	PPV	NPV
Huber et al., 2004	ViziLite	150	PMD	–	–	–	–
Ram and Siar, 2005	ViziLite	40	PMD, OSCC	100%	14.3%	80%	100%
Epstein et al., 2006	ViziLite	134	PMD	–	–	–	–
Kerr et al., 2006	ViziLite	501	OL	–	–	–	–
Farah and McCullough, 2007	ViziLite	55	OL	100%	0%	18.2%	–
Oh and Laskin, 2007	ViziLite	100	Screening	–	–	–	–
Epstein et al., 2008	ViziLite	84	PMD	100%	55%	37%	100%
Mehrotra et al., 2010	ViziLite	102	OL	0%	75.5%	0%	94.8%
Awan et al., 2011b	ViziLite	126	OL	87%	24%	15%	92%
Mojsa et al., 2012	ViziLite	30	PMD	57.6%	37.5%	79.2%	17.6%
Rajmohan et al., 2012	ViziLite	30	PMD, OSCC	85%	100%	100%	76.9%
Ujaoney et al., 2012	ViziLite	44	PMD	59%	78%	–	–
Vashisht et al., 2014	ViziLite	60	PMD, OSCC	95.5%	84.6%	91.3%	91.7%
Kammerer et al., 2015	ViziLite	44	PMD	100%	30%	26%	100%
Chaudhry et al., 2016	ViziLite	100	PMD	84.8%	41.2%	58.3%	70%
Sweeny et al., 2011	Identafi	88	PMD (white)	50%	98%	50%	98%
			(violet)	50%	81%	11%	97%
			(green)	0%	86%	0%	95%
Lane et al., 2012	Identafi	124	PMD	82%	87%	–	–
Messadi et al., 2014	Identafi	21	OL	–	–	–	–
Lalla et al., 2015	Identafi	342	screening	–	–	–	–
Lalla et al., 2016	Identafi	88	OL (white)	100%	100%	100%	100%
			(Violet)	27.5%	96.3%	61.1%	86.4%
			(Green)	40%	71.7%	22.9%	85.1%
McIntosh et al., 2009	Microlux/DL	50	PMD	77.8%	70.7%	36.8%	93.5%
Ibrahim et al., 2014	Microlux/DL	599	Screening	100%	32.4%	17.9%	100%
Moro et al., 2010	GOCCLES	32	PMD, OSCC	100%	93%	92%	100%
Moro et al., 2015	GOCCLES	61	PMD	96.9%	3.1%	50%	50%

OL, oral lesions; PMD, potentially malignant disorders; OSCC, oral squamous cell carcinoma; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value. Where possible, missing data were recalculated.

lasting for 10 min (Liu et al., 2016). A modified version (ViziLite® PLUS) consists of a combination of chemiluminescence and toluidine blue (TB) marking system, an acidophilic dye that selectively stains acidic substances such as DNA. Furthermore, an accessory eyewear has been developed, to allow better isolation of chemiluminescent light (Sambandham et al., 2013). Its clinical use requires a 1-min rinse of 1% acetic acid solution, to desiccate oral tissues, followed by oral examination with 430–580 nm wavelength light. The altered epithelial cells, due to higher nuclear/cytoplasmic ratio, reflect the light and cause the appearance of an "aceto-white" lesion, whereas normal cells appear blue (Nagi et al., 2016).

The first studies regarding ViziLite®, published in 2004–2007, were conducted on subjects with different clinical conditions, ranging from normal mucosa to diagnosed OSCC, with the aim to explore the diagnostic utility of chemiluminescence-based strategies (**Table 2**). In the first reported study, ViziLite® identified a subclinical lesion, suggesting its utility in identifying occult epithelial abnormalities (Huber et al., 2004). In a small cohort of patients with oral lesions, ViziLite® appears to be a better diagnostic tool than TB in detection of OSCC and PMD (Ram and Siar, 2005). Another study highlights the ability of ViziLite® to show brighter and better demarcated lesions than using incandescent light, aiming to enhance the identification of lesions that could be biopsied (Epstein et al., 2006). Unfortunately, these results have not been confirmed, which failed to demonstrate significant improvement in identification and evaluation of oral lesions (Farah and McCullough, 2007; Oh and Laskin, 2007). Interestingly, a cross-sectional study compared ViziLite® and VELscope® to evaluate their clinical utility in diagnosing oral lesions, but the authors failed to demonstrate any superiority to COE (Mehrotra et al., 2010).

For this reason, a new version of this device has been developed (ViziLite® PLUS), aiming to improve the diagnostic power of TB marking system. First results were encouraging, showing that TB reduced the number of false positive cases leaving the false negative rate unchanged (Epstein et al., 2008). On the contrary, ViziLite® PLUS does not seems to be useful to detect malignancies in patients with clearly visible lesions (Mojsa et al., 2012). In fact, some authors described the better diagnostic accuracy of ViziLite® with respect to TB staining alone (Rajmohan et al., 2012; Vashisht et al., 2014), justifying the combined use of these two techniques.

Recently, the results of a clinical study suggested that, although the adjunct of TB to ViziLite® reduced the false positive cases without increasing the number of false negatives, there are little benefits in using this device in general dental practise (Chaudhry et al., 2016).

In conclusion, despite the fact that ViziLite® facilitates the identification of hyperkeratotic areas and may increase the visibility of mucosal lesions, the main limitation is currently the high proportion of false positive and false negative tests, regarding the identification of dysplastic areas rather than hyperkeratosis (Chhabra et al., 2015).

VELscope®

VELscope® (LED Medical Diagnostics, White Rock, BC, Canada) is a hand-held non-magnifying device for direct visualisation of oral mucosa autofluorescence that became commercially available after FDA approval in 2006 (Ayoub et al., 2015). No need of technical measures, such as the use of dimmed light, pre-rinse or lesion-marking solutions, make VELscope® easy to use. It uses a 120 W arc-lamp and a series of philtres and reflectors optimised for producing 400–460 nm wavelength light. The light emitted reaches oral mucosa and excites endogenous autofluorescence substances, called fluorophores (Yamamoto et al., 2017). Preliminary studies, regarding small groups of patients, gave encouraging results (**Table 1**). In the first reported study, 44 patients with confirmed oral dysplasia or OSCC were evaluated with both COE and VELscope®. The results showed that the device can differentiate PMD and OSCC from normal oral mucosa, with high sensitivity and specificity levels (Lane et al., 2006). These results were confirmed in a small OSCC cohort study, in which the use of autofluorescence-guided examination was able to identify subclinical high-risk fields with cancerous changes (Poh et al., 2006). In a study conducted on 60 patients using a semi-quantitative grading system for autofluorescence, VELscope® demonstrate good sensitivity and a better ability to recognise high-grade lesions than COE (Jayaprakash et al., 2009). Another study evaluated 65 subjects with VELscope®, using a specific algorithm based on the ratio of red-to-green fluorescence. The authors found that 405 nm wavelength light was able to discriminate neoplastic and non-neoplastic tissue with high sensitivity and specificity (Roblyer et al., 2009).

In a cross-sectional study, 175 patients with at least one clinical lesion were evaluated using VELscope®. However, despite the good results, the authors warned that this device could lead to overdiagnosis if used by non-specialists (Paderni et al., 2011). In fact, in the following years several studies on patients with PMD or OSCC reported low specificity values, highlighting this as the primary limitation of VELscope® (Awan et al., 2011a; Koch et al., 2011; Scheer et al., 2011). For these reason, other authors concluded that VELscope® examination alone does not provide significant diagnostic benefit beyond COE in screening for PMD and OSCC, also due to interobserver variability (Farah et al., 2012; McNamara et al., 2012). These results were confirmed by a study on 200 patients, limiting the utility of autofluorescence for OSCC screening (Ganga et al., 2017).

One effort to overcome these shortcomings consists of adding the VELscope® exam to the COE. Indeed, as reported by several authors, the combination of COE and VELscope® examination in patients with oral lesions could provide a significative diagnostic yield (Marzouki et al., 2012; Rana et al., 2012; Hanken et al., 2013). However, these results must be interpreted carefully due to the different inclusion and exclusion criteria used to select the patient cohorts, which can influence both sensitivity and specificity.

Other studies focused on combining VELscope® and other diagnostic tests, aiming to find out better approaches to improve detection of PMD and OSCC. For example, the combination approaches of tissue autofluorescence and salivary protoporphyrin IX levels seems to be effective to distinguish between normal mucosa and high-risk lesions (Kaur and Jacobs, 2015). The use of quantitative analysis of autofluorescence were developed to solve the problem of interobserver variability. Novel methods such as quadratic discriminant analysis or luminance ratio were promising, showing a strong concordance with histopathological diagnosis (Huang et al., 2017; Yamamoto et al., 2017). Recently, a retrospective study based on oral photograph was conducted to find colour distribution patterns related to neoplastic lesions. The fluorescence analysis showed differences in the red-to-green ratios of neoplastic areas, suggesting its clinical utility to detect early OSCC (Burian et al., 2017).

Recently, tissue autofluorescence was used to investigate biological aspects of oral carcinogenesis. In the first *in vitro* study, VELscope® was used to investigate the autofluorescence in a rat tongue carcinogenesis model. The results showed significant changes in autofluorescence pattern during progression to dysplasia and carcinoma (Ohnishi et al., 2016). In another study, RNA sequencing technique was used to identify molecular differences related to autofluorescence patterns. Results were encouraging, demonstrating that the autofluorescence-based excision was successful in achieving a clear molecular margin when excising PMD (Farah et al., 2018). These results confirmed those previously reported in literature, in which VELscope® demonstrated that the actual sizes of some lesions are significantly larger than they look clinically (Elvers et al., 2015).

In conclusion, several criticisms have been made about VELscope®, mainly focused to the limited capacity to extend the use of this device in general dental practise. Future research directions are aimed at improving the specificity of this device, allowing wider clinical use of VELscope® in routine general practise (Bhatia et al., 2013).

Identafi®

Identafi® (StarDental - DentalEZ, Lancaster, PA, United States) is a probe-like device designed for multispectral screening of PMD, approved by FDA in 2009 as oral screening device (Vigneswaran et al., 2009). Identafi® has three light sources of different wavelengths: white, violet (405 nm), and green-amber (545 nm) lights, that can be sequentially used in oral examination. While white light provides classical visualisation of oral mucosa, violet light excites endogen fluorophores, enabling the assessment of mucosa autofluorescence, like VELscope®. Green-amber

light, through the reflectance spectroscopy, excites haemoglobin molecules in the blood, with the aim to visualise the vasculature (Messadi et al., 2014). A mirror is attached to the probe to help visualise relatively obscure areas in oral cavity.

The first clinical trial with Identafi® was conducted on 88 patients who were treated previously for OSCC (**Table 2**). Screening results with white, violet, and green lights were compared to each other, showing limited benefits of tissue reflectance and autofluorescence in detecting high-risk lesions (Sweeny et al., 2011). In 2012, was reported a case series of PMD patients with the aim to evaluate the efficacy of Identafi®. Although the results are not clearly described, this device seems to be helpful in identifying characteristics not otherwise visible to the COE (Lane et al., 2012).

In a pilot study, Identafi® was used to evaluate tissue vascularity of PMD and to compare with the histological grading of the lesions using a vascular marker (CD34). The results found a correlation between tissue reflectance and histological assessment of vascular structure, in both OSCC and non-cancerous lesions (Messadi et al., 2014).

Two studies on the effectiveness of Identafi® were conducted on Australian population. In the first one, 342 urban Indigenous community members were screened for oral lesions using reflectance spectroscopy and autofluorescence imaging. Identafi® improved the visibility of oral cavity lesions and was capable to find new lesions not seen during COE, although the prevalence of oral pigmentation in this community could hamper the use of autofluorescence screening systems (Lalla et al., 2015). In the second study, 88 patients were evaluated with Identafi®, showing good specificity, negative predictive value, and accuracy (Lalla et al., 2016).

Taken together, the use of Identafi provide the clinician with more data than COE. Unfortunately, the results interpretation requires high level of experience and clinical training in oral pathology, suggesting that its usage should be limited to reference centres for oral pathology (Lalla et al., 2016).

OTHER DEVICES

Microlux/DL™ (AdDent Inc., Danbury, CT, United States) is a chemiluminescence-based device which became commercially available after FDA approval in 2005. This device has a diffused blue-white LED light source and a fibre optic light guide (McIntosh et al., 2009). It uses the same principles of ViziLite®: after 1-min rinse with 1% acetic acid, oral examination is performed with 460–555 nm wavelength light. Altered epithelial cells cause the appearance of "aceto-white" lesions, and LED light source makes the lesion more easily recognisable. Furthermore, the use of TB can be used in conjunction with Microlux/DL™, to enhance the visualisation of dysplastic areas (Ibrahim et al., 2014). In 2009 was conducted a study on 50 patients with oral white lesions to evaluate the efficacy of Microlux/DL™. The results showed that this device can enhance visualisation of oral mucosa, but no clinical improvement was observed, due to poor ability to distinguish between benign and malignant lesions (McIntosh et al., 2009). Another trial that evaluated the effectiveness of Microlux/DL™ was carried out in 2014. 599 patients were examined with COE and Microlux/DL™ with and without TB, showing high sensitivity but low specificity, indicating that this device is not effective to distinguish between benign and malignant lesions, although seems to be a promising screening test for oral lesions (Ibrahim et al., 2014).

GOCCLES® is a medical device (Pierrel S.p.A, Italy) approved by FDA in 2015. This is a low cost and easy-to-use device consisting in a pair of glasses equipped with special optical philtres that allows autofluorescence detection. Indeed, GOCCLES® was created to provide an easy and low cost mean of identification of autofluorescence abnormalities in oral cavity with the use of any dental curing light (Moro et al., 2015). In 2010 was reported the first study on GOCCLES® in a small cohort of selected patients, showing high sensitivity and specificity (Moro et al., 2010). Five years later, a non-randomised multicentre trial was conducted on patients at risk for OSCC, suggesting the need for further researches to define the diagnostic performance of this device (Moro et al., 2015). Indeed, despite the low cost of GOCCLES® could encourage more careful examinations, its main limitation seems to be the interobserver variability, that could be overcome by proper training.

In recent years, other instruments have been developed and commercialised for facilitate the early identification of oral lesions. Their operating principle is equivalent to the devices described above, using either autofluorescence or chemiluminescence detection. However, their clinical effectiveness is currently hampered by the lack of published studies. For these reasons, they will only be mentioned briefly here. Bio/Screen® (AdDent Inc., Danbury, CT, United States), an instrument with five violet (390–430 nm) high-power LED, designed to enhance the visualisation of mucosal abnormalities through the use of tissue autofluorescence (Kahn et al., 2018).

Orascoptic DK™ system (Orascoptic, Middleton, WI, United States) is another chemiluminescence-based device, designed to improve the visualisation of oral lesions through the use of blue-white LED light and oral rinse of 1% acetic acid solution (Patton et al., 2008).

Sapphire® Plus LD (DenMat Holdings, Lompoc, CA, United States), DentLight DOE™ Oral Exam System (DentLight, Richardson, TX, United States), and OralID™ 2.0 (Forward Science Technologies, Stafford, TX, United States) are other tissue autofluorescence-based devices developed in order to detect oral lesions (Kahn et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

The diagnostic techniques presented here showed great potential for screening and monitoring oral lesions (Liu et al., 2016). Unfortunately, to date several factors hinder an extensive use of these devices: (1) data do not demonstrate clear superiority of these methods compared to COE; (2) there remains the need for well-designed multicentre prospective studies; (3) these devices

exhibit a not-negligible interobserver variability, limiting their use to clinicians with significant experience in oral pathology (Patton et al., 2008; Carreras-Torras and Gay-Escoda, 2015).

However, the current evidence suggests that these devices: (1) seem to be useful in assessing lesion margins that must be biopsied and, therefore, may be useful in surgical management; (2) can be used to investigate biological aspects of oral carcinogenesis, leading to more accurate methods for interpreting data from LBDS; (3) can be enhanced with new approaches used to analyse optical imaging data, with the aim to quantify the results obtained; (4) lowering the costs of these devices could indirectly lead to greater attention for oral lesions among both patients and general dental practitioners, allowing in turn to promote a culture of oral cancer prevention (Carreras-Torras and Gay-Escoda, 2015; Moro et al., 2015); (5) finally, the possibility of implementing LBDS through the use of tissue-marking dyes can in principle allows to develop strategies for the use of nanoparticles. Indeed, nanoparticles can provide molecular targeted imaging, with higher image contrast and resolution. For example, a promising nanotechnology in oral diagnostic research is the quantum dots, consisting in nanometre-sized semiconductor crystals (Walling et al., 2009). The biophysical characteristics of these particles confer several advantages over conventional dyes and fluorescent proteins. The possibility to link the quantum dots to molecules with the ability to target cancer cells make them ideal for diagnostic applications in detecting PMD and OSCC (Chen et al., 2018). Therefore, the use of nanotechnologies could be the next step in the evolution of LBDS, providing devices that can help clinicians to detect and better monitor oral lesions.

AUTHOR CONTRIBUTIONS

MM, AS, and MP conceived the literature review. MM and VT described the VELscope® system. AS and RM described the ViziLite® system. AB and GO described the Identafi® system. AP, MP, and GO wrote the concluding remarks. All authors discussed and approved the final version of the manuscript.

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CHAPTER II:

Restorative Dentistry

RESIN-BASED MATERIALS

More than five hundred million direct dental restorations are carried out every year around the world, representing one of the most prevalent medical interventions in the human body [27]. Nowadays, resin-based materials (RBM) are the elective materials in restorative dentistry. The properties of RBM are material dependent and can be influenced by components such as the chemical and molecular structure of the matrix, and the size and material of the fillers. According to their formulation and particular requirements, RBM can be distinguished into restorative, sealant, cement or provisional materials.

The composition of RBM has evolved significantly since the materials were first introduced to dentistry more than 50 years ago, and they are continuing to change rapidly. Until now, the most important changes have included the reinforcing filler, which has been reduced in size in order to produce materials that are more easily and effectively polished. Other important changes have focused on the polymeric matrix, developing systems with reduced drawbacks, such as polymerization contraction and polymerization shrinkage stress. The interest in these RBM is based on their versatile and different applications. RBM can be classified according to their clinical uses as follows: restorative materials, cavity liners, pit and fissure sealants, cores and build up, luting cements for indirect restoration such as inlays, onlays, crowns, provisional restorations, endodontic sealers, and root canal posts. In addition, RBM together with

the adhesion systems provide the basis of minimally invasive dentistry, in which the preservation of much dental tissue as possible is the final aim.

Resin-Based Materials Composition

RBM are composed of the following elements: polymeric matrix and its monomers, reinforcing fillers, silane coupling agent used for binding the filler to the matrix, and chemical agents which active or modulate the polymerization reaction.

Monomers

The first monomer used for dental composites has been Bis-GMA: it is the ‘’Bowen’’ monomer bisphenol A glycidyl methacrylate (BisGMA). It is synthesized from bisphenol A and glycidyl methacrylate, and is widely used in resin-based composites, because its highly rigid core and relative hydrophobicity. However, due to its high viscosity, Bis-GMA is often used together with Triethylene glycol dimethacrylate (TegGMA) [28].

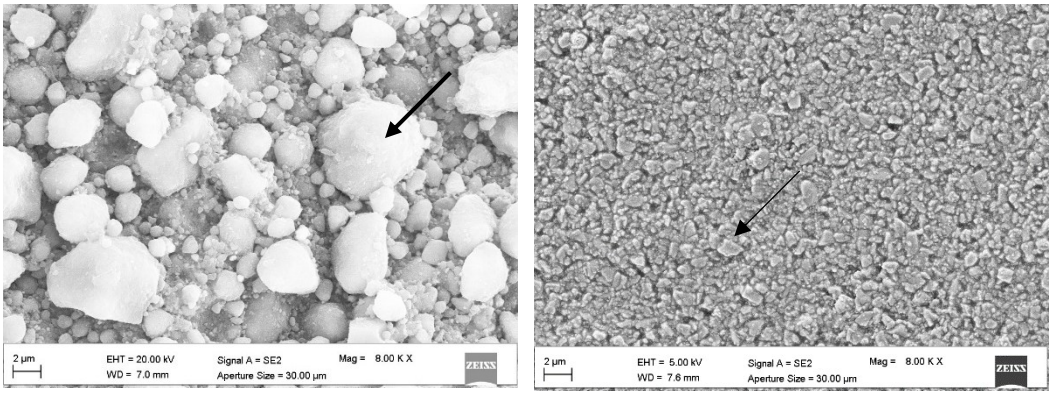
Currently, new blends have replaced Bis-GMA/TegGMA combination, adding or changing the concentration of monomers to overcome their drawbacks: for example, silorane and thiolene systems have been introduced to decrease the polymerization stress. The market is trying to develop new monomers due to the low stability and the release of bisphenol A, which may exert a cytotoxic effect [29,30]. For these reasons, monomers such as urethane dimethacrylate (UDMA) have been developed.

In addition, innovative phosphate monomers have been introduced in the market, such as 10- MDP (methacryloxydecyl dihydrogen phosphate). 10-MDP is a hydrophilic monomer with mild-etching properties pH (~2-3) which can be used in the so-called universal adhesives and specific resin cements [31]. The very peculiar characteristic of these molecules consists in the capacity to chemically react with a wide range of dental substrates and dental materials [32].

Filler content

The filler content is dispersed in the resin matrix in order to increase the mechanical properties of the RBM (Fig. 1). The fillers work as reinforcement; increasing the elastic modulus of the resin composite, providing wear resistance, improving fracture toughness, and giving radiopacity. Moreover, the inorganic filler particles influence handling properties which are adopted for shaping and sculpting during clinical use [33]. The different fillers can be described by their type (glass composition), morphology (size distribution and shape), density, radiopacity, refractive index, and

Fig. 1: Microscopic views of different filler content in resin-based materials. The black arrow indicates filler particles on the surface. Magnification: 8000x. Scale bar 2 µm



surface porosity [34–36]. The first fillers to be introduced were large micron-sized particles (> 10 μm). The need for improved cosmetic results, polish ability, and higher mechanical properties led to a decrease in average particle size (1–10 μm) and more refined particle size distribution. In recent years, RBM with submicron average particle sizes have been marketed (0.2–1 μm). The nanoparticles, typically silica, act in part as viscosity modifiers and display sizes in the range of 10– 40 nm but are rarely observed as discrete, non-agglomerated particles. However, RBM often contain more than one size and type of fillers (mix) .

Functional silane chemistry

In RBM, resin matrix and fillers have different properties and hardness. Therefore, a linker between them is needed in order to achieve more stability of the polymer. For this reason a silane agent (3-methacryloxypropyltrimethoxysilane) is used to promote the interaction and the link between monomers and filler particles, distributing the stress upon both materials [37]. Most of the silane used in dentistry is a bi-functional molecule with two different sides: one side exhibits one or more acrylic reactive group to bind with the resin phase; the other side, hydrolysable $Si(OCH_3)_3$, can react with conventional silica-based glass particles [38].

Initiators in photopolymerization

Generally, RBM are in a gel form enabling clinicians to model them. In order to harden the material, an activator is required to start the curing process, either through

light or a chemical activator:

- The conventional polymerization is a photopolymerization. The camphoroquinone (CMQ) is commonly used as photo-activator: CMQ absorbs over the 400-500 nm range with a pick of absorption at 470 nm and reacts with an initiator, usually a tertiary amine. Then, this combination CMQ-amine starts a free-radical polymerization process. Since the introduction of new curing lamps in the market, different activators have been suggested, such as german-based molecule and phenylbis (2,4,6-trimethylbenzoyl) phosphine oxide. However, the activation system can influence the final shade of RBM: for example, the CMQ imparts a yellowish colour to resin composite [39,40].
- The chemical polymerization is typically found in resin luting cement, mainly where light transmission could be a problem, for example in the post-endodontic restoration or below an indirect restoration. The curing process usually starts when two pastes are mixed, in particular benzoyl peroxide and tertiary amine. Inhibitor molecules could be added to the composition in order to allow an appropriate working time and prevent premature hardening.

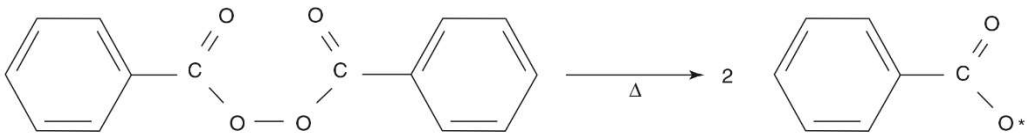
Properties of Resin-Based Materials

The clinical success of RBM depends on their properties, such as the degree of conversion, microhardness, colour stability and biocompatibility [41]. Their brief description will follow:

Degree of conversion

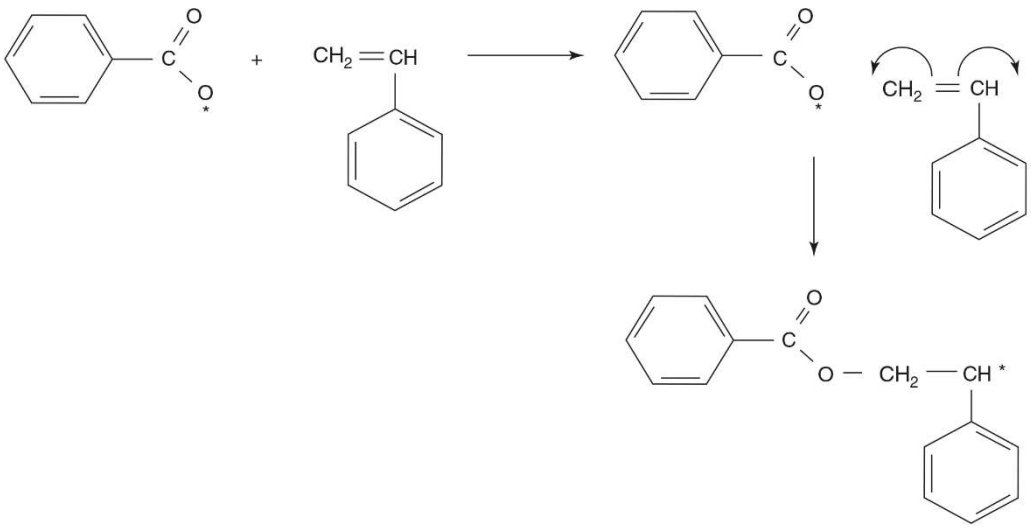
During the polymerization, the carbon double bonds of monomers open and link with other monomers` opened double bonds to form a carbon single bond, converting from monomer to polymer. During the curing process, the total double bond decreases and the material starts to harden. Therefore, the degree of conversion (DC) can help the clinician to evaluate the non-reacted double-bonds. The majority of monomers used in dental materials cure through a chain-growth mechanism, more specifically, a radical chain polymerization [42]. In chain-growth polymerizations, the reaction starts when a chemical or physical activator triggers the initiator. It produces reactive species (free radical, cation, or anion) which can propagate and promotes successive additions of monomer molecules in a chain reaction. Chain-growth reactions take place between monomers containing a single type of functional group, commonly a carbon-carbon double bond (π-bond) [33]. The reactive species opens the π-bond forming a new radical (a macroradical) allowing the addition of a monomer molecules. In this case, the monomers react only with the reactive centre allowing the addition of more monomer molecules in the polymer chain. Therefore, the monomer will form a very large and complex network. However, the impeachment and the rigidity of the network could cause an incomplete monomer conversion [43]. The polymer chain growth is interrupted when the centre is finished by an appropriate reaction depending on the type of reactive centre and the reaction conditions. In general, the polymerization chain shows three phases (Figures from [44]):

1. Initiation: the activator (by thermal, photoactive or chemical factor= **Δ**) activates the initiator producing reactive centres.



