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CONTENTS

COVID 19 and *Pemphigus vulgaris*: a potential correlation

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Autoimmune blistering diseases have been associated with exposure to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In addition, oropharyngeal *Pemphigus vulgaris* appears to be associated with the coronavirus. In order to understand the molecular basis linking SARS-CoV-2 and *Pemphigus vulgaris*, this study explores the molecular mimicry hypothesis and analyzes the peptide sharing between the *Pemphigus vulgaris* autoantigen, i.e., Desmoglein 3 (Dsg-3), and the SARS-CoV-2 proteome. Results indicate a low molecular mimicry level, still immunologically significant, between SARS-CoV-2 and Dsg-3.

Case reports of *Pemphigus vulgaris* (PV) occurring in concomitance with exposure to SARS-CoV-2 have been described (1-5). Such reports highlight the need to define the possible causal link between the virus and the bullous autoimmune disease and, in addition, underline the need for safe therapeutical protocols to treat severe *Pemphigus vulgaris* during the COVID-19 pandemic (6-9).

PV's oral manifestations consist of painful erosions that leave an eroded or ulcerated area in the mucosa. Oral lesions are often the first manifestation of the disease. Furthermore, PV can frequently occur in patients between 45 and 65 years; therefore, it is evident the importance of considering the elderly population as a target that requires particular attention to oral cavity screening (10-12).

Desmoglein 3 (Dsg-3) is a desmosomal adhesion protein targeted by autoantibodies with a specific pathogenic role in *Pemphigus vulgaris* (13-15). The binding of autoantibodies to desmoglein appears to alter intracellular signaling pathways, including components such as p38 mitogen-activated protein kinases (p38 MAPK). The following reduced reorganization of the actin cytoskeleton would then, in turn, exacerbate the loss of desmosomal integrity. Moreover, both caspase-dependent pro-apoptotic pathways are also stimulated upon the binding of anti-Dsg-3 autoantibodies (16, 17).

Previous studies have clearly outlined the role of molecular mimicry in defining immunogenic/cross-reactive sequences in cancer (18-20), infectious diseases (21-29), and autoimmune disorders (30-34). Even more specifically, molecular mimicry appears to be a critical factor in various autoimmune-related aspects of the pathogenesis of COVID-19 (35-37).

We applied computational biology to define whether molecular mimicry and the potential consequent cross-reactivity may play a role in the onset/exacerbation of SARS-CoV-2-associated *Pemphigus vulgaris*. To this aim, the *Pemphigus vulgaris* autoantigen Dsg-3 was analyzed for sharing of minimal immune determinants, i.e., pentapeptides, with the entire SARS-Cov-2 proteome.

Keywords: Desmoglein 3; Covid-19; Pemphigus vulgaris; SARS-Cov-2

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METHODS

The primary sequence of the human protein Dsg-3 (UniProt identifier: P32926), the *Pemphigus vulgaris* antigen that associates with bullous disorders (8), was dissected into pentapeptides offset by one residue (i.e., MMGLF, MGLFP, GLFPR and so on) and the resulting pentapeptides were analyzed to find perfect matches within SARS-CoV-2 proteome (https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2).

Ceramide synthase 3 (CERS3), a human protein that is associated –when altered – with main skin phenotypes such as lamellar ichthyosis and non-bullous congenital ichthyosiform erythroderma (38), was used as a control.

PIR Peptide Match and Peptide Search programs available at UniProt (www.uniprot.org/) were used (39,40). Coronavirus (CoV) controls were as follows, with NCBI:txid in parentheses: Middle East Respiratory Syndrome (MERS)-CoV (1335626); Human (H) CoV-229E (11137); HCoV-NL63 (277944).

RESULTS

Table I shows the peptide sharing between SARS-CoV-2 and the Dsg-3 autoantigen. It can be seen that the level of peptide sharing is low and comparable to that of the control human CERS3 protein; however, it is immunologically noteworthy that the pentapeptide shared by SARS-CoV-2 with Dsg-3 is also present in immunoreactive epitopes derived from viral proteins, as reported in the Immune Epitope Database (IEDB, https://www.iedb.org/). Moreover, the lack of peptide sharing between DSG-3 and the pathogenic MERS-CoV seems interesting.

DISCUSSION

The present study shows a potentially limited, albeit non-zero, impact of molecular mimicry and the consequent cross-reactivity on a possible causal association between exposure to SARS-CoV-2 and *Pemphigus vulgaris*. Only four pentapeptides are shared between the virus and the Pemphigus antigen, although they appear to be of immunologic relevance to mediate an autoimmune response potentially.

The present result suggests that any role of cross-reactivity in the association between COVID-19 and *Pemphigus vulgaris* might be accompanied by other immune-mediated mechanisms. Therefore, other potential possible immunological molecular mechanisms should be investigated to study such an association. Indeed, SARS-CoV-2 infection might trigger loss of immunotolerance through dysregulated cytokine production and hyperinflammatory mechanisms (41), thus potentially triggering latent autoimmunity in *Pemphigus vulgaris*. Being aware of this potential triggering phenomenon could help the clinician, also supported by modern diagnostic devices (42-47), in early intercepting the onset of potentially fatal autoimmune diseases.

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Table I. *Pentapeptide sharing between CoV proteomes and Dsg-3*

CoV	Pentapeptides Shared with CERS3	Pentapeptides Shared with DSG-3
SARS-CoV-2	FLAHI	LAPLL, LLAPL, RGTAV, TSALL
MERS-CoV	–	–
hCoV-229E	TVAGI	AVSIP, PLILT
hCoV-NL63	IVHDV, NLQLM, RHLIP, RLIVF, SRLIV, VLENF	GVDGE, LSSEL, NKDYA, SLVLT

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COVID 19 and autoimmune blistering diseases: is there a link between epidermolysis bullosa acquisita and SARS-CoV-2?

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The blistering disease Epidermolysis bullosa acquisita is a genetic/autoimmune disorder deriving from alterations of the human protein Collagen alpha-1(VII) chain (CO7A1). Exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) promotes a wide variety of autoimmune diseases and might be a risk factor for Epidermolysis bullosa acquisita; in order to further our understanding of the link between this blistering disease and SARS-CoV-2, this study analyzes the peptide-sharing between CO7A1 and SARS-CoV-2 proteome. Results indicate a high level of molecular mimicry between CO7A1 and SARS-CoV-2 and hCoV-229E, and hCoV-NL63, thus suggesting a potential role of COVID-19 as a risk factor for Epidermolysis bullosa acquisita.

Epidermolysis bullosa acquisita (EBA) is a rare autoimmune blistering disease (AIBD) manifesting itself with skin and mucous lesions like vesicles, bullae, and erosions (1). Nowadays, the disease is diagnosed based on the clinical characteristics and serum positivity for autoantibodies (auto-Abs) against collagen VII binding the basement membrane zone, the same auto-Abs that induce mucocutaneous blistering by binding to the anchoring fibril zone (2). Indeed the immunologic origin of EBA allowed for categorizing it as a distinct acquired AIBD, whereas it was initially described as a variant of dystrophic epidermolysis bullosa with adult-onset in light of the clinically indistinguishable presentation (3). Indeed age of onset of EBA may vary widely as opposed to dystrophic epidermolysis bullosa (4,5). Nevertheless, genetic risk factors contribute to EBA, mainly because the HLA-DRB1*15 and HLA-DRB1*16 appear to be overrepresented HLA-DRB1*15:03 allele in black patients of African descent (1).

A recent systematic review of the literature on the impact of covid 19 on AIBD found "published papers that exclusively discussed pemphigus, bullous and mucous membrane pemphigoid, but there seems to have been a lack of published data on the other entities from the AIBD spectrum." (6). Nevertheless, it is plausible to hypothesize an effect of SARS-CoV-2 infection on the risk of acquired epidermolysis bullosa. Indeed, exposure to SARS-CoV-2 and the subsequent associated anti-SARS-CoV-2 immune response may trigger cross-reactivity with the EBA antigen, i.e., CO7A1, leading to the blistering disease. The issue appears relevant and might help address clinical attention towards prevention and care of the bullous disorder (7).

In this context, following previous studies that clarified the possible role of molecular mimicry in defining immunogenic/cross-reactive sequences in autoimmune disorders (8-12), cancer (13-16), and

Keywords: CO7A1; Epidermolysis bullosa acquisita; SARS-Cov-2; Covid-19; autoimmunity; molecular mimicry; cross-reactivity

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infectious diseases (17-24), we sought to investigate here whether molecular mimicry and the potential consequent cross-reactivity might relate to and account for the occurrence of EBA during the SARS-CoV-2 pandemic. To this aim, the CO7A1 protein primary sequence was explored for sharing of minimal immune determinants, i.e., pentapeptides, with the SARS-Cov-2 proteome.

MATERIALS AND METHODS

The primary sequence of the human protein CO7A1 (UniProt identifier: Q02388) that associates with EBA (1) were dissected into pentapeptides offset by one residue (i.e., MTLRL, TLRLL, LRLLV and so on) and the resulting pentapeptides were analyzed to find perfect matches within SARS-CoV-2 proteome (https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2).

Ceramide synthase 3 (CERS3), a human protein that is associated –when altered – with skin phenotypes such as lamellar ichthyosis and non-bullous congenital ichthyosiform erythroderma (23), was used as a control.

PIR Peptide Match and Peptide Search programs available at UniProt (www.uniprot.org/) were used (26,27). Coronavirus (CoV) controls were as follows, with NCBI:txid in parentheses: Middle East Respiratory Syndrome (MERS)-CoV (1335626); Human (H) CoV-229E (11137); HCoV-NL63 (277944). Additionally, Herpes Simplex Virus 1, HSV 1 (10298) and Human Herpes Virus 3, HHV 3 (10335), which have been reported to enhance/reactivate pemphigoid diseases (28,29), were also analyzed.

RESULTS

Table 1 shows the peptide sharing between

Table I. *Pentapeptide sharing between CoV proteomes and CO7A1*

CoV	Pentapeptides Shared with CERS3	Pentapeptides Shared with CO7A1
SARS-CoV-2	FLAHI	GLPGT, GYRVT, IGEAV, IKNAD, KSQDL, LATAL, LLPLV, LLVAA, LPLVS, LPSYA, PGLPG, PGQTG, PSYAA, QGPPG, SRVLG, SSDVL, SVQTF, TGYRV, VQTFF
MERS-CoV	–	-
hCoV-229E	TVAGI	ELPVS, ELSYK, GDKGD, GPPGS, IFSLT, KGDKG, LPGVA, LVLAL, MKGDK, NANRF, PVSIV, QAVVG, QGPPG, RAHVA, SSDVL, VMVLL
hCoV-NL63	IVHDV, NLQLM, RHLIP, RLIVF, SRLIV, VLENF	EVPGS, GDVGP, GPPGS, HSLLV, KGDKG, KGQKG, LPGVA, LVLAL, QGPPG, QLDGL, SRASS, VLLVD, YANSI
HHV 3	-	-
HSV 1		RPGER

SARS-CoV-2 and CO7A1. It can be seen that the extent of peptide sharing between CO7A1 and SARS-CoV-2 is high but not specific, given the high number of peptide commonalities with the control CoVs too. Instead, no common minimal determinant was found between CO7A1 and MERS-CoV, HHV3, and HSV1 proteomes.

DISCUSSION

The present study shows a high level of molecular mimicry between CO7A1 and SARS-CoV-2, hCoV-229E, and hCoV-NL63 CoVs, thus supporting the research hypothesis that cross-reactivity with CO7A1 may occur following exposure to CoVs, in this way determining CO7A1 alterations that can lead to EBA. The result is relevant for improving clinical care of oral pathologic sequelae of COVID-19 and providing insight into the pathogenesis of EBA and warrants further research in this direction. Indeed, the oral pathologist, both supported by modern diagnostic devices (30-35) and aware of this potential triggering phenomenon, could play an essential role in early intercepting the onset of potentially covid-related autoimmune diseases.

Finally, it could be of great interest to investigate whether antecedent autoimmune diseases or the previous use of immunosuppressive agents might affect the COVID-19 clinical presentation. To identify the potential subjects who could fall into the categories most at risk of developing a severe form of COVID19 (which could coincide with the categories that are considered fragile, i.e., elderly, immunosuppressed patients, patients with AIBD) (36-38) would allow for optimizing clinical care algorithms. To date, the research in this regard appears to be inconclusive. While some studies have shown an increased risk of more aggressive forms of COVID-19 in patients with oral autoimmune diseases (39,40), further research is needed to confirm such findings and implement effective preventive and therapeutic strategies.

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Peptide sharing between SARS-CoV-2 and Bullous pemphigoid 180

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Bullous pemphigoid (BP) has been repeatedly reported to occur following exposure to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This study analyzes the molecular mimicry between the 180 kDa bullous pemphigoid antigen 2 (BP180) and the SARS-CoV-2 proteome to further our understanding of the molecular link between the BP and the SARS-CoV-2 infection. Results indicate a high degree of molecular mimicry between BP180, SARS-CoV-2, hCoV-229E and hCoV-NL63.

Bullous pemphigoid (BP) disorders are characterized by subepidermal blistering that clinically may have a potentially life-threatening course (1, 2). Etiologically, BP seems to be triggered by multifactorial genetic-autoimmune damage to hemidesmosomal proteins, namely, BP180 and/or BP230 (3).

Recently, numerous cases of BP have been described in concomitance with SARS-CoV-2 pandemic (4-20), posing the question of what, if any, is the molecular relationship between the virus and the pemphigoid disorders.

In particular, the elderly population is a target that has always required special attention concerning oral screening, and today it is all the more in need of targeted care during the current SARS-CoV-2 pandemic (21-23).

In this study, based on reports on the role of molecular mimicry in defining immunologic cross-reactions due to molecular mimicry in cancer (24-27), infectious diseases (28-33), and autoimmune disorders (34-38), we analyzed the possibility that molecular mimicry may represent a mechanistic link between the BP disorders and SARS-CoV-2 infection. We tested the hypothesis that exposure to SARS-CoV-2 and the subsequent anti-SARS-CoV-2 immune response might trigger cross-reactive attacks against the BP proteins, in this way causing the pemphigoid disease. Here we carried out sequence similarity analyses on the BP180 protein, searching for minimal immune determinants, i.e., pentapeptides, also occurring within the SARS-Cov-2 proteome.

METHODS

The primary sequence of the human protein BP180 (UniProt identifier: Q9UMD9) that associates – when altered - with pemphigoid disorders (1-3) was dissected into pentapeptides offset by one residue (i.e., MDVTK, DVTKK, VTKKN and so on) and the resulting pentapeptides were analyzed as probes to find perfect matches within SARS-CoV-2 proteome (https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2).

Ceramide synthase 3 (CERS3), a human protein that is associated –when altered – with skin phenotypes such as lamellar ichthyosis and non-bullous congenital

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ichthyosiform erythroderma (39), was used as a control.

PIR Peptide Match and Peptide Search programs available at UniProt (www.uniprot.org/) were used (40, 41).

Coronavirus (CoV) controls were as follows, with NCBI:txid in parentheses: Middle East Respiratory Syndrome (MERS)-CoV (1335626); Human (H) CoV-229E (11137); HCoV-NL63 (277944). Additionally, Herpes Simplex Virus 1, HSV 1 (10298) and Human Herpes Virus 3, HHV 3 (10335), which have been reported to enhance/reactivate pemphigoid diseases (42, 43), were also analyzed.

RESULTS

Table 1 shows the pentapeptide sharing between BP180 and SARS-CoV-2. As a control analysis, the sharing between CERS3 and the SARS-CoV-2 was examined and presented in the table. Finally, the sharing between the two human proteins and MERS-CoV, HCoV-229E, HCoV-NL63, HSV-1, and HHV-3 is also described. It can be seen that the extent of peptide sharing between BP180 and SARS-CoV-2 is high but not specific to the new coronavirus, given the high number of peptides shared with the control CoVs too. Instead, no common minimal determinant was found between COL7A1 and MERS-CoV, HHV 3, and HSV1.

DISCUSSION

The present study shows a high level of molecular mimicry and, consequently, a high level of potential cross-reactivity between BP180 and SARS-CoV-2, thus supporting epigenetic autoimmune alterations of BP180 as a possible contributing factor in the etiology of BP. However, an open question remains concerning the high number of sequences shared by BP180 and common cold coronaviruses, comparable to the sharing with the SARS-CoV-2. A possible explanation of this apparent contradiction might be

Table I. *Pentapeptide sharing between CoV proteomes and BP180*

CoV	Pentapeptides Shared with CERS3	Pentapeptides Shared with BP180
SARS-CoV-2	FLAHI	AGNGG, CGSCC, GLPGT, LLSTD, LQGPP, PGLPG, PQGLP, QGPPG, SIAAT, SVGPK, TSPDV, TTADI, TTTTS
MERS-CoV	–	-
hCoV-229E	TVAGI	GDKGD, GPPGS, IQGPP, KGDKG, NGYAK, PPGSG, QGPPG, QNNLA, SRLLS, TTSVQ
hCoV-NL63	IVHDV, NLQLM, RHLIP, RLIVF, SRLIV, VLENF	AKTAS, DYAEL, GPPGS, IQGPP, KGDKG, KGQKG, KTASL, LLSLL, LTWLL, PPGSG, QGPPG, SGALA, SGSPG, SRILS, SSMLT, TPAKK, TTSVQ, VTASS
HHV 3	-	-
HSV 1		GKTTT

found in the broad spectrum of autoinflammatory and autoimmune manifestations of COVID-19. If so, this data would be all the more relevant as it would lay the groundwork for improving clinical care of the oral pathological sequelae of COVID-19, providing information on the correlation between SARs-CoV-2 and BP, justifying further research in this field. The clinician, supported by modern diagnostic devices (44-47) and aware of this potential correlation, could play an essential role in early intercepting the onset of this autoimmune disease.

Indeed it appears that the SARS-CoV-2 not only might be able to induce cross-reactive autoimmunity by way of molecular mimicry (48-50) but also by concomitantly triggering loss of immune tolerance by stimulating pro-inflammatory cytokine production (51). Such complex effects on the immune systems might be peculiar to SARS-CoV-2 but not to common cold coronaviruses, which might not be able to elicit massive autoimmunity and, specifically, BP despite extensive peptide sharing.

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COVID 19 and Bullous Pemphigoid: a hypothesis for a causal link

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Although onset/exacerbation of bullous Pemphigoid (BP) has been reported to occur frequently following exposure to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the link, if any, between BP dermatoses and the viral infection remains obscure. Therefore, searching for possible molecular mechanisms, we hypothesise that molecular mimicry between BP antigens and the SARS-CoV-2 proteins might lead to autoimmune responses cross-reacting with the BP proteins, thus triggering the dermatosis pathologies. Using this research paradigm, we analyzed the Bullous Pemphigoid antigen 1 (BP230) and the SARS-CoV-2 proteome to share minimal immune determinants, i.e., pentapeptides. Results indicate a high level of molecular mimicry between BP230 and SARS-CoV-2, thus supporting the hypothesis of cross-reactivity as a possible major mechanism in the SARS-CoV-2-associated BP etiopathogenesis.

During the COVID-19 outbreak, a conspicuous number of reports (1-11) have illustrated a specific percentage increase of vesiculobullous and autoimmune diseases in the population (12). Especially the elderly population is a target that has always required special attention concerning oral health, and today it is all the more in need of targeted care during the current SARS-CoV-2 pandemic (13-15). However, although a causative role for the SARS-CoV-2 has been proposed (16-19), the issue is under debate (20-23). In particular, the molecular mechanism that might explain the association of SARS-CoV-2 with the bullous dermatoses remains unclear.

Autoimmune bullous dermatoses (AIBD) include heterogeneous disorders, comprising the intraepidermal pemphigus groups, such as the *Pemphigus vulgaris* and *foliaceus* and the subepidermal pemphigoid group, namely bullous and mucous membrane pemphigoid, epidermolysis bullosa acquisita, linear IgA disease, and dermatitis herpetiformis (24). Pemphigoid diseases are vesiculobullous autoimmune diseases clinically characterized by blisters and erosions on the skin and/or mucous membranes. (25).

In this study, taking advantage of studies on the role of molecular mimicry in defining immunogenic/cross-reactive sequences in cancer (26-29), infectious diseases (30-37), and autoimmune disorders (38-43), we analyzed the possibility that molecular mimicry may represent a mechanistic link between the BP disorders and SARS-CoV-2 infection. We tested the hypothesis that -following exposure to SARS-CoV-2 -

Keywords: Bullous pemphigoid; BP230; SARS-Cov-2; Covid-19; autoimmunity; molecular mimicry; cross-reactivity

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the subsequent anti-SARS-CoV-2 immune responses might trigger cross-reactive attacks against the BP proteins, in this way causing the pemphigoid disease. As a preliminary step to prove/disprove this research hypothesis, we carried out sequence similarity analyses on BP230 searching for minimal immune determinants, i.e., pentapeptides (35), also occurring within the SARS-Cov-2 proteome.