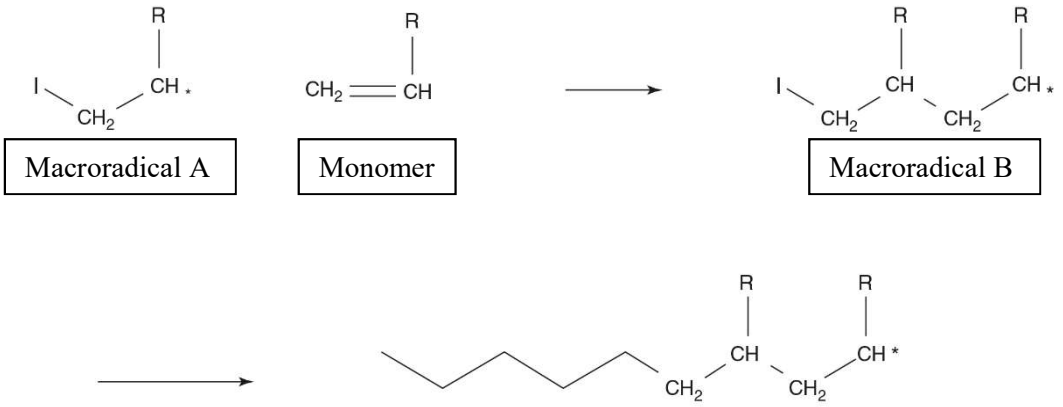
Initiator | Reactive center

The reactive centres react with the monomer molecule, breaking carbon double bonds leading to the formation of a macroradical:



Macroradical

2. Propagation: the reactive centre is propagated between the monomer molecules, and each monomer molecule addition produces a new reactive centre. The polymer chain grows at high speed:



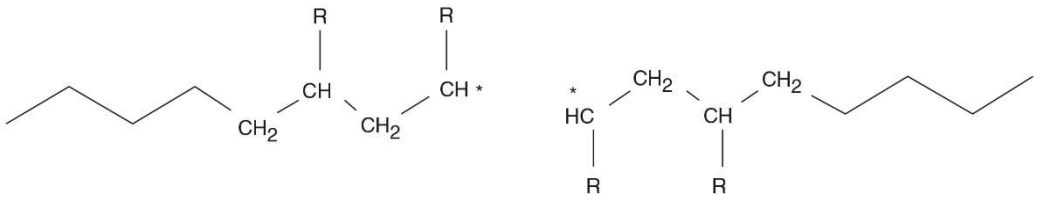
Macroradical A | Monomer | Macroradical B

Polymer chain

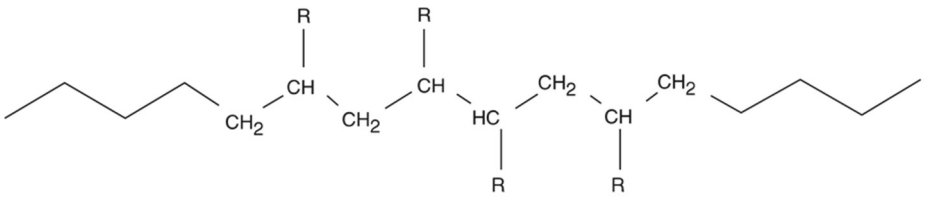
3. Termination: the reaction finishes when a combination of macroradicals or disproportionation reactions happens, assembling a polymer.

Example of possible combinations between two macroradicals:

1)

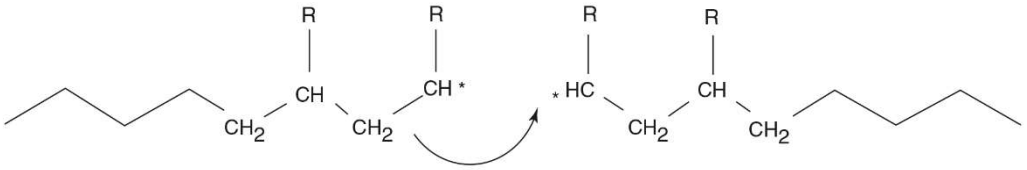


Macroradical A | Macroradical B



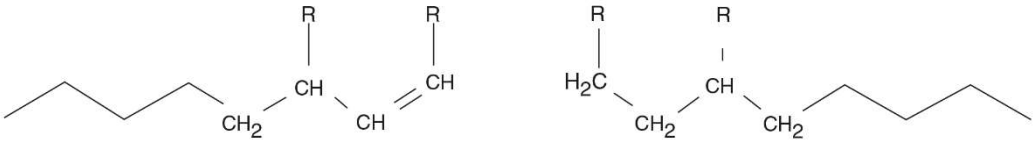
Polymer C

2)



Macroradical A

Macroradical B



Polymer C

Polymer D

The monomer composition influences the mobility of the reactive medium (C=C) during polymerization, consequently influencing the final DC and polymer structure. DC does not mean degree of hardness: two materials can have the same DC but different mechanical properties [45,46]. However, a high DC influences general chemical and physical properties of RBM, as well as reducing the effects of residual monomer in the organism. The DC is a percentage value and, in this study, is evaluated by spectrometer. More formula exists to calculate the DC.

Microhardness

The surface hardness (measured as microhardness, by means of Vickers or Knoop test) is defined as the resistance to permanent indentation or penetration on time. Physical

characteristics of restorative materials strongly influence the clinical longevity of restorations [47–49], mainly when the RBM are on the occlusal surface [50,51]. A low surface hardness value is largely related to inadequate wear resistance and an inclination to scratch, compromising fatigue strength and leading to failure of the restoration [52,53].

Colour and shade stability

In modern society, teeth colour is more relevant than before. The colour of RBM, in particular in resin composites, has acquired an important role in determining restoration success and the colour mismatch between the restoration and the tooth can be considered as failure. The colour is formed by three parameters [54]: hue describes the type of colour whereby we can recognise red from green, blue from yellow; chroma is the degree of colour saturation in the same hue; value is the lightness, which means the degree of luminosity of colour. Value is the most important factor, followed from chroma and hue. One of the papers I authored focused on the tools and tricks to catch the right tooth colour. In clinical practice, the dentist can evaluate the teeth colour by a subjective or objective method [55]. The subjective method evaluates the tooth colour with a shade guide by personal colour perception. For example, the shade guide, the tooth and the restoration material can have different degrees of translucency and can be differently influenced by the background (background effect).

The objective method is another way to evaluate the colour, using instrumental evaluations, such as Digital Camera, Colorimeter and Spectrophotometer. Although

the objective method is an instrumental evaluation, the accuracy of such method is around 80% [56]. However, in both subjective and objective methods, clinicians estimate the tooth colour with an artificial shade guide, which often has a different colour compared to the restorative materials. Our suggestion was to make a custom-made shade guide with the restorative materials in order to have a preview of their colour properties and shade. Moreover, such a guide, if shared, can improve communications between the dentist and the technician. A smartphone app, still not available on the market, will be the new frontier in colour evaluation. The clinician could compare directly on the mobile phone the tooth with a vast variety of different brands of composites, at different thickness. Moreover, it would be a portable, cheap and easy way to evaluate and share the teeth colours with technicians and colleagues.

Biocompatibility

Biocompatibility can be defined as a favourable host response to external materials. In our case the inner part of the tooth, the dental pulp, as well as the gingiva are the appropriate hosts [57]. RBM are composed of various components and, mainly, the monomers of the matrix could be released in an aqueous environment. Therefore, a low DC is detrimental to the biocompatibility of the RBM [58], since unreactive monomers and reactive components are free to react with other systems [59]. High value of DC leaves less free monomer and produces a denser network which reduces water diffusivity [60]. Few studies exist regarding the direct contact between the new RBM and human cells, since a lot of study used mouse cells [61]. Preliminary data

regarding the direct contact between newly-developed dental materials and different human cells were made at Institute of Oral Biology of University of Zurich (see Appendix).

Clinical Uses

RBM are versatile substances. Previous materials, such as amalgam, required a retentive cavity to be mechanically placed. On the other hand, the RBM can be glued to the tooth substrates by an adhesive system and no retention is required. Nowadays, RBM have influenced the entire dental field: endodontic, operative, orthodontic and prosthodontic treatment. The following RBM were analysed in the next paragraph:

-Resin composites: they are used in restorative dentistry to rebuild the tooth after decay, erosion or fracture. These materials can be used to modify the anatomy and fill the cavity. Mainly, the new bulk fill composites can be easily applied using a unique layer (bulk), simplifying tooth restorations in posteriors, thus maintaining good mechanical properties.

-Resin luting agents: these materials are often used to lute indirect restoration to the tooth, filling the space between them. Some of them can be able to cure under different thickness which could attenuate the curing light.

RESIN COMPOSITES

The term “composite material ” means a material with at least two distinct components. Moreover, they should be insoluble in each other but produce a material with different, often, better properties than the individual components alone. The new type of resin composite available in today’s market, which can be recommended especially in posterior regions, is the bulk fill composite.

Focus on Bulk Fill Composites

Bulk fill composites are the last generation of resin composites. The main innovation of these materials is the ability to cure also layers 4 mm-thick. Previous resin composites cured at a maximum 2 mm thickness and required a multi-layering technique, allowing a good light-curing penetration and a low shrinkage stress [62,63]. Such a procedure could cause air bubbles, lack of linkage between material layers and troubles during resin composites placing. Moreover, the placement of such materials require a long chair time, especially in the posterior areas, where the cavities are difficult to fill [64]. The development of bulk fill composites tried to overcome the above-mentioned drawbacks and simplify the restorative procedures.

Chemical and mechanical evaluations

A consistent part of my PhD research project aimed to evaluate the chemical and physical properties of the new bulk fill composites [45]. The performance of different

curing lamps and the DC at different cavity thickness were also evaluated. Five commercial bulk fill composites for posterior restorations were tested. The samples were divided into two groups and cured with different lamps. The DC was evaluated both on top and bottom of the samples to clarify the conversion capacity of the materials and the Vickers Micro Hardness (VMH) was measured on the irradiate surface samples. In summary, all the bulk resin composites can be used at 3.0 mm of thickness. The medium and high viscosity bulk fill materials can safely be used on an occlusal surface since they have a high VMH with both curing lamps. On the other hand, although low viscosity bulk resin composites reached a high DC, they are not suitable for being placed up to the occlusal surface and capping protection is recommended. Moreover, no correlation between DC and VMH was found. This study can be useful to increase the knowledge about both the curing performance of bulk fill composites and different curing lamps.

Effect of polishing procedure

In clinical practice, after the curing phase, the resin composites need to be finished and polished. Finishing procedure indicates the contouring or reduction of restoration in order to obtain an ideal anatomy and polishing procedure reduces the roughness created by finishing and increase the gloss material [65,66]. In clinical practice, clinicians finish and polish resin composite in order to: remove excess material, fit the occlusion, obtain a smooth surface, and improve the aesthetics of resin composites [67]. Moreover, these procedures influence the longevity of restorations [68].

Although a few reports have investigated the chemical and physical properties of resin composites after using modern finishing and polishing systems, no studies have evaluated the timing effect of the polishing system on bulk fill composites containing methacrylate and dimethacrylate monomers [65,68–71]. The aim of our study (presently under review) was to evaluate DC and VMH of different bulk fill composites after immediate and delayed polishing. In summary, polishing procedure influenced the chemical and physical properties of bulk fill composites. This could depend not only on the type and size of the filler but also on the type of monomers. However, the effect of polishing procedure performed immediately after curing does not reduce the chemical and physical properties of the bulk fill composites. In some cases, the immediate polishing procedures may even increase their physical and chemical properties, reaching the same values of delayed polishing procedures. Our results suggest that some composites can be polished immediately after curing in order to finalize the restoration in a one-chair appointment.

RESIN LUTING AGENTS

(Draft Paper: Spectroscopic evaluation of two curing protocols during adhesive cementation using different luting agents)

The development of reliable adhesive systems between the tooth and RBM has led to more conservative dental treatments. Before the introduction of modern adhesive materials, dentists prepared teeth in order to have mechanical retention, and in doing so, often sacrificed healthy dental tissue. The era of adhesive cementation brought about minimally invasive dentistry, in which the clinician could lute indirect restorations using resin luting agent thereby preserving dental tissue.

Resin luting agents are intermediates between the tooth substrate, with or without a bonding agent, and the indirect restoration. They can be divided into two categories: traditional flowable resin-based composites and resin cements. Resin cements can be divided into light, self or dual cured resin cements according to the curing procedure. Each resin cement has its own monomers and formulation.

A free radical reaction allows resin luting agents to move from a viscous to a rigid state in a process called polymerization. During the curing process, the terminal aliphatic C=C bonds are broken and converted to primary C-C covalent bonds between methacrylate monomers, the ratio of this bonds conversion can be described with the degree of conversion (DC). However, the formation of free radical varies with the activator system [72].

Multiple factors can influence the DC of resin cements such as its monomer content, the components of the activation system, and the type of polymerization. The physical and mechanical properties of all RBM are directly influenced by the level of DC achieved during the polymerization [58]; the DC ,therefore affects the longevity of the indirect restoration [73]. Inferior mechanical properties, greater discoloration and degradation are the main drawbacks of a low DC, resulting in a resin luting agent with poor wear resistance and poor colour stability [74,75].

During clinical application, the thickness of the indirect restoration can reduce the amount of curing light. The type of curing, therefore, becomes a fundamental factor for the success of restorations. For example, with a thick inlay/overlay restoration or with deep cavities, clinicians are uncertain whether the resin luting agent has properly polymerized. Moreover, during the luting phase, the clinician adopts a step curing technique in order to fix the indirect restoration: the luting agent is pre-cured for 5 sec, then cured again after a few seconds, allowing the clinician to remove the excess material around the indirect restoration. There are no scientific articles or evidence about the chemical stability after this kind of “step luting” procedure. Knowledge of polymerization kinetics could be relevant, particularly during the cementation of an indirect restoration.

The aim of our study was to analyse the effect of two different curing protocols. The two null hypotheses were: 1) All the tested materials have the same DC; 2) The curing protocols do not influence the DC of tested materials.

Materials and Method

Samples preparation

The following resin luting agents were investigated: Hri Flow, shade UD3 (MF) and pre-heated Hri Micerium, shade UD3 (MH); light cure Nexus Third Generation Yellow (NX3L) and dual-cure Nexus Third Generation, shade Yellow (NX3D); dual cured RelyX Ultimate, shade A3 (RXU) and light cure RelyX Veneers, A3, (RXL). For the pre-heated composite, a compule was put in the oven (Ena Heat, Micerium, Avegno, Genova, Italy) at 55 C°. The composition of the tested materials is described in Tab 1.

For each material, ten samples were made by the same operator: the un-polymerized materials were placed directly on a Kaltek Glass (thickness of 1.1 mm), inside a thin Teflon Ring (0.2 mm height and 20.0 mm diameter) and covered by another thin glass of 0.2 mm, in order to have a sample with a diameter of 20.0 mm and a thickness of 0.2 mm. The samples were divided into two groups: P1, in which five samples were cured for 40 sec; P2, in which five samples were cured for 5 sec, and, after 20 sec, were then cured again for an additional 40 sec. Each sample was evaluated with the spectrophotometer: the first measurement related the bands of the uncured sample and after 5 sec the samples were cured following the protocol of their group and measurements were made.

The samples were cured by Elipar DeepCure S (3M Espe, Seefeld, Germany), with an irradiance of 1470 mW/cm2 ± 20% and a spectrum range between 430–480 nm. Only

Tab. 1: Composition of resin luting agents.

Brand	Manufacturer	Type	Composition	Filler Composition
Enamel Plus HRi Flow	Micerium, Avegno, Genova, Italy	Light-Cure flow resin composite	BisGMA, BDDMA, UDMA, glass filler highly dispersed SiO_2	77% wt Unknown%vol
Enamel Plus HRi	Micerium, Avegno, Genova, Italy	Pre-heated Light cure resin composite	DiUDMA, BisGMA, BDDMA Filler: Particles of zirconium oxide and glass	80 wt% 63 vol%
NX3 light-cure	Kerr, Orange, CA, USA	Light-Cure resin cement	Uncured Methacrylate Ester Monomers, minors filler, pigments, radiopaque agent 20-40%	63 wt % 38 vol%
NX3 dual-cure	Kerr, Orange, CA, USA	Dual-cure resin cement	Uncured Methacrylate Ester Monomers 20-40%	Unknown%wt 47 vol%
RelyX Veneer	3M ESPE, St.Paul, MN	Light-Cure resin cement	TEGDMA/BisGMA; Particles of zirconia/silica and colloidal silica	66. 0 wt % 47.0 vol%
RelyX Ultimate	3M ESPE, St.Paul, MN	Dual-cure resin cement	Methacrylate monomer, alkaline filler, initiator components, stabilizers, pigments, rheological additives, fluorescence dye	67.0wt % 43.0 vol %

Bis-GMA, bisphenol-A glycidyl methacrylate; BDDMA, 1,4-butandioldimethacrylate; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethandimethacrylate

during the curing phases, a prepolymerized composite disk (Filtek Supreme A3B Plus, 3M) of 2.0 mm of thickness and 25.0 mm of diameter was in contact between the tip

lamp and the sample. The composite disk was removed after the curing phase, due to the possibility that its DC could influence the evaluations. The translucency of the composite disk was 10.07 and was calculated using the CIELab space as the difference in color between the disk as it appeared against the standard white background and as it appeared against the standard black background, according to the following equation:

$$TP = [(\mathrm{LW} - \mathrm{LB})\text{^}2 + (\mathrm{aW} - \mathrm{aB})\text{^}2 + (\mathrm{bW} - \mathrm{bB})\text{^}2]\text{^}1/2$$

Values for L* (lightness, where 100 represents white and 0 represents black), a* (red–green chromatic coordinate) and b* (blue–yellow chromatic coordinate) was evaluated by SpectroShade-Micro (MHT S.p.a., Verona, Italy) on white (w) and black (b) background.

Further evaluations were made at day 1, day 2, day 7, day 14 and day 28. Between the measurements, the samples were stored in dry and dark conditions at room temperature.

Degree of conversion evaluations

A Perkin Elmer Spectrum One NTS FT-NIR spectrometer was used for DC evaluation of resin cements [45,76]. For these purposes, spectra were acquired in reflection mode in the 7000-4000 cm^{-1} spectral range. The kinetic evaluations were made by a specific program, TimeBase (PerkinElmer), which continuously recorded the spectra in real

time for a time frame of 5 min (20 evaluations in 5 minutes). All the spectra were interpolated, and the height of specific bands was calculated (Spectrum 10.4 software package, Perkin Elmer). The DC of the resin cements was calculated by comparing the height of specific peaks in the unpolymerized and polymerized samples: one peak related to the unreacted carbon double bonds C=C, directly involved in the reaction (band A); the second peak related to the aromatic carbon double bond into phenol ring which is not involved during the polymerization (band B). For each spectrum, the ratio between band B and band A heights was calculated. In this study after plotting a calibration curve, each band height ratio B/A was converted in DC [45,77].

Statistical analysis

After normality and homogeneity evaluations of the data, One-way ANOVA was performed for DC changes, inside each Protocol. The Tukey HSD test was used for multiple comparisons between groups. Student's t-test was used for the comparisons between the different protocols of the same materials and time points. All tests were performed with $p<.05$, using the statistical package Data Analysis in Excel and R Project.

Results

The DC kinetics of the first 5 minutes are shown in Fig 1 and 2, which highlighted an exponential trend of DC. At the end of the 5 minutes, the DC of P1 was, in decreasing order, as follows: MH>MF>NX3L>RXL>RXU>NX3D ($p<.05$). Instead, the DC of

P2, in decreasing order, was: MF=MH>NX3L=RXL>RXU>NX3D ($p<.05$). After 5 minutes, no statistical differences have been identified between the protocols, except for NX3D: P1, 19.58 ± 0.37 versus P2, 24,04±1,64 ($p=0.004$). The DC evaluations of day 1, 2, 7, 14 and 28 were described in Fig. 4-7 and in details in Appendix Tab. 2-6. On day 1, MF (73.83 ± 1.61), MH (75.59± 2.73) and NX3L (72.73± 4.04) had the highest DC in P1 and MF (74.91 ± 0.99), MH (74.16± 2.82) in P2, while the lowest DC in both groups was recorded by NX3D (P1: 52.24± 0.80; P2: 52.97 ± 1.32). On day 2, MF (78.50± 1.01), MH (79.68± 1.98) and NX3L (76.76 ± 4.66) had the highest DC in P1, while MF (78.50 ± 1.01) and MH (79.68 ± 1.98) had the highest DC in P2. On the other hand, NX3D (58.73 ± 1.29) had the lowest DC in P1, while NX3D (58.19 ± 1.25) and RXU (62.23 ± 5.27) in P2. On day 7, MF, MH, NX3L, RXL showed the highest DC with both the protocols. On day 14, MF, MH, NX3L and RXL had the highest DC in P1 and P2. On day 28, MF, MH, NX3L and RXL had the highest DC in P1 and MH, NX3L e RXL in P2. On day 1, 2, 7, 14 and 28, the effect of P1 and P2 were not statistically different (Fig. 3-6).

Fig. 2: Degree of Conversion kinetics of the tested material with Protocol 1 in the first 5 minutes. MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate; DC, Degree of Conversion; Sec, seconds

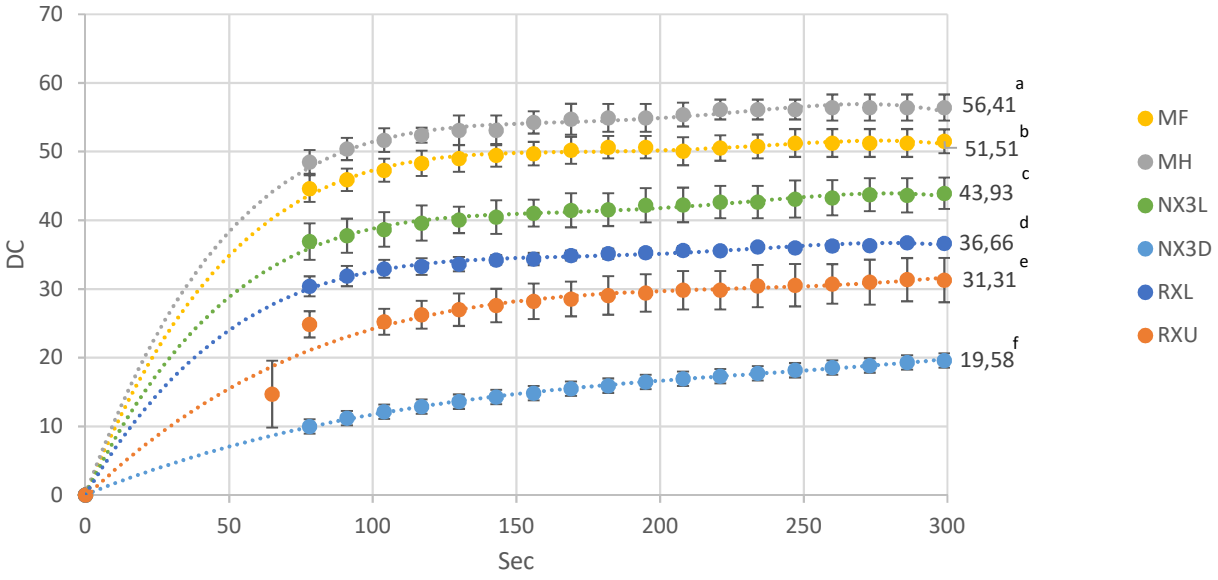


Fig. 3: Degree of Conversion kinetics of the tested material with Protocol 1 in the first 5 minutes. MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate; DC, Degree of Conversion; Sec, seconds

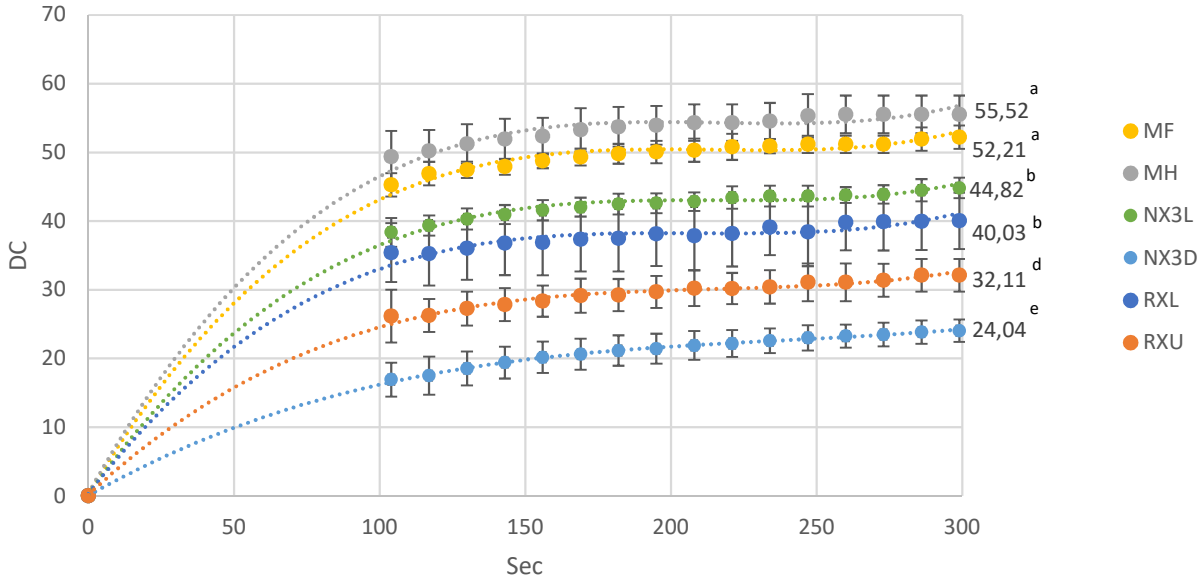


Fig. 4: Degree of Conversion of the tested composites at day 1 and day 28. MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate. P1, Protocol 1; P2, Protocol 2; DC, Degree of Conversion.

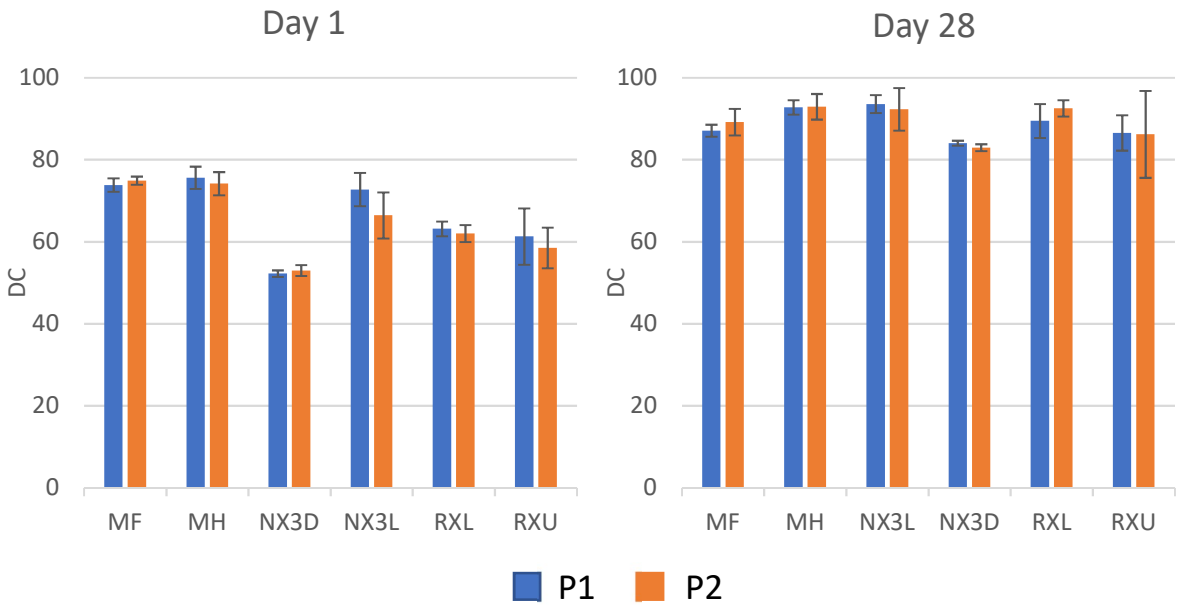


Fig. 5: Degree of Conversion of the tested composites at day 2 and day 28. MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate. P1, Protocol 1; P2, Protocol 2; DC, Degree of Conversion.

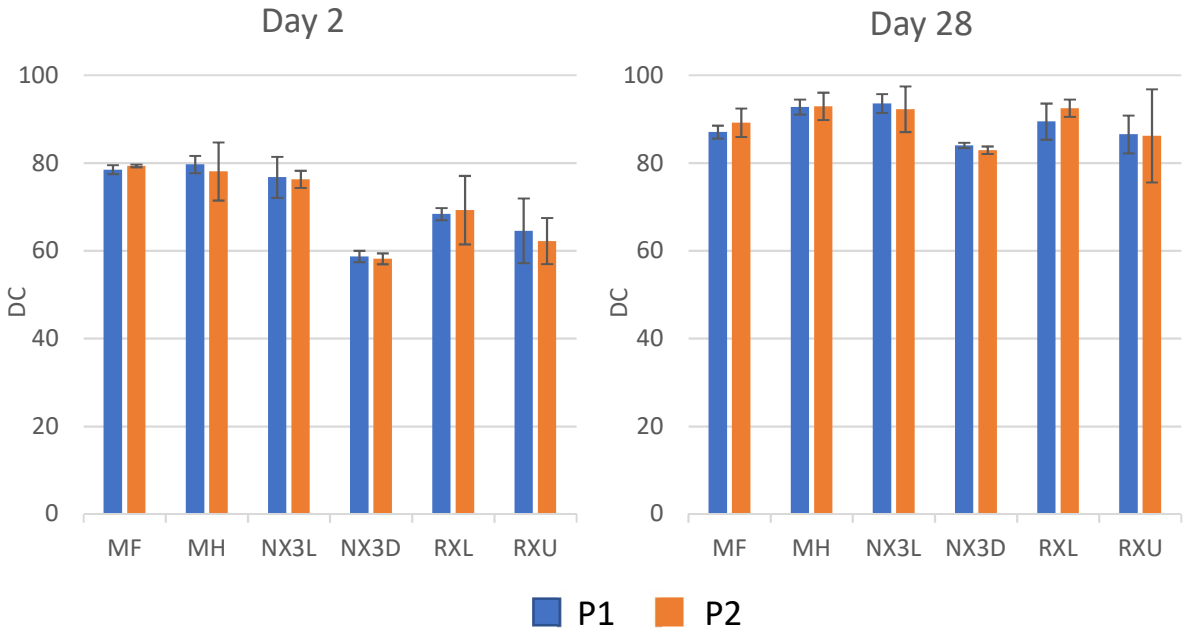


Fig. 6: Degree of Conversion of the tested composites at day 7 and day 28. MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate. P1, Protocol 1; P2, Protocol 2; DC, Degree of Conversion.

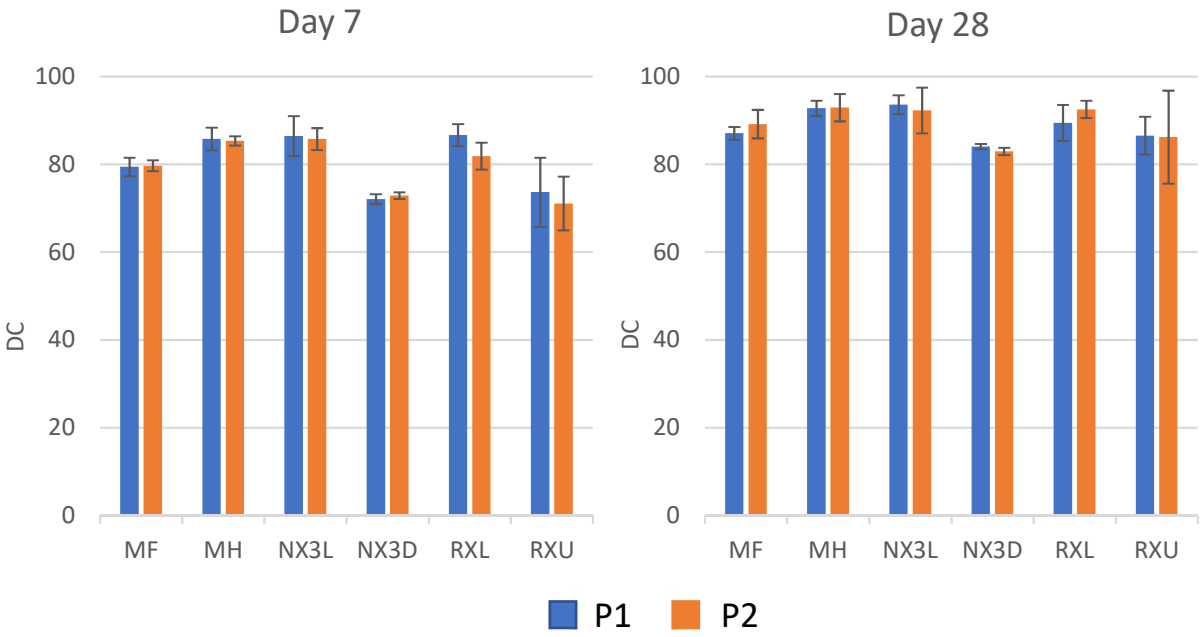
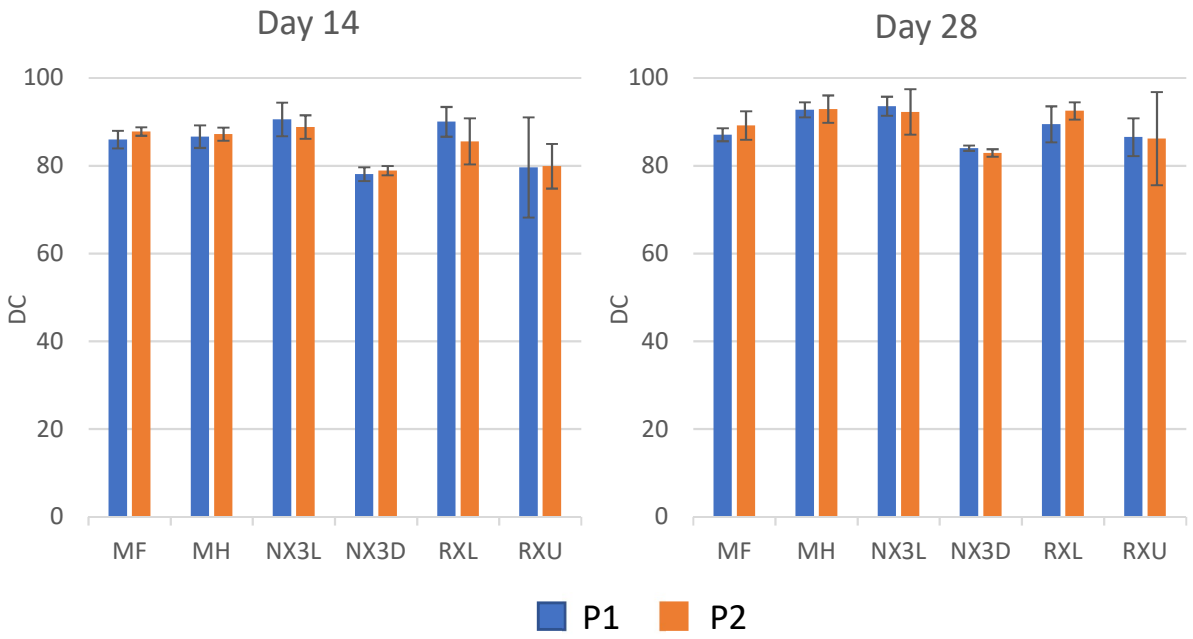


Fig. 7: Degree of Conversion of the tested composites at day 14 and day 28. MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate. P1, Protocol 1; P2, Protocol 2; DC, Degree of Conversion.



Discussion and Conclusion

The longevity of an indirect restoration is directly affected by the resin luting agent [78,79]. In particular, the DC of resin luting agent, as well as traditional resin composites, may influence the chemical and mechanical properties of these materials [80,81]. The DC is a critical factor for biocompatibility and colour stability and is material dependent [74]. A high DC is essential for long-term functionality while an inadequate DC can be detrimental to the success of dental restorations [58,82–84]. In general, the maximum DC reached by resin cements is around 60 % and increases after time [85]. The current results showed that most of the polymerization reactions of light curing materials occurred during the first 5 minutes (Fig. 2,3). In our study we evaluated the DC kinetics of different luting resin agents under a 2.0 mm thick composite disk, simulating an indirect restoration. Furthermore, DC measurements were evaluated over 28 days in order to better understand the chemical effect of step luting protocol on the tested materials. The flow resin composite, MF, and pre-heat high viscosity resin composite, MH, had a higher DC than the other light and dual resin cements after the first 5 minutes. This fact could partly be explained by the percentage of the filler load of MF and MH (77% and 80% respectively), which is higher than the other resin cements. During the polymerization, the resin cement can create a so-called “uncured chamber”: monomers start to cure, and the material starts to become rigid, trapping unreacted monomers in the matrix. For this reason, the curing process needs 1 to 7 days to harden [71,86]. As in MF and MH, a low percentage of monomer content allows a thin layer matrix between monomers and fillers, decreasing

the possibility to create uncured chamber. Our results are in agreement with the findings of Barceleiro et al. which suggest that flowable resin composites are suitable alternative luting agents, when used below a thickness of 2.0 mm or less [87].

In summary, in the first two days MF, MH and NX3L had the highest DC. After 7 and 14 days, MF, MH, NX3L and RXV had the highest DC. However, the DC of dual resin cements were the lowest values at each time point. Although the dual cement is chemically activated, the low contribution of light curing is not enough to reach a high DC, thus requiring more time to reach a high value. Another possible explanation could be the suboptimal concentration of curing inhibitors [88,89]. Inhibitors can be added to resin cement in order to increase the material manipulation and clinical working time [90]. Although the light-cured materials also contain inhibitors, the concentration is relatively lower than the dual-cured materials. Consequently, a good balance between initiators and inhibitors is essential for clinical uses [90].

Our results are in agreement with another study evaluating luting agents when used with ceramic materials instead of composite. Filho et al. found that resin-based cements present low DC when the materials are dually activated through 2.0 mm of reinforced ceramic materials with translucency equal to or less than that of IPS Empress [75]. The translucency of IPS Empress was 10.37 with 2.3 mm of thickness [91]. The translucency of the composite disk used during the polymerisation phases in our study was 10.07 , therefore is less translucent than IPS Empress. Moreover the translucency of our composite disk is lower than some IPS e.max Press, IPS e.max

CAD and Zirconia materials at 2mm [92]. Therefore, our result could relate to indirect restorative materials with a translucency equal or higher than 10.

Regarding the long-term evaluations, the tested protocols were not statistically different. Although different curing modes have been described in the literature, no data exists about DC effect of such "step luting" P2 protocol with a 28-day long evaluation. The P2 can be considered a modify pulse-delay curing, where the polymerization is initiated by a short flash of light followed by a waiting time of several minutes before the final cure is performed. However, in our P2, only 20 seconds were between the pre-curing phase (5 sec) and the final curing phase (40 sec). In the tested P1, the total irradiance was 58800 mW/cm^2 (1470 mW/cm^2 times 40 sec) and no pre curing phase was applied. In the tested P2, to cure the tested material, the total irradiance was 7350 mW/cm^2 for the pre-cure phase (1470 mW/cm^2 times 5 sec), and 58800 mW/cm^2 for the final curing (1470 mW/cm^2 times 40 sec). No difference was noted between the tested curing protocols of P1 and P2 at 1, 2, 7, 14, 28 days.

In the P2 protocol, the low energy (7350 mW/cm^2) of the first 5 sec of curing could initiate the conversion of the luting agent to a semi-solid state and the indirect restoration to be fixed to the tooth not affecting the chemical stability of the material. This finding is in partial agreement with Asmussen et al., although they used different pre-cured phases of 10, 20 and 40 sec followed by 20 sec of final curing, the final DC was not influenced by the low energy density of the pre-cure phase (from 250 mW/cm^2 to 16000 mW/cm^2) which is in accordance with our pre cured energy density of P2

(7350 mW/cm^2) [93]. However, in their study the final curing phase was immediately after the pre-curing phase, without the 20 sec delay.

At a high energy density of the pre-curing phase, the polymerization would proceed at a normal and high rate. While a pre-curing phase at low energy density could start the polymerization process by the formation of limited oligomers, building up discontinuous foci of polymerized material and creating microgel regions [94,95]. This kind of microgel state would allow the clinician to easily remove the excesses when the material is starting to become hard.

Despite the lack of physical and evaluations about adhesion, we can conclude that the light curing resin cements achieved a clinically acceptable DC after 5 minutes. Furthermore, all the luting agents reached more than 50% DC after 1 day. Over the period, the light cured luting cements had the higher DC values than the dual cured ones. In conclusion, we can reject the first null hypothesis but accept the second hypothesis. Moreover, the two protocols do not statistically decrease the DC of the tested materials, also over a long time. Therefore, the clinician can safely use the tested "step luting" protocol (5 sec + 40 sec) to lute the indirect restoration, simplifying the removal of cement excesses, in particular in the interdental space.

Appendix

Degree of conversion of resin cements at different time points

Tab. 2: Degree of Conversion of the tested materials at day 1.

Day 1	P1		P2	
	Mean	SD	Mean	SD
MF	73.83[a]	± 1.61	74.91[1]	± 0.99
MH	75.59[a]	± 2.73	74.16[1]	± 2.82
NX3L	72.73[a]	± 4.04	66.42[2]	± 5.60
NX3D	52.24[b]	± 0.80	52.97[3]	± 1.32
RXL	63.15[c]	± 1.81	61.98[2,4]	± 2.07
RXU	61.27[c]	± 6.87	58.47[2,4]	± 4.96

Tab. 3: Degree of Conversion of the tested materials at day 2.

Day 2	P1		P2	
	Mean	SD	Mean	SD
MF	78.50[a]	± 1.01	79.36[1]	± 0.30
MH	79.68[a]	± 1.98	78.09[1,2]	± 6.63
NX3L	76.76[a]	± 4.66	76.28[2]	± 1.95
NX3D	58.73[b]	± 1.29	58.19[3]	± 1.25
RXL	68.37[c]	± 1.37	69.28[2,4]	± 7.80
RXU	64.56[c]	± 7.38	62.23[3,4]	± 5.27

Tab. 4: Degree of Conversion of the tested materials at day 7.

Day 7	P1		P2	
	Mean	SD	Mean	SD
MF	79.41[ac]	± 2.12	79.66[1]	± 1.24
MH	85.77[a]	± 2.59	85.35[2]	± 1.06
NX3L	86.42[a]	± 4.57	85.75[2]	± 2.50
NX3D	72.09[b]	± 1.12	72.85[3]	± 0.76
RXL	86.64[a]	± 2.52	81.87[1,2]	± 3.06
RXU	73.65[cb]	± 7.88	71.06[3]	± 6.14

Tab. 5: Degree of Conversion of the tested materials at day 14.

Day 14	P1		P2	
	Mean	SD	Mean	SD
MF	85.97[a]	± 2.00	87.82[1]	± 0.97
MH	86.64[a]	± 2.58	87.20[1]	± 1.49
NX3L	90.56[a]	± 3.81	88.83[1]	± 2.69
NX3D	78.08[b]	± 1.57	78.89[2]	± 1.06
RXL	90.02[a]	± 3.39	85.56[1]	± 5.24
RXU	79.65[ab]	± 11.42	79.90[2]	± 5.09

Footnote: MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; RXL, RelyX Veneer; RXU, RelyX Ultimate; NX3L, NX3 light-cure; NX3D, NX3 dual-cure. Different letters and numbers mean statistically difference in the same column ($p<0.05$).

Tab. 5: Degree of Conversion of the tested materials at day 14.

Day 28	P1		P2	
	Mean	SD	Mean	SD
MF	89.16[a]	± 3.24	87.07[1]	± 1.47
MH	92.91[a]	± 3.13	92.75[2]	± 1.73
NX3L	92.27[a]	± 5.19	93.57[2]	± 2.17
NX3D	82.94[b]	± 0.85	84.03[1.3]	± 0.61
RXL	92.51[a]	± 1.98	89.45[1.2]	± 4.12
RXU	86.18[ab]	± 10.60	86.53[1.3]	± 4.31

Footnote: MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; RXL, RelyX Veneer; RXU, RelyX Ultimate; NX3L, NX3 light-cure; NX3D, NX3 dual-cure. Different letters and numbers mean statistically difference in the same column ($p<0.05$).

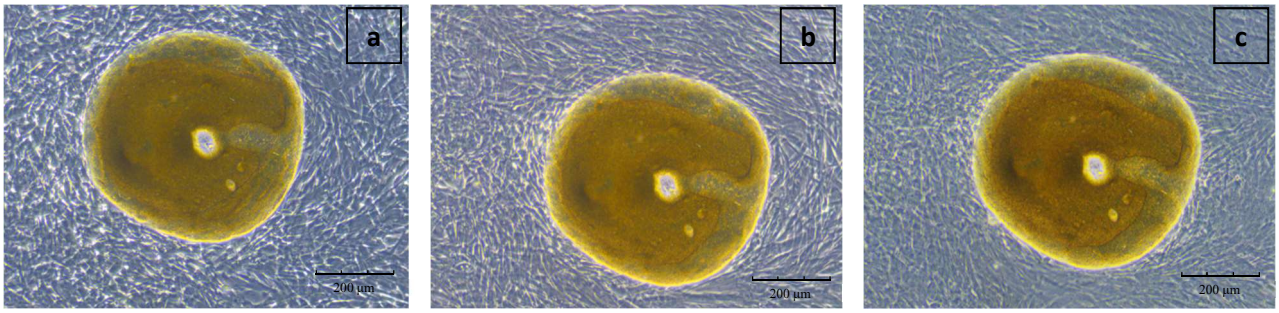
Biocompatibility of Resin-Based Materials

The cytotoxicity tests consist of biological and screening tests based on the observations of the reactions at the interface between cells and materials to estimate cellular growth, reproduction and morphological effects produced by different medical devices [96]. With the continuous development of science and technology, new materials should undergo biocompatibility tests to ensure safe and effective use for humans. Three types of cytotoxicity tests are stated in ISO 10993-5: extract, direct contact and indirect contact tests (including agar overlay assay and filter diffusion). In my study I used the direct contact method between different cell lineages and materials in vitro in order to evaluate their cytotoxic potential. Human dental pulp stem cells (hDPSCs) and human gingival fibroblasts (hGFs) were used in these tests. I used the same collection method of my following study in Chapter III. The 12-well-plates were used for such evaluation. After the material hardening, the medium was put inside the plate and the cells were seeded in it. The medium used for both cell lines consisted of DMEM/F12 (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) with 10% fetal bovine serum (FBS) (PAN Biotech GmbH, Aidenbach, Germany), 1% penicillin/streptomycin (P/S). Every two days, the cells were washed with PBS 10% and medium was changed. The cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The t0 point was established when the cells were attached on the plates. At t0, after 3- and 5-days microscopic pictures were taken. Although no biological assay was done, such as DNA measurements or protein count, my study could be considered a pilot test. Permanence or absence in time of the cells in direct

contact with the material could be a positive or negative factor for their cytotoxic potential. Here are some light microscope images:

-A light curing luting agent, RelyX Veeners (3M ESPE), with hGFs (Fig. 8):

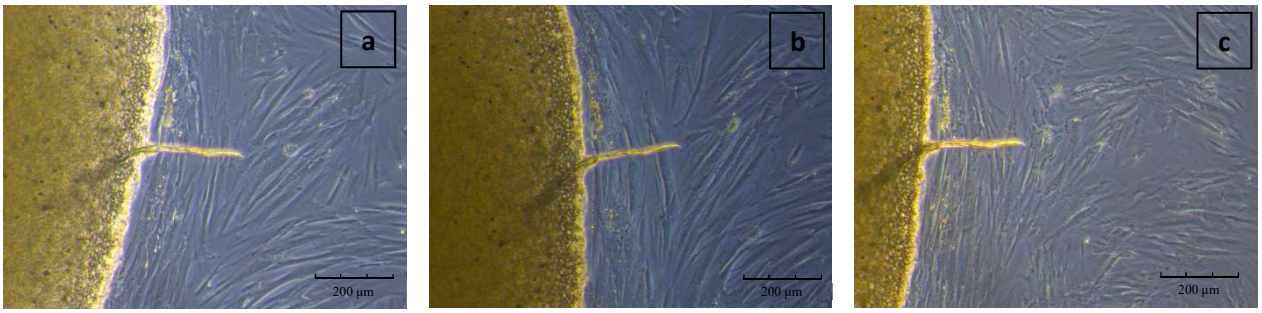
Fig. 8: Light microscope image of human gingival fibroblast in contact with a light resin cement at t0 (a), after 72 hours (b) and after 5 days (c). Scale bar: 200 µm.



After 5 days, no space was evaluated between the tested material and the cells.

- A bulk fill composite, SDR (Dentsply Sirona), and hDPSCs (Fig. 9):

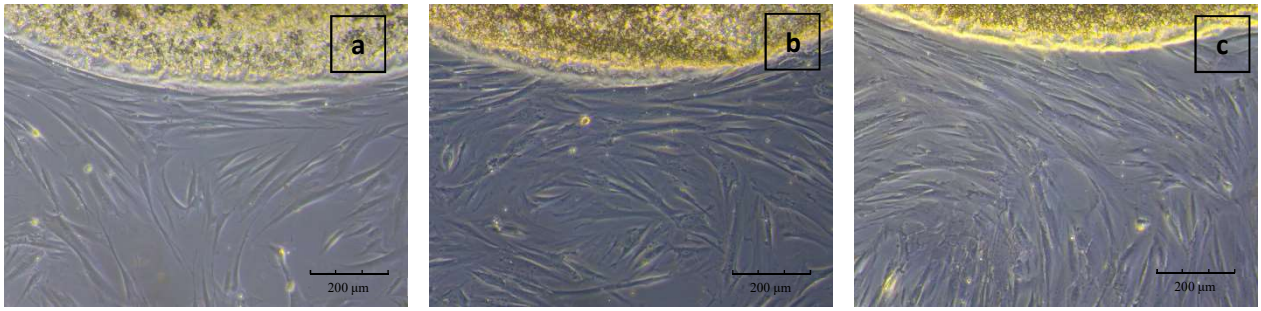
Fig. 9: Light microscope image of human dental pulp stem cells in contact with a flow bulk fill composite at t0 (a), after 72 hours (b) and after 5 days (c). Scale bar: 200 µm.



After 5 days, no space was evaluated between the tested material and the cells.

- A bulk fill composite, SDR (Dentsply Sirona), and hGFs (Fig. 10):

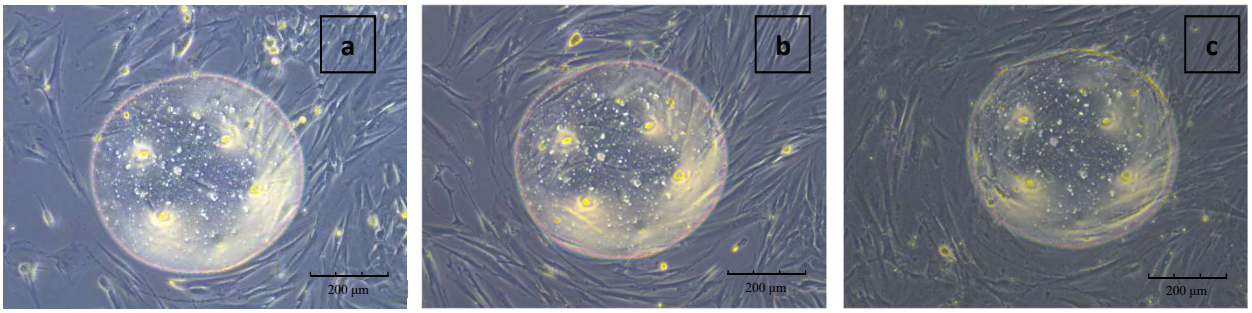
Fig. 10: Light microscope image of human gingival fibroblast in contact with a flow bulk fill composite at t0 (a), after 72 hours (b) and after 5 days (c). Scale bar: 200 µm.



After 5 days, no space was evaluated between the tested material and the cells.

- A prototype bonding agent with hDPSCs (Fig. 11):

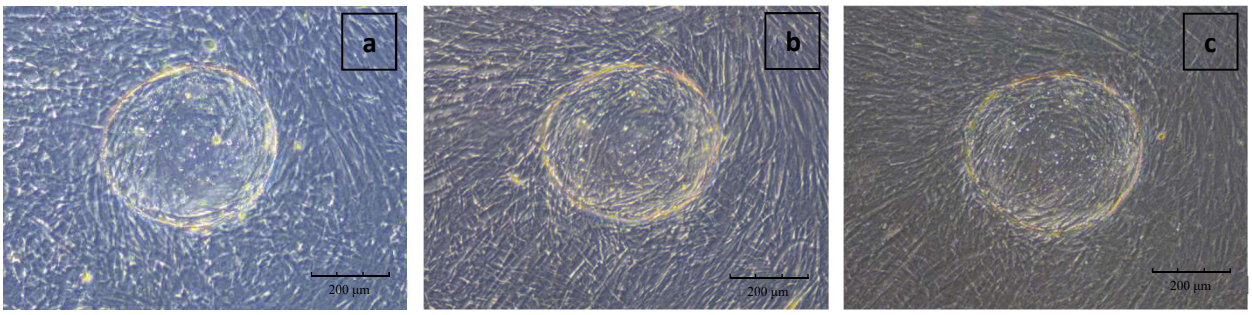
Fig. 11: Light microscope image of human dental pulp stem cells in contact with a prototype bonding agent at t0 (a), after 72 hours (b) and after 5 days (c). Scale bar: 200 µm.



After 5 days, no space was evaluated between the tested material and the cells.

- A prototype bonding agent with hGFs (Fig. 12):

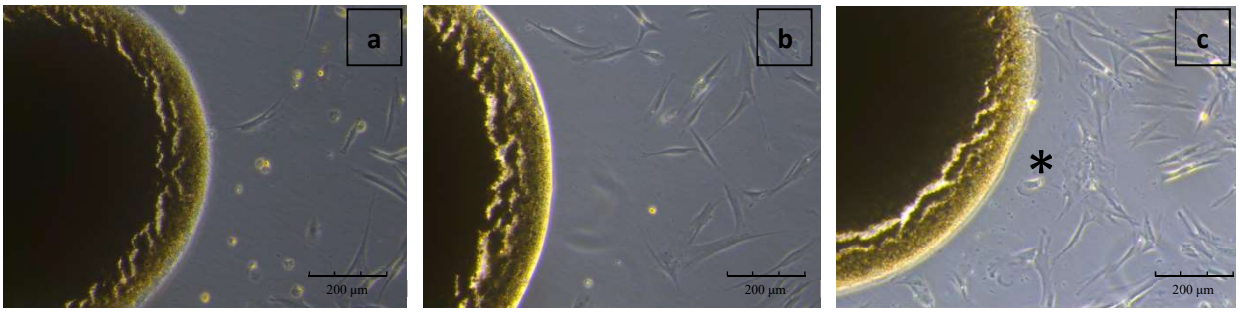
Fig. 12: Light microscope image of human gingival fibroblast in contact with a prototype bonding agent at t0 (a), after 72 hours (b) and after 5 days (c). Scale bar: 200 µm.



After 5 days, no space was evaluated between the tested material and the cells.

- An endodontic cement, Pulp Canal Sealer (Kerr Corp.), with hDPSCs (Fig. 13):

*Fig. 13: : Light microscope image of human dental pulp stem cells in contact with an endodontic cement at t0 (a), after 72 hours (b) and after 5 days (c). Scale bar: 200 µm. *: gap between the tested material and cells.*



After 5 days, although the cells grow far from the material, an empty space could be evaluated between the material and the cells.