MATERIALS AND METHODS

The primary sequence of the human protein BP230

Table I. *Pentapeptide sharing between CoV proteomes and BP230*

CoV	Pentapeptides Shared with CERS3	Pentapeptides Shared with BP230
SARS-CoV-2	FLAHI	AGLIA, ATEET, AVILR, AVKTQ, EAVKT, EDFLE, EEQEE, EESSA, EGCHA, EKALK, EKWES, ELFEN, ELGTD, ELMRE, ENLLL, EQRKQ, GLTSI, GTDLE, IKDTE, INGLM, IQRKY, KALKY, KFCLE, KKKLD, KLCEE, KMKDL, KTLAT, LDKVE, LEETK, LETAQ, LEWLA, LGPLS, LLEWL, LLKSA, LLSVC, LMREL, LNKAT, LNQLT, LPLQL, LTCAT, LTSIK, LTSSS, MKDLS, NFLKE, NNTAS, NVLTL, QLPAP, SALLE, SDVET, SDVLL, SLLSV, TEKSN, TTDPS, TVEEA, TVTLK, VASIK, VDGQV, VELFE, VLSNL, VSSPD, YELQT, YLKRR
MERS-CoV	–	-
hCoV-229E	TVAGI	ALVKL, CAKRF, DEEGG, DENGK, DKPIV, DLVVE, DVEVK, EDALR, EFVAA, EGNYY, ELLKQ, EMVLK, EVELL, EVLLD, FCLEN, GLDAA, IAVIL, ILKQL, ITLKC, KEGNY, KFILA, KILKQ, KTLQE, LAEKF, LASAL, LQDFL, LQRQL, NDAKE, NLPEY, NVDKD, QLNLK, QLPAP, RIDKD, SILKN, SLLSV, SRSEL, TKLSK, VEAVN, VGSLS, VIADK, VIPAT, VKDIE, VQSKL, VSKES
hCoV-NL63	IVHDV, NLQLM, RHLIP, RLIVF, SRLIV, VLENF	AAETI, AEVVK, AKHGD, ALDYL, ALLNG, ATDRF, CAKRF, CSSVY, DEEGG, DGTVE, EALSK, EDALR, EDVPS, EILLS, EKAAL, ETIKA, FCLEN, FLVKN, IEIVN, ITISG, ITYVS, KFILA, KLLDV, KTLQE, KVKLI, LAEVV, LDNQE, LFSKV, LIDYD, LIEGS, LQEFA, LQLGV, LRTLI, LSKLS, NKNVS, PQLAE, QAYED, QLNLK, QLPAP, QTELA, QVPAT, RAGSK, RASSR, RTLID, SDDDF, SILKN, SRASS, SSELK, SSSGV, STVEV, TSVSS, VGSLS, VIPAT, VPSDS, VQSKL, VTLKD, VVEAS, YDVSK
HHV 3	-	-
HSV 1	-	FDQAA, GSRDD, LAKRL

associated with pemphigoid disorders was dissected into pentapeptides offset by one residue (i.e., SLTPG, LTPGG, TPGGA, and so on) and the resulting pentapeptides were analyzed as probes to find perfect matches within the SARS-CoV-2 proteome (https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2). In addition, ceramide synthase 3 (CERS3) is a human protein associated with skin phenotypes such as lamellar ichthyosis and non-bullous congenital ichthyosiform erythroderma (44), was used as a control.

PIR Peptide Match and Peptide Search programs available at UniProt (www.uniprot.org/) were used (45,46).

Coronavirus (CoV) controls were as follows, with NCBI:txid in parentheses: Middle East Respiratory Syndrome (MERS)-CoV (1335626); Human (H) CoV-229E (11137); HCoV-NL63 (277944). Additionally, Herpes Simplex Virus 1, HSV 1 (10298) and Human Herpes Virus 3, HHV 3 (10335), which have been reported to enhance/reactivate pemphigoid diseases (47, 48), were also analyzed.

RESULTS

The peptide sharing between the SARS-CoV-2 proteome and the BP230 protein amounts to 62 peptide commonalities, as reported in Table I. That is, 62 pentapeptides out of 7566 are shared between the SARS-CoV-2 proteome and the BP230 protein.

Table I describes the shared pentapeptides. It can be seen that the extent of peptide sharing between BP230 and SARS-CoV-2 is the highest. Interestingly, a high number of peptides are also shared with the control CoVs, while no peptides or only a few ones are shared with MERS-CoV, HHV 3, and HSV1. In the context of autoimmunity, Table I demonstrates the possibility of cross-reactions between SARS-CoV-2 and the BP230 protein.

DISCUSSION

The present study shows a high level of molecular mimicry and, consequently, a high level of potential cross-reactivity between BP230 and SARS-CoV-2, thus supporting the hypothesis of a causal link between exposure to the virus and bullous pemphigoid onset/exacerbation.

In fact, in the current SARS-CoV-2 pandemic, cross-reactivity has been proposed as a mechanism that might explain the immunopathologies associated with the coronavirus infection (49). The hypothesis is that peptide sharing between SARS-CoV-2 and human proteins might trigger immune responses able to attack the virus and human proteins with consequent autoimmune sequelae.

The likewise high peptide sharing with the control HCoV-229E and HCoV-NL63 might add to the potential cross-reactivity between BP230 and SARS-CoV-2. The data presented here might address scientific research to define targets and mechanisms in analyzing the potential association of SARS-CoV-2 and coronaviruses in general with bullous dermatoses.

Finally, it is also noteworthy to underline that the potential risk of cross-reactivity between SARS-CoV-2 and human proteins is undervalued since the present analyses have been conducted on linear pentapeptide sequences. Actually, by considering the possibility of conformational epitopes (50), the viral versus human commonalities would be exponentially higher. Based on these considerations, these data might be a starting point for future scientific studies and clinical investigations and suggest possible protein targets of autoimmunity in SARS-CoV-2 infection. From an experimental point of view, the results seem to be the basis for further studies to verify the presence of autoantibodies against these protein targets in patients' sera. Clinically, the results warrant stringent surveillance on the future pathologic sequelae of the current SARS-CoV-2 pandemic. Indeed, the clinician, reinforced by modern diagnostic devices (51-53) and alert to this possible link, could play a critical part in early intercepting the start of this autoimmune disease.

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The potential role of peptides in cancer, infectious diseases, and autoimmunity: a narrative review

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In the last two decades, peptidomics, which can be defined as the science of studying the biological relevance of peptides, has grown in relevance. Today, we can 1) define disease-associated–proteins in terms of short peptide modules, 2) precisely target cancer cells by hitting peptide sequences of tumour-associated antigens, and 3) inactivate harmful cross-reactive autoantibodies, among others; indeed, scientific progress has been made in epitope definition, and we can precisely identify the amino acid (aa) sites involved in immunological reactions and locate antigenicity and immunogenicity within minimal immune pentapeptide determinants. Clinically, peptides offer new approaches for fighting the spread and re-emergence of infectious pathogens and provide new strategies for safe therapies in cancer and autoimmune pathologies. Here, this paper carries out a historical review of the role of peptides in medicine in the last decades.

Scientific research focused on proteomics and peptides profoundly modified the immunological context that has been dominant since the 1950s (1). Indeed, peptidomics generated bioactive peptide databases and catalogues of experimental data on antibody and T cell epitopes studied in humans, non-human primates, and other animal species in infectious disease, allergy, autoimmunity, and transplantation as the Immune Epitope Database (IEDB) (2).

Comparative immunoanalysis at the peptide level of pathogen and human proteins coupled to the exploration of epitopic sequences by using IEDB resource (2) introduced the peptide epitope concept as a minimal immune determinant dimensionally formed by a grouping of 5-6 aa, mathematically defined by a low number of occurrences, and physicochemically characterized by hydrophobicity (3-6). As thoroughly discussed (1, 6, and further references therein), hydrophobicity is the main property that defines immunotolerance versus immunogenicity. Hydrophobicity is the filter by which the terminal hydrophobic sequence of the germline B-cell receptor avoids frequent, hydrophilic sequences and binds rare, hydrophobic pentapeptide determinants, protecting the self from the nonself. Hence, procedures and substances that alter/manipulate the cellular hydrophobicity (i.e., adjuvants) (6) can trigger immune cross-reactions against the self with consequent severe autoimmune sequelae. Here, we analyze the role of peptides in (immuno) therapeutical approaches addressed at fighting infectious diseases, cancer, and autoimmunity.

Infectious diseases: walking along the peptide immunome

A few examples of peptide-based vaccine usage in the fight against infectious diseases are the following:

- an elevated serum titer against Bordetella pertussis was obtained upon vaccination with peptides (15-22 aa long) from the S1 subunit of pertussis toxin (7):
- synthetic peptides were used as antigens for the

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detection of humoral immunity to Plasmodium falciparum (8);
- virosome-formulated synthetic peptides were proposed as malaria vaccines (9) and were used to induce parasite growth-inhibitory antibodies (10). Moreover, a synthetic peptide from Plasmodium vivax merozoite surface protein-9, sequence EAAPENAEPVHENA, was found to be highly immunogenic in mice, thus appearing as a promising vaccine target (11);
- peptides from hepatitis C virus (HCV) 2a (aa pos: 432-441, 576-584, 627-635, 716-724, 1085-1094, and 2251-2260) efficiently induced peptide-specific CTLs from HLA-A24(+) HCV2a-infected patients as well as healthy donors, thus resulting suitable for peptide-based immunotherapy of HLA-A2+ HCV2a-infected patients. (12,13). Likewise, numerous reports documented positive results by using peptide-based HCV vaccines in controlled trials (14-17);
- a synthetic glycopeptide acted as an HIV broadly neutralizing antibody epitope (18) and reduced viral burden followed HIV-1 p24 peptide-based therapeutic immunization (19-21);
- vaccination with a synthetic peptide from the Influenza virus hemagglutinin protects against distinct viral subtypes (22) and, in addition, potent peptidic fusion inhibitors of the Influenza virus have been described (23);
- dendrimer-conjugated peptide vaccine enables the clearance of Chlamydia trachomatis genital infection (24).

Peptides in cancer immunotherapy

Clinically, research at the epitope level progressively impacted the cancer immunotherapy field. Indeed, immunotherapies to fight malignancies began with whole cells or cell lysates and dendritic cells or genetically engineered vaccines capable of inducing an enhanced immune response in the organism. Whole-cell vaccines consisted of X-irradiated autologous or allogeneic tumor cells co-administered with adjuvants. Dendritic cell vaccines were prepared by transfection or transduction with disease antigen-encoding DNA – a prelude to the current nucleic acid-based vaccines - or by pulsing the cells with antigenic peptides in vitro for subsequent re-injection into the patient (25,26). Humoral and T cell-mediated immune reactions to autologous tumor cells were generated ex novo and/or enhanced in immunized patients but did not correlate with clinical response. Generally, no clinical benefit occurred (27-29).

Then, as depicted in Figure 1, attention gradually shifted from whole cells to antigens (30-32) and eventually to antigen-derived peptides such as human epidermal receptor kinase (HER-2/neu) peptide ILHDGAYSIL, which was shown to induce effective antitumor immunity against HER-2/neu-expressing tumor cells (33-35). After which, numerous scientific and clinical studies followed and documented the potential of short peptide modules for developing anti-cancer vaccines. Among the many:
- sera from breast/prostate cancer patients recognize a set of extracellular EC HER-2/neu sequences (namely, CSPMCKGS, ACPYNYLST, VNARHCLP, SYMPIWKFP, and PINCTHSCVD) that consequently were proposed as a specific and efficacious peptide platform in developing anti-HER-2/neu vaccines for fighting breast and prostate cancers (36);
- mutated Ras peptides are effective in specific immunotherapy in patients with pancreatic and colorectal carcinomas (37);
- epitope mapping of the Melan-A/MART-1 oncoprotein showed that a B cell epitope is located in the 5-mer peptide PAYEK of the sequence 101-115 (PPAY-EKLSAEQSPPP) of the Melan-A/MART-1 (38); successively, Madoulet's lab demonstrated in vivo prophylactic activity of the 101-115 peptide-based vaccine in controlling melanoma growth (39);
- the monoclonal antibody (MAb) ED17 raised against the full-length HPV16 E7 oncoprotein recognizes a short linear determinant (namely, AHYNIVTFCCKC) (40);
- vaccination with tyrosinase peptides can induce long-term freedom from recurrence in melanoma patients (41);
- the linear antigenic determinant of the Mab T311 raised against the melanoma/vitiligo tyrosinase autoantigen is located in the low similarity 15-mer aa sequence tyrosinase 233-247 IP-

YWDWRDAEKCDIC, within the fragment 237-247 (42);
- folate receptor alpha peptide vaccine generates immunity in breast and ovarian cancer patients (43);
- Peptides allowed to build a phage-displayed library (44).

In brief, evidentiary data on the peptide utilization in cancer immunotherapy documents the possibility of opening a successful new chapter in oncology.

Peptides in autoimmune diseases: from Hepatitis C Virus to Pemphigus Vulgaris.

Peptide-based immunotherapies also lay the ground to reduce/eliminate autoimmune diseases. Indeed taking advantage of the concept of "target replacement", it has been demonstrated that:
- peptide fragments from human acetylcholine receptor suppress experimental autoimmune myasthenia gravis (45);
- glutamic acid decarboxylase peptide 524-543 delays the onset of diabetes (46);
- cell cycle inhibiting peptide therapy was successfully used to inhibit the progression of the lupus-like syndrome in mice with the levels of anti-dsDNA IgG autoantibodies and the incidence and severity of renal involvement lower in treated mice than in non-treated mice, thus opening new avenues for the treatment of systemic lupus erythematosus(47);
- proteomic scan for tyrosinase peptide antigenic pattern in vitiligo and melanoma allowed to distinguish the epitopic peptide sequences associated with melanoma from those related to vitiligo autoantibodies, defined the linear B epitope pattern on tyrosinase autoantigen, and provided definitive evidence of humoral immune responses against tyrosinase (48);
- epitope definition by proteomic similarity analysis led to identifying the linear determinant of the anti-desmoglein 3 (Dsg3) MAb 5H10, i.e., the Dsg349-60REWVKFAKPCRE peptide (49), to define a Dsg linear determinant common to Pemphigus vulgaris and Pemphigus foliaceous (50), and to realize the first peptide-based Pemphigus vulgaris treatment(51);
- copolymer 1, a synthetic peptide, suppresses experimental autoimmune encephalomyelitis, i.e., a model for multiple sclerosis(52), by acting against the immunodominant epitope 82-100 of myelin basic protein (53);
- the pentapeptide MTADV suppresses chronic inflammations and autoimmune pathologies in mouse models of rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis(54,55).

DISCUSSION

Two main points stand out from the present review: first, the central role of peptides in furthering our knowledge of the basic tenets of immunology, and second, the high therapeutic potential of peptides in

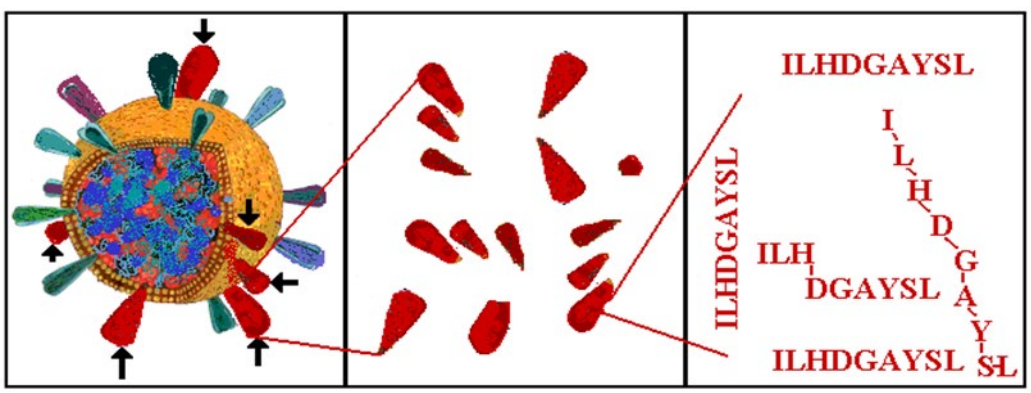

Fig. 1. *The vaccine target: from whole cells to specific peptide sequences via antigen proteins: HER-2/neu 435-443ILHDGAYSIL as an example. Left: a breast cancer cell hyperexpressing HER-2/neu antigens (indicated by black arrows). Middle: HER-2/neu protein antigen. Right: HER-2/neu 435-443ILHDGAYSIL peptide.*

various diseases. Indeed, peptide-based vaccines have been proposed with highly positive results promising new perspectives on infectious diseases (56-59) and malignant diseases and autoimmune disorders (60-63), which remain crucial health issues for the human population. Moreover, the very convenient safety profile of such therapeutics should be noted.

Vaccine formulations based on peptides, especially fragments which are absent or rarely occurring in the human host, whilst being present in the infectious agent or the cancerous cell, are expected to specifically hit the noxious targets without collateral harmful cross-reactions (64, 65); this is even more relevant when considering the elderly population, which is a target that has always required special care and attention (66-69). Useless to say, such an approach opens the doors to advances in vaccinology. Finally, peptide-based specific immunomodulation might limit treatments based on broad-acting immunosuppressants burdened with various undesired side effects (70,71).

Conflicts of Interest:

The authors declare no conflict of interest.

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Oral Microbioma and *Pemphigus vulgaris*

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OBJECTIVE: *Pemphigus Vulgaris* (PV) is a mucocutaneous autoimmune disease mediated by autoantibodies that often affects oral mucosa. However, little is known about the connection between oral infections and PV. The present study addresses the hypothesis that immune responses following bacterial infections may cross-react with PV autoantigens, thus providing the pathogenic stimulus, eventually leading to clinical manifestations.

METHODS: Available proteomic resources and immunologic data were explored. Searching for common peptides that underlie immune cross-reactions, the analyses focused on pathogenic oral bacteria and PV autoantigens, i.e., Desmoglein-3 (Dsg3).

RESULTS: It was found that the analysed bacteria share numerous immunoreactive heptapeptide sequences with Dsg3.

CONCLUSION: These data seem to support the hypothesis that the oral microbiome may also contribute to the pathogenesis of PV, with important implications for the treatment of this disease.

Pemphigus Vulgaris (PV) is a mucocutaneous autoimmune disease mediated by autoantibodies (1). Oral lesions are the first signs of disease that may precede cutaneous lesions. (2, 3). Clinically, PV causes painful erosion, preceded by blisters which burst at the minimal trauma and leave an eroded or ulcerated area in the oral mucosa. A recent meta-analysis has reported that 90.3% of patients with PV presented oral lesions either isolated or concurrent with lesions on the skin or other mucosa and that 50.8% of patients had PV lesions affecting the oral mucosa exclusively (4). Moreover, PV can arise in most patients aged 45-65 yrs, thus highlighting the importance of considering the elderly population as a target that requires particular attention regarding oral health (5-7).

The main autoantigen targeted by pemphigus autoimmunity has been identified as Desmoglein-3 (Dsg3), a component of desmosomes that participates in the adhesion of keratinocytes (8).

Dsg3 contains an extracellular domain, a short transmembrane domain, and a cytoplasmic domain. In PV, autoantibodies are directed toward the extracellular domain (9, 10). Although the concept of pemphigus's pathobiology is widely accepted, there is a debate about the PV etiology (9,11).

Keywords: oral microbioma; desmoglein-3; Pemphigus vulgaris; cross-reactivity; autoimmunity

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PV develops due to interaction between predisposing genetic and environmental factors (1, 12), thus raising the question of whether infectious agents might represent a trigger factor for the disease. Consequently, this study aims to investigate a possible relationship between the immune response directed against pathogenic bacteria that colonise the oral cavity and the etiopathogenesis of PV. In other words, could immune responses against these pathogenic agents cross-react with the PV autoantigen (Dsg3)?

Given this research question, we used UniProt resource (13) to interrogate the proteomes relative to periodontopathogenic agents searching for peptide sequences shared with the PV autoantigen and found that oral pathogens share numerous heptapeptide sequences with Dsg3. Noteworthy, many of the shared heptapeptides are also of immunologic relevance, as revealed by inspection of the Immune Epitope DataBase (IEDB) (14), thus supporting the hypothesis of autoimmune cross-reactivity as a mechanistic basis that might contribute to our understanding of oral PV. In addition, the data encourage further wide-microbiome analyses that could contribute to developing immunotherapies for preventing/blocking PV.

MATERIALS AND METHODS

Bacterial proteomes were searched for heptapeptide matching with the PV autoantigen protein, Dsg3, as follows: the primary amino acid (aa) sequence of Dsg3 (UniProt identifier: P32926) was dissected into heptapeptides overlapping each other by six aa (for example MMGLFPR, MGLFPRT, GLFPRTT, and so forth). Centromere protein C (CENP-C, UniProt identifier: Q03188-1) was used as autoantigen control. The two autoantigens are described in UniProt resource (http://www.uniprot.org/) (13).

Successively, each heptapeptide was analysed for occurrences in the bacterial proteomes under analysis by using Pir Peptide Match program (15). The following bacterial proteomes were explored (with NCBI Taxonomy Id in parentheses): *Aggregatibacter actinomycetemcomitans* (NCBI txid: 714); *Campylobacter rectus* (NCBI txid: 203); *Eikenella corrodens* (NCBI txid: 539); *Fusobacterium nucleatum* (NCBI txid: 851); *Parvimonas micra* (NCBI txid: 33033) *Porphyromonas gingivalis* (NCBItxid: 242619); *Prevotella intermedia* (NCBI txid: 28131); *Tannerella forsythia* (NCBItxid: 203275). *Rickettsia rickettsii* (NCBI txid: 452659) was used as bacterial control.

Finally, the immunologic potential of the heptapeptides shared with Dsg3 was analysed by searching for their occurrence(s) in immunoreactive epitopes catalogued at the Immune Epitope Database and Analysis Resources (IEDB; www.immuneepitope.org) (14). IEDB is an authoritative, curated database that reports experimentally validated cell epitopes.

RESULTS

Table 1 describes the peptide overlap between eight oral bacteria chosen as representatives of pathogenic bacterial species (16) and Dsg3, with *R. rickettsii* and the autoantigen Centromere protein C (CENP-C) used as a bacterial control and an unrelated autoantigen control, respectively.

Table 1 shows that:

- the PV autoantigen and the eight periodontopathogenic bacterial proteomes share 23 heptapeptides (including multiple occurrences), while the extent of the heptapeptide overlap with the control CENP-C amounts to 16 heptapeptides;
- the extent of the sharing is extremely impressive, considering that in terms of probability, the chances that two proteins share a heptapeptide is equal to 20^{-7} (or 1 in 1,280,000,000 or 0.00000000078125). That is, it is close to zero;
- the bacterial proteomes most involved in the sharing are *E. corrodens, F. nucleatum, and T. forsythia;*
- none of the 16 heptapeptides shared between the CENP-C autoantigen control, and the bacterial proteome control *R. rickettsii* was found to be part of immunoreactive sequences;
- numerous heptapeptides shared between the Dsg3 autoantigen, and the bacterial proteomes are of immunologic importance by being present in epitopic sequences experimentally validated as immunoreactive (14) (as shown in Table 2).

DISCUSSION

Research in oral microbiology is furthering our knowledge of the role of infections in oral pathology, thus opening new scenarios for therapeutic and

preventive approaches to oral diseases (17,18). Progressively, the scientific and clinical data accumulated on the oral microbiome also reveals the molecular basis and the mechanisms that might connect infections to oral diseases (19). Furthermore, previous studies have correlated the microbiome with other autoimmune diseases of the oral cavity (20-24) and oral conditions, such as orthodontic appliances(25). Indeed, there is an interest in highlighting any other correlations between orthodontic appliances and the modifications of the oral microbiota, like predisposing factors for the onset of periodontal diseases, caries, and oral diseases (26-34).

The specialist in oral care should be aware of this correlation to be able to intercept early the beginning of several oral diseases, supported by modern diagnostic devices (35-39). Furthermore, links between the oral microbiome and diseases are not restricted to oral involvements but also affect systemic diseases (40-42).

In this study, taking advantage of data obtained on the role of molecular mimicry in defining immunogenic/cross-reactive sequences in autoimmune disorders (10,43-46), infectious diseases (47-53), and cancer (54-57), we explored peptide sharing and the potential consequent cross-reactivity between pathogenic bacteria and PV Dsg3 autoantigen.

Table I. *Heptapeptide sharing between periodontopathogenic bacteria and Dsg3 autoantigens*

BACTERIA[1]	**CENP-C**	**Dsg3**[3,4]
Rickettsia rickettsii (452659)	AKENIGK	LLLLLLA
Aggregatibacter actinomycetemcomitans (714)	KSEESPV, NLGIPLG, STALLQG	TLSGSQG
Campylobacter rectus (203)	DEISRCS	GAATGVG
Eikenella corrodens (539)	AESTALL, SNKKRIC	AAIGLLL, EISLGVD, LLLGLLL, **LLLLGLL**, NKAASNV
Fusobacterium nucleatum (851)	EVSVKTK, NKKSNKK, TPDSKKI, VNKKSNK	DKEITNI, KLAEISL, **NTGDINI**, **SIAVKNK**, **TLTNSLD**
Parvimonas micra (33033)	–	**AVCSSSP**,, KDGEGLS, LAIFVVV
Porphyromonas gingivalis (242619)	KDPETRE, KNIRKKT	GLLLLLL, —
Prevotella intermedia (28131)	QNVIPSS, RKSESSP, SLKQRTI	FMESSEV, GLLLLLL, **TITAEVL**
Tannerella forsythia (203275)	KILATDV	GLLLLLL, LLLGLLL, QTLSGSQ, **RNTGEVR**

1Representatives of pathogenic bacterial species (16) listed with NBCI Taxon Id in parentheses: R.rickettsii was used as a control.

2Bacterial proteomes were searched for heptapeptide matching with Dsg3. The autoantigen Centromere protein C (CENP-C) was used as a control. The four autoantigens are described at UniProt resource (http://www.uniprot.org/) (12). The autoantigen primary sequences were dissected into heptapeptides overlapping each other by six residues. Then, each autoantigen-derived heptapeptide was used to search the bacterial proteomes for exact matches using Pir Peptide Match program (15).

3Shared heptapeptides with immunologic potential marked bold and underlined.

4The immunologic potential was explored searching for shared heptapeptide occurrence(s) in immunoreactive epitopes catalogued at IEDB (www.immuneepitope.org) (14).

Table II. *Immunologic potential of the peptide matching between periodontal bacteria and Dsg3*
A) *Epitopes derived from CENP-C and containing peptides shared between CENP-C and the bacterial proteomes1*
None
B) *Epitopes derived from Dsg3 and containing peptides shared between Dsg3 and the bacterial proteomes2*

ID[3]	EPITOPES[4]
123574	pfgifvvdkNTGDINIt
125568	svklSIAVKNKaefhqs
168942	kaldyeqlqsvklSIAVKNKaefhqsvisryrv
434756	lSIAVKNKaefhqs
132902	sqepagtpmfllsRNTGEVRTLTNSLDreq
135573	llsRNTGEVRTLTNSLD
230446	AVCSSSPsvvvsart
125545	rdstfivnkTITAEVLa
108166	tpmfllsRNTGEVRt
125433	gtpmfllsRNTGEVRtl
132902	sqepagtpmfllsRNTGEVRTLTNSLDreq
135573	llsRNTGEVRTLTNSLD
230486	llsRNTGEVRtltns
434753	llsRNTGEVRtlt
434758	mfllsRNTGEVRtlt
434772	tpmfllsRNTGEVRtlt

1 CENP-C was used as autoantigen control
2 Data from IEDB (14)
3 Epitopes listed according to the IEDB ID (14)
4 Shared sequences in capital letters.

The intense peptide sharing that we described might explain the coexistence of bacterial infections and autoimmune PV in the oral cavity. As a conclusive note, it is important to underline that autoimmune cross-reactivity deriving from molecular mimicry phenomena (58-60) seems to play a main pathogenic role in several diseases – from cardiovascular diseases (58) to oral erythema multiforme (59) and rheumatoid arthritis (60) – that associate with oral infections.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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In vivo confocal microscopy in oral pathology: a step towards optical biopsy

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A wide variety of primitive and secondary conditions, pathologies, and lesions may affect the oral mucosa. For most of them, biopsy and histopathology are mandatory for diagnostic and prognostic purposes. Chronic inflammatory and dysimmune diseases require long-life follow-up and various biopsies along with the time, as well in the case of tumors, where the timing of early diagnosis and the discovery of relapses may be slowed down by patient and work delays. In vivo confocal microscopy (CM) offers additional real-time information on the cytological and histological features of the epithelial lesions. The present work summarized the CM application in oral pathology and its future perspectives. In vivo CM could be usefully introduced in the clinical practice to noninvasively monitor any histological changes occurring in the oral mucosa, thus shortening the diagnostic times, and improving the patients' compliance to follow-ups.

The oral cavity may suffer a wide variety of primitive and secondary conditions, pathologies, and lesions of different nature, behavior, evolution, and prognosis, which require specific diagnostic protocols and treatments (1-7). Chronic inflammatory/dysimmune diseases affect the patient's well-being, while, in the case of tumors, expectations and quality of life are compromised proportionally to the time of diagnosis (8).

The diagnostic delay with which oral carcinomas are often recognized (9,10) is responsible for high mortality and poor long-term survival, more destructive therapeutic approaches, and residual physical, mental, and relational disabilities (11).

Conversely, early diagnosis makes it possible to identify the potentially malignant oral diseases (OPMDs) (12) and early-stage tumors in the bud, allowing more conservative therapeutic approaches and a greater expectation and quality of life of the affected patient.

Histopathology assessment is the gold standard for diagnosing OPMDs, oral squamous cell carcinomas (OSCCs), and other nontumoral oral mucosal diseases. It requires one or more specimens obtained by incisional or excisional biopsies, planned at the end of the diagnostic pathway.

Currently, the diagnosis comes after a minimum of 3 weeks to over two months. On average, the routine diagnostic process includes a series of progressive steps, as follows:

- a first visit of the patient, with the eventual request for instrumental investigations
- the re-evaluation of the lesion at 14 days to exclude other nature
- the planning of the diagnostic biopsy
- the waiting for the histopathological assessment.

Only at this point, when no less than three weeks have passed, the eventual neoplasm can be approached with the proper treatment.

These various obliged steps may contribute to a

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diagnostic delay (8), which is partly attributable to the patient who does not recognize the early, nonspecific symptoms (patient's delay), partly to clinicians not correctly informed (misdiagnosis, doctor's delay), and partly to the clinical process of classification of the suspected lesions (work's delay), sometimes approached with "watchful waiting approaches (13).

Furthermore, the suspicious lesions and conditions may require repeated observations. Thus, two or more weeks may pass before the incisional or excisional diagnostic biopsy (14), followed by a further therapeutic surgery with the removal of the primary tumor and any lymph node chains affected by metastases (15) in case of malignancy ascertained by the ex vivo routine histopathological examination (Treatment planning delay).

Various instrumental investigations, mainly RM, CT scan, US, PET, and NBI (16-23), used in clinical practice, provide helpful information for tumor staging. At the same time, the diagnostic setting culminates with planning an exploratory biopsy to preliminarily establish the nature and histological characteristics of the suspicious lesion. Furthermore, a pre-surgical mapping is necessary to evaluate the lesion histology in the various points of multifocal or extensive lesions. In these cases, the selection of sites is currently extremely operator-dependent and uncertain and may benefit from adjunctive procedures, such as vital stainings and tissue autofluorescence (24-30).

These low-cost procedures have been proven to be more accurate than the clinical inspection alone in defining the margins of the epithelial neoplasms and their malignant nature. However, the possibility to face false positives/negatives or the need for two-weeks re-evaluations induce the need for clinically obtaining further details to perfect and shorten the diagnostic times. None of the adjunctive tests can replace tissue biopsy and histological assessment (31).

In vivo confocal microscopy of oral mucosa

The introduction into clinical practice of non-invasive imaging side-chair devices that offer additional real-time information on the cytological and histological features of the suspected neoplasm is the solution to reduce the need to re-evaluate the patient and guide to a biopsy targeted, precise, reasoned diagnostics, tailored to each case. In this way, the quality of the diagnostic process is improved, the diagnostic time is shortened, and the costs arising from each patient's access to the structures decrease.

Among the non-invasive imaging tools to pursue cytological and histological information from living oral mucosal lesions (32), the literature has reported, in recent years, the flourishing of in vivo confocal microscopy (CM) for optical tissue biopsy.

A confocal microscope for clinical use integrated with specific software allows obtaining and real-time visualizing on dedicated screens the virtual histological sections of the living tissue at a microscopic resolution close to conventional histology by scanning point by point the tissue itself (30). The confocal microscopes, currently available for clinical use on humans, work using incident lights at specific wavelengths, to whom the subcellular compounds of the tissues react by producing a refractive (Reflectance Confocal Microscopy, RCM) (33) or fluorescent (Fluorescence Confocal Microscopy, FCM) (34) light.

Then, a detector collects the emitted light point by point, and an integrated software rebuilds, pixel by pixel, the image of the scanned section on a screen in real-time.

The lights used are not harmful to the tissues, and the procedure does not require anesthesia.

Confocal microscopes for in vivo use scan tissues on horizontal planes, up to the average depth of 300-600 μm, and it is to be taken into account for histological comparison, as it requires a reinterpretation of the so-called "confocal criteria," compared to conventional histological or ex vivo imaging which is based on the evaluation of tissue sectioned on transverse planes (35-37).

Today, the in vivo imaging by CM is widely considered in dermatology (38) as an excellent supportive adjunct in various clinical steps, as follows:

- to reduce the use of biopsy sampling;
- to assist the surgeon in the pre-surgical mapping of the lesion to be removed;
- to noninvasive follow-up of relapses, and
- to monitor the healing processes and the therapeutic responses to the treatments.

Conversely, CM in stomatology has been only recently considered (39). After pilot studies on healthy mucous membranes for the validation of the method

(40-42), further research with promising results has focused on the definition of "confocal" criteria found in a series of pathologies of the oral mucosa.

Chronic inflammatory, dysimmune and autoimmune diseases

Literature reports the use of CM in defining the confocal features of several oral conditions and pathologies of inflammatory, dysimmune, and autoimmune etiology. Oral lichen planus (OLP) is a chronic inflammatory disease of uncertain etiology (43-46), which may affect the oral mucosa with or without the co-occurring involvement of skin and other mucosae (47-49). It is characterized by chronic T-cell mediated immunological dysfunction and reactive, regenerative, or reparative epithelial changes (50). RCM has been widely applied to image lichen planus from different sites (51-53) and the confocal criteria of OLP under RCM imaging (55-57). The imaging by in vivo CM allows the follow-up of the patients with more compliance and less need for repeated biopsies.

Similarly, vesicular-bullous autoimmune diseases, such as pemphigus vulgaris, pemphigoids, and their variants (58-62) have been advantageously approached by in vivo CM, thus allowing the real-time identification of the histological and cellular details, thus orienting the differential diagnosis between them and other vesicular-bullous erosive diseases, both in the skin and oral mucosa (55,63-66). It reduces the times for diagnosis and allows to establish the treatment required earlier.

Oral mucosa pigmentations

The oral mucosa may present brownish, blue, or black macules due to endogenous pigments (nevi, melanoma, melanin deposits, and blood derivatives) or exogenous substances (amalgam tattoo, graphite, inks). Their differential diagnosis is challenging and requires biopsy due to the variable etiopathogenesis and lack of clinical pathognomonic signs (67). Also in these kinds of lesions, CM has proven its utility to distinguish them, thus shortening and ameliorating the diagnostic procedure and the therapeutic approach (68-71).

Precancerous and tumoral oral lesions

The in vivo CM imaging of oral malignancies and their precursors is reported in the literature with promising results since it allows discovery in real-time microscopic signs of cancer and considerably shortens the work delay to treat the patients. Various works depicted the confocal differential criteria for oral carcinomas and other tumoral affections, with details close to the conventional histology (72-76).

Oral Infectious diseases

Oral diseases of infectious origin have not yet been investigated under CM, despite preliminary studies on skin and genital mucosa affected by warts, and other HPV-related lesions have been preliminarily reported (77-82).

Dental enamel imaging

The microscopic dental architecture, and its defects, due to caries, demineralization, or inadequate fillings, may have been imaged only in vitro, and it requires to process of the extracted teeth. However, recent works report the usefulness of in vivo CM on dental surfaces, with flourishing works reporting the confocal features of healthy and unhealthy surface enamel up to 300 μm (83-86). This revolutionary approach could help establish the appropriateness of dental filling and the responsiveness to remineralizing therapies or the effects of orthodontic procedures on the enamel by in vivo imaging with good compliance from the patients and a lack of invasive procedures in children and special needs patients (87-92).

DISCUSSION

A wide variety of oral lesions and diseases require biopsy and histopathological analysis for definitive diagnosis. However, the planning of the biopsy requires preliminary observations, instrumental examinations, and presurgical decisions on where and what part of large or multifocal lesions need to be included.

Oral squamous cell carcinoma (OSCC) is the most frequent oral malignancy and, together with other kinds of oral neoplasia (93-95), is associated with poor overall survival and a high risk of metastasis in the advanced stages (96). Furthermore, biological tissue and salivary markers have been widely investigated

but not yet considered in clinical practice to predict or intercept early signs of malignant transformation of OPMDs (97-103).

An adjunctive noninvasive technique, as CM is, is auspicial to be introduced in the clinical practice, thus allowing in vivo and real-time definition of the microscopic features of each kind of lesion.

Furthermore, in vivo CM does not require anesthesia or drugs and offers to shorten the diagnostic time for quick therapeutic establishment. In vivo CM has made it possible to identify, at microscopic resolution, the cell morphology and the architecture of the tissue, layer by layer up to the subepithelial connective tissue, at a maximum depth of just over 300-600 μm, which is the penetration limit of the incident laser beam through the dental surface and the oral mucosa, respectively.

Although we are currently far from routine application in clinical support like dermatology, the prospects are revolutionary in oral pathology and oncology, where the diagnostic process needs to be shortened and perfect.

It is desirable that in the future, in vivo CM should be usefully widely applied in oral pathology and medicine, as in dermatology, not only to reduce and ameliorate the biopsy sampling, thus assisting the surgeon in the pre-surgical mapping of the lesions, but also for the following purposes:

- to noninvasive follow-up, and evaluate the responsiveness to treatments and the onset of relapses, as well as the healing processes of such chronic oral diseases affecting the patients' mouth (104-109)
- to evaluate in vivo the pathobiological events underlying the onset of oral diseases (58,59, 110-114)
- to study infectious diseases and the search for indicators of viral, bacterial, or fungal etiology (115-122)
- correlate microscopic CM signs with biological tissue and salivary markers suggestive of specific diseases for diagnostic and prognostic purposes (123-132)
- to study the effects on oral epithelia and teeth of novel drugs, substances, and treatments (133-142).

Meanwhile, the gold standard for diagnosing oral cancer remains conventional histopathology biopsy supported by proven staining methods or other molecular biology.

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In vivo Optical Coherence Tomography in oral cavity: an update

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Optical Coherence Tomography (OCT) technique is based on broadband light, using a low coherence interferometry system. The OCT technology, in the last decades, has been widely employed in various medical branches, showing as a diagnostic mean capable of providing high resolution macroscopic and microscopic images of biological tissues. Nowadays, alongside with other *in vivo* diagnostic means, OCT has been introduced also in dentistry and oral medicine demonstrating as a device of considerable interest both for hard and soft tissue of oral cavity. Current literature shows off hundreds of studies about feasibility of OCT application for early diagnosis of oral disorders. The purpose of this paper is to analyze and summarize the evidence present in the literature, providing an update on the state of the art of the *in vivo* application of OCT on hard and soft tissues of the oral mucosa.

In the past decades Optical Coherence Tomography (OCT) has raised as an *in vivo* prospective non-invasive and non-radioactive adjunct tool, for the advance frontiers of imaging of biological tissue layers (1, 2). The OCT technology is based on interferometers, and it allows to provide real-time cross-sectional sub-surface tissue images, conventionally defined as tomographic images, being capable to generate real-time depth-resolved two-dimensional (2D) and three-dimensional (3D) images, at excellent spatial resolution (10-20 μm) and adequate depth of penetration into the tissues (1-2 mm) (3).

Although OCT and ultrasonography display the same tissue image cut, ultrasound uses sound waves, while OCT technique is based on broadband light, employing a low coherence interferometry system (4). A near-infrared (IR) ray of light (an electromagnetic wave) is thrown directed inside the tissues: the light reflects and diffuses at the level of the different tissues in a different way, and interference signal is acquired by charge-coupled device (5). The machine receives these differences translated into a delay time of the echo of light, that is reflected or backscattered by the different structures, measuring the delay by using the principle of interferometry itself (6). Very low coherence interferometry allows to measure the range between structures with extreme accuracy, measuring the light rebounded and comparing it with a light beam thrown on a reference path (7). The application of broad-bandwidth light sources in OCT enables to display tissue microstructures morphology to be imaged in detail at depths significantly greater than the penetration depth offered by other conventional diagnostic tools, as reflectance confocal microscopy (8).

There have been introduced two OCT modalities: Time-Domain (TD)-OCT and Frequency-Domain (FD)-OCT. FD-OCT is the second generation of OCT technology, and there are two different methods: Spectral-Domain (SD)-OTC and Swept-Source (SS)-OCT, basing on the output characteristics and the

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structure of the receiving components of the light source (9). FD-OCT provides an implementation of the low coherence interferometry principle, since it involves spectral information to generate scans, without the need for a mechanical scan of the optical path length; this technology increases both the speed and the resolution of the images compared to those of traditional TD-OCT (10). Over the years, many functional upgrading has been made, starting from OCT Doppler (DOCT), polarization sensitive OCT (PS-OCT), endoscopic OCT and acoustic OCT (11).

First OCT medical applications were experimented *in vitro* in human retina and in atherosclerotic plaque (12). Then, thanks to the progressive improvement of the performances both optical specifications and of the system capabilities, the OCT boasts great usefulness in various fields of research and clinical applications, up to be reputed as a sort of "optical biopsy" (13). Novel applications have been conducted in dentistry and oral medicine, in order to evaluate the OCT feasibility as a useful technological tool both for hard and soft tissues of the oral cavity, aiming to validate OCT device alongside with other *in vivo* diagnostic means (14, 15).

The purpose of this work is to synthetize main evidence of literature concerning *in vivo* application of OCT in oral cavity.

Oral hard tissues optical coherence tomography

First *in vivo* OCT dental microstructures exam was experimented in 1998 by researchers from the Laboratory of Medical Technology of Livermore, California (16, 17). The works from Colston and colleagues displayed dental hard tissues images 3 mm depth and soft oral tissues images 1.5 mm depth; they shown the feasibility to obtain dental scans, allowing to appreciate real-time *in vivo* images of enamel, enamel-dentin junction, composite restoration, gum margin, and periodontal pockets, through the OCT infrared beam of light (17).

Further studies mainly have deepened the opportunity to endorse OCT for the detection of enamel erosions, quantifying the severity of tooth demineralization and identify dental decay, in order to perform the early diagnosis of caries and to compare OCT images with conventional x-ray exams (18-20).

In the 2014 a study by Shimada *et al*. observed 86 dental surfaces in order to detect demineralizations and decays by using *in vivo* an SS-OCT device (21). Interestingly, authors compared their OCT results with radiographic images, affirming that OCT sensitivity and specificity for detecting enamel demineralization, cavitated enamel lesions and dentin caries were higher than those of the radiography.

A work by Lai and colleagues demonstrated the OCT usefulness in monitoring periodontal disease, evaluating with *in vivo* scanning both the gumline and alveolar line in order to obtain precise measurements (22). Their results showed promising data for the effectiveness of OCT exam as a valid aid in the diagnosis and management of various periodontal disease stages (23-25).