In conclusion, different reactions at the interface between cells and materials can be detected. The following possible explanations could be done. For RBM, biocompatibility may be mainly influenced by the monomer and filler type and the DC of the material [58,90]. For example, in SDR (Dentsply), a flow bulk fill composite, the hDPSCs and hGFs remained in contact on the samples surface after 5 days. Other tested RBM showed no gab between the material and the cells, and in Fig. 11 and 12 some cells seem to stay on the material for 5 days. However, for other dental materials, the biocompatibility could be influenced by their ingredients. In an endodontic cement, Pulp Canal Sealer (Kerr Dental), after 5 days, no cells were in contact with the material, maybe because of the eugenol content in the cement mixture.

Although numerous articles refer to the toxicity of dental materials and monomer resin agents, further studies should be done on new resin monomers and materials in order to better understand the cytotoxicity reason and to develop new and safe dental materials.

PAPERS REFERRING TO CHAPTER II

New Trends of Colour and Background Effect in Restorative Dentistry

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Abstract: Evaluation of the right colour is an important step in restorative dentistry. In history, clinicians started to take the colour with subjective methods. For instance, shade guides were used to compare the teeth with their colour tabs and choose the right one. However, this method presents some issues related to the clinician: everyone perceives colours in different ways, not only because humans differ from each other, but also because they can be affected by local, physiological, physical and psychologically uncontrolled factors, such as fatigue, aging, emotions and lighting conditions. All these factors together contribute to make the subjective method unpredictable. For this reason, new instruments need to be exploited by clinicians in order to describe teeth colour in a more accurate and objective manner, thus applying the objective method. The digital camera, the colorimeter and the spectrophotometer are some of the instruments that can be used to reach this purpose. In both the subjective and objective methods, during determination of the colour, the clinician often focuses on teeth and forgets what surrounds it, like the black background of the mouth or the environmental light. These elements may influence the perception of the colour and, mainly in clinicians with a low level of experience, they could lead to a wrong evaluation of right shades. In order to solve these issues, different strategies can be applied by clinicians, such as making their own shade guide, mixing the objective and subjective methods, or use new devices.

Keywords: Spectrophotometer, Background effect, Tooth colour, Shade guide, Colorimeter.

1. INTRODUCTION

Red, blue, green… these are some of the colours that we can attribute to objects without knowing whether they are the right ones: colour is an aspect that our visual system attributes to them. Many disciplines were interested in the phenomenon of colour. Dentistry also searched to identify it correctly, mainly, when clinicians try to replicate teeth using direct or indirect restorations.

Before going ahead, we have to identify and resume what is colour. It "was born" from the light ("lux" in Latin), it is the visible portion of the electromagnetic spectrum: visible light ranges from 380 nm to 760 nm, or in frequency from 790 THz to 435 THz. The light interacts with matter mainly by scattering and absorption, thus colour is born by this interaction: it is the portion of the scattering light reaching the observer [1], in other words, the human eye. Visible radiations coming to human eyes are processed by retina (rods and cones); they can convert a photon into an electrical signal. Then, the brain elaborates it and allows us to perceive colours [2]. Everyone perceives the colour in different ways, it is the subjective way; not only because humans differ from each other, but because colour perception can be affected by several factors: personal experience, aging, emotions, light condition and fatigue. Even the same person can perceive the same colour differently in different time of the day, depending on the attitude too [3]. This subjective perception of colours gave rise to debates and, indeed, there were several attempts to describe it objectively.

The artist Albert H. Munsell was one of the first scientist to describe the colour with three parameters [4]:

- hue: it describes the type of colour whereby we can recognise red from green, blue from yellow;
- chroma: it is the degree of colour saturation in the same hue;
- value: it is the lightness, the degree of luminosity of colour.

These parameters had some interpretation problems, and, in 1976, CIE (Commission international de l'Eclairage) established parameters to measure and communicate colour: three mathematical parameters were used to create absolute colour spaces, some of these spaces are CIELAB space and CIELCh space [5,6]. By means of these spaces and their mathematical parameters, it is objectively possible to describe all of colours. The colour space CIELAB is not a linear space and is determined by three coordinates L*, a*, b*:

- L* represents the lightness, it runs from 0, that is the absolute black, to 100, that is absolute white;
- a* represents the quantity of green or red, it ranges from –a (green) to +a (red);

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- b* represents the quantity of blue/yellow, it ranges from –b (blue) to +b (yellow).

On the other hand, the colour space CIELCh is a cylindrical shape space and is determined by three coordinates L*, C* and h*:

- L* is the same of CIELAB;
- C* represents the chroma and it is linear measure;
- h* represents the hue and it is an angle value.

In these colour spaces, it is possible by means of the coordinates to: locate the colour precisely in the space; recognize it accurately; make it transmittable [7] and evaluate the spatial and perceptive difference between two colours by using dE. dE is the distance between two points (colours) in colour space and it is linked with eyes perception of colour: dE values more than 1.7 dE identify two visually different colours, if dE values is less than this value, no perceptive difference is noticed by human eye [8].

2. DISCUSSION

In dentistry, one of the attempts to establish the colour of teeth was to compare them with samples of difference shades. The first attempts were Vitapan Classical and Vitapan 3D Master shade guides. Nowadays, shade guides are used and the clinician can compare their colour tabs with the teeth and choose the colour. Therefore, it is a subjective method and the main issue is the colour perception among clinicians [9], which can be affected by local, physiological, physical and psychologically uncontrolled factors, such as fatigue, aging, emotions, lighting conditions [10] and metamerism [11]. All these factors are variable, thus making the subjective method unpredictable.

As a short-sighted person needs glasses to see better, so the clinician can use new instruments to describe faster, more correctly and more objectively (objective method) the tooth colour.

2.1. Digital Camera

Digital cameras take pictures using three sensors, which measure red, blue and green wavelength. The photograph picture is based on RGB colour model, in which these three colours are added together in different ways to reproduce all colour range. The digital picture can be used by clinician to obtain important information about the shape of teeth and its characterization through post-processing changes, consisting on increasing contrast or decreasing saturation. Some authors [12] describe digital camera as a useful instrument in dentistry to document and seize the details of anatomy and colours of teeth, which are not perceived with naked eye. The digital camera could be a basic approach to evaluate colours and its details, and may help clinicians to realise an aesthetic dental restoration. Furthermore, the clinician should also consider sending the digital images to technicians: that can visualize the initial situation and the smile of the patient, and send back pictures of the potential restorations that can be performed [13]. However, this system cannot post elaborate the colour. The following two different systems are available to do that:

2.2. Colorimeter

It evaluates the colour of objects in a similar way as the human eye does; filtering the electromagnetic wave with three optics (red, green and blue) [14]. The colours are described with CIELab or CIELCh. In the literature some authors [15] criticize the accuracy of colorimeter because it changes with value and chroma and with deterioration of the filters [16].

2.3. Spectrophotometer

The spectrophotometer takes a pictures using 5500K° illumination, which represents the daylight and ideal light [17]: it can decrease or delete the metamerism effect, in which the colours of different objects match under one light source but appear different under another one [18]. Also by means of spectrophotometer it is possible to describe colour with CIELab and CIELCh spaces and evaluate dE. Furthermore, some of the dental spectrophotometers can compare the colour of restorations with default colours of Vitapan Classic or Vitapan 3D Master, or can assess difference between images. Besides, with both colorimeter and spectrophotometer, the clinician can save the images and the evaluations in his own PC, sending them to the technician to give him more information about the restoration to be performed. The technician could also use this method to communicate with the dentist and show him the working progress.

In dentistry, these last instruments are not often used, because of their bulky dimension and low accuracy, around 80% [19], which make their findings unpredictable. In addition, all these methods can be influenced by a hidden effect, which is underestimated many times from the clinician:

2.4. Background Effect

Sometimes the clinician only focuses on teeth during determination of tooth colour and he forgets what surrounds it, like the black background of the mouth, or the environmental light. It is important not to forget that anterior teeth have a thin thickness, with some translucent areas, and so they can be influenced by environment: the cervical area by gum, middle and incisal area by the black background of the mouth. We can see "the background effect" in colour evaluation in both subjective methods and instrumental methods and it is of paramount importance to determine the background of intra-oral situation, also to mimic the oral environment in the laboratory [20].

In the subjective method, the clinician often put the sample-shades of the shade guide near the mouth, and then he determines the colour. This step could bring clinicians in a wrong shades taking (mainly if they have little experience), because they compared two different materials which, in addition, have different degree of translucency and so they are influenced by background in different ways. The background affects also instrumental evaluation, but, it is less crucial than in visual method, because the spectrophotometer works with ideal condition, like 5500 k° illumination [21], and can describe objectively the colour using three parameters, moreover, they can evaluate even only one area of the tooth or define the background. On the other hand, the influence of background on colour is still a controversial issue and there is not a consensus in the scientific literature about the ideal background to use by clinicians or technicians [20].

3. CONCLUSION

"Pares cum paribus facillime congregantur" [22], literally translated from latin: "equals with equals are very easily associate (themselves)". In both subjective and objective methods, we use samples of shade guides with different properties than teeth, like translucency and opacity. It makes difficult to catch the right colour of the teeth, mainly if the clinician has a low level of experience.

Making personalised shade guide using their own materials of reconstruction (like resin-based composites) could be the right solution, even because nowadays dental composites have the same properties of dental hard tissues [23]. Indeed:

1) clinicians could create discs, shells or tooth shape samples of different thickness and types (dentin, enamel or body composites). They could be mixed and superimposed near the tooth which should be reconstructed, so that both shade guide samples and tooth have the same background; 2) the custom-made shade guides, with a variable thickness width could help the clinician to better understand the material's behaviour, the correspondence between thickness and colour [23]. The laboratory material also could be used with the same custom-made shade guide, improving the communication between clinician and technician.

Another reason to use custom made shade guide, is that, even though many manufactures already have shade guides of their composites, the clinician should carefully use them because of the mismatch between their colour and VITA correspondence: the manufacturers decide the composite colour with different backgrounds (black or white) which can influence the colour itself. Regarding the instrumental evaluation, using personalised shade guides could help the clinician to compare it directly with the image of a tooth by software, in order to delete the problems of subjective methods. Clinically, trusting in instrumental evaluation of colours with personalised composite shade guides could be useful to solve the colour problem and safely increasing their own experience. The new potential trend, still not available on the market, is to use the mobile phone itself to choose the colour. The clinician could compare directly on mobile phone the tooth with most of different trends of composite at different thickness, catch the right shade and use the right composite thickness. Therefore, clinicians could use their own composite and its thickness, to make the right "recipe". Indeed, it would be a portable and easy way to evaluate and shear the teeth colours. Although these new tools and trends could help the clinician to catch and achieve the right colour, there are not ideal materials: best aesthetic materials can give bad results if they are used incorrectly, so as worst aesthetic materials can give good results in good hands.

CONFLICT-OF-INTEREST STATEMENT

The authors declare no conflict of interest related to this publication.

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Spectroscopic and Mechanical Properties of a New Generation of Bulk Fill Composites

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Objectives: The aims of this study were to *in vitro* evaluate the degree of conversion and the microhardness properties of five bulk fill resin composites; in addition, the performance of two curing lamps, used for composites polymerization, was also analyzed.

Materials and Methods: The following five resin-based bulk fill composites were tested: SureFil SDR®, Fill Up!™ , Filtek™ , SonicFill™ , and SonicFill2™ . Samples of 4 mm in thickness were prepared using Teflon molds filled in one increment and light-polymerized using two LED power units. Ten samples for each composite were cured using Elipar S10 and 10 using Demi Ultra. Additional samples of SonicFill2, (3 and 5 mm-thick) were also tested. The degree of conversion (DC) was determined by Raman spectroscopy, while the Vickers microhardness (VMH) was evaluated using a microhardness tester. The experimental evaluation was carried out on top and bottom sides, immediately after curing (t0), and, on bottom, after 24 h (t24). Two-ways analysis of variance was applied to evaluate DC and VMH-values. In all analyses, the level of significance was set at $p < 0.05$.

Results: All bulk fill resin composites recorded satisfactory DCs on top and bottom sides. At t0, the top of SDR and SonicFill2 showed the highest DCs-values (85.56 ± 9.52 and 85.47 ± 1.90, respectively), when cured using Elipar S10; using Demi Ultra, SonicFill2 showed the highest DCs-values (90.53 ± 2.18). At t0, the highest DCs-values of bottom sides were recorded by SDR (84.64 ± 11.68), when cured using Elipar S10, and Filtek (81.52 ± 4.14), using Demi Ultra. On top sides, Demi Ultra lamp showed significant higher DCs compared to the Elipar S10 ($p < 0.05$). SonicFill2 reached suitable DCs also on bottom of 5 mm-thick samples. At t0, VMH-values ranged between 24.4 and 69.18 for Elipar S10, and between 26.5 and 67.3 for Demi Ultra. Using both lamps, the lowest VMH-values were shown by SDR, while the highest values by SonicFill2. At t24, all DC and VMH values significantly increased.

Conclusions: Differences in DC and VMH among materials are suggested to be material and curing lamp dependent. Even at t0, the three high viscosity bulk composites showed higher VMH than the flowable or dual curing composites.

Keywords: bulk fill resin composites, degree of conversion, surface microhardness, curing lamps, spectroscopy

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INTRODUCTION

In nowadays dentistry, resin-based composites have been and are widely used for dental restorations, even if with some disadvantages: shrinkage, shrinkage stress, micro cracks (in the dental structure or in the resin material), debonding, and secondary caries (Ilie et al., 2007; Ilie and Hickel, 2011; Tantbirojn et al., 2011; El-Saftya et al., 2012; Van Ende et al., 2012; Czasch and Ilie, 2013).

In the time, the multi-layer technique tried to reduce these drawbacks: the fact to place composites in dental cavities, using thin increments of 2 mm or less, allowed a good penetration of the light-curing, thus lowering the shrinkage stress (El-Saftya et al., 2012). Nevertheless, this procedure might afford air bubbles, no linkage between layers, troubles during composites placing, especially in the posterior areas, in which it is sometimes difficult to fill the deep cavities and it is often required a long chair time (Abbas et al., 2003; Sarrett, 2005).

To solve these problems, the manufacturers introduced bulk fill resin composites, consisting in new chemical monomers and fillers with an enhancement of their translucency and, consequently, with the potentiality of obtaining an optimal degree of conversion (DC), even in the bottom of the cavities, where it is more difficult to reach high DC. It has been demonstrated that there are several factors affecting the mechanical properties of a resin composite: chemical composition, amount of emitted radiation, distance from the tip of the light source, and photo-activation mode (Da Silva et al., 2008b). Recent improvements in nanotechnology have led to an optimal filler content of this new generation composites, by the addition of free nanosized spherical particles and clusters, which can act as a single unit, thus significantly improving their mechanical properties, also after finishing and polishing procedures (Beun et al., 2007; Jung et al., 2007; Czasch and Ilie, 2013). Furthermore, the benefit of these materials consists in the fact that they can be cured to a maximal increment thickness of 4–6 mm with a limited shrinkage, due to their high translucency, thus allowing the clinicians to rapidly fill the cavity, shortening the chair time (Van Ende et al., 2012). However, even if the first generation bulk filling materials (introduced by Dentsply with a product called SDR) presented a limited shrinkage stress, they showed unsatisfactory mechanical properties, due to the low percentage of fillers, thus requiring the use of a conventional resin composite, acting as an enamel-top capping layer (Campodonico et al., 2011; Ilie and Hickel, 2011; Van Dijken and Pallesen, 2014).

These limitations were improved with the introduction of high viscosity bulk fill composites, which can fill up the occlusal area in a unique step (as single bulk increment), cured and hence sculpted, without the need of an additional top capping layer. Indeed, innovative composites have been recently available on the market, such as SonicFill, which, for instance, uses the sonic energy to decrease viscosity; once the sonic energy has been removed, the resin composite gradually returns to the starting high viscosity status, assuring good mechanical properties (Ahmad, 2013).

The recent large use of high viscosity bulk fill composites have been due to the following positive factors: simplified procedures, increase of the filler percentage, high depth of cure, acceptable translucency, negligible shrinkage stress after polymerization, and satisfactory cavity adaptation (Ahmad, 2013).

As every modern resin-based system, also bulk fill composites necessitate the light curing process to be polymerized. It is noteworthy that clinicians have the tendency to overestimate the properties of bulk fill resin composites, giving an incorrect evaluation of the volumetric shrinkage, shrinkage stress, and DC. Moreover, other factors such as modulus of elasticity, rate of polymerization, polymerization kinetics, initiator chemistry, gel point, type of filler and monomer, development and intensity of the curing stress have to be taken into account (Ferracane et al., 2014). In fact, even if bulk filling restorations were recommended also in cavities up to 4 mm deep, it turned out that many practitioners are still improperly doubtful on their suitability in the clinical field (Czasch and Ilie, 2013).

The clinical performance of posterior resin-based composite restorations can be strongly affected by various parameters as DC and surface hardness. A high DC can determine good mechanical properties, chemical stability and longevity of the restoration (Da Silva et al., 2008a); it has been measured using different methods, such as Raman, NIR (near-infrared) and MIR (middle-infrared) spectroscopies, in order to evaluate changes of aliphatic/aromatic double bond ratio. A satisfactory polymerization degree is essential for the success of the restoration and can require even more than 24 h (Yoon et al., 2002; Miyazaki et al., 2003; Conti et al., 2005).

The surface hardness (measured as microhardness, by means of Vickers or Knoops tests) is defined as the resistance to permanent indentation or penetration on time, and it has to be evaluated in finishing and polishing phases, or when the resin composites are placed on large areas of masticatory force (Galvao et al., 2013; Tarle et al., 2015).

In order to evaluate the acceptable values of DC and surface microhardness, it is worldwide accepted to refer to the International Standard Test ISO 4049-2009, introduced for dental polymer-based restorative materials. In fact, to consider the suitability of a dental restoration (Flury et al., 2012), it is mandatory to take into account the determination of the depth of cure of a dental composite, referring to both DC and Vickers microhardness (VMH) as fundamental key tests (Flury et al., 2012; Leprince et al., 2012).

The primary aim of this study was to analyse the mechanical and spectroscopic properties of one low viscosity bulk fill composite (SDR), one medium viscosity/dual curing bulk fill composite (Fill Up!), and three high viscosity bulk fill composites (Filtek, SonicFill, SonicFill2), by means of DC and VMH measures. In addition, the potential correlation between DC and VMH was also evaluated.

The secondary aim was to evaluate the performance of two curing lamps in the photo-polymerization process of the different composite samples.

The null hypotheses were: (1) DC values and VMH values do not significantly change within the tested bulk fill composites and there is a correlation between DC and VMH; (2) there are no difference in the performance of the two tested curing lamps.

MATERIALS AND METHODS

Five commercial (shade 3) bulk fill composites for posterior restorations were tested (**Table 1**):

(1) SureFil® SDR® (from now on called SDR), a low viscosity flowable composite (Smart Dentin Replacement, Dentsply Caulk, Milford, DE, USA), which shows, in the manufacturer instructions, to need a final top capping layer.
(2) Fill Up!™(Coltène Whaledent AG, Altstätten, Switzerland), a medium-viscosity/dual curing bulk composite, which requires one step increment and no top capping layer, as shown in the manufacturer instructions.
(3) Filtek™ Bulk Fill Posterior Restorative (3M ESPE, St Paul MN, USA) a high viscosity bulk composite, which requires one step increment, no top capping layer as shown in the manufacturer instructions.
(4) SonicFill™ (Kerr Corp. Orange, CA, USA), and
(5) SonicFill2™ (Kerr Corp. Orange, CA, USA). Both SonicFill systems combine a flowable resin composite with an universal resin composite by using a hand piece which enables sonic activation. As shown by the manufacturer instructions, SonicFill2™ presents improved mechanical properties compared to SonicFill™.

The two light-curing lamps used for the polymerization of resin-composite samples presented the following technical details. Elipar S10 (3M ESPE) had an energy output 1200 mW/cm^2 and spectrum between 430 and 480 nm; on the other hand, Demi Ultra (Kerr Corp.) had variable energy output from 1100 to 1330 mW/cm^2 and spectrum between 450 and 470 nm, the energy intensity gradually changing from 1100 until 1330 mW/cm^2.

Degree of Conversion (DC)

To evaluate DC, homemade Teflon cylinders (of 4 mm in height and 6 mm of internal diameter) were used; for each curing lamp, 10 disk-shaped specimens of each of five resin composites were obtained and photo-polymerized in bulk for 20 s. During the photo-polymerization, to exclude oxygen contamination, each sample was covered with a mylar strip on both surfaces. It has to be noted that the sample mass simulates the amount of resin composite usually used to fill up a dental cavity of recurrent dimensions. All the samples were measured on top side (top) and on bottom side (bottom), immediately once cured (t0), and, only on bottom, after 24 h (t24), too. In addition, for SonicFill2, the DC measurements were performed also in 10 3 mm-thick and 10 5 mm-thick samples, and finally, a last measurement was performed after 240 h (t240) only in SonifFill2 4 mm-thick samples.

A DXR FT Raman spectrometer (Thermo Fischer Scientific, Zug, Switzerland) was used to obtain the Raman spectrum of bulk composites. In Raman determinations, DC was evaluated by comparing the ratio of the alkene carbon-carbon double bond (1638 cm^{-1}, reaction band B), which was formed during the polymerization, with the one of the aromatic benzene ring (1610 cm^{-1}), whose intensity does not change during the polymerization (reference band A). To evaluate the DC, calibration curves were plotted assuming that the ratio A/B on top of the no cured material may represent the 0% of polymerization, while the same ratio on the top at t24, may be taken as 100% of polymerization. For this reason, the top DC-values will not be described in the results. **Figure 1** shows a typical Raman spectrum with the decrease of olefinic C = C mode at 1635 cm^{-1} (reaction band) during the curing of both surfaces.

Vickers Microhardness

Vickers microhardness was determined with Leitz Micro-Hardness (Wetzal GMBH, Wetzlar, Germany) tester on the same samples used for the DC measurements. The method consisted in indenting the sample by a diamond indenter with the form of a right pyramid. In our case, a 50 g load was applied for

TABLE 1 | Chemical composition of the tested bulk fill composites.

Materials	Manufacturer	Type	Composition
SureFil® SDR®	Dentsply Caulk	Bulk-fill flowable composite	modified UDMA, TEGDMA, EBPDMA, pigment, photoinitiator, barium and strontium alumino-fluoro-silicate glasses, Silicon Dioxide—Amorphous, Strontium. Aluminosilicate Glass. Filler load: 68 wt%; 45 vol%.
Fill up!™	Coltène/Whaledent AG	Dual curing bulk composite	TMPTMA, UDMA, bis-GMA, TEGDMA, dibenzoyl peroxide; benzoyl peroxide, Zinc oxide coated. Filler load: 65 wt%; 49 vol%.
Filtek™ bulk fill posterior restorative	3M/ ESPE, St. Paul, MN, USA	Bulk-fill paste composite	Bis-GMA, bis-EMA, UDMA, zirconia, Filler load:76.5 wt%, 58.4 vol%.
SonicFill™	Kerr Corporation, CA, USA	Bulk-fill paste composite activated	Resin: EBADMA, BisphenolA-bis-(2-hydroxy-3-mehacryloxypropyl) ether, TEGDMA, 3-trimethoxysilylpropyl methacrylate, SiO_2, Glass, oxide, chemicals. Filler load: 83.5 wt%; 83 vol%.
SonicFill2™	Kerr Corporation, CA, USA	Bulk-fill paste composite activated	Poly(oxy-1,2-ethanediyl), α,α′-[(1-methylethylidene)di-4, 1-phenylene]bis[ω-[(2-methyl-1-oxo-2-propen-1-yl)oxy]-Not available. 2,2′-ethylenedioxydiethyl dimethacrylate. Filler load: 81.3% wt % unreported.

Bis-GMA, bisphenol-Aglycidyldimethacrylate; bis-EMA, ethoxylated bisphenol-A-dimethacrylate; UDMA, urethane dimethacrylate; TMPTMA, trimethylolpropane Trimethacrylate; TEGDMA, triethylene glycol dimethacrylate; TEGDMA, triethylene glycol dimethacrylate; EBADMA, ethoxylated bisphenol-A dimethacrylate; EBPDMA, ethoxylated Bis-GMA; SiO_2, silicon dioxide; wt%, weight percentage; vol%, volume percentage.

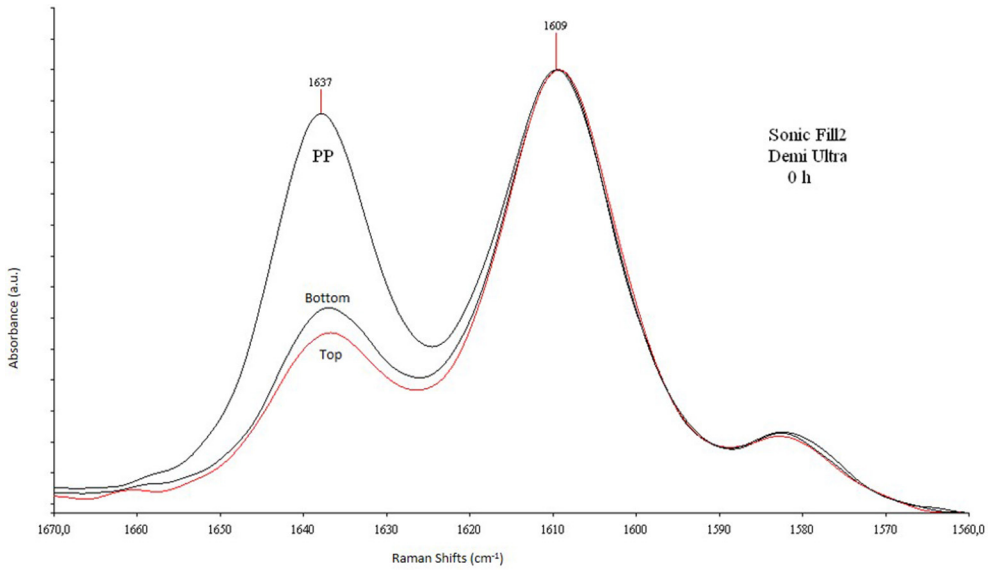

FIGURE 1 | Raman spectra between the reaction and reference bands. Raman spectra in the region 1670–1560 cm^{-1} of SonicFill2, 4 mm-thick, cured with Demi Ultra.

15 s. Once the load was removed, it was possible to evaluate (by using a microscope) the corresponding average value of the two indentation diagonals, to get the area of the sloping surface and, hence, to determine the corresponding hardness value.

The measurements of VMH were achieved at t0 and at t24 on the irradiated top surface of the samples. Three measurements were made for each sample: on the middle, on 0.15 mm and on 0.3 mm from the center. Calculations were made by using a computer software (Hardness-Course Vickers/Brinell/Rockwell copyright IBS 2012 version 10.4.4) (Fleming et al., 2008; Roberts et al., 2009; Nagi et al., 2015).

Statistical Analyses of DC and VMH Data

Statistical analyses were performed by means of R Project for Statistical Computing 3.3.0 (https://www.r-project.org/) and Microsoft Excel 2013. Normality of data distribution and homogeneity of group variances were verified by Kolmogorov-Smirnov test and Levene test, respectively. Differences of DC and VMH-values among groups and at different time intervals were evaluated by two-ways analyses of variance (Two-Ways ANOVA). The Tukey test was applied for *post-hoc* comparisons. In all analyses, the level of significance was set at $p < 0.05$.

RESULTS

Descriptive statistics of DC-values are reported in **Table 2**. Two different curing lamps were used in this study, Elipar S10 and Demi Ultra. At t0, they recorded statistically significant different DC-values between top and bottom sides ($p < 0.05$).

On top sides, at t0, DC-values of tested composites ranged between 70.40 and 85.56% using Elipar S10. On the other hand, using Demi Ultra lamp, top DC-values at t0 ranged between 82.30 and 90.53%. At t0, top DC-values obtained by Demi Ultra were significantly higher than the ones obtained using Elipar S10 ($p < 0.05$). SDR and SonicFill2 had higher DC-values than the other composites cured using Elipar S10; SonicFill2 recorded the highest DC-values when cured using Demi Ultra.

TABLE 2 | Degree of Conversion (DC).

Composites	*N*	t0 top		t0 bottom		t24 bottom	
		DC%	SD%	DC%	SD%	DC%	SD%
ELIPAR S10							
SDR	10	85.56	9.52	84.64	11.68	93.17	8.04
FILL UP!	10	82.79	3.20	65.03	6.57	94.71	7.96
FILTEK	10	78.27	3.95	74.32	7.30	92.20	9.01
SONICFILL	10	70.40	10.66	68.12	6.29	78.76	8.13
SONICFILL2	10	85.47	1.90	77.01	8.47	78.62	9.23
DEMI ULTRA							
SDR	10	84.93	2.26	75.67	2.20	91.94	10.02
FILL UP!	10	88.75	3.89	65.45	11.36	75.45	14.05
FILTEK	10	86.54	2.15	81.52	4.14	98.84	4.17
SONICFILL	10	82.30	6.03	78.78	5.90	90.95	8.92
SONICFILL2	10	90.53	2.18	75.44	3.53	80.25	7.32

N, number of samples; DC, Degree of Conversion; SD, Standard Deviation.
Mean of DC-values with Standard Deviation at t0, on top and bottom, together with t24 bottom values of samples cured using Elipar S10, and Demi Ultra lamps, respectively.

On bottom side, at t0, DC-values ranged between 65.03 and 84.64%, using Elipar S10. On the other hand, using Demi Ultra, they ranged between 65.45 and 81.52%. At t0, the highest bottom DC-values were recorded by SDR cured by Elipar S10 and Filtek cured by Demi Ultra ($p < 0.05$). After 24 h, all DC-values significantly increased ($p < 0.05$). Both at t0 and t24, the values recorded by the two curing lamps were not statistically different ($p > 0.05$). An additional DC evaluation was performed on

SonicFill2 samples of different thickness (3 and 5 mm-thick) at t0, using both lamps. **Figure 2** shows means of DC-values on top side of 3, 4, and 5 mm-thick samples, that were not statistically different ($p > 0.05$). On the other hand, on bottom side, the mean DC-values in the 3 mm (84.2%) and 5-thick samples (68.7%) were statistically different ($p < 0.05$). The last evaluation of SoniFill2 samples (4 mm-thick) after 240 h showed no significant difference of DC-values on top between t24 and t240 ($p > 0.05$).

The VMH-values recorded on the top of the different specimens by using the two curing lamps were statistically different (**Figure 3**). Demi Ultra showed higher VMH-values than Elipar S10 ($p < 0.05$). The mean of VMH measurements (from t0 to t24) showed the following significant increase ($p < 0.05$): 51.9 at t0 vs. 61.04 at t24 (using Elipar S10); 53.28 at t0 vs. 61.91 at t24 (using Demi Ultra). SonicFill2 had the highest VMH-values ($p < 0.05$): being 69.18 ± 3.15 at t0, using Demi Ultra, and 75.2 ± 1.69 at t24; using Elipar S10, 67.3 ± 3.7 at t0 and 71.12 ± 1.52, at t24. On the other hand, SDR showed the lowest VMH-values ($p < 0.05$): being 28.4 ± 2.34 at t0 and 35.28 ± 1.2 at t24, using Demi Ultra; using Elipar S10, 26.5 ± 3.71 at t0 and 36.73 ± 1.48 at 24.

Finally, Pearson's Test indicated that there is no correlation between DC and VMH, using both curing lamps (0.24 for Demi Ultra, 0.016 for Elipar S10).

DISCUSSION

Nowadays, it is becoming of growing tendency to use bulk fill resin composite materials because of their simplified procedures for filling in a single increment posterior restorations compared to the multi-increments techniques required by conventional

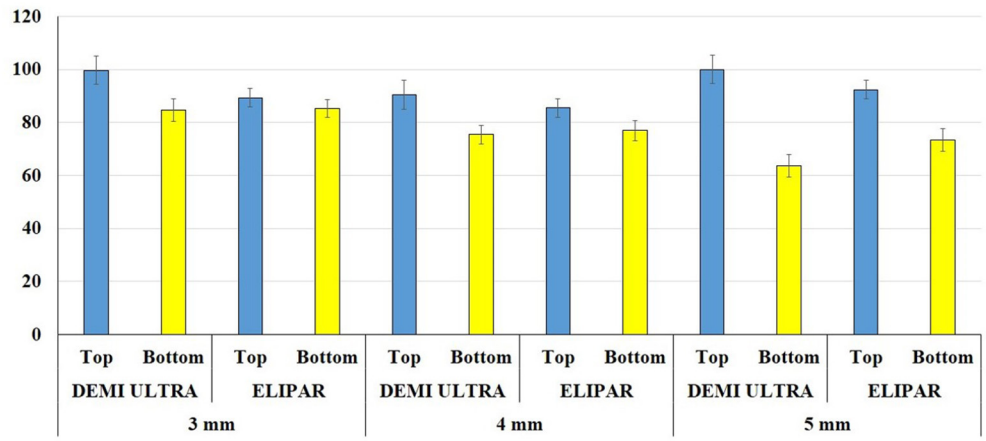

FIGURE 2 | Degree of Conversion (CD) comparison between top and bottom at different time. Mean of DC top and bottom values at t0 of SonicFill2 in 3, 4, and 5 mm-thick samples, cured using Demi Ultra and Elipar S10 lamps.

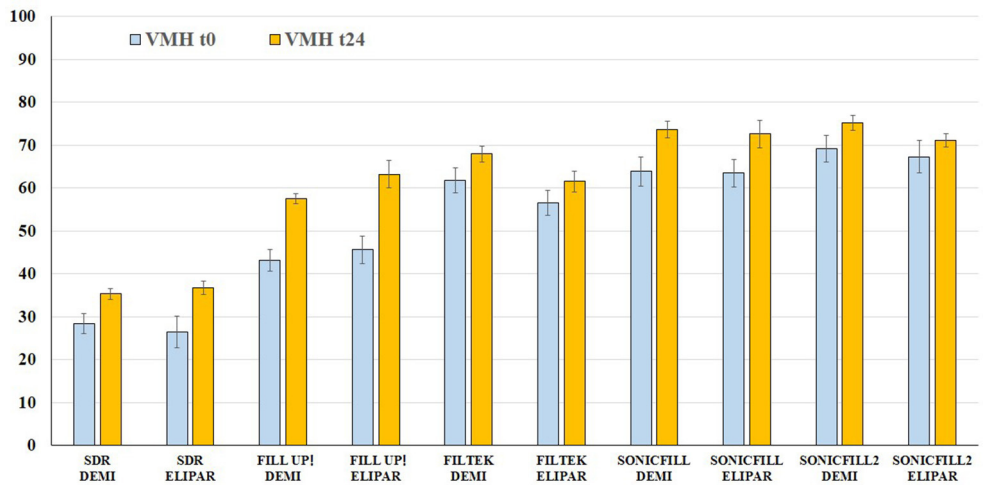

FIGURE 3 | Vickers microhardness (VMH) comparison on top at different time intervals. Mean and Standard Deviation of VMH-values of the five composites at t0 and t24 using Demi Ultra (DEMI) and Elipar S10 (ELIPAR) lamps.

resin composites. Indeed, manufacturers and recent scientific reports demonstrate that the main advantages of this restorative procedure consist in an increased depth of cure and a low polymerization shrinkage (Ilie et al., 2013; Leprince et al., 2014). Dental restorative composites polymerize to a certain depth, depending on the light beam penetration of the curing lamp inside the mass (Leloup et al., 2002), and a suitable polymerization of the whole composite mass remains one of the main important factors influencing the clinical success (Czasch and Ilie, 2013). In fact, high DC-values are important to assess optimal physical, mechanical and biological properties of resin composites; on the other hand, when there is not an optimal DC incomplete polymerization (unreacted—dangerous—monomer), marginal microleakage, discoloration, decrease of bonding strength and low mechanical properties can occur (Yap et al., 2004; Kusgoz et al., 2011; Alonso et al., 2013; Galvao et al., 2013). For a clinically acceptable restoration, some authors indicate that the DC-value should reach at least the 55% of it, however, even if resin-based dental materials are properly cured, they generally exhibit a significant amount of unreacted monomers (Soares et al., 2007; Galvao et al., 2013). High values of DC, up to 60%, can be due to improvements in the resin matrix (flexibility and viscosity of the starting monomer), to the composition and size of fillers and to the irradiance intensity (Dickens et al., 2003; Turssi et al., 2005).

In this study, in order to evaluate the DC-values of the tested bulk fill composites, which are based on the use of prepolymerized resins, it was assumed that the ratio 1635/1610 (described above) of the starting material, before the curing, may represent the 0% of polymerization, whereas the ratio of 100% of polymerization is assumed at t24 on the samples top sides (Ferracane, 1995; Blackham et al., 2009). For this reason, at t24, the DC-values on the top were not evaluated. Indeed, due to the overlapping of the reaction bands at 1635 cm^{-1} at t24 with the one at 240 h (e.g., SonicFill2), it has been hypothesized that after 24 h the polymerization process can be reasonably concluded. Therefore, these two reference points allow us to establish the evolution in time of the polymerization process: it was between t0 and t24, or between 0 and 100%. Raman spectroscopic evaluation shows that different DC-values are recorded between top and bottom at t0 for all composites, using the two curing lamps ($p < 0.05$). On top and at t0, Demi Ultra seems to cure better than Elipar S10 ($p < 0.05$), while on bottom and at t0, the two curing lamps are not statistically different to cure SDR and Filtek, which show the highest DC-values using Elipar S10 and Demi Ultra, respectively. After 24 h, bottom DC-values increase using both Elipar S10 and Demi Ultra, with no significant differences between the two curing lamps: once again SDR and Filtek show the best performance. In agreement with literature reports, all 4 mm-thick tested samples show high DC-values, mainly for t0 bottom determinations (Goracci et al., 2014; Leprince et al., 2014; Marovic et al., 2015). In the case of SDR, the high DC-values can be due to the high fluidity and transparency of this resin, even if, it is noteworthy again to remark, that a flowable resin composite, like SDR, needs an additional hardening top layer. Literature data concerning SonicFill and SDR evidence some discordance and, in general, lower DC-values than our data, mainly for t0 bottom determinations (57.9 and 50.3%, respectively, when using Demi Ultra lamp, with an output of 1100 mW/cm^2; see Goracci et al., 2014). Indeed some reports show that SDR DC-values on top ranged from the 77% (t0), using a light unit output of 1200 mW/cm^2 (Guimaraes et al., 2013), to the 67%, reported by other authors (Van Ende et al., 2013; Marovic et al., 2015). Among the high viscosity samples, SonicFill2 shows the highest DC-value with both curing lamps (at t0, on top), being Demi Ultra the best unit to polymerize it. However, both curing lamps result in a satisfactory performance, being Demi Ultra slightly superior than Elipar S10 in top surfaces curing ($p < 0.05$).

As mentioned above, DC-values of SonicFill2 have been determined also on 3 and 5 mm-thick samples at t0 (**Figure 2**). The bottom DC mean values of these samples at different thickness are statistically different, thus suggesting that the thickness of samples could condition the DC. However, it is noteworthy to underline that acceptable DC-values are registered even on the bottom side of 5 mm-thick samples.

Finally, the fact that there is no difference in DC SonicFill 2 values between t24 and t240 means that a satisfactory degree of polymerization can be reached after 24 h, in agreement with other literature reports (Ferracane, 1995; Blackham et al., 2009).

Hardness and microhardness measure the resistance to plastic deformation and indicates the resistance to indentation under functional stresses: a high value can be indicative of the ease of finishing and polishing of a restoration (Rahiotis et al., 2004; David et al., 2007). It has already been reported that hardness values may be related to the DC of carbon double bonds of a resin composite; even if with some divergence, it has been shown that a conversion around 90% of the resin may correspond to a bottom/top VMH ratio of 80% (Bouschlicher et al., 2004). Moreover, the microhardness of a resin composite depends also on the thickness: an increase in thickness causes a microhardness lowering.

In recent years, modulator interacting with camphorquinone (as in the case of the dual-curing composite Fill Up!), transparency enhancements and/or technological up grades (as in the case of high viscosity bulk fill composites) have been added to the resin composites, thus resulting in satisfactory microhardness values even in 4 mm-thick samples. Indeed, some authors reported that high viscosity bulk fill composites might exhibit high microhardness values because of the high percentage of fillers content (Barabanti et al., 2015; Nagi et al., 2015). **Figure 3** reports mean and SD of VMH top values at t0 and t24, using the two curing lamps. As expected, after 24 h, all samples show significant VMH increases ($p < 0.05$). Among the five materials, the lowest VMH mean value is shown by SDR, while a considerable increase is found for the medium viscosity dual curing Fill Up! (57 and 63 vs. 51 VMH, as claimed by the manufacturer Coltene); noteworthy is that at t0, SonicFill2 shows the highest VMH-value, using both curing lamps.

Several studies have tried to find a correlation between the DC and VMH for some authors no correlation may be drawn, while, for others, a negative trend occurs between the two parameters (Ferracane, 1985; De Wald and Ferracane, 1987; Santos et al.,

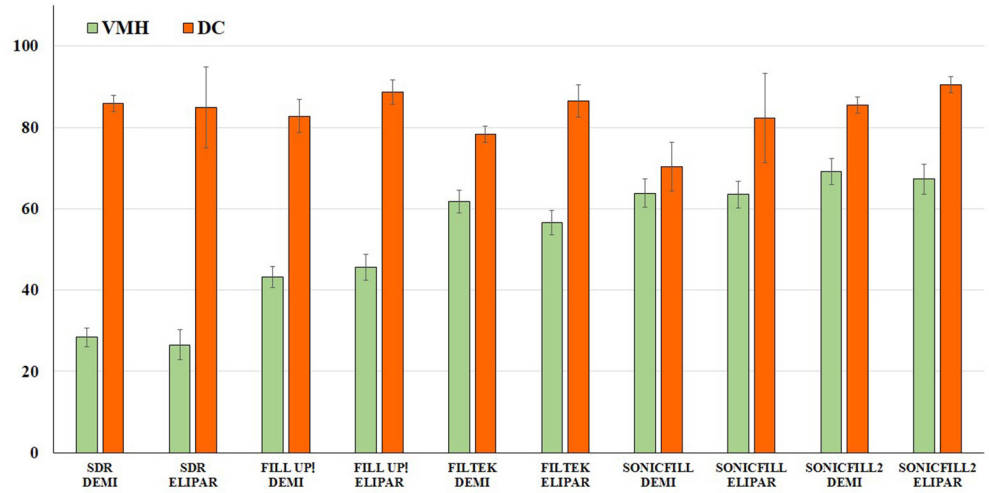

FIGURE 4 | Correlation between Vickers microhardness (VMH) and Degree of Conversion (DC). Comparison between mean VMH and DC-values (top t0) of the samples cured using Demi Ultra (DEMI) and Elipar S10 (ELIPAR) lamps.

2007; Da Silva et al., 2008a). In our case, the lack of any correlation is evident (Pearson's correlation coefficient: 0.24 for Demi Ultra, −0.016 for Elipar S10), meaning that, even if high values of two single parameters may point out a satisfactory dental curing, a linear correlation is lacking (**Figure 4**). This fact may be due to the different contents and viscosity of the five composites (**Table 1**).

In the attempt to analyse the behavior of a reasonable range of new bulk fill dental composites, a significant number of samples have been evaluated in the present work, however, other experiments are needed to make definitive remarks and, therefore, future analyses will aim to continue to analyse newly introduced materials. In definitive, differences in DC and VMH-values between our study and previous literature reports can be ascribed to improvements of fillers (composition and particles size distribution, percentage of filler load), of the resin matrix (monomer type and its chemical structure), and also to the enhanced performance of the new curing lamps. Indeed, it is important to underline the great impact that nanotechnology has produced in terms of development and progressing of dental materials science. Therefore, the present study can be considered relevant since there are no previous reports evaluating all the five bulk fill resin composites, using vibrational techniques, such as Raman spectroscopy, and also because this is the first time in which the performance of two new generation curing lamps have been analyzed.

CONCLUSIONS

In the light of the presented results, the two null hypotheses can be rejected: firstly, because there are differences in DC and VMH within the tested bulk fill composites, and no correlation can be drawn between DC and VMH; secondly, because the behavior of the two curing units is slightly different. Within the limits of the current study, it can be concluded that the five resin composites recorded satisfactory polymerization degree on both top and bottom sides.

To summarize, the flowable SDR shows high DC-values with both lamps, while, among high viscosity samples, SonicFill systems (and especially SonicFill2), combining the advantages of a flowable dental material with a universal resin composite, by using sonic activation, demonstrate excellent DC-values. In particular, SonicFill2 showed the highest DC-value using both curing lamps (at t0, on top), and an acceptable DC, even in case of 5 mm-thick samples.

Significant differences in VMH-values among the five tested materials are found: the lowest value has been evidenced by the flowable SDR, reinforcing the need of a top capping layer, while, in agreement with the literature, an appreciable increase has been found for the dual curing Fill Up!. Among the high viscosity resin composites, a satisfactory performance has been demonstrated by SonicFill and, mainly, by SonicFill2. Both DC and VMH determinations appear clinically significant to make a prevision of the future performances of restorations. The use of medium and, mainly, high viscosity bulk fill materials may also be important to avoid a further capping application. Moreover, this study can be useful to increase the knowledge of clinicians in understanding the curing performance of the tested lamps.

Our upcoming aim will be to increase the number of the tested dental composites, as well as to further study by means of vibrational techniques both dental and composites surfaces after finishing and polishing.

AUTHOR CONTRIBUTIONS

RM contributed to the research protocol, performed the experiments as part of his Ph.D. project, contributed to

the results analyses and writing. GO contributed to the idea, the research protocol, and the writing of the present manuscript. GT contributed to the correct developing of the research idea, the writing, the technical background of the entire manuscript. CC contributed to the technical idea and support all experimental phases. VL contributed to the Raman facilities use and data interpretation, and to the discussion finalization. MP contributed to the idea and the updated results organization of the manuscript. AP contributed to the idea, the findings interpretation, and the editing of the manuscript.

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Correlation between chemical-physical properties of methacrylate and dimethacrylate resin composites after different polishing time (Paper in review)

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Abstract

Purpose: This study aimed to analyse the effects of polishing on different methacrylate and dimethacrylate resin composites at different time points.

Methods and Materials: Ten samples of each tested composites were prepared, cured and divided into two two groups: in Group I (immediate), five samples were polished immediately after curing; in Group D (delayed), five samples were polished after 24 h from curing. Degree of conversion (DC) and Vickers microhardness (VMH) were evaluated both before and after polishing, immediately (t0) and 24 hours from curing (t24). `

Results: The DC and VMH of the tested materials were statistically different ($p < 0.05$): at t0, in both composites, DC statistically increased after polishing, while at t24, Group I and Group D were not statistically different. For VMH, at t0, the behaviour of the two composites was statistically different. At t24, VMH of Group I and Group D of each composite was not statistically different.

Conclusions: DC and VMH of the tested materials increased after polishing and, in dimethacrylate composite, they reached at t0 the same values as the ones at t24. Restorations filled with dimethacrylate can be finalized immediately after their curing.
Clinical relevance: Polishing procedure, immediately after curing, can improve the DC and VMH of the tested resin composites. The clinician could safely polish the tested methacrylate and dimethacrylate bulk composite in one-chair appointment.

INTRODUCTION

The light polymerization of dental composites leads to crosslinked networks between monomers, making the composite hard [1]. The formation of these crosslinked networks, evaluated by degree of conversion (DC), together with the Vickers micro hardness (VMH) of materials have a great importance in the success and longevity of dental restorations [2, 3]. It is well known that materials with low DC and VMH enhance the failure rate of reconstruction: a low DC can cause a progressive worsening of the superficial surface or a low biocompatibility, while a low VMH can be linked with low wear resistance and scratches on the reconstruction surface [2, 4]. DC and VMH properties are not correlated with each other, then DC of different materials can give different VMH [5, 6]. In clinical practice, clinicians should finish and polish them in order to: remove excess of material, fit the occlusion, obtain a smooth surface, and improve the aesthetic of resin composites [7].

Finishing indicates the contouring or a reduction of restoration to obtain an ideal anatomy. Polishing reduces the roughness created by finishing [8, 9]. The roughness is one of the causes of plaque accumulation, gingival inflammation, superficial staining and secondary caries: all of these are some of the frequent failure reasons of teeth reconstructions; an appropriate finishing and polishing are critical clinical procedures that enhance the longevity of restorations [10]. However, polishing and finishing effects depend on the type of resin

composites and can influence their chemical and physical properties [11].

In the last few years the so-called bulk fill resin composites have been introduced on the market: they consist in a combination of new chemical monomers (methacrylate or dimethacrylate) and fillers high translucency in order to gain an optimal DC [10, 12].A lot of articles concern about the effects of polishing on traditional resin composites [8, 13] but, although a plethora of new polishing systems are available in the market, there are no detailed studies concerning their timing effect on the properties of bulk fill composites.

Therefore, the aim of this study was to evaluate the effects of immediate and delayed (after 24 hours) polishing on the DC and VMH of a methacrylate and dimethacrylate bulk fill composites. The null hypotheses were: 1) the timing of the polishing does not influence the DC; 2) the timing of the polishing does not influence VMH.

MATERIALS & METHODS

The following two bulk fill resin composites were used: a low viscosity bulk fill resin. The following two bulk fill resin ccomposites, Estelite Bulk-Fill Flow shade A2 (Tokuyama Dental Inc., Encinitas, CA), a methacrylate bulk fill resin composite (MET), and a high viscosity bulk fill composites, Filtek One Bulk Fill Restorative shade A2 (3M, St. Paul, MN, USA), a dimethacrylate bulk fill resin composites (DMET). The compositions of the tested materials are shown in Table 1. For each resin composite, ten samples were prepared by using homemade Teflon cylinders (3.0 mm in height and 6.0 mm in internal diameter).

All samples were photo-polymerized in bulk using samples were photo-polymerized in bulk using Elipar DeepCure S lamp (3M, St. Paul, MN, USA) for 20 secs with an irradiance around 1470 mW/cm^2 and a spectrum range between 430-480 nm. During the photo-polymerization, samples were covered with a Mylar strip to exclude oxygen inhibition. After curing, the samples were divided into two groups: MET Group I (I: immediate) and DMET Group I, in which five samples were finished and polished

Table.1: Composition of the tested materials.

Type	Brand	Composition	Filler load
Bulk Fill low viscosity methacrylate composite	Estelite Bulk Fill Flow (Tokuyama Dental)	Bis-GMA, Bis-MPEPP, TEGDMA, 200nm spherical silica and zirconia $SiO2-ZrO_2$ 200nm spherical SiO_2-ZrO_2	70.0 wt% 56.0 vol%
Bulk Fill high viscosity dimethacrylate composite	Filtek™ One Bulk Fill Restorative (3M ESPE)	AFM, AUDMA, UDMA, DDDMA, non-agglomerated/non-aggregated 20nm silica filler, a non-agglomerated/non-aggregated 4 to 11nm zirconia filler, an aggregated zirconia/silica cluster filler (comprised of 20nm silica and 4 to 11nm zirconia particles) and an YbF_3 filler consisting of agglomerate 100nm particles	76.5 wt% 58.5 vol %

wt%, weight percentage; vol% volume percentage; AFM, addition-fragmentation monomer; AUDMA, aromatic urethane dimethacrylate; Bis-GMA, bisphenol A glycidyl dimethacrylate; Bis-MPEPP, bisphenol A polyethoxy methacrylate; DDDMA 12-dodecane- dimethacrylate; DMA, dimethacrylate; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate; YbF_3, ytterbium trifluoride.

immediatelly after curing (t0); MET Group D (D: delayed) and DMET Group D, in which other five samples were finished and polished after 24 h (t24) from curing. The unpolished samples of each groups were considered as internal control both at t0 and t24: MET Group C and DMET Group C (C: Control). DC and VMH evaluations were performed before and after each finishing and polishing procedure at both t0 and t24. Two different disks covered by aluminium oxide (Sof-Lex Disks , 3M, St. aul, MN, USA) were used to finish the samples: a final contouring medium disk (30 µm abrasive particle size) and a finishing fine disk (14 µm abrasive particle size). In this study, these different disk sizes were used in order to simulate the effect of finishing burs which can be used both in anterior or posterior reconstructions. Successively, the elastomer wheels

impregnated with aluminium oxide (Spiral Wheels system, 3M, St. Paul, MN, USA USA) were used for the polishing procedures. Each Sof -Lex disk was used in circular motion applying a light pressure for 10 secs with a slow speed handpiece at 7500 rounds/min. Then, the samples were polished using a Spiral Wheels system and diamond paste (Shiny paste C, Enamel Plus, Genova, Italy): they were used with a slow handpiece at 6000 rounds/min. All the procedures were performed by a single dental operator to minimize operator changes in variability. Right after each use, each finishing and polishing instrument was discarded. Before evaluations, the samples were washed with distilled water and dried. Then, all groups were stored for 24 hours at 37°C.

Degree of conversion analysis

A Perkin Elmer Spectrum One NTS FT-NIR spectrometer equipped with a FR-DTGS detector was used for DC evaluation [5, 14]. The spectra were acquired in reflection mode in the 10000-4000 cm^{-1} spectral range on the top of each sample by using the Near Infrared Reflectance accessory (NIRA) (Perkin Elmer). All the spectra were interpolated, and two points baseline linear fitted in the 6300-5300 cm^{-1}, and the height of the bands at 6165 cm^{-1} and 5990 cm^{-1} was calculated (Spectrum 10.4 software package, Perkin Elmer). The band at 6165 cm^{-1}, corresponding to the alkene carbon–carbon double bond vibration (band B), decreased during the polymerization process, while that at 5990 cm^{-1}, corresponding to the vibrational modes of the aromatic benzene ring, did not change in intensity (band A). For each spectrum, the ratio between band B and band A heights was calculated (band height ratio B/A). After plotting a calibration curve, each band height ratio B/A was converted in DC [5, 15].

Microhardness (VMH) analysis

On the same surface of the samples analysed by FT-NIR spectroscopy, the VMH was determined by the Leitz Micro-Hardness tester (Wetzalr GMBH, Wetzlar, Germany). Themethod consisted of indenting the sample by using a pyramid-shaped diamond with a load of 50 g for 15 secs.

For each sample, three measurements were performed, respectively on the middle of the sample, at 0.15 mm and 0.3 mm from the centre. After removing the load, the values of the two indentation diagonals were evaluated by using a microscope; the area of the sloping surface was obtained and used to determine the corresponding hardness value. Calculations were made by using Hardness-Course Vickers/ Brinell/ Rockwell copyright IBS 2012 version 10.4.4 software package [16–18].

Surface evaluation

A Field Emission Scanning Electron Microscopy Zeiss Supra 40 (SEM), using a power of 3000 Kvolt and a 400x magnification, was used for a further surface evaluation of both groups.

Statistical analyses

Statistical analysis was performed with R Project for Statistical Computing 3.3.0 (https://www.r-project.org/) and Microsoft Excel 2013. Normality of data distribution and homogeneity of group variances were verified by the Kolmogorov-Smirnov test and the Levene's test, respectively. Given the normality and homogeneity of the distribution, one-way analyses of variance (One-Way ANOVA) and Tukey's test for comparisons between groups , were chosen ($p< 0.05$). Pearson test was used to evaluate the correlation between the DC and VMH of each material.