A very recent randomized controlled trial, in 2021, has shown the great OCT versatility: Şen *et al*. employed OCT for in vivo evaluating orthodontic surface sealant layer thickness and integrity (26). OCT values made it possible to appreciate the significant difference in the thickness of the sealant between the beginning and the end of the orthodontic treatment (27-31).

Dental hard tissues are often subject to disorders related to aging, health status and syndromic pictures which lead to anomalies of the shape or structure of the teeth (32-35). Taleb *et al*. in 2018 reported some cases of dentinogenesis imperfecta (DI), which involves dentin development, determining loss of the overlying enamel (36). Authors examined the teeth of a subject affected by DI, clearly appreciating demarcated areas of hypomineralization close the dentin-enamel junction.

Lastly, OCT improvement has followed hand in hand advanced in dentistry and prosthodontic, up to be considered as an aid in implantology diagnostic system (37). Kikuchi *et al*. in 2014 have investigated the reliability of OCT scanning for the detection of marginal gaps at the implant–abutment interface (38). The authors concluded that SS-OCT appeared as a potential non-invasive and non-radioactive tool capable of monitoring the misfit interface between an implant and its abutment.

Oral mucosa optical coherence tomography

Oral mucosa represents an argue diagnostic topic

since it is involved in several autoimmune, infective, neuropathic, systemic, preneoplastic and neoplastic disorders (39-47).

The anatomotopographic features of the oral mucosa have been widely explored through the *in vivo* evaluation by OCT images of the soft tissues in healthy, dysplastic and malignant mucosa, using different image interpretation methods (48). An *in vivo* OCT imaging of healthy oral mucosa enables the macroscopic identification of the stratified squamous epithelium, of the underlying lamina propria with uniform intensity and of the underlying intact basement membrane (49).

One of the most sensible topic for oral pathologist is represented by oral cancer and oral potentially malignant disorders (50, 51). A study by Tsai *et al*. compared the images obtained by *in vivo* OCT of healthy oral mucosa with those of subjects suffering from oral malignant and oral potentially malignant disorders (52). The samples were placed in four different groups, basing on pathological diagnosis, ranging from healthy mucosa (control group), to arrive at squamous cell carcinoma of the oral cavity (OSCC), passing through epithelial hyperplasia and epithelial dysplasia. The authors then compared the histological pictures with OCT scans, taking as reference three diagnostic parameters: standard deviation (SD) of a scan signal profile, the exponential decay constant (α) of a scanning and epithelial spatial frequency spectrum thickness (T). The obtained values showed that the standard deviation increases, the parameter α decreases and the epithelium becomes thicker in epithelial hyperplasia, epithelial dysplasia and OSCC samples (three study groups) compared to the control group. A more recent study by the same group of authors used SS-OCT to identify in vivo microstructural and microvascular features at different sites of healthy oral mucosa in order to consider as potential markers for the diagnosis of oral malignant and potentially malignant lesions (53).

In 2009 Wilder-Smith *et al*. performed a clinical examination, followed by OCT imaging and then biopsy and histological examination in 50 patients with healthy mucosa, dysplastic lesions and malignant lesions. Each lesion was first diagnosed based on the *in vivo* OCT evaluation and then on histopathology (54). Real-time OCT images of dysplastic lesions showed epithelial thickening with loss of stratification in the epithelial layers deeper than in healthy oral mucosa. In the OCT images of OSCC, peculiar features have been highlighted, such as heterogeneity of the epithelial thickness, the presence of erosive areas and dripping in the subepithelial layers, with loss of integrity of the basement membrane. The results obtained showed a significative correspondence between the diagnosis based on the *in vivo* OCT imaging and the one based on histological pictures.

Oral mucosa is involved in a wide range of infective, systemic and autoimmune disorders, which represent demanding challenges for the clinicians in need of technological support (55-62). In this perspective, the efficacy of OCT in vivo has also been explored in immune-mediated diseases, thanks to the peculiar pathological characteristics of these oral pathologies (i.e., oral lichen planus, pemphigus vulgaris and muco-membranous pemphigoid) (63-66). A very recent study by Panzarella et al. (66) enrolled patients suffering from desquamative gingivitis; moreover, several authors have identified, through the analysis of OCT images, morphological patterns peculiar to oral lichen planus, pemphigus vulgaris, and muco-membranous pemphigoid (48, 67, 68)

DISCUSSION

Despite optical coherence tomography devices have shown a wide range of application and feasibility in various branches of dentistry, quality of scanning has proven to remain operator-sensitive, as well as various technological diagnostic aids (69, 70).

Based on the available data, OCT shows as an upward technique for detecting and monitoring various pathological conditions, such as tooth lesions, periodontal and peri implant disorders (70-77). Current knowledges about *in vivo* OCT application for oral hard tissue need further investigations but should be considered promising, as well as most real-time non-radioactive imaging technologies (78-82). Optical coherence tomography arises in the diagnostic path system to monitor the healing or worsening of the oral cavity clinical condition in malignant and potentially malignant disorders (83-89).

Although biopsy followed by histological examination still remains the "gold standard" for a reliable diagnosis, further studies are needful to validate the OCT technique as a valid *in vivo*, non-invasive and real time examination for the early detection of malignant and potentially malignant disorders of the oral cavity.

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State-of-the-art of topical Photodynamic Therapy in oral benign, malignant and potentially malignant disorders: a scoping review

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Photodynamic therapy (PDT) is a non-invasive tool based on the use of a photosensitizer (PS) and a light source that can activate the PS and lead to the production of Reactive Oxygen Species (ROS). It is currently used in different fields of medicine such as gynecology, dermatology, oncology, and dentistry. In dental practice, the main branches involved are periodontology, oral pathology, orthodontics, and endodontics. PDT finds its spot against antibiotic and antimycotic resistance. Oral pathology is involved in the treatment of several oral diseases such as Oral Carcinoma, Oral Lichen Planus, Oral Candidiasis, Oral Leukoplakia, and Actinic Cheilitis. Because of its non-invasiveness, PDT has already found a place in oral pathologists' daily practice. In this study, we aimed to analyze the main papers published regarding PDT and oral pathology or oral medicine to give a detailed picture of the state-of-the-art and to detect its possible role in the next years.

Photodynamic Therapy sinks its roots at the beginning of the '900 when some researchers found that the combination of light and some chemicals could induce cell death, and Jesionek started treating skin tumors with the combination of eosin and white light (1). Topical Photodynamic therapy (PDT) is a non-invasive tool that relies on the use of a photosensitizing agent selectively accumulated in lesion tissue and a light source. PDT has already been described as a useful therapeutic option in different medical fields such as dermatology, gynecology, and oncology, whilst in dentistry in periodontology, endodontics, and oral pathology (2,3). Oral Pathology is the part of Dental Medicine involved in the treatment of autoimmune diseases such as Pemphigus Vulgaris and Bullous Pemphigoid (4–10), infective diseases such as oral candidiasis, HSV, AIDS, or HCV-related lesions (11–17), Oral Potentially Malignant Disorders (OPMD) such as Oral Lichen Planus (OLP) (18–20), Oral Leukoplakia (OL) (21), Actinic Cheilitis, in oral manifestations of systemic diseases (22–24) such as gastroesophageal reflux (25), inflammatory bowel diseases (26–28) and in the early diagnosis of Oral Squamous Cell Carcinoma (OSCC) (29). Nowadays in oral medicine PDT represents a validated treatment option for some pathological conditions such as OL, OLP, Oral Candidiasis, and OSCC (30-32). The interaction of the photosensitizing agents, involving oxygen, leads to the production of Reactive Oxygen Species (ROS) and the subsequent toxic effect on the selected substrate not harming the surrounding healthy tissue (33,34). The main photosensitizers (PS) described are based on a porphyrin-like nucleus and include molecules such as 5-aminolevulinic acid (ALA), methylene blue (MB), indocyanine green, and toluidine blue (TB) (35). The light source involved with the wavelength emitted in the PS absorption spectrum activates the reaction and

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produces ROS (36). The Reactive Oxygen Species have been described as activating different pathways to produce cellular death through apoptosis, necrosis, or autophagy. Direct action of PDT, which seems the main involved in the therapeutical outcome, produces cellular necrosis resulting from cellular swelling and a rapid loss of plasma membrane integrity. Cellular apoptosis involves 40 different kinds of molecules that undergo 30 different reactions that activate two different pathways (37). PDT seems also to directly damage the tumor vascular portion which leads to microvascular collapse and subsequent cellular necrosis due to hypoxia or anoxia. The use of different photosensitizing molecules has already been described as the key to targeting the cytotoxic damage to several pathological conditions.

Oral Benign Disorders: Oral Candidiasis

Oral Candidiasis is a mycotic disease in most cases caused by Candida albicans (38), but at least other seven species have been attributed to the disease in the oral cavity such as C. kruesi, C. guillermondii, C. glabrata, C. lusitaniae, C. parapsilosis, C. pseudotropicalis, C. stellatoidea, and C. tropicalis (39). Different factors like chronic corticosteroidal therapy, AIDS, and diabetes could predispose or worsen the pathological picture (40). In Oral Medicine, based on the clinical features, oral candidiasis is divided into white or erythematous candidiasis, with further subtypes for each category (41,42). De Oliveira Mima et al. Compared topical antifungal therapy with Photogem® mediated PDT highlighting a significant reduction in fungal colonization in both therapies with a clinical success rate in the nystatin group of 53% and 45% for the group treated with PDT. Despite a clinical superimposable result immediately after the therapy (15 days) the recurrence of palatal inflammation was observed in 75% in the Nystatin group and in 78% of the patients belonging to the PDT group, this result seems to be ascribable to the impossibility of removing the causal factor that caused the primary infection (43). A systematic review by Javed et al. assessed the use of PDT against oral fungal infections by analyzing 15 studies that met the inclusion criteria. Six of the studies were performed on animal models, five studies were performed on in vitro models, and one study was performed on denture models. The diode laser wavelengths ranged from 450 nm to 660 nm. The main PS involved were Porphyrin-based PS, TB, or MB. The authors recorded that 93% of the studies that met all the inclusion criteria exhibited antifungal effects, the main advantages seem to be the effectiveness even against azole-resistant fungi and the selective exposition of microbes to ROS. The main disadvantage seems to be the lack of standardization in PDT protocols: the use of different PS at different concentrations, the use of different source light resulting in different wavelengths, and the time of exposition that is highly varied in all the studies included (44). Prazmo et al. in their literature review examined the different studies that used PDT to treat chronic C. albicans infections in an animal in vivo models. The use of a high concentration of MB as PS seemed to have a photodisinfection role in rat models. The authors also showed as the main critical point the large differences in every PDT protocol that can lead to very different clinical results and different recurrence ratio (45). Rojz et al. reported a case in which a 12-year-old male patient underwent head and neck radiotherapy (HNRT) and showed a non-responsive mycotic infection due to the immunodeficiency caused by cancer treatment. After a single PDT session, the authors reported the lesion and symptoms remission (46).

Infective diseases

Microbiome alterations seem to play a key role in different fields of dentistry such as in oral pathology (47,48), periodontology in periodontal disease (49–52) and in periimplantitis (53,54). Several authors have assessed the usefulness of PDT in orthodontics: El Shehawy et al. evaluated MB mediated PDT effect on orthodontic leveling and alignment using a 635 nm gallium aluminum arsenide laser applied in 10 sites for 10 seconds. Clinical outcome was quantified using Little's Irregularity Index (LII) and the statistical analysis revealed a significant LII decrease in both PDT and control groups, no statistical difference was found between the two groups' LII scores (55), demonstrating the inaudible effect of PDT on orthodontic treatments (56–60). Plotino et al. analyzed the state-of-the-art regarding PDT in endodontics. The

authors found a large number of studies in vitro, but only a few studies regarding its effectiveness in vivo. The results seem to suggest a significant reduction following root canal treatment with adjunct PDT compared to conventional treatment (61).

Oral Potentially Malignant Disorders (OPMD): Oral Lichen Planus (OLP)

OLP is a disimmune disease involving T-cell mediated chronic inflammation (62–64). Its clinical appearance can range from white striae (also known as Wickham's Striae) to erosive-bullous and ulcerative lesions (65,66). The main therapeutic alternatives described are topical corticosteroids, calcineurin inhibitors, and PDT (67,68). In 2018 Akram et al. published a systematic review focusing on "Is PDT effective in the treatment of symptomatic OLP?". The research yielded six studies that included PDT protocols with a light source with a wavelength ranging from 320 to 660 nm and an exposure time ranging from 70 to 150 seconds. The patients' follow-ups ranged from 4 to 48 weeks. Three of the six studies included used clinical scores to assess OLP clinical features, in two of them the use of corticosteroids showed a statistically significant improvement compared to PDT and in one of them PDT showed a comparable outcome between PDT and steroid use. The main discrepancies seem to be the comorbidities and the smoking status that could further worsen the clinical outcomes (69). Wang et al. included in their systematic review and meta-analysis nine RCTs and seven studies for their meta-analysis. The authors showed in the statistical analysis no differences between topical corticosteroids and PDT for sign scores (using Thongprasom score) and for symptoms scores (using VAS score) (70). Hesse et al. published their paper to "demonstrate the heterogeneity in PDT of OLP". The authors found seventeen clinical studies that used five different PS, the light source's wavelength ranged from 420 to 682 nm and the number of treatments ranged from one to ten. The patients' follow-ups ranged from 0 to 48 months. In all controlled studies the PDT was at least equal in effectiveness compared to conventional treatment and no adverse effects were recorded. The authors found also a lack of studies that tried to examine the PDT on a cellular level (71).

Oral Leukoplakia (OL)

OL is described by WHO as "white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" (72). Based on the clinical features it can be distinguished into homogeneous, non-homogeneous, and verrucous proliferative leukoplakia (PVL) (73). Due to its clinical nature, OL remains a challenging condition for the clinician (74). Li et al. published the only systematic review analyzing the use of PDT in the treatment of oral leukoplakia. The review included sixteen studies and a total of 352 patients, the main PS involved were Photofrin, MB, ALA, and chlorine-e6, the wavelength used ranged from 420-660 nm, and the follow-up period varied from 1 to 119 months. The OL mean recurrence ratio ranged from 0 to 60%. (75)

Actinic Cheilitis

Lai et al. performed a systematic review to identify the best therapies for actinic cheilitis. The study included 49 papers and 789 patients. The most frequent therapies found were laser therapy and PDT. The most used PS was MAL. The results showed that the best clinical results were reached when partial surgery and laser therapy were performed alone or combined with PDT. In this case, PDT alone showed non-comparable results when compared with topical therapy (76).

Oral Malignant Disorders: Oral Squamous Cell Carcinoma (OSCC)

OSCC is the most common oral cavity malignant tumor (about 90%) (77). Different factors seem involved in its pathogenesis such as smoking, alcohol consumption, and betel nut chewing (78,79). Cerrati et al. performed a literature review and meta-analysis to compare the efficacy of PDT to surgical resection in the treatment of adults with early stages of OSCC. The study included 24 papers that met the inclusion criteria, 12 included in the PDT group and 12 included in the surgery group, at the end of the data extraction 411 patients were the ones who underwent PDT, and 740 were the ones that underwent surgery. The patients' follow-up ranged from 4 to 189,5 months and the results showed an efficacy rate of 86% for PDT and 80% for surgery. The statistical analysis

revealed no statistical differences in the efficacy between the groups. The recurrence ratio was analyzed and the results showed a 12% ratio in recurrence for patients that underwent PDT and 23% for patients that underwent surgical resection, also in this case the statistical analysis revealed no differences between the groups (80).

Photodynamic diagnosis

Photodynamic Diagnosis (PDD) represents a non-invasive in vivo tool for diagnostic and surgical procedures. Due to its non-radioactive nature, it could have a role in clinical examination and follow-up (81–83). Jurczyszyn et al. (84), in fact, evaluated photodynamic activity as a potential diagnostic mean, alongside many other tools (85–91). The authors described PDD as a useful tool for the visualization of irregular lesion margins more precisely than a classical white-light examination and a further diagnostic aid could be fractal dimension analysis. The authors enrolled 35 patients suffering from OLP and OL that underwent ALA mediated PDD and PDT. Statistical analysis showed that fractal dimensions of leukoplakia foci of the tongue in a white-light examination were significantly lower in PDD.

DISCUSSION

Photodynamic therapy seems to have a potential role in different fields belonging to dental practice (92,93). Most of the studies highlighted as main advantages of the technique the high selectivity, the absence of recorded side effects, and the fact that it is easy to perform the technique (94). These reasons suggest that PDT has its target in medically-complex patients in which surgery could be contraindicated (95-97). The main disadvantage is certainly the lack of standardization in the PDT protocols (time of exposure, PS selection, sessions' number and clinical evaluation) (33).

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An overview of High-Definition Ultrasounds applied in oral diseases

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OBJECTIVE: Ultrasonography (US) is a non-invasive and real-time medical imaging technique based on the reflection of sound waves and the specific impedance of the body's tissues. It has been used as a diagnostic aid in oral diseases both with an intraoral and extraoral approach in recent years. The fields of application have been varied, from oral squamous cell carcinoma (OSCC) to vesiculobullous diseases. It is added to several other imaging technologies such as optical coherence tomography, narrow-band imaging, tissue fluorescence, and reflectance microscopy. This study aims to provide an overview of the technique and fields of application in the oral cavity.

METHODS: Narrative overview of the literature synthesizing the findings of literature retrieved from searches of PubMed, Scopus, and Web of Science database.

RESULTS: Most studies in the literature analyze the feasibility of the US in OSCC. In OSCC, the technique allows for identifying the lesion in the surrounding tissue context; furthermore, the high dimensional accuracy allows for measuring the tumor thickness, giving diagnostic and prognostic information. Ultrasonography has been applied also to oral lichen planus (OLP), Pemphigus Vulgaris (PV), Mucous membrane pemphigoid (MMP), and temporomandibular joint (TMJ).

CONCLUSION: The US represents a promising imaging technique in vivo and real-time for the characterization of oral diseases. Further studies are needed on the topic.

Ultrasound imaging (US) is based on the reflection of the waves off of the tissues, resulting in different shades of grey, from white to black (1). In the last twenty years, the interest in using this technique in the oral district has been growing hand in hand with the technological increase (2). To date, US joins other imaging techniques used in oral pathologies, such as optical coherence tomography, confocal reflectance microscope (RCM), narrow-band imaging (NBI), and tissue fluorescence (3–6). For most of the oral diseases, the diagnosis gold standard is represented by the histopathological microscopic examination, however, the imaging techniques seem to be a promising diagnostic tool for the clinician (7). These techniques, in fact, make it possible to help the clinician both in diagnostic and operational terms (8, 9). All these techniques have been applied in the prevention and management of oral squamous cell carcinoma (OSCC) and for the oral mucosa's inflammatory and autoimmune diseases (10–13). Regarding the OSCC, these non-invasive methods are placed together with other screening methods for an early diagnosis and, therefore, an improvement in prognostic terms (9, 14–17). Immediately comparable to the US are

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RCM and OCT (18, 19). RCM is an in vivo confocal laser microscope and can be used on soft and hard tissues, returning images with a microscopic or submicroscopic resolution by "cutting" the tissue on the horizontal plane (20–23). One of the advantages is that its application is not limited to the oral mucosa but also to the teeth; This also broadens the range in the evaluation of enamel defects in patients with systemic diseases or the enamel damage subsequent to a dental treatment (19, 24–28). However, the technique is limited by a small scan depth (3, 25). OCT can also be used in hard tissues to obtain a vertical stack of the analyzed tissue. Unlike the US, the technique is based on the backscatter of infrared light and has a high spatial resolution (29). The limits are also represented here by the high cost, poor ergonomics, and the lack of a variety of probes suitable for the oral cavity (30–33). The US has characteristics ideal for producing vertical sections images in vivo and in real-time of the soft tissues of the oral cavity, through a variable resolution (based on the frequency emission) and discrete ergonomics, thanks to the wide choice of probes (34–36). In head and neck areas, ultrasonography has been used to diagnose neck masses, salivary gland tumors, tongue carcinoma, and other oral lesions (2).

In the present work, scientific literature has been reviewed to overview the current state of the art in the use of ultrasonography for diseases affecting the oral mucosa.

MATERIALS AND METHODS

A narrative overview of the literature has been performed. PubMed, Scopus, and Web of Science (WOS) were used as search databases to investigate literature and review Ultrasonography in the oral cavity. The keywords used were "ultrasound" AND/OR "ultrasonography" AND "oral cavity" AND/OR "oral mucosa" AND/OR "oral diseases" AND/OR "temporomandibular joint". Results were then restricted to papers on in vivo or in vitro studies in humans, that had been published. Relevant studies have been selected.

Technical features

Ultrasound waves are emitted from piezoelectric crystals contained in a probe placed over the structures of interest and introduced into the body with a frequency ranging between 1.6 and 50 MHz; the ones that are reflected produce an echo headed to the transducer in the probe (1). High-frequency transducers have a frequency range from 10 to 100 MHz (37).

Ultrasound waves are emitted perpendicular to the surface of the transducer. It is possible to widen the deep sonographic field by bending the surface of the transducer (convex array transducer) (36, 38). Waves will be parallel to each other when the probe surface is flat (linear array transducer). Linear array transducers usually have higher frequencies (10-12 MHz), less penetration, and excellent resolution. The ultrasound images obtained by a linear array transducer will be rectangular, while those obtained with a convex array transducer will be wider with increased depth because this probe does not delineate the actual tumor shape (39, 40).

Color Doppler

Color Doppler sonography extends the range of ultrasound diagnosis by allowing assessment of the vascularity of superficial lymph nodes. It combines gray-scale ultrasound and the pulsed Doppler technique. Flow blood is displayed in color superimposed on the gray-scale image in real-time B-mode scanning. Color code reflects the frequency shift dependent on flow velocity and the angle of the sound beam with the blood flow (41, 42).