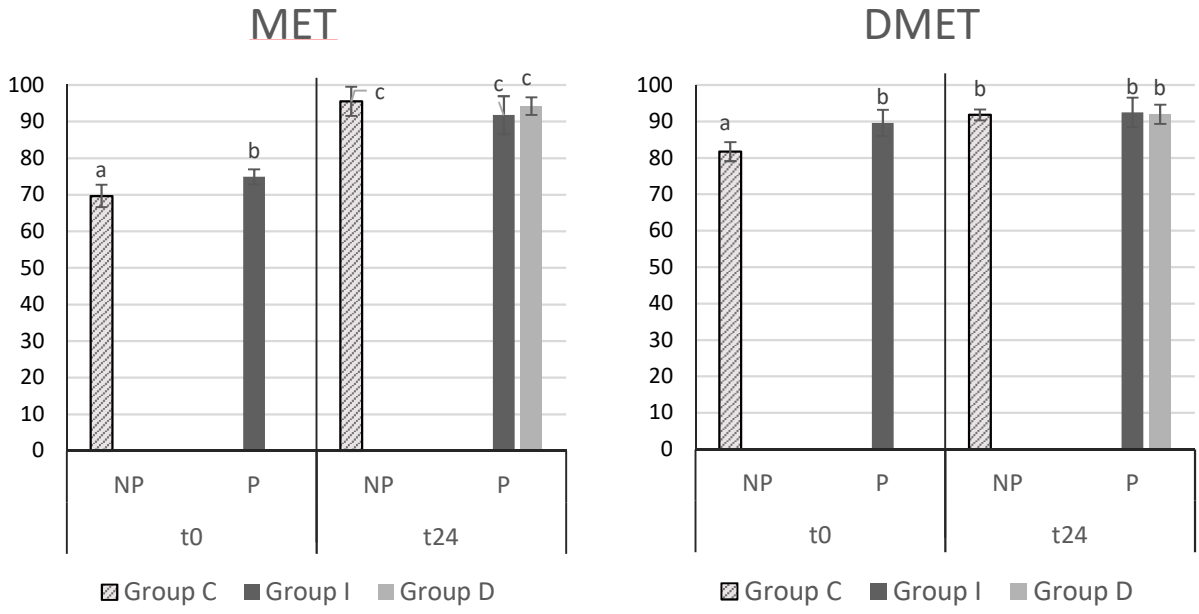
RESULTS

The statistical evaluations of DC and VMH are reported in Figures 1-2, respectively. ANOVA

test showed that DC and VMH of the tested

materials recorded significant differences. At t0, the DC of MET Group C (69.68 ± 3.90) was statistically different from MET Group I (74.88 ± 2.06) ($p=0.03$); at t24, all the groups, were not statistically different (MET Group C: 95.53 ± 4.01; MET Group I: 91.73 ± 5.21; MET Group D: 94.21 ± 2.43). At t0, DC of both DMET Group were statistically higher than the ones recorded with MET. At t0, DC of DMET Group C (81.70 ± 2.63) was statistically different from DMET Group I after polishing (89.57 ± 3.62)

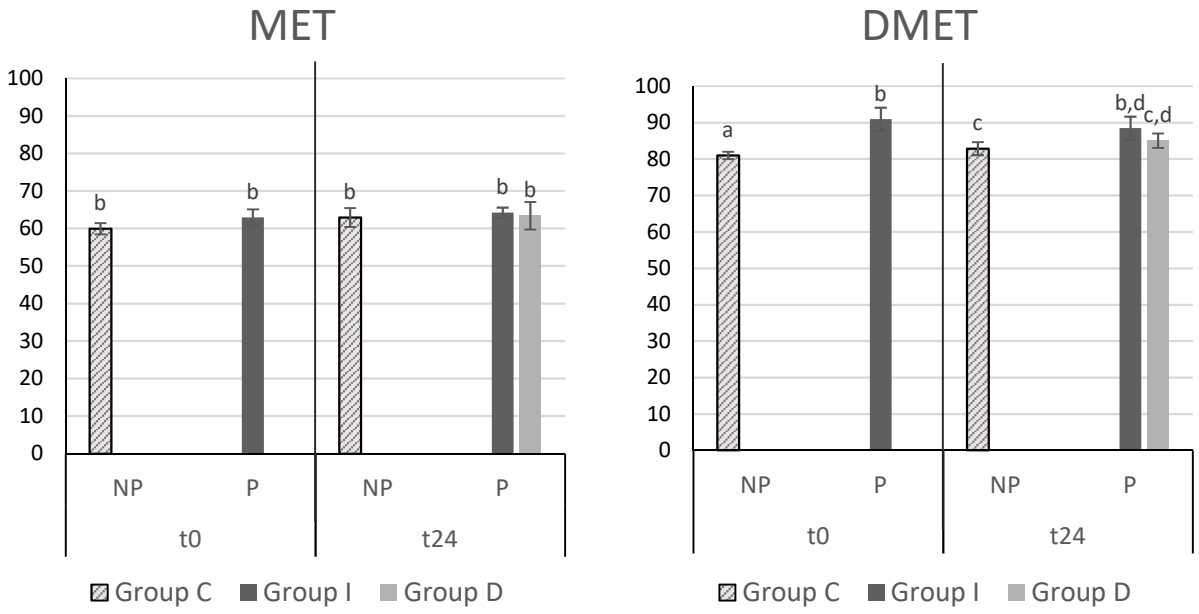
Figure 1: Degree of conversion calculated at t0 and t24, with different polishing time. Estelite Bulk-Fill Flow (MET); Filtek One Bulk Fill (DMET) composites.



*Student test, different superscript letters (a, b, c) represent statistically significant difference ($p < .05$); NP: the samples were not polished; P: the samples were polished; Group I: the samples were cured, finished and polished immediately after curing; Group D: the samples were cured at t0 but finished and polished after 24 hours. Group C: the samples were not polished, it works as internal control group.

($p=0.03$); at t24, DC of DMET Group C increased (91.78 ± 1.48), but not statistically differences were found between the other groups (DMET Group I: 92.48 ± 4.02; DMET Group D: 91.96 ± 2.62). Therefore, all DMET groups at t24 were not statistically different compared to DMET Group I at t0. However, at t24, all DC of both composites were not statistically different. Regarding VMH (Figure 2), although the VMH increased after 24 hours, the VMH values of all groups, both at t0 and t24, were not statistically different. At t0, the VMH of DMET Group I (90.90 ± 3.20) resulted statistically higher than DMET Group C (78.01 ± 4.13; $p=0.02$). At t24, VMH of DMET Group I (90.9 ± 3.20) was not statistically different from DMET Group I at t0 (88.43 ± 6.17; $p=0.57$) and from DMET Group D at t24 (85.03 ± 1.98; $p=0.29$). All VMH of DMET groups resulted statistically higher than

Figure 2:Vickers micro hardness calculated at t0 and t24, with different polishing time. Estelite Bulk-Fill Flow (MET); Filtek One Bulk Fill (DMET) composites.



*Student test, different superscript letters (a, b, c) represent statistically significant difference ($p < .05$); NP: the samples were not polished; P: the samples were polished; Group I: the samples were cured, finished and polished immediately after curing; Group D: the samples were cured at t0 but finished and polished after 24 hours. Group C: the samples were not polished, it works as internal control group.

both MET groups. No correlation was found between VMH and DC, both in MET (correlation value: 0.16) and DMET (correlation value: 0.40). SEM results showed that in the tested materials different roughness patterns may be detected. In MET, the lowest viscosity resin composite, both Groups I and D showed an irregular surface (Figures 3A, 3B). On the other hand, DMET Group I showed a surface smoother and more regular than DMET Group D (Figures 4A, 4B).

DISCUSSION

It is well known that the properties of resin composites are material dependent [2, 5, 8]. In clinical practice, considering the oxygen inhibition on the surface, finishing and polishing procedures are important for improving DC and VMH of resin composites and, hence, the success of a restoration [19]. Finishing and polishing steps are used

Figure 3. SEM images of Estelite: A) MET Group I: polished immediately after curing; B) MET Group D: polished after 24 hours from curing.

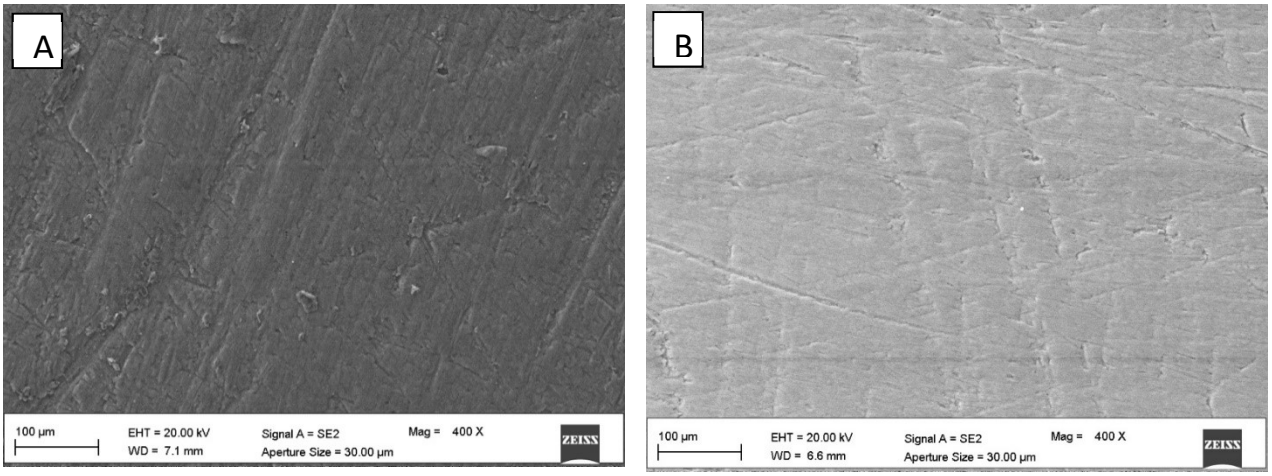
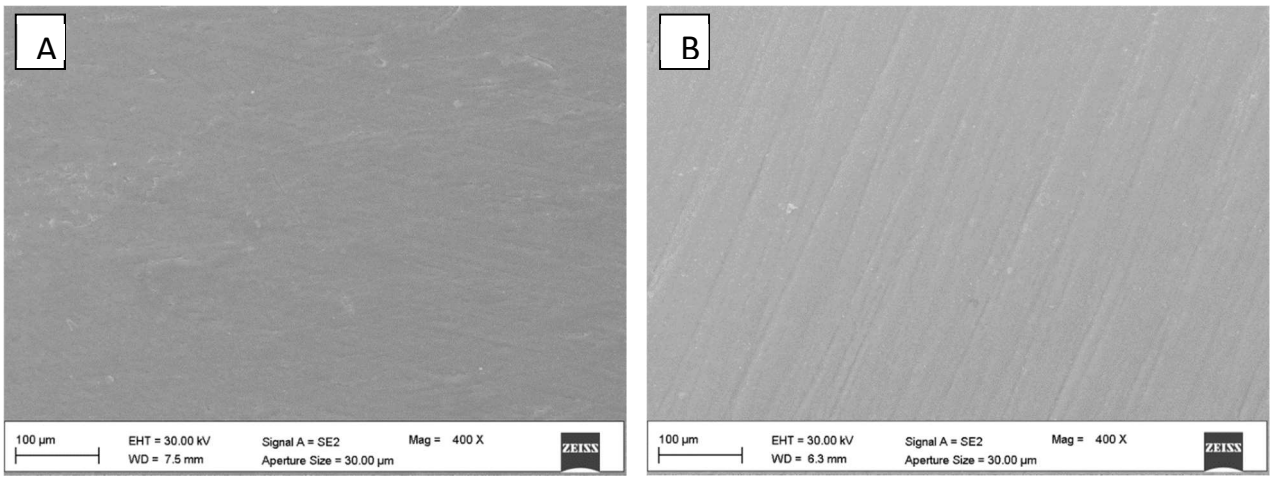


Figure 4. SEM images of Filtek One Bulk Fill Restorative composite: A) DMET Group I: polished immediately after curing; B) DMET Group D: polished after 24 hours from curing.



to modify and improve the profile of a direct composite reconstruction: its success depends on both the composite used and the system adopted to finalize it [11, 19]. Although, a few reports have investigated the chemical-physical properties of resin composites after using modern finishing and polishing systems, no studies have evaluated the timing effect of new polishing system on bulk fill composites containing methacrylate and dimethacrylate monomers [10, 13, 20–22]. In the present study, DC and

VMH of tested resin composites were evaluated after polishing procedures performed at different time. In summary, immediately after curing, for DMET of our study, polishing procedure helped the resin composite to immediately reach the same DC recorded at t24. On the other hand, for MET, the polishing procedures increased the DC at t0; however, it did not reach the t24 values. As hypothesized by the scientific literature, the process behind this phenomenon could be explained by the fact that brushing, during polishing, could improve DC because the heat, generated by the rotary instruments, catalyses the polymerization process on the surface layer [23]. However, monomer content could explain the DC difference between MET and DMET: DMET resin composite has an addition-fragmentation monomer, which can promote the increase of DC better than dimethacrylate monomers during the polishing procedures. However, in our study, the polishing procedures influenced DC more in DMET than MET composite. Another important property concerning the dental composite and its clinical use is the hardness. It describes the deformation degree of the material and it is a valuable parameter of comparison between tooth structure and masticatory forces, especially in the posterior stress-bearing areas [24], thus being considered an important property for resin composites. The use of materials with a hardness similar to the one of dentin is mandatory to achieve an optimum clinical restoration performance, and this holds mainly for bulk fill resin composites [25]. Nowadays, clinicians often use bulk fill resin composites in posterior areas where their placement is difficult and occlusal forces are more relevant than in anterior areas [25]. Indeed, clinicians seldom leave resin composite restorations unfinished and unpolished, because surface roughness is the most frequently used parameter in assessing the surface quality of several composite materials [26].

Our results revealed that VMH values of DMET Group I increased after polishing ($p<.05$) and they were not statistically different from DMET Group D at t24 ($p>.05$). Regarding

MET VMH of all the Groups, the values were not statistically different (p>.05). Some authors have hypothesized that, immediately after curing, the surface layer, mostly composed of the organic matrix, may further polymerize during polishing, thus increasing the resistance of the surface layer [13, 27]. However, in our results, the two tested composites are different not only for the monomers type, but also in vol% and wt%, as well as in filler dimensions. DMET contains a thicker filler (DMET: 4-20 nm versus MET: 200 nm) and a higher wt% and vol% than MET, and consequently, it contains more filler surface to be linked by the matrix monomers and silanes. Maybe, the friction temperature of the polishing procedure could improve the silanes action [28–30]. The temperature could increase the ability of silanes to link filler and matrix, improving the hardness. Then, DMET could be more influenced by the temperature than MET, having a higher filler surface area (more vol% and small filler size) which one linked by the silanes MET. Although Chinellatti et al [31] proved that delayed finishing and polishing procedures generally result in a surface similar to or even harder than the one obtained with immediate finishing and polishing procedures, we underline that, in their study, they used flowable, microfilled and minifilled composites, and no bulk fill composites were evaluated. Partially in disagreement with our results, Cenci et al [21] assessed that the polishing procedure, immediately after polymerization, causes an incomplete curing, and that the stress generated by rotary instruments may influence the integrity of the restoration, being the range of filler particles a possible explanation for this discrepancy. However, their study dealt only with microfilled or microhybrid resin composites, whereas neither bulk fill composites, nor monomers, nor nanofilled composites were evaluated. The performance of finishing and polishing procedures are often qualitatively verified using SEM, by evaluating shape and contour changes that may not be observed with a profilometer (which gives an objective and quantitative evaluation) [32, 33]. The SEM images of the tested

composites appear different: MET Groups show more irregular surfaces (> 0.2 μm) than DMET ones (Figures 3,4), mainly when polishing was performed at t0.

The surface roughness of a resin composite depends on several material factors, such as the type, shape, size and distribution of the inorganic filler, and can increase the risk of plaque formation [34–37]. In our case, the presence of thinner filler size results in a reduced matrix interparticle spacing, meaning more protection of the organic matrix and a reduced filler grabbing (as in DMET, with a higher ratio nano filler/organic matrix): more the organic matrix than filler influences the surface roughness because the matrix, less hard than filler, can show some irregularities after polishing.

Finally, as the previous traditional resin composites, both in MET and DMET resin composites, no correlation between DC and VMH was found. It means that not only the DC but also the resin composition influence the mechanical properties of the new bulk fill composites.

Although our study presents several limitations, such as the limited number of resin composites and unique polishing system used, the present study can represent the first report on the influence of polishing timing on chemical and mechanical properties of the methacrylate and dimethacrylate bulk fill materials.

In summary, the first hypothesis can be rejected. In Group I of both composites, polishing procedures increase the DC at t0, reaching, mainly in DMET, the same DC of t24. Furthermore, the second hypothesis can be partially rejected. In DMET Group, the polishing procedure lead an improvement of VMH, both if it was performed at t0 and t24. In MET, polishing procedures do not statistically affect VMH values in both groups.

Our clinical advises may be applied for the above tested high (DMET) and low (MET) viscosity composites: in the case of DMET, the clinicians with an immediate polishing can achieve better chemical-physical properties and a smoother surface than the ones reached with a delayed polishing. On the other hand, in case of MET, although the

polishing improves the DC, we recommend waiting 24 hours before polishing procedures, because the surface roughness is worse in immediate polishing.

CONCLUSION

In the bulk fill composites, not only the type and size of the filler, but also the type of monomers and silane content could influence the chemical and physical properties of resin materials after polishing procedures. However, the effect of polishing procedures is material dependent.

CONFLICT OF INTEREST

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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CHAPTER III:

Regenerative Dentistry

In clinical dental practice, the treatment of carious lesions consists on the removal of the damaged tissue and its replacement with composite resins [33,97]. If bacterial infection spread until the pulp chamber, the whole dental pulp is removed and the treatment of choice is the root canal therapy, in which the pulp chamber and roots are filled with synthetic materials [98]. When the tooth is lost, dental implants are used to replace the missing teeth. Nowadays, all current treatments are reparative and no dental regenerative treatments are available.

The tooth can be divided in the crown and the root. Enamel and dentin are the main mineralized components of the crown and the root is constituted of cementum and dentin. In dental crown, the enamel-dentin complex provides rigidity and stability under occlusal forces; however, some external factors, such as mechanical trauma, chemicals, and bacterial infections can damage the tooth. The components of tooth have different embryonic origin: dentin (odontoblasts), dental pulp, cementum, periodontal ligament, and alveolar bone have a cranial neural crest origin (mesenchymal part). Ameloblasts, which produce proteins for enamel formation, have an ectodermal origin. When the formation of tooth crown is complete, ameloblasts undergo programmed death, losing their ability to repair enamel. In dental lesions, if the damage is minimal, odontoblast progenitors or stem cells of the pulp are able to differentiate into odontoblasts and form amorphic dentin. This is a reparative dentin and is constituted by irregular dentinal tubules embedded in dentin matrix [99,100].

Instead of using resin composite or implants, regenerative dentistry could bring alternative solutions in repairing dental tissues. Combination of different scaffolds

with stem cells could be one of these regenerative solutions [97,101–103]. Stem cells play an essential role in tissue homeostasis, repair and regeneration. They have high proliferation capacity and ability to self-renew and to differentiate into different cell lineages [104,105]. In recent years different stem cell lineages have been used in clinics for cell-based therapies in order to regenerate damaged tissues. Stem cells are put into the site of a possible injury in order to regenerate the damaged tissue. In addition, they can differentiate by using the local environment information. This kind of autologous transplantation removes the need for immunosuppression and decreases the transplant rejection [97].

Stem cells can be classified as Embryonic Stem Cells (ESCs) and Adult Stem Cells (ASCs), according to their origin. The ESCs can differentiate into all cell types of human body while the differentiation potential of ASCs is restricted to certain cell lineages [106]. ASCs have been identified in adult tissues from both epithelial and mesenchymal origin such as skin [107], bone marrow [108], adipose tissue [109], peripheral blood [110], cartilage [111], intestine [112], and periosteum [113]. ASCs are more applicable than ESCs in stem cell-mediated therapies and regenerative medicine because of their ethical concerns. Since they have low immunogenicity and less tumorigenic potency than ESCs, they are promising candidates for regenerative therapies [114]. The first ASCs were taken from bone marrow or blood, but they demonstrated drawbacks caused by the stem cells stimulatory drugs [115]. Since then, several other populations of ASCs have been identified, even in various dental tissues. Dental stem cells (DSCs) are considered a promising source of ASCs since they are

easily accessible. DSCs have been identified in the pulp of deciduous and permanent teeth [116,117].

Classification of Dental Stem Cells

Different DSCs have been isolated and characterized [101,118,119]: Dental Pulp Stem Cells (DPSCs); Stem cells from Human Exfoliated Deciduous teeth (SHED); Periodontal Ligament Stem Cells (PDLSCs); Dental Follicle Progenitor Cells (DFPCs); and Stem Cells from Apical Papilla (SCAP). DPSCs, SHED, and SCAP are the dental pulp-related stem cells, PDLSCs and DFPCs are periodontium-related stem cells [120]. Other dental-related stem cells have been identified later; they are Gingival Mesenchymal Stem Cells (GMSCs) and human Natal Dental Pulp Stem Cells (NDP-SCs) [121,122]. DPSCs were first identified from adult human dental pulp by Gronthos and colleagues in 2000 [117]. However, the existence of stem cells in dental pulp has been reported by Yamamura in 1985 [123].

The differentiation potential of three cells lineages were evaluated at Institute of Oral Biology of University of Zurich: human DPSCs (hDPSCs), human gingival fibroblast (hGFs) and human foreskin fibroblasts (hFFs).

Dental pulp stem cells

The hDPSCs can be extracted from common non-invasive surgical practice that can be performed in adults, such as tooth extraction. Dental pulp tissue and hDPSCs can be easily harvested in the human third molars, since these teeth are often discarded

after the extraction. hDPSCs are considered similar to mesenchymal stem cells (MSCs) not only because they have a good proliferative potential, but also for their ability to differentiate into multiple cell lineages in vitro [105].

Gingival fibroblasts

Gingiva is a component of oral mucosa and is involved in biological mucosal barrier [122]. It is the oral tissue surrounding the tooth and attached to the alveolar bone. Regenerative and wound healing capacity are the main properties of gingiva tissue: it is characterized by a rapid healing and little scarring, in contrast to the common scar formation present in skin [124]. Histologically, gingiva is composed of three layers: an epithelial layer, a basal layer, and a lower spinous layer that is similar to the dermis of skin. MSCs and hGFs have several common properties in terms of morphology, cell-surface marker, gene expression patterns, and differentiation potential [125,126]. hGFs demonstrated a multilineage differentiation potential into adipogenic, osteogenic, and chondrogenic cell lineages in vitro [125,126]. The major advantage of using gingiva is the possibility to achieve the cells without sacrificing the tooth. Moreover, gingival tissues can be obtained from minimally invasive procedures at any time in life. Due to these properties, fibroblasts could be a valid alternative for regenerative treatments.

Foreskin fibroblasts

hFFs can differentiate into bone, cartilage and fat [127] they are accessible after biopsies. Several studies have indicated that foreskin tissues contain subpopulations of mesenchymal progenitor/stem cells that allow cytodifferentiation into multiple lineages [128–130].

In our study, hDPSCs, hGFs and hFFs cells were cultured in two different mediums: osteogenic and adipogenic medium. Cells were cultured for 21 days and harvested at day 0 (plating day), day 7, day 14 and day 21. Alizarin Red S and Oil Red O were performed to respectively identify extracellular calcium and lipids. After collecting the cells and performing the RNA extraction, DNA synthesis and quantitative real-time PCR were performed in order to evaluate the expression changes of the following transcription factors:

- Nanog and Octamer-binding transcription factor 4 (OCT4) are transcription factors required to maintain the pluripotency and self-renewal of ESCs. They regulate genes involved in coordinating multiple cellular functions [131,132].

- Runt-related transcription factors 2 (RUNX2) is an osteoblast transcription factor and Osterix SP7 (OSX/SP7) is a transcription factor required for osteoblast differentiation. Both of them are transcription factors which are involved in the osteoblastic differentiation [133].

- Peroxisome Proliferator-Activated Receptor (PPAR-γ2), a nuclear receptor that is almost exclusively expressed in adipocytes and lipoprotein lipase (LPL), an enzyme

of lipoprotein triglyceride metabolism which is expressed in preadipocytes [134], are involved in adipogenic differentiation [135].

- Alkaline phosphatase (ALP) is involved with the initial phase of dentin matrix biomineralization and with osteogenic differentiation [136].
- Dentin sialophosphoprotein (DSPP) are involved in osteoblastic and odontogenic differentiation [137].

Low levels of NANOG and OCT4 was identified in basal conditions in hDPSCs, hGFs and hFF. Mostly during osteogenic differentiation, NANOG and OCT4 expression initially upregulated and modulated in all three cell populations. Importantly, the dynamic of the modulation of these genes in the three cell types was very different, showing a cell-type-specific regulation of stemness-related genes upon differentiation. This indicates that the expression of NANOG and OCT-4 is not simply increased or decreased in equal measure as differentiation occurs or subsides. These results apparently contradict previous findings showing down-regulation of this gene during cytodifferentiation [138,139].

In osteogenic conditions, both hDPSCs and hFFs showed sustained RUNX2 expression, a moderate peak of SP7/OSX expression at day 14, and progressive upregulation of ALP. hGFs failed to maintain RUNX2 expression and to upregulate ALP. However, hGFs showed a striking peak in SP7/OSX expression at day 14, associated with a major upregulation of OCT4 and NANOG.

In adipogenic culture conditions, significant upregulation of PPAR-γ2 and LPL expression was observed in all experimental groups at early stages. This upregulation was particularly pronounced in hFFs. LPL expression was significantly higher in hFFs but not in hGF, when compared to hDPSCs, despite the higher adipogenic potential of both these fibroblastic populations. Fibroblasts expressed significantly higher levels of PPAR-γ2 already in basal conditions, with hGFs expressing over 20-fold more PPAR-γ2 than hDPSCs. These results indicate that hFFs and hGFs possess a significantly higher adipogenic potential compared to hDPSCs.

Importantly, hDPSCs were the only cell type upregulating DSPP expression when cultured in osteogenic conditions. This is in accordance with their known ability to give rise to odontoblasts [140,141]. The observed dynamic modulation of RUNX2, OSX and ALP also correlates with an odontoblastic differentiation program. During murine tooth development, RUNX2 and OSX are highly expressed in immature odontoblasts, while RUNX2 is downregulated upon terminal differentiation [142]. ALP, on the contrary, is expressed at high levels also in mature odontoblasts [143]. In conclusion, the present results support the idea of using fibroblasts for regenerative purposes based on their multilineage differentiation potential. Both hGFs and hFFs contain multipotential progenitors that are able to form osteogenic and adipogenic tissues. Moreover, they are more prone towards adipogenic differentiation when compared to hDPSCs. However, hDPSCs might represent a more appropriate cell population for regenerative purposes involving bone and dental tissues.

PAPER REFERRING TO CHAPTER III

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A comparative *in vitro* study of the osteogenic and adipogenic potential of human dental pulp stem cells, gingival fibroblasts and foreskin fibroblasts

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Human teeth contain a variety of mesenchymal stem cell populations that could be used for cell-based regenerative therapies. However, the isolation and potential use of these cells in the clinics require the extraction of functional teeth, a process that may represent a significant barrier to such treatments. Fibroblasts are highly accessible and might represent a viable alternative to dental stem cells. We thus investigated and compared the *in vitro* differentiation potential of human dental pulp stem cells (hDPSCs), gingival fibroblasts (hGFs) and foreskin fibroblasts (hFFs). These cell populations were cultured in osteogenic and adipogenic differentiation media, followed by Alizarin Red S and Oil Red O staining to visualize cytodifferentiation. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed to assess the expression of markers specific for stem cells (*NANOG*, *OCT-4*), osteogenic (*RUNX2*, *ALP*, *SP7/OSX*) and adipogenic (*PPAR-γ2*, *LPL*) differentiation. While fibroblasts are more prone towards adipogenic differentiation, hDPSCs exhibit a higher osteogenic potential. These results indicate that although fibroblasts possess a certain mineralization capability, hDPSCs represent the most appropriate cell population for regenerative purposes involving bone and dental tissues.