Yamamoto et al. proposed using doppler ultrasonography images of the invasion front of the OSCC along the length of their tumor boundaries to assess three vascular indexes for the quantitative analysis of the blood flow signal area (43). The associated tumor thickness (TT) and quantitative analyses of vascular findings seem related to the pathological grade of malignancy and cervical lymph node metastasis.

Ultrasound artifacts

Artifacts may distort the size, position, and shape of the studied structures or even show structures that are not present with a negative influence on measurement accuracy (44). However, some artifacts are handy for diagnosing different conditions. For example, ultrasounds are less able to penetrate through denser tissue, for example, an oral fibroma or a solid structure like implants (composed of titanium or titanium alloy), teeth, calcifications, or salivary stones (45–47). This causes a shadow artifact behind the solid structures (48).

Posterior enhancement artifact may occur when imaging

fluid-filled structures (like an abscess or a mucocele). More ultrasound waves will penetrate the fluid-filled structure, and a white enhancement area will appear behind it. This artifact is essential in diagnosing and ultrasonographic classification of salivary gland tumors (49, 50).

The refraction artifact occurs when an ultrasound beam refracts at the edge of a rounded structure. This artifact may disappear when changing the angle of the ultrasound beam clarifying the nature of the artifact (38).

The mirror artifact appears when a high acoustic impedance tissue redirects the ultrasound waves. The mirror artifact will mimic a virtual object on the opposite side of the structure with a more hypoechoic, blurred, and distorted image (51). Reverberation artifact occurs when ultrasound bounds between two interfaces with significant acoustic impedance (52). The waves move forward and backward among these interfaces. The device will recognize these waves as parallel lines with equivalent distances between them, reducing density for the lower lines because the reflected waves are lesser in number. This fallout is in a striped configuration with dark and sharp lines (39).

Intraoral or extraoral approach

In examining oral lesions, ultrasonography was carried out by placing a transducer on an extraoral site, but it was not easy to obtain detailed data. The intraoral ultrasonographic examination is non-invasive, rapid, and easily repeated; the number of possible applications has thus increased (2).

According to Chan and coworkers (45), the two approaches are superimposable and must be chosen based on the anatomical position of the area to scan with ultrasound. It is preferable to perform both approaches in some regions to have complete information.

An extraoral approach has some limitations: it does not provide adequate images, especially in the tongue, oral floor, and palate region, because air spaces within the oral cavity attenuate acoustic waves, and ultrasound does not penetrate the bone well. Moreover, the acoustic attenuation caused by the distance from the lesion to the skin could become problematic (50). However, the extraoral approach is necessary for some applications such as temporomandibular joint disorders (53, 54).

DISCUSSION

Ultrasonography has been used in various branches of medicine such as gynecology, gastroenterology, cardiology, angiology, and, concerning the head and neck region, to detect salivary glands abnormalities and malignancy(52, 55). This technique has been used in the oral cavity for different purposes: from the study of periodontal tissues to the clinical and presurgical characterization of benign and malignant lesions of the oral mucosa (48, 56, 57).

Some recent ex vivo and in vivo studies suggest that high-frequency ultrasounds can discern tissue layers based on image contrast. This contrast results from acoustic backscatter, which is directly proportional to the change in density found in the boundaries between tissue layers (58).

Several exploratory studies have evaluated the application of ultrasonography on several pathologies of the oral mucosa. Only one study, by Izzetti et al., evaluated the potential role of intraoral high-frequency US (HFUS) imaging in the diagnosis of Pemphigus Vulgaris (PV) and Mucous membrane pemphigoid (MMP) (59). Various imaging techniques have been used to evaluate this family of pathologies (such as OCT) in vivo and real time (29, 60). The gold standard to diagnose vesiculobullous diseases is histology and direct immunofluorescence (DIF), which aim to assess the localization of the bullae and the antibody target (61, 62). Through the US in b-mode and c-mode, it was possible to identify not only the position of the bubble - below the epithelium for MMP and intra-epithelial for PV and the characteristics of echogenicity and homogeneity of the mucous and submucosal layers. Although sensitivity and specificity, compared to histology, have not always been satisfactory, the application of US as a diagnostic aid for this group of pathologies appears promising.

Another study by the same authors evaluated the application of the US in the oral lichen planus (OLP) using B□mode and the pattern of vascularization (color doppler) (63). OLP is a disease characterized by a complex etiology that affects oral mucosae by T-cell mediated chronic inflammation (64). Extra-oral sites (i.e., scalp, skin, nails, conjunctiva, esophagus, larynx urethra, vulva and vagina, and perianal area) can be involved (65–67). OLP diagnosis is made by evaluating both clinical and histological criteria and the differential diagnosis must be made with

an extensive range of pathologies (68–70). Usually, OLP lesions are multiple, symmetrical, and bilateral. OLP is generally classified into six clinical variants: reticular, papular, plaque-like, erosive, atrophic, and bullous (71, 72). Some other imaging techniques, such as RCM, have been evaluated as a tool for the 'in vivo' characterization of OLP in non-invasive and real-time (73). OLP lesions presented a thick, hypoechoic superficial layer at the US exams, corresponding to the mucosa. Vascularization could be more intense in the superficial layers than in the deeper layer. According to the authors, it would also be possible to distinguish the different ones from a US point of view: for example, in the erosive-ulcerative form, the mucosal layer disruption in the center with on the sides a mucous thickening and an intact submucosal layer have been observed.

Another clinical application is TMJ imaging. TMJ plays a central role in clinical practice both for the generalist dentist and the orthodontist. MRI is the gold standard, however, cannot be carried out in some patients (e.g. pacemaker wearers, claustrophobic patients), and it is a high-cost procedure also in terms of time (74). Several studies have analyzed disc displacement with US in symptomatic and asymptomatic patients (75, 76) In a 2018 study by Kalyan et al. it was possible to determine the distance between the articular capsule and mandibular condyle in symptomatic and asymptomatic patients by US. This is of great importance since the trauma of the TMJ could be observed in some orthodontic treatments (77–80).

Oral Squamous cell carcinoma (OSCC)

Intraoral ultrasound (US) has been particularly useful in determining the interface between tumors and the surrounding myo-architecture at the deep margin (11, 81). The ultrasonographic pattern of most of the invasive carcinomas was a relatively well-defined hypoechoic lesion, while the pattern of the surrounding tissues was homogeneous echogenic (82).

Some authors, contrariwise, hypoechoic lesions were reported with irregularly shaped margins and not well circumscribed in the majority of cases. This study investigated ultrasonography (US) to identify the primary tumor in head and neck squamous cell cancers (HNSCC) of unknown primary sites. Transcervical and intraoral ultrasonography were performed by using convex (3.75–6.0 MHz and 5–7.5 MHz) transducers (38). The same group of authors in a precedent study described the base of the tongue tumors as hypoechoic with irregular margins (38). This difference could probably be due to the transcervical probe, with a low frequency and a convex type of image, so it is not easy to be compared to the images obtained with a linear probe. Moreover, tumors with increased invasive mode could show irregular and unclear margins on US images (83).

The tongue is the most often examined region because it is easily accessible compared to other oral cavity sites. Furthermore, it is easy to distinguish the tumor from the homogenous muscular composition of the tongue while other disturbing anatomical structures are absent (40, 84).

Accuracy of ultrasound technique

The primary consideration in assessing the accuracy of an imaging technique is the grade to which that procedure matches directly with histopathology. In OSCC, the ultrasonic images allowed to distinguish the tumor from the surrounding tissues; moreover, it is possible to measure the tumor thickness with a strong correlation between the values obtained with ultrasound scanning and histological sections (11). The clinical routine magnetic resonance (MRI) and computed tomography (TC) represent the diagnostic imaging techniques of choice. However, these are more expensive, and in the case of CT, they expose the patient to a dose of radiation (85). Moreover, according to some authors, ultrasonography would have greater dimensional accuracy in OSCC at early stages with a thickness <5 mm.

A meta-analysis of studies correlating US tumor thickness (TT) measurements to histopathology concluded that US measures are highly accurate and only slightly overestimate TT values (84). Another review reaffirms the relationship of US and TT with histopathologic TT and further discusses the potential utility of different US parameters in oral tongue cancer management and the use of US as an intraoperative adjunct to resection.

The high diagnostic accuracy of US could also be

useful in the diagnosis of hyperplastic pathologies or other non-neoplastic lesions (34, 86, 87).

Limitation of the technique

The main technical limitation of intraoral US is when large or posterior lesions are present, primarily due to difficulty accessing the posterior tongue with the probe and the induction of vomiting (82). As an intrinsic limit of this technique, intraoral US is a strongly operator-dependent live examination: consequently, different radiologists with variable experiences may report different information (88). If the patients have a limitation in mouth opening, then intraoral sonography is challenging to perform (35, 89).

Conclusions

The US represents a promising imaging technique in vivo and real-time characterization of oral mucosal diseases. Some considerations must be made regarding the advantages of this technique: high accuracy in dimensional determination, ease of execution, and good patient compliance. Another thing to consider is the low costs of the examination to guarantee access to treatment for the entire population, especially in terms of primary prevention (90–95). Further studies are needed to confirm the feasibility of the US in the diagnosis and management of oral mucosa diseases.

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Oral Lichen Planus and Epstein-Barr virus: a narrative review

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Oral Lichen planus (LP) is an immune-mediated disease of unknown etiology that affects the oral mucous membranes. Six clinical forms of OLP lesions can be detected individually or associated: papular, reticular, plaque-like, atrophic, erosive, and bullous. The antigen that triggers the inflammatory immune reaction in OLP lesions is yet unclear. The correlation between Epstein-Barr virus (EBV) infections and Oral Lichen Planus has been reported in the literature. The present work summarized the evidence to establish if EBV can play a role in the etiopathogenesis of the disease.

Oral Lichen planus (OLP) is an immune-mediated disease of unknown etiology that affects the oral mucous membranes, with an estimated prevalence of 0.5-3% (1). It is a disease in the middle-aged (30-60 years old range) and is more common among women (female/male sex ratio is 1.5 to 3) (2).

Six clinical forms of OLP lesions can be detected individually or associated: papular, reticular, plaque-like, atrophic, erosive, and bullous (3,4).

The antigen that triggers the inflammatory immune reaction in OLP lesions is yet unclear. This trigger might be a self-antigen or an exogenous antigen (5,6). Autoimmune-like damage in OLP lesions results from T CD8+lymphocytes-induced apoptosis of the basal epithelial cells (7,8).

The most investigated virus in association with OLP is the Hepatitis C virus. Several studies (9-13) suggest a strong link between OLP and HCV, which found that OLP patients have a higher risk than controls of being HCV seropositive. However, the authors underlined a large variability linked to geographical differences. This association is evident in high endemic areas for HCV infection (14-16).

A recent study (17), based on previous data that have outlined the role of molecular mimicry in defining immunogenic/cross-reactive sequences in autoimmune disorders (18-22) and not only (23-34), showed that four potentially immunogenic heptapeptides (SSSSSSS, QEQLEKA, LLLLLLA, MLSGNAG) are shared not only between HCV but also with Epstein-Barr virus (EBV) and three human proteins. These proteins are involved in the interactions of plaque proteins and intermediate filaments. These four heptapeptides are part of validated epitopic sequences. It seems feasible that they might have a role in the immune crossreactive attack(s) leading to the inflammatory and altered cellular status that characterizes oral lichen. Starting from this theoretical proposal, we decided to review the available evidence of a correlation between the EBV and OLP.

EBV prevalence in the oral lichen planus lesions

Several studies estimated EBV prevalence in tissue specimens obtained from OLP lesions using polymerase chain reaction (PCR) (35-41). Results

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were very conflicted, as the positivity range varied from a minimum of 0% (38) to a maximum of 62.5% (41). In addition, Vieira Rda et al. (41) investigated, through PCR, the EBV DNA presence in blood and saliva samples and exfoliated cells obtained both from OLP patients and healthy controls: they found a high prevalence of the virus in saliva and exfoliated cells in both the cases and controls. However, no significant results were found at a significativity of 5% on EBV status in blood samples of both groups. The non-atrophic-erosive cases (i.e., reticular, plaque, and mixed types of OLP lesions) showed the highest prevalence in the cases, and they were also the most affected by the EBV virus. These results agree with the literature that, in most cases, showed the highest prevalence of EBV infection in the reticular and plaque-type (36). Sand et al. found a significant difference between EBV prevalence in OLP patients compared to control subjects (38). They also studied the influence of smoking, snuff use, and alcohol consumption on EBV prevalence, finding no differences or correlations. Kis et al. showed that patients with OLP carried EBV DNA more frequently than controls in the lesion but not in the adjacent healthy mucosa (40).

DISCUSSION

OLP is a potentially malignant disorder. In the last decades, considerable progress has been made in early diagnosis thanks to innovative imaging methods that help the clinician in the differential diagnosis (42-45) and monitor suspicious lesions (46-54).

Despite this, there is still a scarcity of clinical, histological, and molecular predictors to define malignant development for OLP (55,56). In this context of malignant transformation, assessing the presence or absence of EBV infection could provide valuable data and that could also be integrated into the analysis of OLP lesions.

EBV is a herpes virus, first isolated from the cells in Burkitt's lymphoma (57). It binds to receptors on epithelial cells of the oropharynx and salivary glands, but also lingual epithelium has receptors for EBV, and the virus can infect even cervical and lacrimal epithelium (58). EBV infects mainly the basal and intermediate layers, where it remains latent. The EBV receptor is lost as the epithelial cells differentiate and move towards the luminal surface. The EBV receptors are identical to receptors for the complement component C3d: this receptor is known as CD21, and it is up-regulated in OLP patients (59,60).

A recent study found that the heptapeptide LLLLLLA is familiar to EBV, HCV and the Pemphigus Vulgaris desmoglein-3 antigen (18,19). This data is interesting because immunoreactivity against desmoglein-3 has previously been described in patients with oral lichen planus (61). Moreover, the EBV heptapeptide MLSGNAG is shared with plectin, a hemidesmosome protein involved in cross-linking and stabilizing cytoskeletal intermediate filaments networks and regulating their dynamics (62). Hence, a down-regulation of plectin could disrupt the intermediary filaments network (63,64).

Some epithelial cancers are strongly associated with EBV infection (e.g., nasopharyngeal carcinoma and a subset of gastric and lymphoepithelioma-like cancers) (65,66). However, the results are very unclear, with the prevalence of EBV presence in OLP specimens ranging from 0% (38) to a maximum of 62.5% (40). A recent meta-analysis reported preliminary results that seem to support a statistically association between EBV infection and risk of OLP (67). The Authors conclude that additional investigations are required to explore the association between EBV and OLP further. Maybe prospective cohort studies focusing on a long-term follow-up of OLP patients positive for EBV should be performed to assess its potential malignancy.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Correlation between Oral Lichen Planus and HPV infection

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Oral Lichen planus (LP) is an immune-mediated disease of unknown etiology involving oral mucous. OLP lesions can be single or multiple and with the following clinical variants: papular, reticular, plaque-like, atrophic, erosive, and bullous. The antigen that activates the inflammatory immune reaction in OLP lesions is unknown. The literature reports a correlation between Human papillomaviruses (HPV) infections and Oral Lichen Planus. The present paper recapped the evidence to establish if HPV can be a player in the etiopathogenesis of the disease.

Oral Lichen Planus is an inflammatory disease characterized by a cell-mediated autoimmune reaction in which lesions result from T CD8+lymphocytes-induced apoptosis of the epithelial basal cells (1,2). Papular, reticular, plaque-like, atrophic, erosive, and bullous are the clinical forms of OLP lesions. (3,4). The antigen that triggers the inflammatory immune reaction in OLP lesions is unclear. This trigger might be a self-antigen or an exogenous antigen (5, 6). Several findings support the evidence of autoimmune-like damage in OLP lesions: adult-onset, female prevalence, chronicity of the disease, association with other autoimmune diseases, demonstration of autocytoxic T cells in OLP lesions, development of OLP like lesions in patients with GVH disease, and effectiveness of immunosuppressor therapy (7-11). Among the exogenous agents, the role played by several viruses has been investigated with ambivalent results (12).

A particle of epidemiological evidence supports the association between OLP and HCV infection. A correlation theory argues that the virus, as it would replicate in the oral mucosa, may attract HCV-specific T lymphocytes (13, 14).

Etiopathogenesis of OLP has also been associated with the other viruses (15), Human papillomaviruses (HPV) (in particular the 16 genotype) is one of the most extensively investigated viruses (16). The purpose of this paper is to analyze and summarize the pieces of evidence present in the literature, providing an update on the state of the art of the correlation between HPV and OLP.

Prevalence of different HPV genotypes in OLP lesions

A large number of studies investigated HPV detection in OLP lesions. Kato et al. (17) examined the largest group of OLP cases and found an HPV DNA prevalence of 41.5% (83/200). The most investigated and retrieved genotypes are the "high risk" 16 (18-22) and 18, (21-23) that are considered to be the most potentially malignant. No significant differences in the prevalence of viral infection were described among

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the various clinical and histological presentations of OLP lesions. Syrjänen et al. were not able to detect HPV antigens in two oral lichen planus lesions by means of the immunoperoxidase (IP-PAP) analysis even if the two OLP specimens were examined positive for HPV DNA (17). On the other hand, Kashima et al. examinated twenty-two oral lichen planus specimens finding contrasting results: 4 of 22 OLP specimens were positive for HPV capsid antigen (all women and two of them showed koilocytes) using an immunoperoxidase examination, while none was positive for HPV DNA by means of in situ hybridization (24). Cox et al. found positivity to HPV DNA in 3 of 4 OLP lesions examined and co-infection by HPV-16 and HSV-1 in two of these specimens (19,20). Other studies found evidence of co-infection by HPV and other viruses in OLP specimens (23) and also mixed HPV genotypes infections (21-23). Interestingly, Mattila et al. found a prevalence of 15.9% (13/82) of HPV infection in the examined OLP lesions: five of these patients evolved to cancer even if, in the original biopsies used for the study, no dysplasia was present. Another point of interest in this study is that none of the five lesions which progressed to invasive carcinoma resulted positive for the "high risk" HPV genotypes, while they were found positive for the considered "low risk" HPV 6 and 11 genotypes (25). Sahebjamiee et al. retrieved higher values of HPV DNA prevalence in OLP patients with dysplasia (4/7, 57%) than in OLP patients without dysplasia (5/33, 15%). Furthermore, the prevalence of HPV 16 in OLP lesions showing dysplasia was significantly higher (3/7, 43%) than in OLP patients without dysplasia (0/33, 0%) (26).

Lastly, a novel approach was presented by Viguier et al., who examined the specificity of lesional CD8+ cytotoxic T lymphocytes in oral lichen planus patients, which has never been analyzed before. They studied the diversity and antigen specificity of the TCR expressed by CD8+ T cells in 10 OLP patients, 6 of whom presented past HPV infection, and three were found to be still HPV positive. 2 of 3 presented HPV16-specific IgG. Results showed that a notable proportion of HPV16-specific clonal CD8+ blood T cells of OLP patients infiltrate mucosal and/or skin lesions, decreasing and disappearing following clinical remission (27).

DISCUSSION

HPV are small DNA viruses showing epithelial tropism and can induce hyperplastic, papillomatous, and verrucous squamous cell aberrations in the stratified squamous epithelia (28,29). One of the most peculiar characteristics of the papillomaviruses is their genotype-specific host restriction and the preference of particular papillomavirus types for specific anatomical sites, where they cause lesions with distinctive clinical pathologies (30).

A recent study (17), based on numerous data that have outlined the part of molecular mimicry in identifying epitopic sequences in autoimmune disorders (31-35) among others (36-46), showed that four potentially immunogenic heptapeptides are in common between HCV, EBV, HHV-7, HSV-1, CMV (but not HPV16) and three human proteins (pinin, desmoglein-3, and plectin). These proteins are implicated in the interactions of plaque proteins and intermediate filaments. Given that these heptapeptides are a portion of validated epitopic sequences, it seems likely that they might have a function in the immune cross-reactive attack(s) conducting to the inflammatory and altered cellular status that denotes oral lichen.

An enormous variability of the data available on this matter in literature is present: according to some authors, the extent of the link between OLP and HPV would be ample (OR: 8.83),21 while according to others, it seems overestimated (16).

The variance in HPV DNA prevalence among all the studies presented may be attributed to the differences in sensitivity of the applied molecular methods, sampling methodologies, patient characteristics, and the types of studied specimens (e.g., biopsy tissue, oral mucosal brushing, saliva, and blood samples). Even if the "high risk" genotypes as HPV 16 and 18 (which are established risk factors in oral cancer development (47)) have been the most founded viral type in the OLP specimens examined, there is no sufficient evidence at the present moment that they could be involved in the etiopathogenesis of OLP neither in the malignant transformation of the lesions.

OLP is a potentially malignant disorder. In the last years, improvement has been achieved in early diagnosis with the aid of innovative imaging methods that

assist the clinician in the differential diagnosis (48-52) and monitor suspicious lesions. (53-60).

Nevertheless, there is yet an insufficiency of clinical, histological, and molecular predictors to define the malignant outcome for OLP (61,62). In this context of malignant transformation, further studies that evaluate the long-term course and evolution of the OLP disease considering the HPV presence would be attractive.

Conflicts of interest:

The authors declare that they have no conflict of interests.

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Aesthetic and functional aspects of incisal guidance: a systematic review

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This systematic review of the literature aims to assess the scientific evidence regarding incisal guidance from both aesthetic and functional perspectives. The ultimate aim was to provide the clinician with evidence-based guidelines to assist in routine dental practice. The Medline database (PubMed) was searched for relevant articles using the keywords "incisal guidance" and "anterior dental guidance" and no time constraints. Types of studies selected were randomised clinical trials (RTCs); prospective, retrospective and transversal studies; controlled clinical trials; and comparative studies. This search furnished 252 articles, but only eight were deemed suitable for review after application of the inclusion and exclusion criteria. The final sample contained no RCTs, comprising only transversal clinical trials. No study included a previous sample size estimation, and none performed method error analysis. None of the subjects analysed dropped out. Only one article featured blind measurements, and only one a randomly selected sample. The description of the characteristics of the test subjects was judged to be adequate in five studies, intermediate in one, and poor in two. The quality of the articles was deemed to be low in three cases, and intermediate in the remaining five. Given the heterogeneity of the articles deemed suitable for review and the poor statistical power of several, it was not possible to formulate evidence-based guidelines. Nevertheless, several indications useful for clinical practice are suggested, particularly as regards combined orthodontic-restorative treatments.

Incisal guidance is the path on the lingual surface of the upper anterior teeth along which the lower anterior teeth slide when a person is in neutral or distoclusion. If the anterior teeth are in mesioclusion, the lingual surfaces of the lower teeth slide along the incisal margin and/or the labial surfaces of the upper teeth. Together with the TMJ, the muscles and correlated neuromuscular system, the incisor guide helps to control the anterior, posterior, and sometimes even lateral movements of the lower jaw, protecting the posterior teeth. If the forces are excessive, and if the periodontal tissues are susceptible, unfavourable incisal guidance contributes significantly to the loss of alveolar bone and thereby promotes dental mobility.

Incisal guidance is essentially the product of four components: the overjet, overbite, incisor level, and labiolingual curve (1). Nevertheless, the relationship between the upper and lower anterior teeth is not the only determining factor in incisal guidance, and aesthetics, phonetics, and movement of the condylar margin are also key (2).

In 1976, a group of gnathologists met to examine all the various theories on occlusion, and to endeavour to establish a set of requisites for functional incisal guidance, with a view to translating theoretical concepts into practical advice for routine clinical practice (3). The central question was, and remains, to discover what steps a clinician should take to ensure optimal aesthetics and function. With this in mind the present review was performed to analyse the available scientific research regarding the aesthetic and functional aspects of incisal guidance, in order to compile a set of evidence-based guidelines for clinical dental practice.

MATERIALS AND METHODS

Research strategy

This systematic review was largely performed

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according to indications issued by the National Health Service (NHS) Centre for Reviews and Dissemination (4). The reference search performed was focussed on retrieving all articles concerning aesthetic and functional aspects of incisal guidance on the Medline database (PubMed). No time constraints were applied to the search, and all articles yielded by the search terms "anterior dental guidance" and "incisal guidance", after setting the search parameters to include "clinical trials", "controlled clinical trials" "randomised controlled trials" and "comparative studies", were considered.

Selection criteria

The types of article included were randomised clinical trials (RTCs); prospective, retrospective and transversal studies; controlled clinical trials; and comparative studies. No constraints were placed on the sample size. All clinical trials that focussed exclusively on analysis of the canine

Table I. *Type of study*

Article	**Study Design**
Kubein-Meeseinburg D et al. (8)	T, CT
Pelletier et al. (9)	T, CT
Donegan SJ (14)	T, CT
Ogawa T et al. (10)	T, CT
Otake et al. (11)	T, CT
Moreno et al. (15)	T, CT
Lamontagne et al. (13)	T, CT
Bauer et al. (12)	T, CT

T: transversal; CT: clinical trial

Table II. *Quantitative analysis of data*

Article	Both incisal and canine /posterior guidance	Tools for simulating/measuring the occlusion	Effects of occlusal patterns on muscles	Evaluation of anterior and posterior disclusion angles	Reference plane used	Type of movement examined	Scope
Kubein-Meeseinburg et al. (8)	Yes, incisal and canine guidance	Plaster models, axiograph, x-ray	no	yes	-	Protrusion	To study the biomechanical relationship between incisal and condylar guidance
Pelletier et al. (9)	yes	Arbitrary modified facebow + axiograph	no	yes	Axis–ala plane	Protrusion	To evaluate the relationship between anterior and posterior disclusion angles
Donegan et al. (14)	yes	Radial gauges	no	no	Occlusal plane	Centric occlusion	To measure the curvature & inclination of lingual surfaces of upper anterior teeth
Ogawa (10)	yes	3D mandibular movement analysing system; metallic bite registration strips	no	yes	Camper plane	Protrusion	To measure condyle points and inclination and sagittal pathways of incisors, canine and first & second molars
Otake (11)	yes	Metal overlays on upper canines; gnathohexagraph; frontal & lateral cephalograms	no	yes	Frontal plane	Lateral	To assess the role of condylar guidance in incisor path angles during lateral excursions, modifying the canine guide
Moreno et al. (15)	Yes, normal guidance, overbite, anterior openbite	—	Yes, via electro-myography	no	Frankfort plane parallel to floor	Masticatory	To evaluate differences in swallowing and chewing forces or maximum stress
Lamontagne et al. (13)	yes	Arbitrary earbow, semi-adjustable articulator, adjustable incisal plate	no	yes	Frontal plane	Lateral	To evaluate any relationship between preferred chewing side & anterior guide angle
Bauer at al. (12)	no	Electronic axiograph; arbitrary earbow; articulator with individual values	no	yes	Axis–orbital plane/Frankfort plane Horizontal and inclination of condyle	Protrusion	To evaluate any association between degree of incisor wear & incisal/condylar guidance

guide were rejected, as were those that included subjects with joint pathologies or malocclusions caused by vertical skeletal issues (open bite).

Data collection and analysis

The following aspects of the data collected were assessed: analysis of both incisal and canine/posterior guides, tools for measuring/simulating the occlusion, effects of occlusal patterns on the muscles, evaluation of disclusion angles, plane of reference used, type of movement examined, and scope of study. In addition, to document the methodological value of every article, a qualitative analysis was performed according to the methods described by Antczak et al. and Jadad et al., conforming to the pre-established characteristics (5, 6). Thus the following parameters were examined: type of study, sample size and prior sample size estimation, description of sample characteristics, drop-out rate, method validity, method error

Table III. *Sample characteristics*

	Number of subjects	Gender distinction	Orthodontic treatment	Parafunctions	Initial evaluation of presence or absence of incisal guidance
Kubein-Meeseinburg et al. (8)	14 patients	No	Not specified	Not specified	No
Pelletier et al. (9)	50 patients (45 with + 5 without anterior guidance)	Yes	Yes, 44%	No	Yes
Donegan et al. (14)	Plaster models of 32 patients	No	Not specified	Not specified	No
Ogawa et al. (10)	54 (27M+ 27F)	Yes, influences the results	No	Not specified	Yes
Otake (11)	5	Only males	Not specified	Not specified	Evaluation of the presence of guidance of function in lateral movement
Moreno et al. (15)	45 (12M + 33F)	Yes, but does not influence the results	Not specified	Not specified	Yes
Lamontagne et al. (13)	40 (35F + 5 M)	Yes, but does not influence the results	No	No	No
Bauer et al. (12)	85 orthodontic patients (53 F + 32 M); 58 with incisal guidance	Yes, but does not influence the results	Yes	Yes, bruxism	Yes

Table IV. *Qualitative analysis*

Article	Previous estimate of sample size	Selection description	Randomization	Drop-outs	Valid method	Method error analysis	Blinding in measurement	Adequate statistics provided	Quality standard
Kubein-Meeseinburg et al. [8]	no	+	no	no	yes	no	no	no	poor
Pelletier et al. [9]	no	+++	no	no	yes	no	no	no	intermediate
Donegan et al. [14]	no	+	no	no	yes	no	no	yes	intermediate
Ogawa [10]	no	+++	yes	no	yes	no	no	yes	intermediate
Otake [11]	no	+++	no	no	yes	no	no	yes	poor
Moreno I et al. [15]	no	+++	no	no	yes	no	no	no	poor
Lamontagne et al. [13]	no	+++	no	no	yes, partially	no	yes	no	intermediate
Bauer et al. [12]	no	++	no	no	yes	no	no	yes	intermediate

analysis, blind measurement, and appropriate statistical analysis. The quality of each study was thereby defined as poor, intermediate or high (7).

RESULTS

Type of study

The initial search yielded 252 articles, and after application of the inclusion and exclusion criteria, a total of 8 were reviewed. The type of study reported in each article is described in Table 1, while the results of the review are outlined in Tables 2, 3 and 4. All articles analysed were transversal studies, in that they all collected measurements and assessments on patient samples or plaster models of such at one particular time point. No RCTs, studies with control groups, prospective, retrospective or comparative studies were found by the search.

Study objectives

Five out of eight studies (8-12) analysed the relationship between incisal guidance and the condylar guide. Of these five, one in particular (12) focussed on a functional/aesthetic aspect, in that it evaluated the presence of a possible association between the degree of wear on the incisors and incisal and condylar guidance. The study by Lamontagne et al. (13) also examined the degree of wear on the incisor guides, in this case, however, correlating it with the preferred chewing side. Another article (14) performed static measurements of the inclination of the lingual surface of the anterior teeth, while Moreno et al. (15) investigated the influence of various factors, including incisal guidance, on muscle activity.

Sample characteristics

In two out of eight studies, no reference was made to patient gender, and in only one out of the remaining six articles was the influence of this aspect on the results mentioned (8, 10, 14). In four out of eight articles (8, 11, 14, 15), whether or not the patients had undergone prior orthodontic treatment was not specified, while the remaining four comprised two specifying that the patients had not been previously treated (10, 13), and two (9, 12) that they had. Only one (12) took parafunctions into account, while the remaining seven (8, 10, 11, 14, 15) made no mention of whether or not patients were affected. Finally, half of the articles (9, 10, 12, 15) described an analysis of the presence or absence of incisal guidance, and only one (11) assessed the presence of the guide in the function of the group in lateral movement.

Tools for simulating/measuring the occlusion

The axiograph was used in three out of eight studies. (8, 9, 12) In that by Kubein et al. (8), plaster models and radiographs were analysed without specifying the type of radiograph, articulator, or facebow employed. Pelletier et al. (9) used a modified facebow, analysed in the first part of the study, using the axis–ala plane as a reference. Bauer et al. (12), instead, considered the axis–orbit plane. One study used an electronic axiograph and an arbitrary earbow with an individual measurement articulator. Donegan et al.'s (14) measurements of plaster models were made using purpose-built gauges, taking the occlusal plane as the plane of reference. Ogawa et al. (10) used three-dimensional analysis of mandibular movement, enabling them to record movements in the six degrees of freedom. They used the Camper plane as the horizontal reference plane. Otake et al. (11) fitted metal overlays on the upper canines to evaluate the effect of alterations in the inclination of the canine guide on condylar kinetics and the incisal paths with respect to the frontal plane. They also made use of a semi-adjustable articulator with facebow and a gnathohexagraph with six degrees of freedom to measure mandibular movements. Lamontagne et al. (13) also used a semi-adjustable articulator with earbow, measuring the incisal guidance with respect to the frontal plane by means of an adjustable plate. Only one study (15) evaluated the correlation between occlusal and muscle patterns, in this case via electromyography.

Movement and guidance types examined

In seven out of eight articles, (8-11, 13-15) not only the incisal guidance but also the canine guide was taken into consideration, while the other (12) focused solely on the incisor guides. Four out of eight studies (8-10, 12) examined protrusive movements, while of the remaining four clinical trials, only one performed

measurements in centric occlusion (14), two studied lateral movement (11, 13) and one (15) the mastication movement as a whole – the investigation was centred on the muscle forces that developed.

Evaluation and correlation of anterior and/or posterior disclusion angles

Seven studies out of eight (8-14) evaluated the anterior and/or posterior disclusion angles and the correlation between the two. Kubein-Meesenburg et al. (8) described the biomechanical system between incisal and condylar guidance as akin to a "link quadrangle", which fulfills Grashoff's condition under normal physiological conditions. This study states that the condylar guide has an angle of 0º to 45º and identifies 22º as the maximum angle of movement of the hinge axis that describes the mandible in protrusion. These values exist in a condition of equilibrium in which alterations are manifested as stomatognathic system dysfunction. Those authors also state that each point on the incisal margin can represent the guide point of incisal guidance and that the biomechanical system of incisal/condylar guidance is not affected by growth.

Pelletier et al. (9) noted that 77.8% of subjects with clinically significant incisal guidance possessed an anterior disclusion angle in protrusion 13.5° greater on average than the corresponding posterior disclusion angle. Nevertheless, the remaining 22.2% of patients had anterior disclusion angles smaller than the corresponding posterior disclusion angles, and no interference of the posterior teeth in protrusion. In 64% of the sample, differences in disclusion angle between left and right sides ranging from 0º to 3º were noted. However, those authors found no statistically significant correlation between the anterior and posterior disclusion angles. In their opinion, the intra-articular tissues of the temporomandibular joint play a significant role in the determining the relationship between the skeletal components.

Donegan et al. (14) were the only group to perform static measurements on plaster models, concentrating on the curvature and inclination of the lingual surfaces of the anterior teeth from the transition point on the cingulum to the incisal margin. They claimed that the incisor function and curvature varies considerably from that of the canines, and that overcontouring of the lingual surfaces of the upper incisors to obtain contact may be harmful.

Ogawa et al. (10) stated that the major determining factors behind every dental path in protrusive movement are condylar guidance and gender. The incisal path has a greater influence on the path of the anterior teeth, while the condylar path has a greater influence on the path of the posterior teeth. Furthermore, the incisal model has a greater influence the condylar path, but there is no significant correlation between the inclination of the two guides. In females, who display a shorter mandible with less distance between the condyle and dentition than men, the effect of condylar guidance is greater. In contrast, the inclination of the dental paths displayed no gender-related differences, and is greater than the inclination of the condylar path. Moreover, the paths of the anterior teeth are greater than those of the posterior teeth.

Otake et al. (11) found that when the incisal path angle becomes steeper than the natural dental guide, the distances of the non-working side condyle paths (centre of condyle) decreased significantly, while those of the working side condyle paths remained unchanged. The non-working side condyle has an active role during lateral excursions, while the working-side condyle follows the consequent movement, and is susceptible to variations in the incisal path during lateral excursions.

Lamontagne et al. (13), on the other hand, showed that the majority of individuals with a preferred chewing side had a smaller angulation of the lower incisal guide on that side, i.e., this behaviour manifests clinically as greater abrasion of the anterior teeth, although this difference was not found to be statistically significant.

Bauer et al. (12) reported that the manifestation of a parafunction is independent of the incisal guidance angles, and of the incisor inclination and relationship. Their results also revealed a slightly greater tendency to incisor wear when there is a normal or excessive functional space than when this space is reduced. Furthermore, in the absence of incisal guidance, they reported a greater tendency for TMJ pain in both the muscle and joint itself.

Only one study (15) did not involve evaluation of the disclusion angles, instead focussing on the force that develops in various occlusal situations, including normal incisal guidance, increased overbite and anterior open bite, during deglutition, mastication and maximum effort. They found no difference in muscle activity when comparing different types of incisal guidance.

Qualitative analysis

Evaluation of the quality of the studies took into consideration the sample size, the presence or absence of method error analysis, the presence or absence of blind measurements, distortions and contradictory variables, and the suitability or absence of statistical methods. No study proposed a prior estimation of sample size, and none performed method error analysis. Similarly, no drop-outs were reported in any article, only one study reported blind measurements (13), and only Ogawa et al. (10) studied a randomly selected sample. Description of the sample characteristics was judged adequate in five articles, (9-13) medium in one, (15) and poor in two (8, 14). Likewise, the overall quality of the studies was deemed to be poor in three cases (8, 11, 15) and only intermediate in the remaining five. (9, 10, 12-14)

Kubein-Meeseinburg et al.'s (8) study was found to be of poor quality because the sample was very small and inadequately described. Furthermore insufficient information was provided regarding the apparatus used to make the measurements. Similarly, Otake et al. (11) also studied a very small sample, while Moreno et al. (15), despite providing a detailed description of their sample and method of assessment, performed no stress testing in protrusion, which would have highlighted the muscle activity in the various types of incisal guidance.

Of the studies judged to be of intermediate quality (9, 10, 12-14) Pelletier et al. (9), although thoroughly describing a sample of more than adequate size, used the axis–ala as a reference plane, which had a greater influence on the numerical data than the use of the axis–orbital or occlusal planes. Donegan et al.'s work (14), on the other hand, displayed a defect in the measurement technique, relying on convex or concave radial gauges, which serve to measure the curvature radius of objects but are not ideal for measuring the lingual surfaces of the teeth, forcing the researchers to approximate measurements using 'best-fit' gauges. The sample in Ogawa (10) was also adequately described, and indeed randomly selected and assessed as to the presence or absence of incisal guidance. Furthermore, mandibular movements were analysed by means of a three-dimensional system, thereby overcoming the problem of the fixed relationship between the incisal plate and the condyles of the articulators. However, that study could only be considered of medium quality as they made no use of method error analysis, nor a facebow, which would have minimised the errors due to inter-individual variation in dentocranial morphology. Lamontagne et al. (13) were the only researchers in this sample to include blind measurements, examining the incisal guidance angle with no prior knowledge of the preferred chewing side. However, this factor was established subjectively by an observer, and could therefore represent a source of bias. Finally, in Bauer et al. (12), it is important to recognise that the degree of bruxism is difficult to quantify, and that those affected display different patterns of laterotrusive movement, a fact that was only mentioned in passing. Hence, even though the patients selected unequivocally presented this parafunction, the authors failed to provide a detailed quantification of the problem, and the article quality cannot therefore be considered as high.

DISCUSSION

Evaluation of incisal guidance and measurement method

The aim of this systematic review was to analyse the scientific evidence available regarding the incisal guide from both aesthetic and functional perspectives, in order to provide guidelines for routine clinical practice. However, application of the inclusion and exclusion criteria yielded only eight articles for review, none of which detailed a randomised clinical trial. Only five of the eight studies (8-12) analysed the relationship between incisal and condylar guidance. Four of these (8-10, 12) examined protrusive movements, and only one performed measurements in centric occlusion (14). Likewise, only two studied lateral movements, and one (15) looked at the mastication movements as

a whole, being a study focussed on the muscle force that develops. Similarly, only four out of eight studies (9)(10)(12)(15) performed an initial assessment of the presence or absence of incisal guidance.

Each study relied on different methods of measurement, each with their own limitations, but the most influential factor on the numerical data was the choice of reference plane. For instance, one set of authors (9) elected to use the axis–ala plane, which can yield different values with respect to the axis–orbital or occlusal planes. A further two important aspects to consider are the influence of gender on the results of each investigation, and any prior orthodontic treatment that the sample may have undergone. In one study alone (10) was gender found to influence the results, whereas two articles stated that some (9) or all (12) of the subjects in the sample had previously been treated orthodontically. The effect of orthodontic treatment should not be underestimated when evaluating the quality of an investigation, as it may bring about alterations that can heavily influence the results.

Study quality

The greatest limitation affecting the articles selected for review was the time factor. Indeed, no article reported data obtained at different time points, and all studies were transversal, i.e., gathering data at a particular point in time. Another important factor to consider is the "statistical power" of the trials reviewed, which depends on the number of patients involved and has a major influence on the ability of the study to furnish meaningful results. Put simply, the greater the number of participants, the greater the statistical power of a study. In our sample, only one study (11) was based on an extremely small sample, but even in those investigating a greater number of participants, results were not found to be statistically significant (8, 9, 13, 15).

Furthermore, no study was found to provide a previous estimation of the sample size, and none calculated the method error. Furthermore, no drop-outs were reported in any study, only one relied on blind measurements (13), and only one (10) a randomly selected sample. Hence the study quality was judged to be low in 3 cases (8, 11, 15) and intermediate in the remainder (9, 10, 12-14).

Clinical relevance

The incisal guide is one of the main goal of a dental treatment even if a clear correlation with the health of the TMJ is not demonstrated (16-25). A lot of studies evaluated the position of upper and lower incisors by CBCT scans (26-31) confirming the importance of anterior disclusion for a correct occlusion. Prosthetic treatments have often been planned in order to respect the incisal guide (32-41) but the orthodontic treatment are always associated with a variation of the position of anterior teeth (42-44). Recently new archwires were introduced in order to generate low and constant forces on the teeth (45-49) and to avoid excessive labial inclination of upper and lower incisors (50-56). Aligners (57-65) and lingual appliances (66-76) are usually planned on digital set up and the management of the the relationship of anterior teeth is potentially more reliable. Also the use of rapid palatal expander can increase the intra-arch diameter creating space for the teeth alignment and reducing the risk of a not stable incisal guide (77-82). However, the severity of some malocclusion as open bite (83-85) or missing upper lateral incisors (86-87) may require the use of skeletal anchorage (88-95) or inter proximal reduction (96) to achieve a correct contact between anterior teeth in the respect of function and esthetics of adults and growing patients (97-105).

Quality analysis of the articles selected enables us to conclude that:

The literature contains no RCT on incisal guidance.

Various means of measuring incisal guidance have been employed, each with their own limitations.

The majority of findings in the literature do not reach statistical significance.

No study contained a prior estimate of sample size, and none calculated method error. No study featured drop-outs, only one relied on blind measurements (14), and only one on random sample selection (10).

Given the heterogeneity of the studies and their low statistical power, it is impossible to propose evidence-based guidelines, but the review did reveal indications that may be useful for the clinician, especially in combined orthodontic-restorative treatment.

In particular, to establish the incisal guidance of a patient, the first step is for the clinician to determine the posterior protrusive disclusion angles using an

axiograph, and to transfer these angles to an articulator using a kinematic facebow (9). As an alternative to the axiograph, a 3D system of analysing mandibular movement (10), or the gnathohexagraph (11) can be used. Each point on the incisal margin may represent a guide point for incisal guidance (8).