Stem cells play an essential role in tissue homeostasis, repair and regeneration due to their high proliferation capacity, their ability to self-renew and to differentiate towards different cell lineages[1]. Stem cells reside in specific niches, which represent specialized microenvironments that maintain the ability of stem cells to self-renew and influence their fate[2]. In recent years a variety of stem cell populations have started to be used in the clinics for regenerative/repair purposes. The fundamental idea of these cell-based therapies is that stem cells administered to the injury site can differentiate into cells that are specific to the local environment, thus supporting the repair/regeneration of the damaged tissue[3]. Mesenchymal stem cells (MSCs) can give rise to cells of different mesodermal lineages such as osteoblasts, adipocytes, chondrocytes[4], cardiomyocytes, pericytes, endothelial cells and smooth muscle cells[3]. This potential, combined with the fact that they are easily isolated, manipulated and expanded *in vitro*[5], make them a precious tool for successful regenerative medical trials. According to the International Society for Cellular Therapy, three minimal requirements are defined for human MSCs: (1) they have to be plastic-adherent under normal culture conditions, (2) they should express CD73, CD90 and CD105, but not CD11b or CD14, CD19 or CD79α, CD45, CD34 and HLA Class II surface markers, (3) they must be able to differentiate into osteoblasts, chondroblasts and adipocytes *in vitro*[6].

MSCs represent inhomogeneous cell populations with varying degrees of lineage commitment[7]. Adult bone marrow was the first source of MSCs identified and in more recent years other stem cell sources were discovered in a variety of tissues and organs such as periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and teeth[3]. Dental MSCs were initially isolated from the pulp of human third molars in 2000[8] and since then

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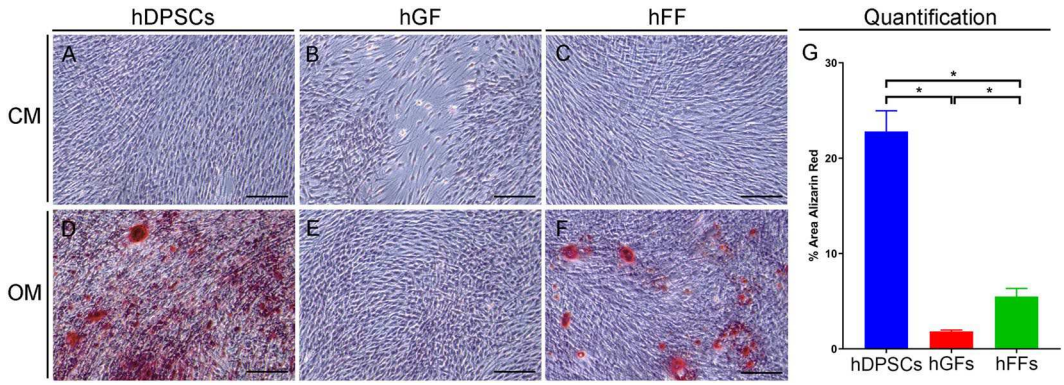

Figure 1. Microscopic views of Alizarin Red S staining of cultured human dental pulp stem cells (hDPSCs), human gingival fibroblasts (hGFs) and human foreskin fibroblasts (hFFs) cultured for 21 days with control medium (CM; **A**–**C**) and osteogenic medium (OM; **D**–**F**). Calcium deposits (red color) are evident in hDPSCs (**D**) and hFFs (**F**), but not in hGFs (**E**), cultured in OM. (**G**) Quantification of Alizarin Red staining as proportion of Alizarin-red-positive surface (in %). Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, $*p < 0.05$; $**p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: CM, control medium; hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium. Scale bars: 100 μm.

MSCs were also isolated from the pulp of exfoliated deciduous teeth, apical papilla, dental follicle, periodontal ligament[9] and periapical cysts[10]. However, these dental-derived MSC populations vary in their expression of stem cell surface markers and in their ability to differentiate into distinctive cell lineages[11]. Human dental pulp stem cells (hDPSCs) have been extensively studied over the past years and constitute very attractive candidates regarding cell-based regenerative therapies for a variety of reasons: they can be conveniently collected from extracted adult teeth without ethical concerns[8], they possess immunosuppressive activity[12] and can be safely cryopreserved without affecting their differentiation properties[13]. Consequently, hDPSCs may be isolated from a patient, stored and transplanted autologously at a later date, therefore making allogenic grafting and immunosuppression redundant[14]. hDPSCs are able to differentiate into odontogenic, osteogenic, chondrogenic, adipogenic, vascular, myogenic and neurogenic lineages[11,15]. Among adipose tissue, bone marrow and dental-derived MSCs, hDPSCs produce the greatest volume of mineralized matrix and therefore have greater potential for future applications in the regeneration of damaged tooth structures or bone in mandibular defects[16].

Fibroblasts are ubiquitously distributed in connective tissues and play a major role in synthesis and secretion of extracellular matrix, as well as in inflammation, wound healing and fibrosis[17]. MSCs and fibroblasts share several common properties in terms of morphology, cell-surface marker and gene expression patterns, as well as differentiation potential[18,19]. Indeed, *in vitro* studies have shown that fibroblasts are also plastic-adherent and capable of differentiating into bone, fat and cartilage, while expressing all of the MSC surface markers[20]. Additionally, similar to MSCs, fibroblasts are able to suppress mitogenic and allogeneic lymphocyte proliferation[21]. Because of these properties, fibroblasts hold the potential for clinical application in the treatment of many diseases and constitute a very appealing alternative for regenerative applications due to their high accessibility and availability.

Therefore, in this study we assessed the differentiation potential of two human fibroblastic populations, foreskin (hFFs) and gingival (hGFs) fibroblasts, and compared them to hDPSCs by means of *in vitro* differentiation assays, complemented with expression of specific stem cell, osteogenic and adipogenic marker genes.

Results

In vitro differentiation assays. *Osteogenic differentiation assay.* Staining with Alizarin Red S allows visualization of extracellular calcium deposits in a bright orange-red colour. Staining revealed that hDPSCs, hGFs and hFFs cultured in control medium (CM) were not able to form mineralized nodules. When cultured for 21 days in presence of osteogenic medium (OM) hDPSCs formed a dense mineralized plexus (Fig. 1A,D). hFFs also displayed unequally distributed mineralized nodules when cultured in OM (Fig. 1C,F), whereas no mineral deposits were visible in cultures of hGFs with OM (Fig. 1B,E). Quantification of the alizarin red staining confirmed the observations, showing a significantly higher Alizarin Red-staining in hDPSCs when compared to hGFs and hFFs (Fig. 1G), as well as in hFFs compared to hGFs.

Adipogenic differentiation assay. To monitor the adipogenic differentiation progress, cultured cell populations were dyed with Oil Red O, which stains lipid droplets in red colour. Staining revealed that after 21 days of culture in adipogenic medium (AM) hGFs and hFFs formed scattered lipid droplets (Fig. 2E,F), while no lipid droplets were observable in cultures of hDPSCs (Fig. 2D). The observation was confirmed by quantification of Oil Red O staining, which displayed significantly higher areas of Oil Red O staining in both hGFs and hFFs compared to hDPSCs (Fig. 2G). Furthermore, cells of the fibroblastic groups (Fig. 2E,F) exhibited a more spherical shape than hDPSCs (Fig. 2D).

Gene expression analysis. The expression levels of each analysed gene within the three groups (i.e., hDPSCs, hGFs and hFFs) are shown individually after different days of incubation in osteogenic and adipogenic

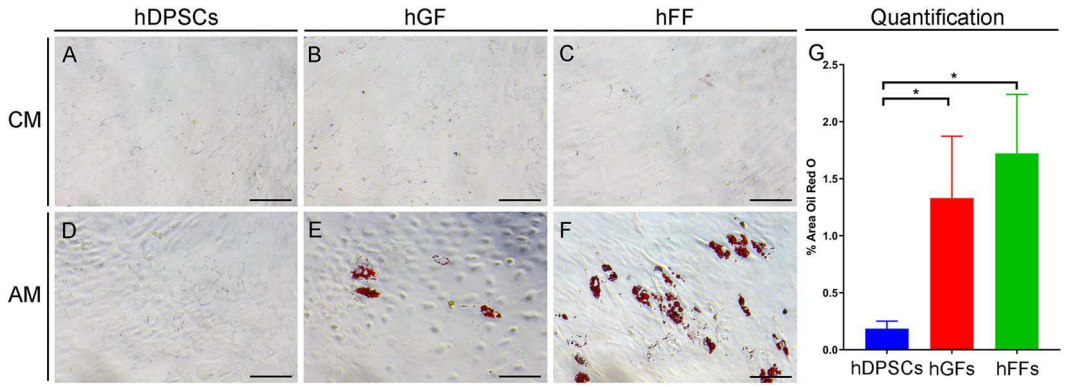

Figure 2. Microscopic views of Oil Red O staining of cultured hDPSCs, hGFs and hFFs on day 21 of incubation with control medium (CM; **A**–**C**) and adipogenic medium (AM; **D**–**F**). Lipid droplets in red color are visible in hGFs (**E**) and hFFs (**F**), but not in hDPSCs (**D**), cultured in the presence of AM. (**G**) Quantification of oil red O staining as proportion of oil red 0-positive surface (in %). Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: AM, adipogenic medium; CM, control medium; hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts. Scale bars: 100 μm.

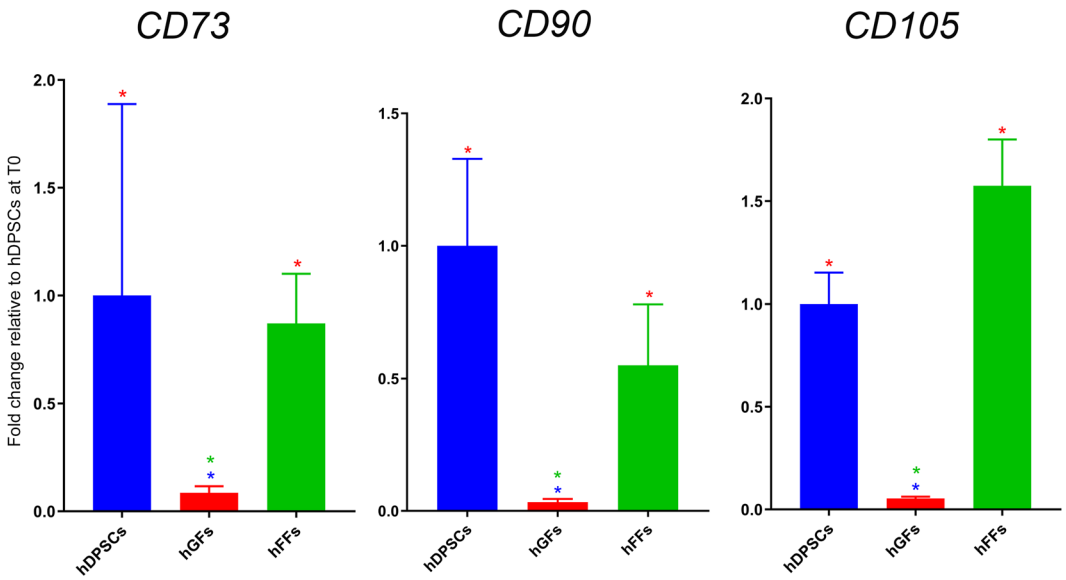

Figure 3. Gene expression levels of CD73, CD90 and CD105 in hDPSCs, hGFs and hFFs cultured in control conditions. Fold change is normalized to hDPSCs. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts.

medium. The gene expression levels of the samples within each group (cell type) are presented relative to the gene expression level on day one of the respective group (A-C in all panels), as well as normalized for the expression of the gene in hDPSCs on day 0 (before treatment; figure D in all panels).

Gene expression analysis of stem cell markers. We first verified and quantified the expression of genes used as markers for mesenchymal stem cells, namely *CD73*, *CD90* and *CD105*[6], in hDPSCs, hGFs and hFFs cultured in control conditions. Expression of these three genes was detected in all three cell populations analysed (Fig. 3). Of notice, hDPSCs and hFFs expressed comparable levels of *CD73*, *CD90* and *CD105*, while hGFs displayed significantly lower levels of these mRNAs (Fig. 3).

We then analysed the expression of *NANOG* and the Octamer-binding transcription factor 4 (*OCT-4*), recognized stem cell markers that play pivotal roles in the maintenance of self-renewal and pluripotency[22–27].

NANOG: *NANOG* was surprisingly upregulated in all cell types upon treatment with differentiation media. Incubation of hDPSCs in osteogenic conditions led to a significant upregulation of the expression of *NANOG* already after one week, followed by its progressive downregulation at day 14 and 21 (Fig. 4A). A similar pattern

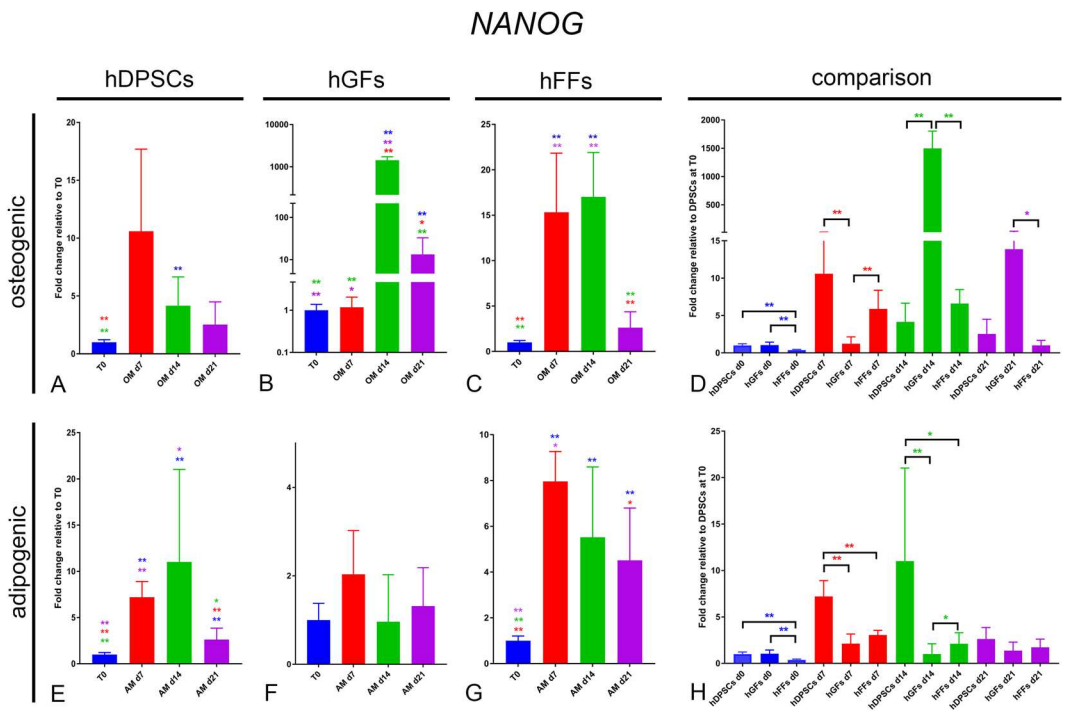

Figure 4. Gene expression levels of NANOG in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of OM and AM, relative to the respective expression levels at T0. D) Expression levels of NANOG normalized for NANOG expression in hDPSCs at T0 Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium; AM, adipogenic medium.

was observed in hFF, where *NANOG* expression increased by day 7 of culture in OM and significantly decreased by day 21 (Fig. 4C). hGFs displayed a different response, as they did not upregulate *NANOG* expression by day 7. However, in these cells we detected an extremely high *NANOG* expression at day 14, followed by its abrupt downregulation by day 21 (Fig. 4B). Less dramatic fluctuations were detected in the expression of *NANOG* in all cell types cultured in adipogenic conditions (Fig. 4E–G). hDPSCs and hFFs displayed an upregulation of *NANOG* expression by day 7, followed by its downregulation by day 21 (Fig. 4E,G), similarly to what was observed upon incubation with OM. Of notice, the upregulation of *NANOG* in hFFs in AM was much less pronounced than that observed in OM (Fig. 4C,G). hGF cultured in AM displayed a modest modulation of *NANOG* expression, characterized by a mild, upregulation at day 7 and downregulation at day 14. The observed difference in the levels of *NANOG* expression in control conditions at day 0, with hFFs expressing lower levels (approx. 50% less) of NANOG compared to hDPSCs and hGFs, do not obviously correlate with the modulation of the expression of this gene throughout osteogenic and adipogenic differentiation (Fig. 4D,H).

OCT-4: *OCT4* expression displayed trends grossly similar to those observed for *NANOG* expression. In hDPSCs, an increase, although variable, in *OCT4* expression at day 7 in OM was followed, at the subsequent timepoints, by its generalized maintenance at levels 4 times higher than in control conditions (Fig. 5A). Interestingly, culture in AM led to a strong upregulation of *OCT4* expression by day 14, followed by its significant downregulation by day 21 (Fig. 5E). Expression of *OCT4* in hFFs showed a trend similar to that observed analysing *NANOG* expression (Fig. 5C). In this case, however, the modulation of *OCT4* expression between the different time points in hFFs was less pronounced (Fig. 5C). Similar to *NANOG*, *OCT4* expression in hGFs cultured in OM displayed a great increase at day 14, followed by a sudden downregulation at day 21 (Fig. 5B). hGF cultured in AM displayed an opposite but much more modest modulation of *OCT4* expression (Fig. 5F). In these cells, *OCT4* was mildly upregulated at day 7, downregulated at day 14 and upregulated again at day 21 (Fig. 4B). In contrast to what observed for *NANOG*, at the basal level, hFFs displayed a 2-fold higher expression of *OCT4*, compared to hDPSCs and hGFs (Fig. 5D,H).

Gene expression analysis of osteogenic and odontogenic markers. Runt Related Transcription Factor 2 (*RUNX2*), alkaline phosphatase (*ALP*) and osterix (*SP7/OSX*) are well-established osteogenic markers[28–30]. RUNX2 is crucial for the formation of mineralized tissues, and is generally used as a marker of early phases of osteogenic differentiation. *ALP* is a widely expressed hydrolase enzyme and plays a key role in the mineralization of hard tissues, as the hydrolysis of phosphate esters supplies free phosphate that is required for the creation of hydroxyapatite crystals[31,32]. OSX is encoded by the *SP7* gene and is an osteoblast-specific transcription factor[29,33]. *DSPP* codes for dentin sialophosphoprotein, a key component of the dentin matrix, expressed at highest levels by odontoblasts[34].

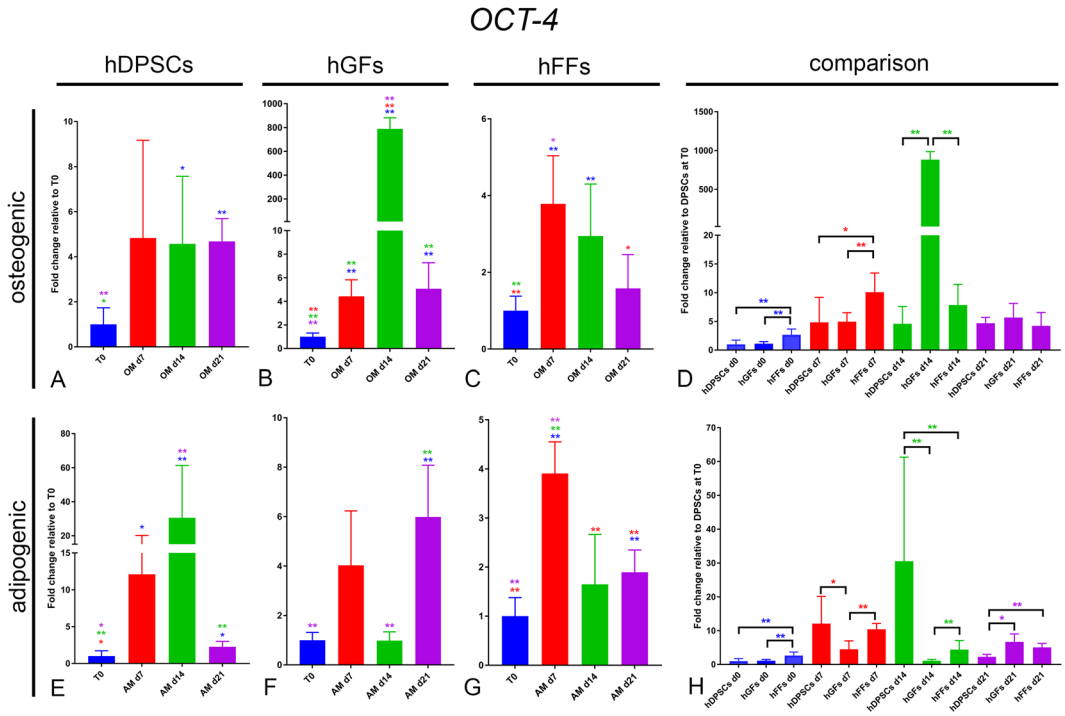

Figure 5. Gene expression levels of *OCT*-4 in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of OM and AM, relative to the respective expression levels at T0. (**D**) Expression levels of *OCT*-4 normalized for *OCT*-4 expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium; AM, adipogenic medium.

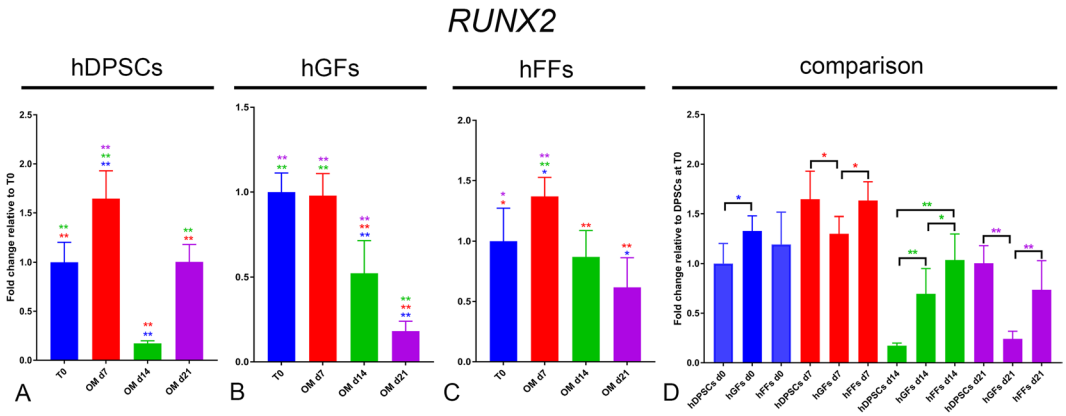

Figure 6. Gene expression levels of *RUNX2* in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of OM, relative to the respective expression levels at T0. (**D**) Expression levels of *RUNX2* normalized for *RUNX2* expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium.

RUNX2: hDPSCs and hFFs incubated with OM exhibited upregulation of *RUNX*2 expression levels at day 7. In both groups *RUNX*2 expression decreased at day 14, and increased subsequently in DPSCs at day 21 (Fig. 6A,C,D). In hGFs, incubation in OM did not lead to any increase in *RUNX2* expression, and ultimately led to its significant downregulation at day 14 and 21 (Fig. 6B). At basal levels the expression of *RUNX2* was comparable in all groups, while it was surprisingly lower in hDPSCs at day 14 (Fig. 6D). At day 21, RUNX2 expression was significantly higher in hDPSCs and hFFs, compared to hGFs (Fig. 6D).

ALP: An increase in *ALP* expression was observed in all cell groups cultured in presence of OM (Fig. 7). In hDPSCs *ALP* upregulation was already significant at day7, and reached a >100-fold increase by day 14, maintained

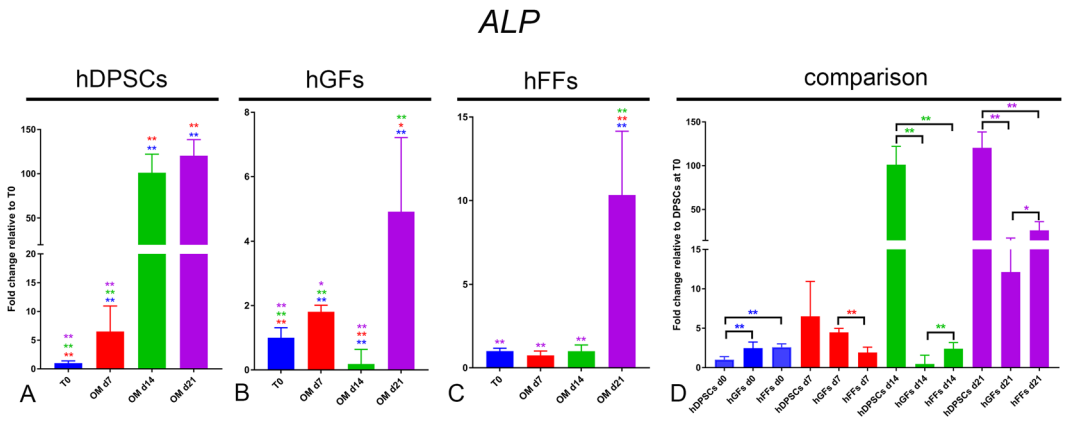

Figure 7. Gene expression levels of *ALP* in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of OM, relative to the respective expression levels at T0. (**D**) Expression levels of *ALP* normalized for *ALP* expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, $*p < 0.05$; $**p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium.

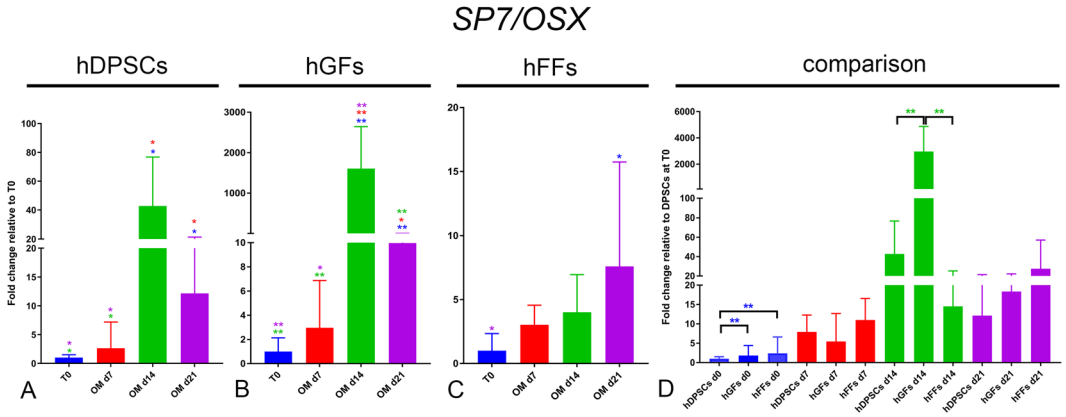

Figure 8. Gene expression levels of *OSX* in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of OM, relative to the respective expression levels at T0. (**D**) Expression levels of *OSX* normalized for *OSX* expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, $*p < 0.05$; $**p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium.

at day 21 (Fig. 7A). The increase was much more modest in hGFs and hFFs, where *ALP* expression reached a maximum of a 5-fold and 10-fold increase compared to time 0, respectively (Fig. 7B,C). Basal levels of *ALP* were comparable among all groups, although hGFs and hFFs displayed a mildly higher expression when compared to hDPSCs. From day 14, *ALP* levels were significantly higher in hDPSCs than in hFFs and hGFs (Fig. 7D).