In protrusion:

The angle of inclination of the condyle guide ranges between 0° and 45°, and the angle of movement of the hinge axis that describes the mandible in protrusion is no greater than 22° (8);

There is no statistically significant correlation between the anterior and posterior disclusion angles, but the former are 13.5° greater than the latter (9).

Condylar guidance is individual and immutable. Only the anterior guide, not the posterior, can be modified to improve masticatory function. The influence of incisal and condylar guidance on the path of protrusive movement varies according to the type of tooth and the gender of the individual (10).

The onset of a parafunction is independent of the incisal guidance angles and the incisor inclination and relationship. There is a slightly greater tendency to incisor wear when the functional space is normal or increased than when it is reduced. When incisal guidance is absent, there is a greater tendency to TMJ pain (12).

In lateral movement:

When the incisal path angle becomes steeper than the natural dental guide, the distances from the centre of the condyle on the balancing side decrease whereas those on the working side remained unchanged (11).

The majority of individuals with a preferred chewing side have a smaller angulation of the incisal guide on that side, i.e., a clinical manifestation of greater abrasion of the anterior teeth (13).

In centric occlusion:

Overcontouring of the lingual surface of the upper incisors to obtain contact may be harmful (14).

During mastication:

Variations in incisal guidance do not affect the activity of the masticatory muscles (15).

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Carriere Motion® 3D™ appliance followed by Self-ligating brackets System in the correction of Class II malocclusion: dentoalveolar and skeletal effects compared to the use of intermaxillary elastics

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OBJECTIVES: The aim of the study was to analyze the skeletal and dentoalveolar effects of Carriere Motion® 3D™ appliance (CMA; Henry Schein Orthodontics, Carlsbad, Calif) followed by full fixed multibracket appliance. The results were then compared to a sample treated with class II elastics and full fixed multibracket appliance.

MATERIALS AND METHODS: Treatment records of 30 Caucasian patients with initial class II malocclusion were retrospectively selected and divided into two groups. In Group 1, patients were treated with the Carriere Motion® 3D™ device, and in Group 2 with class II elastics, both followed by fixed multibrackets appliance. Digital dental casts were analyzed using the VAM (Vectra, Canfield Scientific, Fairfield, NJ, USA) software to gather in-out, tip and torque values to compare the data between the two groups. Lateral cephalograms before and after treatments were analyzed.

RESULTS: The dental cast analysis revealed a reduced tip of the upper canine and upper first molar, mainly in Group 1, meaning that the Carriere Motion® 3D™ appliance causes a distal inclination of the crowns to which it is bonded. From lateral cephalograms analysis emerged that skeletal values changes were not statistically significant. Indeed, we observed mainly dento-alveolar variations in both groups.

CONCLUSIONS: The Carriere Motion® 3D™ appliance was not able to determine a bodily distal movement of upper molars, but a combination of lower and upper dental effects. No statistically significant differences were found in the correction of class II malocclusion when compared to the group treated with intermaxillary elastics.

The etiology of Class II comprises several factors, skeletal, dentoalveolar or combination of both. The most common one is mandibular retrusion (1). There are many methods for class II correction, one of the most common is fixed multibracket appliance (FMA) in association with intermaxillary elastics (IE) (2-20). Another valid and common alternative treatment option is maxillary molar distalization (21-24); in this solution the use of miniscrews could offer anchorage control and side effects reduction (25-48).

One device for Class II correction available for all orthodontists is the Carriere Motion® 3D™ appliance (CMA); Henry Schein Orthodontics, Carlsbad, Calif) (49). It has become very popular due to its efficiency and versatily (50–52). It can be used to establish a dental Class I relationship at the beginning of the orthodontic treatment. Whereas elastics for class II correction are used at the end of the treatment with FMA and require full-day cooperation, the CMA is used in the first phase of treatment, when patient collaboration is higher and it simplifies the correction of malocclusion during the

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following step of the treatment (53).

The appliance consists of a stainless steel bar, bonded to the maxillary canine or upper first premolar and upper first molar. The canine pad has a hook for engaging the IE. The upper molar pad presents a ball-and-socket design to allow distalization, tipping and rotation of the tooth and it is designed to stop its function once the upper first molar rotation is corrected (Fig. 1) (49).

IE are applied from upper canine or upper first premolar to mandibular first molar. Mandibular anchorage may consist in removable Essix-type clear retainer (modified to accommodate bonded buccal tubes or hooks), lower lingual arch, temporary anchorage devices, and FMA (54).

Class II correction with the CMA appliance typically requires 4-5 months to be completed. (54, 55) After dental Class I is achieved, treatment can continue with FMA or with aligners.

Limited investigations on treatment effects produced by the CMA are available. Kim-Berman et al. examined the skeletal and dentoalveolar treatment effects produced in Class II patients by the CMA followed by FMA stating that correction was due mainly to dentoalveolar movements (55).

Areepong et al. evaluated three-dimensional treatment changes produced by the CMA in adolescent patients with Class I and Class II skeletal relationships and they found that Class II correction was due to distal tipping and rotational movements (56). Yin et al. compared the treatment effects of CMA, IE and a Forsus appliance. They declared that CMA did not cause any skeletal effects and that it was not more effective than other Class II treatment methods (57).

Given the increasing popularity of the CMA, the aim of this study was to evaluate the skeletal and dentoalveolar treatment effects of this appliance in patients with a skeletal and dental Class II malocclusion. A group treated with CMA followed by a FMA was compared to another one treated with Class II elastics associated with FMA.

MATERIALS AND METHODS

Subjects

Treatment records of 30 Caucasian patients were retrospectively selected from the archive of Postgraduate School of Orthodontics in Ferrara. They were divided into two groups composed by 15 patients each. The study was approved by the Postgraduate School of Orthodontics Ethics Committee (number FUPSO2222020). The first group (G1=15, 6 males a 9 females, mean age 14,46 ± 1,24 years) was treated with CMA followed by self-ligating brackets Damon 3MX (Ormco, USA) (Fig. 1). The second group (G2=15, 7 males and 8 females, mean age 14 ± 1,96 years) was treated with IE and the same brackets as Group 1 (Fig. 2).

The inclusion criteria were 1) late mixed dentition or permanent dentition; 2) Skeletal class II due to mandibular retrusion; 3) Bilateral Edge-to-Edge or Full Class II division 1 malocclusion patients; (4) no previous

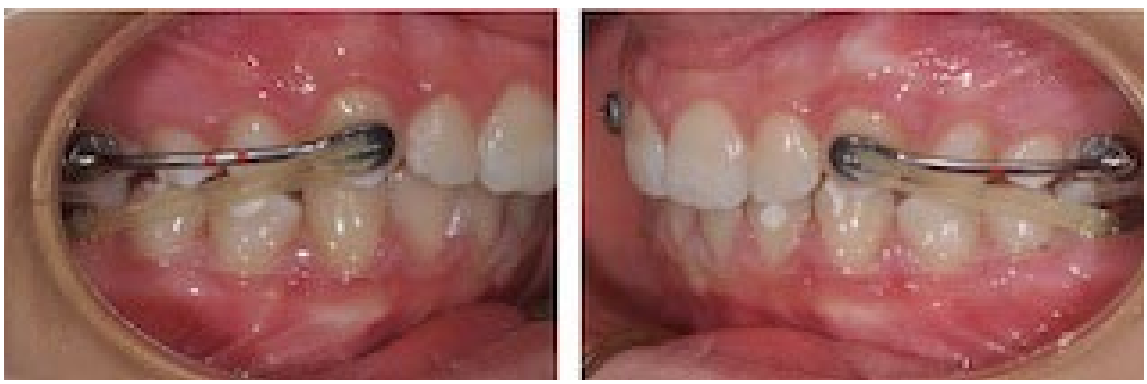

Fig. 1. *Carriere Motion® 3D™ appliance*

orthodontic treatment; (5) complete pre- and post-treatment records; (6) non-extraction treatment plan; and (7) no dental anomalies.

Data Collection

Each patients' pre-treatment and post-treatment lateral cephalograms were collected. Cephalometric analysis was made using Dolphin Imaging (Chatsworth, Calif) software. The following parameters were taken into consideration: U1-PP, IMPA, SNA, SNB, ANB, SN-GoGn, SN-Po, U6-Ptv (Table I) (Fig. 3).

Each patients' digital models were analyzed in .stl format using VAM software (Vectra, Canfield Scientific, Fairfield, NJ, USA). This allowed the identification of anatomical reference points, occlusal planes and axes on the digital models, required for calculation of the angulation, inclination and buccal prominence of each tooth. (58) Measurements were based on a method originally involving the identification of a total of 60 reference points per model (excluding second molars). However, in this case, second molars were also included in the study, expanding the number of reference points to 100 per model. (Fig. 4).

Once the 100 reference points had been marked,

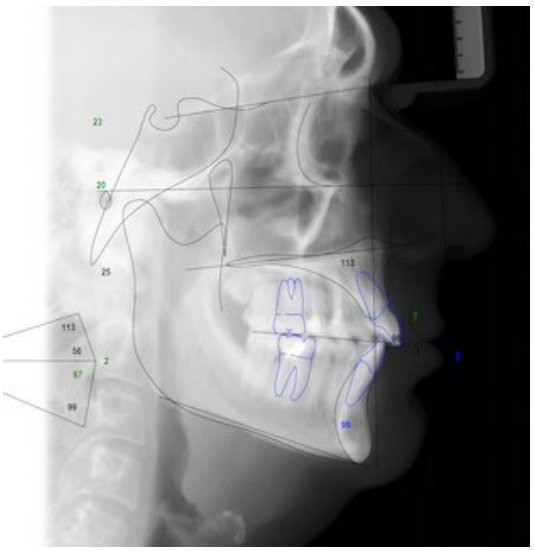

Fig. 3. *Cephalometric tracing*

Table I. *Cephalometric parameters*

Parameters	Definition
U1-PP °	Angle between the upper incisor axis and the palatal plane (Anterior Nasal Spine-Posterior Nasal Spine)
IMPA°	Angle between the lower incisor axis and the mandibular plane
SNA°	Angle between Sella Nasion line and Nasion-point A line
SNB°	Angle between Sella Nasion Line and Nasion-poin B line
ANB°	Difference between SNA and SNB angles
SN-Po°	Angle between SN and pogonion.
SN-GoGn°	Angle between the SN line and the Gonion-Gnathion line.
U6 to PTV Plane,mm	Distance in mm between the upper molar and a plane passing through the Pterygomaxillary point of the pterygomaxillary fissure.

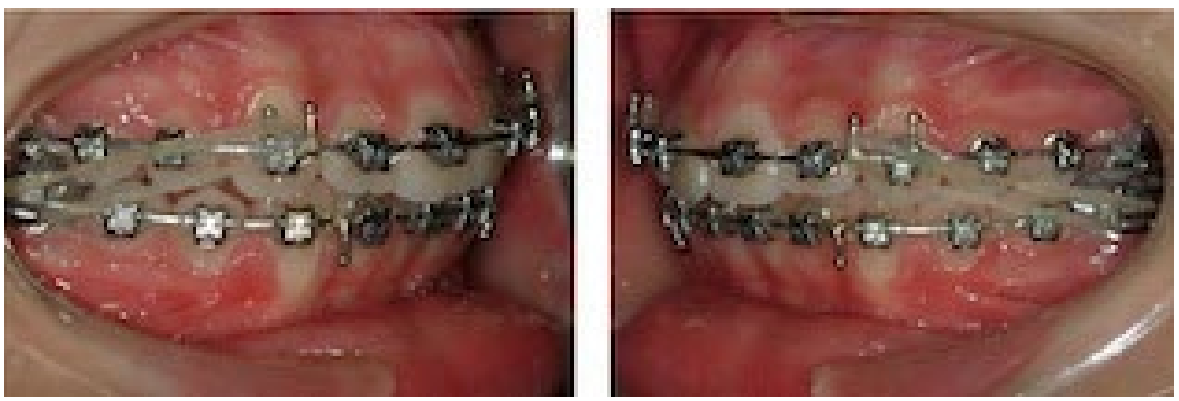

Fig. 2. *Class II elastics with fixed multibracket appliance*

their three-dimensional coordinates were extrapolated and exported, first into a .txt file, and then in a dedicated spreadsheet provided with the software. This spreadsheet enabled extrapolation of the first (in/out), second (tip) and third (torque) order values for each tooth with respect to a 3D Cartesian grid based on the occlusal reference plane, which was obtained by means of the following points:

Reference points at the mesiobuccal cusps of teeth 16 in the maxilla and 46 in the mandible

Reference points at the mesiobuccal cusps of teeth 26 in the maxilla and 36 in the mandible

The centroid of all occlusal points of the FACC (the facial axis of the clinical crown) of all remaining maxillary and mandibular teeth; canines were excluded from this calculation as their occlusal FACC point is generally outside the occlusal plane identified by the other teeth (58).

Six weeks after the analysis was made, the same operator repeated the analysis on 11 randomly selected patients in order to evaluate the method repeatability by calculating their absolute mean error and generalized estimating equation (GEE) and linear regression.

The study could not be blind because both the doctor and the patient knew which type of therapy they were receiving. However, the operator who developed the analysis of data was blind.

The treatment was considered to be completed when Andrew's six keys of occlusion were obtained (59).

Data obtained for Group 1 and Group 2 where then compared with each other concerning first, second and third order values and cephalometric measurements.

Statistical Analysis

Data collection procedures were carried out by a single researcher. All data were tested for normal distribution using the Shapiro-Wilk test by another researcher. Also, variance equality was assessed using the F test. After considering the two tests, the Student's t test was utilized to make the comparisons between groups: Aggregati, Mann-Whitney or Satterthwaite adjustment of the Student's t test was employed based on the acceptance of normal distribution and variance equity. The statistical significance threshold was set at $p<0.05$ (**). A $p<0.1$ (*) was not considered, a $p<0.01$ (***) was considered highly significant.

All statistical computations were made using the RStudio® Shiny® software. The sample size analysis showed that the two groups, composed by 15 members each, had an alpha value of 0.05 and a power of 0.8.

RESULTS

The mean absolute error for first, second and third order values is shown in Table II. The GEE linear regression for first, second and third order values is reported in Table III.

Second maxillary molars and second mandibular molars were analyzed following the complete procedure of analysis through the VAM software but their values were removed from the results because in most cases they weren't fully erupted in initial dental casts.

The treatment required 1,64 ±0,4 years to be completed in G1 and 1,96±0,6 years in G2. Post-treatment first, second and third order values are presented in Table IV.

Comparison between groups showed statistically significant (SS) differences in U1(***), L3 (***), L6 (***). Comparison between groups showed no SS differences, except for L4 (**). Comparison between groups showed SS differences for U3(**), U4 (***), L4 (***), L5 (***).

Table II. *Mean absolute error for first, second and third values*

Measure	Mean absolute error	STD
In/out	0.2207	0.6313
Tip	2.0421	7.5777
Torque	1.7686	19.6687

Fig. 4. *Identification of a total of 100 reference points per model using VAM software*

Cephalometric values

Pre- and post-treatment cephalometric measurements are presented in Tables V and VI.

No SS differences were found in the variations of U1-PP, SNA and SNB. There were SS differences in ΔANB in the comparisons between G1 and G2 (**). The same SS differences were found in the comparisons of ΔSN-Po between G1 and G2 (**). No SS differences in terms of ΔSN-Gogn and ΔU6-ptv were found. The ΔIMPA difference between the two groups was not SS but it was increased in both samples.

DISCUSSION

Both CMA followed by FMA and IE associated with FMA were effective for class II correction but in Group 1 the treatment time was lower with respect to Group 2 one, probably due to the higher cooperation of the patient at the beginning of the orthodontic treatment in G1. (49)

Similar Class II treatment duration was found by Kim-Berman et al who reported a total treatment time with CMA followed by a FMA of 18.2 months (± 4.8 months). (55) Yin et al reported different treatment time in class II cases treated with CMA followed by FMA and class II elastics. (57) In order to compare first-, second-, and third-order values we decided to use a digital method of measurement, because of its greater precision and low mean error reported by the literature. (58) At first we calculated the mean absolute error and the generalized estimating equation (GEE) linear regression and had positive results on the method's validity.

A key factor in making a precise analysis was the definition of the occlusal plane, stable and unchanged for all second- and third- order measurements.

For what concerns in/out values, the comparison between groups showed few statistically significant differences but they are so small that are clinically not significant.

Concerning Tip values, the comparison between groups showed no significant differences. A previous

Table III. *GEE linear regression for first, second and third values*

Analysis Of GEE Parameter Estimates							
Empirical Standard Error Estimates							
	Parameter	Estimate	Standard	95% Confidence Limits		Z	Pr > |Z|
In/Out	Intercept	0.0185	0.0761	0.0059	0.0311	0.2436	0.8072
	II_measure	0.9696	0.0464	0.9616	0.9771	20.8768	0
Tip	Intercept	-0.2655	0.2612	-0.2223	-0.3088	-1.0166	0.3092
	II measure	0.9509	0.0328	0.9455	0.9563	28.9979	0
Torque	Intercept	0.2049	0.2574	0.2475	0.1623	0.7959	0.4261
	II measure	1.0009	0.0136	0.9986	1.0032	73.501	0

Table IV. *Descriptive statistics and statistical comparison of in-out, tip and torque values.*

	IN-OUT						TIP						TORQUE					
	G1		G2		G1 vs G2		G1		G2		G1 vs G2		G1		G2		G1 vs G2	
Variables	Mean	SD	Mean	SD	Diff	P	Mean	SD	Mean	SD	Diff	P	Mean	SD	Mean	SD	Diff	P
U1	0,90	0,36	0,56	0,27	0,34	0,006827	5,11	3,55	6,64	3,14	-1,53	0,2218	4,97	3,26	6,28	4,84	-1,31	0,3921
U2	0,85	0,25	0,7	0,36	0,15	0,2032	9,39	5,51	10,02	2,83	-0,63	0,6978	0,5	4,03	3,27	4,91	-2,77	0,1034
U3	1,71	0,27	1,5	0,41	0,21	0,0912	5,62	4,43	8,19	4,57	-2,57	0,1293	-7,4	5,46	-3,48	4,91	-3,92	0,04342
U4	1,56	0,16	1,54	0,3	0,02	0,2101	1,31	6,93	4,32	3,69	-3,01	0,1531	-12,7	5,08	-6,84	4,31	-5,86	0,003938
U5	1,49	0,23	1,43	0,29	0,06	0,2271	2,27	4,39	6,15	4,99	-3,88	0,05119	-14,6	6,46	-11,1	5,84	-3,50	0,1332
U6	1,20	0,33	1,06	0,62	0,14	0,06424	-1,57	4,63	1,76	7,05	-3,33	0,1375	-20,54	10,93	-19,17	4,77	-1,37	0,7715
L1	0,66	0,37	0,44	0,26	0,22	0,07124	0,57	1,73	0,42	2,39	0,15	0,8491	2,95	6,87	4,94	5,77	-1,99	0,397
L2	0,73	0,26	0,6	0,3	0,13	0,2418	0,91	4,02	1,51	3,36	-0,60	0,6593	-0,88	5,67	2,66	5,04	-3,54	0,08073
L3	1,88	0,32	1,55	0,3	0,33	0,008616	2,34	5,21	4,22	4,77	-1,88	0,5897	-9,21	5,5	-5,91	5,56	-3,30	0,1137
L4	2,11	0,27	2,05	0,37	0,06	0,582	1,92	3,39	4,58	3,43	-2,66	0,04169	-21,31	5,37	-13,88	6,82	-7,43	0,002567
L5	2,01	0,22	2	0,34	0,01	0,9262	6,53	4,82	5,22	4,34	1,31	0,4427	-26,63	5,21	-19,71	6,48	-6,92	0,009875
L6	2,38	0,42	1,95	0,34	0,43	0,005075	2,93	6,23	3,95	4,73	-1,02	0,6187	-40,02	10,53	-35,4	7,62	-4,62	0,2132

SD: standard deviation; Diff: difference; P: P-value

study evaluated the effects of the appliance on the dentition by means of 3D CBCT and found that both the upper canine and upper first molar moved with a distal tipping of the crown and a distal rotation of the same. (56) So it is possible that in the sample analyzed in this study the effects were the same if we compare our results with Andrew's ideal tip of upper canines and upper first molar. (60)

Taylor et al. studied the U6 inclination of a Pendulum treatment followed by FMA: our study's results can be comparable with a Pendulum treatment, whereas Taylor et al. found a U6 distal inclination of 2,6° ± 5,8° (61).

The difference in third order values found in the comparison between groups are SS but they are so small that they have no clinical relevance.

CMA and other auxiliaries for Class II correction could be used in combination with aligner therapy; (62-80) however, the selected group for this study the different groups needed to change only one variable and maintain the same type of appliance.

Different appliances express different types of biomechanics and therefore the side effects could be different; in fact, lingual appliance can obtain a better control of the lower incisor and a slightly different arch shape. (81-107)

In cephalometric analysis, IMPA values did not show significant differences. However, a slight proclination of lower incisors was observed. The use of Class II elastics still determines side effects in the lower arch, regardless the anchorage method used. Other authors have reported an increase of the proclination of the lower incisor using the CMA. (54–56)

No statistically significant differences in the variations of U1-PP, SNA and SNB were found. The variations of SNA (-1,06°) and SNB (0,14°) in G1 are in line with Kim-Berman et al. (55) This means that the skeletal correction induced by the CMA in growing patients is not clinically significant, as Yin et al. already reported in 2019. (57) According to these data Class II correction is not obtained by means of skeletal effects.

G1 showed a decrease of vertical dimension (-0,58°), in line with what was found by Yin et al. (-1,6°). (57)

There are no differences in terms of U6-Pvt therefore no bodily distalization of the first upper molar in none of the study groups happened.