SP7/OSX: Incubation with OM induced a significant upregulation of *SP7* expression already at day 7 in hDPSCs and hGFs (Fig. 8A,B). At day 14, hDPSCs and hGFs showed a massive *SP7* upregulation (Fig. 8A,B), while the levels detected in hFFs remained constant (Fig. 8C). At day 21, hDPSCs and hGFs showed a downregulation of *SP7* expression, which however remained significantly higher than that observed at T0 (Fig. 8A–C). At basal levels, hDPSCs displayed a slightly lower expression of *SP7* (Fig. 8D).

DSPP: *DSPP* expression was significantly and progressively upregulated in hDPSCs at 7 and 14 days of culture in osteogenic conditions, and it was then downregulated to basal levels by day 21 (Fig. 9A). No significant increase in *DSPP* expression could be detected in hGFs and hFFs (Fig. 9B,C). On the contrary, *DSPP* expression became transiently undetectable in hGFs upon incubation in OM (Fig. 9B). At all stages from day 7 to day 21, *DSPP* levels were significantly higher in hDPSCs compared to both hGFs and hFFs (Fig. 9D).

Gene expression analysis of adipogenic markers. Peroxisome proliferator-activated receptor γ2 (*PPAR-γ2*) and lipoprotein lipase (*LPL*) are used as adipogenic differentiation marker genes. PPAR-γ2 is considered as a master regulator of adipogenesis[28], while LPL is involved in lipid transport and provides glycerol and free fatty acids by catalysing the hydrolysis of triglycerides[35].

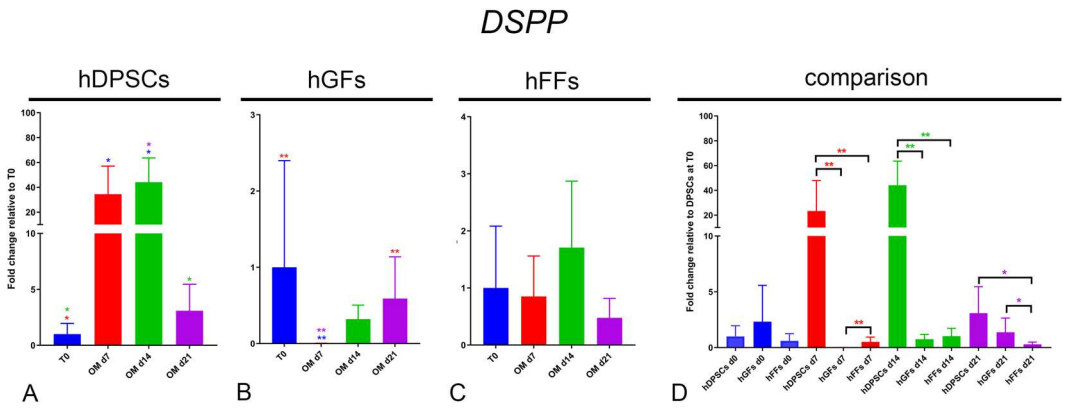

Figure 9. Gene expression levels of *DSPP* in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of OM, relative to the respective expression levels at T0. (D) Expression levels of *DSPP* normalized for *OSX* expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; OM, osteogenic medium.

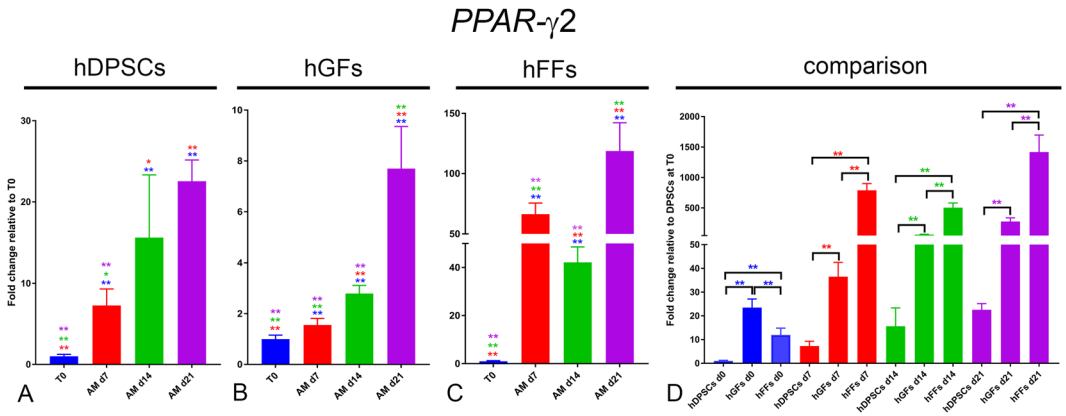

Figure 10. Gene expression levels of *PPAR-γ2* in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 1, 7, 14 and 21 days in presence of AM, relative to the respective expression levels at T0. (**D**) Expression levels of *PPAR-γ2* normalized for *PPAR-γ2* expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, *$p < 0.05$; **$p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; AM, adipogenic medium.

PPAR-γ2: *PPAR-γ2* expression was upregulated in all groups cultured in the presence of AM (Fig. 10). This upregulation was extremely pronounced in hFFs, where *PPAR-γ2* expression reached levels 70-fold higher than at T0 already after 7 days, and remained high throughout the differentiation period, with a decrease at day 14 (Fig. 10C). *PPAR-γ2* was also upregulated in hDPSCs and hGFs cultured in AM, albeit to a lower extent. In these two groups, *PPAR-γ2* expression increased progressively from day 0 to day 21 (Fig. 10A,B). Interestingly, expression of *PPAR-γ2* was significantly higher in fibroblasts compared to hDPSCs already at time 0, with *PPAR-γ2* expression in hGFs even >20-fold higher than that detected in hDPSCs (Fig. 10D).

LPL: *LPL* expression was upregulated in all three groups cultured in AM (Fig. 11). In hDPSCs *LPL* expression peaked at day 14, to then decrease at day 21 (Fig. 11A). In hGFs the upregulation observed at day 7 was followed by a progressive downregulation at day 14 and day 21 (Fig. 11B). hFFs showed an opposite trend, as they displayed a continuos upregulation of *LPL* expression from day 0 to day 21, with a peak of 400-fold increase at the latest timepoint (Fig. 11C). In contrast to what was observed with *PPAR-γ2*, the expression levels of *LPL* before any treatment were comparable among the three groups (Fig. 11D).

Discussion

Carious and periodontal diseases such as periodontitis, fractures, genetic defects or aging can lead to tooth damage and loss, thus decreasing the quality of life[36]. So far in dental clinical practice the treatment of choice for replacing missing teeth are osseointegrated dental implants[37], while the treatment of a carious lesion consists of the removal of the infected hard tissue part and its replacement by composite resins[38]. When the bacterial infection reaches the dental pulp, root canal therapy is the treatment of choice, in which the pulp is substituted

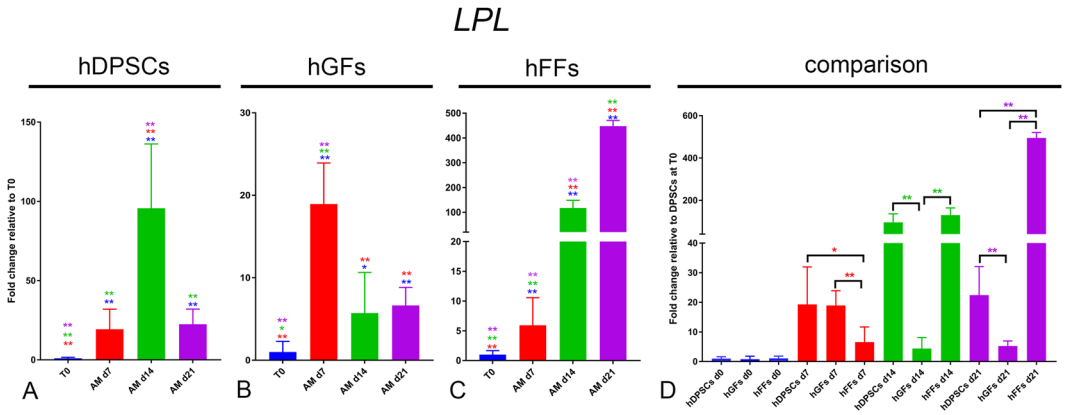

Figure 11. Gene expression levels of *LPL* in cultured hDPSCs (**A**), hGFs (**B**) and hFFs (**C**) for 0, 7, 14 and 21 days in presence of AM, relative to the respective expression levels at T0. (**D**) Expression levels of *LPL* normalized for *LPL* expression in hDPSCs at T0. Asterisks: Mann Whitney – U/Wilcox Rank Sum Test, $*p < 0.05$; $**p < 0.01$. Colour of asterisks onto each column indicates the column used for the comparison. Abbreviations: hDPSCs, human dental pulp stem cells; hFF, human foreskin fibroblasts; hGF, human gingival fibroblasts; AM, adipogenic medium.

by synthetic filling materials[39]. Regenerative dentistry represents an alternative solution for the repair of dental tissues using a variety of techniques and therapeutic approaches[40]. For example, methods used nowadays include injection of stem cells and/or soluble molecules such as growth factors[41]. Therefore, the goal of regenerative dentistry is to achieve partial or complete regeneration of the damaged or missing dental tissues, thus restoring their biological function and structure[40]. Various promising *in vivo* studies have already demonstrated the potential of human dental pulp stem cells (hDPSCs) for regenerative purposes, such as alveolar and mandibular bone regeneration in patients or the reestablishment of dental pulp and mineralized tissues in dogs[42,43]. However, there is a need for alternatives since teeth do not always constitute an ideal cell source: the supply of hDPSCs is limited and bound to the extraction of the respective, healthy tooth[8]. Fibroblasts from different organs can be more accessible, as they can be obtained from a plethora of surgical procedures, and have been shown to share many similarities with mesenchymal stem cells (MSCs) such as the multilineage differentiation potential[19]. Fibroblasts for various sources were already analysed for their regenerative potential. Human gingival fibroblasts (hGFs) are easily obtainable from the gingiva that is often resected during general dental treatments[44]. We have recently shown that both hGFs and hDPSCs are able to attract vessels when seeded into silk fibroin scaffolds, and therefore may improve healing and regeneration of damaged tissues[45]. While there is still little information on the stem cell properties and differentiation potential of hGFs[46], several *in vitro* studies have shown that these cells are able to form osteogenic, chondrogenic and adipogenic tissues[47,48]. Human foreskin fibroblasts (hFFs) are accessible after circumcision/biopsies and are also capable to differentiate into bone, cartilage and fat[49]. Several studies have indicated that both gingival and foreskin tissues contain subpopulations of mesenchymal progenitor/stem cells that allow cytodifferentiation into multiple lineages[50–52]. However, it is not clear if distinct cell types exist within these two tissues, since hGFs and hFFs are barely distinguishable from MSCs regarding phenotype, expression of specific markers and immunosuppression responses[53,54].

Cell differentiation follows a shift in gene expression, a process involving the coordinated action of transcription factors, non-coding microRNA, DNA methylation, histone modifications and other chromatin remodeling activities[55,56]. *NANOG* and *OCT-4* are both well-established embryonic stem cell (ESC) markers. They play a major role in the maintenance of the pluripotent state of ESCs and are down-regulated as the cells become more committed[22,27]. Although previous studies suggested that these genes were no longer expressed in adult stem cells[23], more recent findings showed that their expression persists in MSCs[24]. We detected low levels of *NANOG* and *OCT4* in basal conditions in hDPSCs, hGFs and hFF, followed by a significant initial upregulation and successive modulation of their expression mostly during osteogenic differentiation in all three cell populations. Importantly, the dynamic of the modulation of these genes in the three cell types was very diverse, showing a cell-type-specific regulation of stemness-related genes upon differentiation, thus indicating that the expression of *NANOG* and *OCT-4* is not simply increased or decreased in equal measure as differentiation occurs or subsides. These results apparently contradict previous findings showing down-regulation of this gene during cytodifferentiation[23,57]. However, recent studies suggested that increased *OCT-4* expression enhances the ability of MSCs to differentiate into osteogenic and adipogenic lineages[58,59], possibly priming loci coding for factors fundamental for lineage commitment[60]. MSCs have been shown to differ in the expression of ESC markers depending on the source from which they were obtained[58]. It is likely that the decision if a cell will differentiate or remain quiescent and self-renew is the result of the interplay with many other transcription factors and pathways and a question of increased and decreased expression of genes over the course of time rather than a simple switch on or off[22,61]. In this regard, all groups modulated the expression of *OCT4* and *NANOG* both in osteogenic and adipogenic conditions. hGFs displayed a striking synchronized increase in the expression of these two genes after two weeks of incubation in osteogenic medium, a dynamic completely different from that of hDPSCs and hFF, which might

be correlated with the observed differences in differentiation potential. In fact, hDPSCs and hFFs, but not hGFs, were able to form mineralization nodules upon osteogenic induction *in vitro*. This correlates with the different modulation of osteogenic markers observed in hDPSCs and hFF, and hGFs. *RUNX2* is known as a master control gene in osteoblastic differentiation as it plays a crucial role in the differentiation of MSCs into preosteoblasts[62]. *SP7/OSX* is involved in the maturation of preosteoblasts into mature osteoblasts[29]. ALP plays an important role in the mineralization process[31] and is often used as a marker for osteogenic differentiation. In osteogenic conditions, both hDPSCs and hFF showed sustained *RUNX2* expression, a moderate peak of *SP7/OSX* expression at day 14, and progressive upregulation of *ALP*. hGFs failed to maintain *RUNX2* expression and to upregulate *ALP*. However, hGFs showed a striking peak in *SP7/OSX* expression at day14, associated with a major upregulation of *OCT4* and *NANOG*. Although previous *in vitro* studies have demonstrated that hGFs are capable to form mineralized deposits in osteogenic media concentrations that differ from those used for the present study[46], the observed expression patterns might be indicative of a cell-specific incapability to pursue the full osteogenic differentiation path. In this regard, it has been shown that *SP7/OSX* overexpression can induce osteogenic differentiation in murine embryonic stem cells and murine bone marrow stromal cells, but not in fibroblasts[63]. Importantly, hDPSCs were the only cell type upregulating DSPP expression when cultured in osteogenic conditions. This is in accordance with their tissue of origin and their known ability to give rise to odontoblasts[64,65]. The observed dynamic modulation of *RUNX2*, *OSX* and *ALP* also correlates with an odontoblastic differentiation program. During murine tooth development, *RUNX2* and *OSX* are highly expressed in immature odontoblasts, while *RUNX2* is downregulated upon terminal differentiation[66]. *ALP*, on the contrary, is expressed at high levels also in mature odontoblasts[67]. These observations suggest that hDPSCs cultured in osteogenic conditions actually show an odontoblastic-like differentiation dynamic. The expression of *PPAR-γ2*, a transcription factor essential in the formation of adipocytes[28], and *LPL*, which is expressed in preadipocytes and plays a crucial role in lipid metabolism and concentration of triglycerides[35], was analyzed in order to assess the adipogenic differentiation potential of the three cell populations. In adipogenic culture conditions, significant upregulation of *PPAR-γ2* and *LPL* expression was observed in all experimental groups already at early time points. This upregulation was particularly pronounced in hFFs. Histological staining with Oil Red O revealed lipid droplets in hFFs and hGFs, but not in hDPSCs. Similarly, only hFFs and hGFs showed a shift from fibroblastic/spindle to spherical cell shape, which represents a clear sign of adipogenic differentiation[28,35,68]. *LPL* expression was significantly higher in hFFs, but not in hGF, when compared to hDPSCs, despite the clearly higher adipogenic potential of both these fibroblastic populations (as indicated by Oil Red O stainings). Fibroblasts expressed significantly higher levels of *PPAR-γ2* already in basal conditions, with hGFs expressing over 20-fold more *PPAR-γ2* than hDPSCs. This higher expression was then maintained throughout the differentiation period. These results thus indicate that hFFs and hGFs possess a significantly higher adipogenic potential compared to hDPSCs. Nevertheless, *in vitro* differentiation assays do not constitute a physiological environment and therefore it is not clear whether the observed changes during cytodifferentiation are rather caused by a temporary up-regulation of tissue-specific genes due to artificial *in vitro* concentrations of substances and if they can truly be translated to an *in vivo* situation[18]. In conclusion, the present findings support the idea of using fibroblasts for regenerative purposes based on their multilineage differentiation potential. Both hGFs and hFFs contain multipotent progenitors that are able to form osteogenic and adipogenic tissues and are more prone towards adipogenic differentiation when compared to hDPSCs. However, hDPSCs might represent a more appropriate cell population for regenerative purposes involving bone and dental tissues.

Materials and Methods

Collection of human cells. The procedure for anonymized human dental pulp stem cells (hDPSCs) and human gingival fibroblasts (hGFs) collection at the Zentrum für Zahnmedizin, Zürich, was approved by the Kantonale Ethikkommission of Zurich (reference number 2012–0588) and the patients gave their written informed consent. All procedures were performed according to the current guidelines. All surgical procedures and tooth extractions were performed by professional surgeons and dentists. Human foreskin fibroblasts (hFFs) were purchased from ATCC (ATCC, Manassas VA, USA). Human dental pulp stem cells (hDPSCs) were isolated from the dental pulp of extracted wisdom teeth of healthy patients as previously described[45]. The dental pulps were enzymatically digested for one hour at 37 °C in a solution of collagenase (3 mg/mL; Life Technologies Europe BV, Zug ZG, Switzerland) and dispase (4 mg/mL; Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland). A filtered single-cell suspension was plated in a 40 mm Petri dish with hDPSC growth medium containing DMEM/F12 (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) with 10% fetal bovine serum (FBS) (PAN Biotech GmbH, Aidenbach, Germany), 1% penicillin/streptomycin (P/S) (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland), 1% L-glutamine (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland), and 0.5 μg/ml fungizone (Life Technologies Europe BV, Zug ZG, Switzerland) after washing away the enzyme solution. Cells were passaged at 80–90% confluence and expanded in the same growth medium. Gingival fibroblasts (hGFs) were isolated from healthy parts of gingiva collected from biopsies, as previously described[45]. Gingival tissues were washed in phosphate buffered saline (PBS) (Life Technologies Europe B.V., Zug ZG, Switzerland), sectioned into small pieces and placed in 35 mm Petri dishes (TPP Techno Plastic Products AG, Trasadingen SH, Switzerland) for the outgrowth of human gingival fibroblasts (hGFs). The fibroblast growth medium was composed by high glucose DMEM/F12 (Life Technologies Europe B.V., Zug ZG, Switzerland), 10% FBS, 1% P/S, and 1% HEPES (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland). hGFs were expanded in DMEM high glucose (Thermo Fisher Scientific AG, Reinach BL, Switzerland) supplemented with 10% FBS, 1% P/S, and 1% HEPES (Thermo Fisher Scientific AG, Reinach BL, Switzerland). Cells were passaged at 80–90% confluence.

Cell culture and differentiation. Cells were expanded as monolayers on T-150 culture flasks (Sarsted AG, Switzerland) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; ThermoFisher/Life

Technologies, Switzerland) supplemented with 10% Foetal Bovine Serum (FBS, Bioswisstech AG, Switzerland), 100 U/ml penicillin/streptomycin (Sigma-Aldrich/Merck, Darmstadt, Germany), and Amphotericin B 0.25 μg/μL (ThermoFisher Scientific, Switzerland) incubated at 37 °C in 5% CO_2. The medium was replaced every second day. Cells were passaged once a confluence of 70–80% was reached. Cells were washed once with phosphate buffered saline (PBS) before trypsin was added for 3 min at 37 °C for their detachment. Trypsin was blocked by addition of 5 volumes of DMEM/F12 supplemented with 10% FBS. The cells were then centrifuged and seeded into T25 flasks (Sarsted AG, Switzerland) for the differentiation assays. 20'000 cells per well were seeded onto 24-well-plates for histological staining, while for gene expression analysis, 250'000 cells were seeded onto T25 plates.

The osteogenic differentiation medium consisted of DMEM supplemented with Ascorbic Acid (200 μM), β-Glycerolphosphate (10 mM), Dexamethasone (10 nM) (Sigma-Aldrich/Merck, Darmstadt, Germany), and Amphotericin B 0.25 μg/μL (ThermoFisher Scientific, Switzerland). The adipogenic differentiation medium consisted of DMEM (1 ml) supplemented with Dexamethasone (1 μM), IBMX (0.5 mM), Indomethacin (200 μM), Insulin (10 μM) (Sigma-Aldrich/Merck, Darmstadt, Germany) and Amphotericin B 0.25 μg/μL. Cells were cultured for 21 days in osteogenic medium (OM) and adipogenic medium (AM). Cells were collected from the T25 flasks on day 0 (plating day), 7, 14 and 21 and used for RNA extraction. Cells cultured on 24-wells plates were cultured for 21 days, stained (see following paragraph) and examined under a bright-field microscope.

Stainings. *Alizarin Red S* staining was performed to identify extracellular calcium deposits of cells differentiated into osteoblasts. Alizarin Red S powder was dissolved in distilled water, pH 4.2. Cells were washed with PBS, fixed with 4% PFA for 30 min, washed with distilled water and finally Alizarin Red S staining solution was added to each well for 45 min at room temperature in the dark. Thereafter wells were washed with deionized water and then PBS was added. The cells were viewed under a bright-field microscope, where calcium deposits exhibited a bright orange-red color. *Oil Red O* staining was performed to identify lipids in cells differentiated into adipocytes. 300 mg of Oil Red O powder were added to 100 ml of 99% isopropanol and then mixed with deionized water and filtered through a funnel. Cells were washed with PBS, fixed with 4% PFA for 30 min, washed again with deionized water, 60% isopropanol was added for 2–5 min and after isopropanol aspiration Oil Red O was added for 5 min. Thereafter, the wells were rinsed with tap water, hematoxylin counterstain was performed for 1 min and cells were rinsed with warm tap water. The cells were viewed under a bright-field microscope, where lipids exhibited a red color while the nuclei of cells were blue.

Stainings were quantified by measuring the proportion of Alizarin-Red and Oil-Red-O positive area over the total area imaged, using Fiji[69]. 3 independent samples were analysed for each cell type.

Gene expression analysis. *Collection of cells and snap-freezing.* Cells were collected by trypsinization at day 0, 7, 14 and 21, snap-frozen in liquid nitrogen and stored at −80 °C.

RNA isolation and purification. The RNA isolation on snap-frozen cells from the differentiation assays using the RNeasy Plus Universal Mini Kit was performed according to the instructions given (Qiagen AG, Hombrechtikon ZH, Switzerland).

cDNA synthesis. Reverse transcription of the isolated RNA was performed using the iScript™ cDNA synthesis Kit and according to the instructions given (Bio-Rad Laboratories AG, Cressier FR, Switzerland). Briefly, 1000 ng of RNA were used for reverse transcription into cDNA. Nuclease-free water was added to add up to a total of 15 μl. 4 μl of 5x iScript reaction mix and 1 μl of iScript reverse transcriptase were added per sample in order to obtain a total volume of 20 μl. The reaction mix was then incubated for 5 min at 25 °C, for 30 min at 42 °C and for 5 min at 85 °C using a Biometra TPersonal Thermocycler (Biometra AG, Göttingen, Germany).

Quantitative real-time PCR. The 3-step quantitative real-time PCRs were performed using an Eco Real-Time PCR System (Illumina Inc., San Diego CA, USA). Expression level analysis of *GAPDH* (housekeeping gene), *ALP*, *SP7*, *RUNX2*, *PPAR-γ2*, *LPL*, *OCT-4* and *NANOG* were carried out using the SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad CA, USA) in combination with oligonucleotide primers (Table 1). Using MicroAmp® Fast Optical 48-Well Reaction Plates (Applied Biosystems, Carlsbad CA, USA), 5 μl of SYBR® Green PCR Master Mix reverse and forward primers (200 nM), and 2 ng of template cDNA were added to each well. The thermocycling conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 55 °C for 30 sec and 60 °C for 1 min. Melt curve analysis was performed at 95 °C for 15 sec, 55 °C for 15 sec and 95 °C for 15 sec. Expression levels were calculated by the comparative ΔΔCt method ($2^{-\Delta\Delta Ct}$ formula), after being normalized to the Ct-value of the *GAPDH* housekeeping gene. Gene expression analysis was performed on 6 independent samples per condition. Samples were always compared one-vs-one using the Mann Whitney - U/Wilcox Rank Sum Test (Graph Pad Prism 8.0).

Ethical approval and informed consent. The procedure for anonymized human dental pulp stem cells (hDPSCs) and human gingival fibroblasts (hGFs) collection at the Zentrum für Zahnmedizin, Zürich, was approved by the Kantonale Ethikkommission of Zurich (reference number 2012–0588; confirmed 2017–00932) and the patients gave their written informed consent. All procedures were performed according to the current guidelines. All surgical procedures and tooth extractions were performed by professional surgeons and dentists. Human foreskin fibroblasts (hFFs) were purchased from ATCC (ATCC, Manassas VA, USA).

Gene	Accession no.	Forward primer 5′-3′	Reverse primer 5′-3′	Amplicon length (bps)
GAPDH	NM_002046.5	AGGGCTGCTTTTAACTCTGGT	CCCCACTTGATTTTGGAGGGA	205
NANOG	NM_024865.3	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG	115
OCT-4	NM_002701.5	CTTTCTCAGGGGGACCAGTG	GGGACCGAGGAGTACAGTGC	102
RUNX2	NG_008020.1	GCCAGGGTCTAGGAGTTGTT	ACCCACCACCCTATTTCCTG	212
ALP	NM_000478.5	ATGAAGGAAAAGCCAAGCAG	ATGGAGACATTCTCTCGTTC	276
SP7 (OSX)	NM_152860.1	CCTCTGCGGGACTCAACAAC	AGCCCATTAGTGCTTGTAAAGG	127
DSPP	NM_014208.3	TTTGGGCAGTAGCATGGG	CCATCTTGGGTATTCTCT	181
PPAR-γ2	NM_138712.3	GAACGACCAAGTAACTCTCC	CGCAGGCTCTTTAGAAACTCC	137
LPL	NM_000237.2	ACGGCATGTGAATTCTGTGA	GGATGTGCTATTTGGCCACT	200

Table 1. List of the oligonucleotide primers used. Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *OCT*-4, Octamer-binding transcription factor 4; RUNX2, Runt-related transcription factor 2; ALP, Alkaline phosphatase; SP7(OSX), Sp7 Transcription factor; DSPP, Dentin Sialophosphoprotein; PPAR-γ2, Peroxisome proliferator-activated receptor; LPL, Lipoprotein lipase.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

M.B., R.M. Collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. A.W., P.P. Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript. G.O. Data interpretation, manuscript writing and editing, final approval of the manuscript. T.A.M. Conception and design, data analysis and interpretation, manuscript writing, critical reading and editing of the manuscript, final approval of manuscript, financial support.

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CONCLUSIONS

The research project of my doctorate has accomplished the general aim to evaluate different strategies to save and replace the tooth tissues. Firstly, innovative tools for preventive dentistry were investigated. New oral care tool and oral diagnostic devices were described in order to improve oral health status of the patient. Secondly, innovative restorative resin-based materials, that are frequently used for tooth reconstruction, were evaluated to better understand their behaviours and their mechanical and chemical properties. Furthermore, polishing procedures for resin-based composites and luting protocols for resin cements were investigated as helpful instruments to achieve successful clinical outcomes. However, it is noteworthy to highlight that all dental materials placed in the mouth have a precise deadline. The mouth is a particular environment in which chemical and physical factors reduce the survival rate of every rehabilitation, from a simple restoration to a complex case of implants. For this reason, regenerative dentistry could be the real innovative approach to either repair or replace the tooth. Thirdly, the differentiation potentials of different cell lineages were evaluated. Although human gingival and foreskin fibroblast could be able to differentiate in osteogenic and adipogenic tissues, dental pulp stem cells remain the gold standard cell lineage to regenerate dental tissues

In conclusion, Conservative dentistry is not only a discipline in which innovative tools, highly performing materials and regenerative strategies could simplify and enhance restorative procedures, but also a way of being a good clinician that performs the best rehabilitation with the minimal biological cost.

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