Both CMA followed by FMA and IE associated with FMA showed to be efficient ways to correct class II malocclusion.

CMA showed a predominance of dentoalveolar effects. None of the groups showed clinically relevant changes in cephalometric values at the end of treatment. Treatment time of Group 1was shorter because of the higher cooperation of the patients at the beginning of the treatment.

Dental correction of Class II relationship is achieved by a combination of maxillary and mandibular dentoalveolar effects. No bodily distalization of the upper first molar was observed in either of the groups.

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Comparative SEM analysis of metal ions release of four types of brackets before and after aging: an *in vitro* study

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BACKGROUND: The aim of the study was to evaluate the metal ion release from new brackets of different origins, manufacture and composition and the metal ion release of used brackets after at least 12 months of treatment. At the same time, corrosion tests were performed to assess whether the difference in geographical origin and the marketing price are correlated to a better quality of the product. METHODS: Four different brackets were selected: Damon MX3 (Ormco, California), Victory SS (Monrovia, California), Equilibrium 2 (Dentaurum, Germany) and Hangzhou ORJ (Hangzhou ORJ Medical Instrument & Material Co.,Ltd. Sandun-China). Thirty brackets for each brand were randomly selected from new packs. Brackets from the oral cavity were sterilised at 134° for 35 min. An artificial saliva was prepared according to the Meyer-Fusayama recipe at three different pH 2.5/4.5/7.0. The brackets, divided into the three different pH were immersed in artificial saliva at 37° for 28 days. In addition, potentiodynamic electrochemical tests were carried out to analyse the behaviour of the brackets when forced to corrode. RESULTS: Ion releases are higher at pH 2.5, being critical nickel and copper releases from the Hangzhou ORJ. In pH 4.5 the Hangzhou ORJ bracket shows significant nickel and copper release, far from the guard levels. At pH 7 Hangzhou ORJ bracket shows some nickel release while other attachments are inert. CONCLUSION: the brackets on the market are safe and comply with the recommended safety standards. If the patient is suspected of being sensitive to nickel or chromium, it is prudent not to use materials with critical releases of these elements.

In the field of dentistry, the race for technology to develop new materials with ever better characteristics and potential must consider the safety of these materials. Brackets, bands and archwire are among the most used instruments in fixed orthodontics and are made of metal alloys containing nickel, cobalt and chromium in different percentages (1-4). The same materials are also used in lingual fixed appliances, with different characteristics and manufacture (5-12). Stainless steel is one of the most used materials in orthodontics for the manufacture of orthodontic brackets. The types of steel used are numerous and chosen on the basis of their characteristics of workability and resistance in particular to corrosion (13-18). The oral cavity is a very aggressive environment by its nature as it begins the degradation-digestion of the food introduced. Saliva has a pH between 5.8 and 7.1 and contains sodium (240-920 mg/L), potassium (560-1640 mg/L), chlorides (525-1085 mg/L), bicarbonate (122- 793 mg/l). Obviously, the composition is very variable over time, both for physiological reasons and for the introduction of food and drinks of different nature, as well as for the presence of microorganisms of the oral cavity and bacterial plaque (19-20). Metals are particularly sensitive to pH and the presence of chlorides, as well as to thermal variations (21). The

Key words: metal ions, SEM analysis, orthodontic appliances, orthodontic brackets

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aggressiveness of the oral cavity causes corrosion of the metal alloys of the orthodontic devices. For example, implants or miniscrews are much less reactive to this process (22-29). Indeed, at pH 7, all the miniscrews are biocompatible while the eluates obtained at pH 4 showed significant cytotoxicity response (30-35).

The release of metal ions due to corrosion of orthodontic appliances can cause both local and systemic adverse effects (36). Some ions like Cr and Ni are potentially allergic and capable of inducing a late-phase, type IV hypersensitivity reaction, typified by symptoms such as gum growth, burning sensation in the mouth, metallic taste, angular cheilitis and labial desquamation in the oral cavity (37). It must be considered that many orthodontic patients have hypersensitivity to Ni. Because it's widely used in daily practice, (38-40) doctors should be prepared to handle a possible reaction (41). The aim of the study is to use alternative, but equally valid tests. In this research, the release of metal ions from new orthodontic brackets of different geographical origin, different manufacture, different composition and the release of the same metal ions from brackets that have been worn inside the oral cavity for at least 12 months are taken into consideration and compared. At the same time, corrosion tests are conducted to assess whether the difference in geographical origin and market price of the above-mentioned brackets correlates with a better quality of the proposed product in terms of the materials used and the manufacturing process. Different orthodontic brackets have been evaluated, both traditional and self-ligating brackets, as they are widely used by clinical practice (42-43).

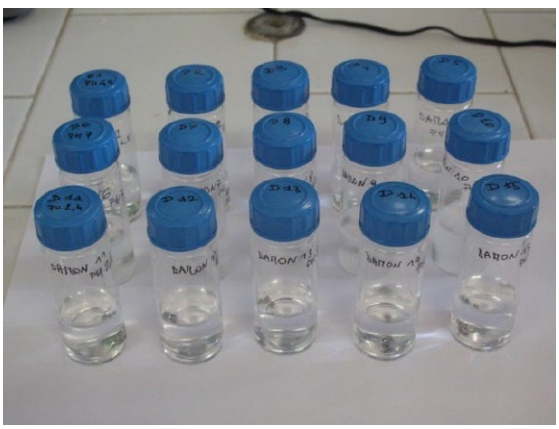

Fig. 1. *Brackets weighed with a precision balance and divided into three pH; for each pH were made 5 pairs immersed in plastic containers.*

MATERIALS AND METHODS

In the present study four types of brackets with different origins, manufacture and type of alloy were examined:

- Damon MX3 (Ormco USA, California). AISI 17-4 PH with the following composition in percentage by weight C 0,07/ 1 Mn/1 Si/ Cr 15,5-17,5/ Ni 3,0-5,0/ Cu 3,0-5,0/ Nb 0,15-0,45/ P 0,04/ S 0,03/ Fe);
- Victory SS, MBT prescription (3M Unitek, Monrovia, Calif.); AISI 17-4 PH with the following composition in percentage by weight: Cr 10%-20% / Ni ≤ 4%;
- Equilibrium 2, Roth prescription (Dentaurum, Germania) DIN 1.4435 (AISI 316L) PH with the following composition in percentage by weight: C ≤ 0,03 / Si ≤ 1,0 / Mn ≤ 2,0 / Cr 17,0-19,0 / Ni 12,5-15,0 / Mo 2,5-3,0 / P ≤ 0,045 / S ≤ 0,025 / N ≤ 0,11 / Fe.
- Hangzhou ORJ (Hangzhou ORJ Medical Instrument & Material Co.,Ltd. Sandun-China).

Thirty premolar and canine brackets for each brand were taken randomly from new packs. Brackets were purchased from normal distribution channels. Brackets from the oral cavity were considered as well, debonded after at least 12 months of use (average about 18 months) and placed in glass containers with distilled water. Deformed samples or affected by debonding procedures were discarded from the study. These brackets were cleaned of plaque residue with a rotary brush at very low speed and without abrasive paste and then bagged and steam sterilized at 134° for 35 min. This temperature is far from the temperatures that cause phase changes in the steel alloys of orthodontic brackets so the treatment should not affect the release of ions.

First, the brackets were examined under an electron microscope (Philips-ESEM-XL30), to highlight the macroscopic characteristics of manufacture and to identify points of vulnerability to corrosion factors. Spectra were performed in at least two locations to verify the stated composition of the metal alloy using a spectrograph connected to the SEM.

Then, the bases of all brackets, both new and used, were coated with bonding resin. This procedure was

performed because the bonding bases have a complex geometry and are generally sandblasted to increase their retention. This type of preparation greatly increases their susceptibility to corrosion, so we consider it essential to take this into account in in-vitro studies because the bonding plate is not exposed to the oral environment being covered by the adhesive and applied to the tooth surface. In our study, the attachments were resin-coated with Transbond XT (3M Unitek).

Artificial saliva was prepared according to the Meyer-Fusayama recipe: Kcl 0,4 g/l., NaCl 0,4 g/l., $CaCl_2 \cdot 2H_2O$ 0,906 g/l. , $NaH_2PO_4 \cdot 2H_2O$ 0,69 g/l., urea 1 g/l. This artificial saliva was prepared at three different pH:

- pH 2.5 was made to force corrosion and test the quality of the material by emphasizing the weak points;
- pH 4.5 is present in the oral cavity when certain foods are introduced but can also be present for long intervals of time when complex microenvironments with bacterial plaque are formed.
- Neutral pH should represent an ideal condition of inertness of the tested material.

Brackets were weighed with a precision balance then divided into three pH; for each pH were made 5 pairs immersed in plastic containers, to have more measurements (not sample dependent) with 100 ml of artificial saliva (Fig. 1). Plastic containers with the bracket were immersed in the saliva bath where a constant temperature was kept via an immersion heat source connected to a thermostat set at 37°C for 28 days (Fig. 2).

In addition, potentiodynamic electrochemical tests were done to analyze the behavior of brackets when forced to corrode. These tests were performed on both new and used samples: the brackets were connected with a copper electrical wire via resin (Transbond XT) to the potentiostat electrode (Potentiostat 273-Princeton Applied Research) and immersed in artificial saliva at pH 4.5. Two tests were performed per type of bracket and the graphs were superimposed to compare the different behaviors of the tested brackets.

The test is used to assess the corrosion resistance, especially pitting, of the device and evaluates the ability of the device to re-passivate when the polarization cycle is reversed.

Fig. 2. *The samples were immersed at 37 ° C for 30 days.*

RESULTS

Results of electron microscopy (SEM) analysis

The images obtained from the SEM analysis of new brackets highlight the complexity and microstructure of all analyzed brackets (Fig. 3).

On the other hand, the images obtained from the used brackets evaluation (Fig. 4) point out the areas of irregularity around the slot and the area of the soldering to the base which appears lighter in color. This color is due to the refraction of gold since the weld has been covered with a golden layer to protect it from corrosion. It can also be appreciated that the surface finish is not very homogeneous.

If the spectra carried out on the etching are realized, its inhomogeneity is realized in the specific case we intend superficial as they compare with regard to corrosion. By carefully evaluating them, surface components with different composition percentages of the alloy elements are detected. The base of the bracket has a different composition compared to the slot, for example.

Results of potentiodynamic electrochemical test

The later the corrosion starts on the graph, the more resistant the material will be. The faster the re-passivation occurs, the more resistant the material will be. In these graphs, the wording "European" stands for the Dentaurum attack (Germany), while the word "chinese" stands for the Hangzhou ORJ attack (China) for the sake of brevity.

The Victory and Equilibrium new brackets showed a higher potential to free corrosion than the others

(Fig.5). The lowest passivity current values were recorded for the Victory bracket characteristic that the material is "self-protecting"), the Equilibrium bracket also showed low values, even higher than the Victory. Pitting starts for both at 0.5 - 0.6V.

Both the Damon and the Hanghzou have free-corrosion values around 0, indicating a lower nobility of the material or of some parts of the finished part (e.g. weld or pin, as can be seen from the photos) and do not resist pitting, with pit corrosion starting in both cases as early as 50 mV.

From Fig. 6 it can be seen that the resistance test show less difference in behavior if used brackets are considered.

For all, the initial resistance to corrosive attack has decreased; the Victory bracket resists a little longer at the beginning and maintains passivation for longer before starting to corrode again. The Dentaurum (Eu) bracket resists the most before reaching the critical pitting point and then has a similar trend to the Victory. The Damon one behaves better when used than new. This can be explained by assuming that since the weld of the flap is already completely corroded, the weak points have practically exhausted their ability to corrode.

The Hangzhou Orj bracket worsens its situation even if it shows a slight repassivation capacity probably due to the fact that much of the possible corrosion has already occurred, the surface has already given up a significant amount of its available ions.

Results of immersion in artificial saliva

Measurements were obtained using the ICP-OES (Inductively coupled Plasma- Optical Emission Spectroscopy) method (Spectro Germany, Ciros). For each pair of couplings, three measurements were made from which the average was derived (Table I-II). There are five pairs in order to remove instrumental error from the measurements.

Measurements are in parts per million, but the sensitivity of the test is in parts per billion and the limit of detection is 10 to 50 ppb. The statistically significant measurement is to the second decimal place of the measurement.

The greatest ion releases were recorded at ph 2.5; the Hangzhou bracket showed the greatest releases, nickel and copper releases being critical, while chromium release is about half of the daily dietary intake.

Significant releases also occurred for the Damon bracket, especially for nickel and chromium, always at pH 2.5. pH 2.5 is present in the oral cavity in oxygen-

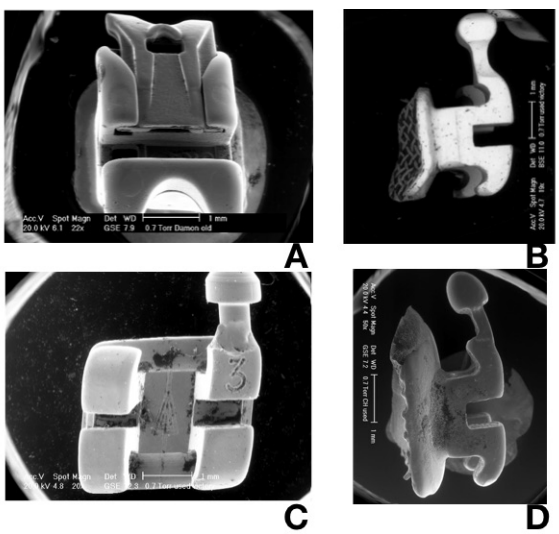

Fig. 4. *Frontal and lateral view of the used brackets: A) Damon; B) Victory; C) Equilibrium; D) Hangzhou.*

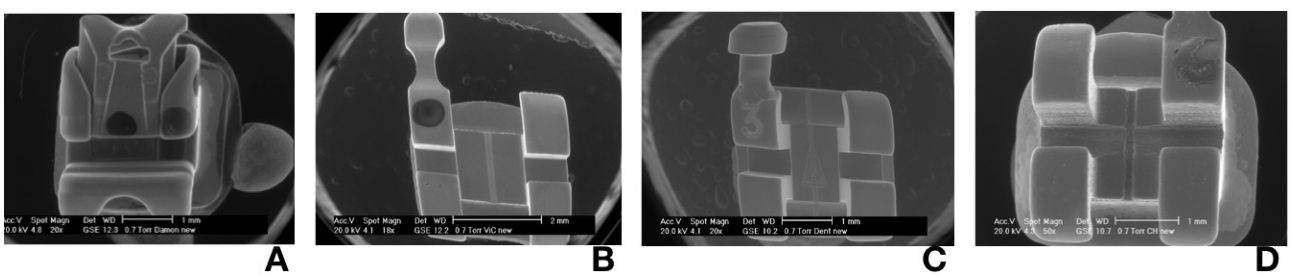

Fig. 3. *General frontal view of the new brackets: A) Damon; B) Victory; C) Equilibrium; D) Hangzhou.*

poor microenvironments such as under ligatures or under accumulations of fresh bacterial plaque. In pH 4.5 Hangzhou ORJ bracket shows significant nickel and copper release although far from the guard levels. The Damon bracket shows minimal releases, while the Victory and Dentaurum brackets release practically nothing. At pH 7 only Hangzhou ORJ bracket shows some nickel release while the other brackets are practically inert. Following, the results of used brackets are described in Table III.

DISCUSSION

The only orthodontic treatment that does not cause the release of metal ions in the oral cavity is the one which involve metal-free appliances, like clear

Table I. *ppm in 10 mL released per unit of mass (ppm / g)*

PH		Fe	Ni	Cu	Cr
2,5	**Ch**	37,647	866,652	14,046	11,156
	Eu	2,112	0,244	0,187	0,122
	V	1,007	0,078	-1,000	0,000
	D	253,092	148,185	0,035	21,271
4,5	**Ch**	0,086	31,750	1,000	0,127
	Eu	0,000	0,000	0,000	0,000
	V	0,000	0,000	0,000	2,000
	D	0,000	0,000	0,000	2,000
7	**Ch**	0,065	9,143	1,000	0,000
	Eu	0,000	0,000	0,000	0,000
	V	0,000	0,000	0,000	0,000
	D	0,000	0,000	0,000	2,000

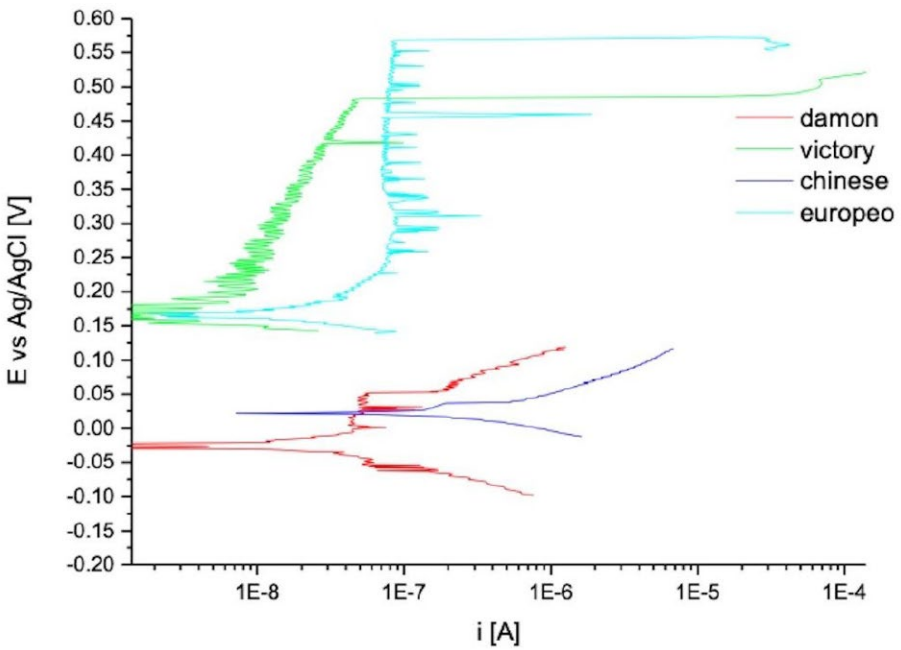

Fig. 5. *Dynamics potentia graphs performed on new brackets*

aligners (44-49) and some resin removable activators (50). It has been demonstrated that clear aligners can be as efficient as fixed appliances in correcting milt-to-moderate malocclusion (51-59). Miniscrews can also be safely used because less susceptible to metal ions release (60-62). This is the reason why there could be a great indication in using these other appliances, especially when hypersensitivity to Ni is diagnosed. The decision is the result of a diagnostic initial approach which involves numerous considerations about the patient's malocclusion (60-80). From this in-vitro study, two types of behavior were observed: Hangzhou Orj and Damon resealed more ions when new, otherwise Victory and Equilibrium released more metal ions after use.

As reported in previous study, this result can be explained by the fact that the greatest releases often occur in the first two months of treatment. Then, it stabilizes at a low level of release due to the phenomenon of ion depletion of the metal surface, to the effect of the salivary biofilm and solid deposits that shield the surface from corrosive attack (81-83). It can be assumed that the weld of the Chinese brackets has been corroded so much during treatment

Table II. *Total micrograms released by 1 pair (mg)*

PH		Fe	Ni	Cu	Cr
2,5	**Ch**	58,188	1337,200	21,698	17,234
	Eu	3,424	0,396	0,304	0,198
	V	1,392	0,108	0,038	0,000
	D	496,400	290,600	0,068	41,720
4,5	**Ch**	0,134	49,800	1,760	0,000
	Eu	0,000	0,000	0,000	0,000
	V	0,000	0,030	0,000	0,000
	D	0,000	0,188	0,000	0,000
7	**Ch**	0,100	14,240	0,164	0,000
	Eu	0,000	0,000	0,000	0,000
	V	0,000	0,000	0,000	0,000
	D	0,000	0,033	0,000	0,000

Table III. *Results from the immersion in artificial saliva performed for used samples.*

	Ppm	Cr 205.552	Cr 267.716	Cu 219.226	Cu 324.754	Fe 239.562	Fe 259.941	Ni 231.604	Ni 341.476
	2,3	0,034	0,032	0,019	0,029	0,409	0,398	0,045	0,045
	4,5	0,002	0,001	0,000	0,000	0,009	0,009	0,008	0,008
Eu	7	0,002	0,000	0,000	0,000	0,009	0,008	0,004	0,006
	2,3	1,452	1,412	0,168	0,388	>16.620	>16.620	13,340	12,692
	4,5	0,002	0,001	0,000	0,001	0,009	0,009	0,008	0,008
Dem	7	0,002	0,000	0,005	0,003	0,009	0,008	0,005	0,006
	2,3	0,078	0,077	0,023	0,026	0,189	0,187	0,088	0,087
	4,5	0,002	0,002	0,034	0,035	0,009	0,009	0,025	0,027
Vic	7	0,002	0,000	-0,002	0,002	0,009	0,009	0,006	0,007
	2,3	0,690	0,655	3,150	2,960	2,465	2,375	9,740	9,090
	4,5	0,019	0,017	0,359	0,328	0,759	0,747	7,763	7,353
Ch	7	0,003	0,001	0,207	0,201	0,060	0,059	0,110	0,107

that it has already released a lot of ions and reached an equilibrium. In this case, probability most of the surface ions have already been released and the biofilm is not sufficient to completely stop the corrosive process. For this type of brackets, in addition to the ions detected in this research, ions will also have been released from the weld material, silver and tin ions. This type of release is known to be cytotoxic.

The Damon brackets has a critical composition weld at the level of the flap, it is assumed that at the beginning it releases most of the surface ions at acid pH, but since the weld is very small, the release decreases at a later stage due to ion depletion. In fact, at ph 7 of the sample used, the increases in release are similar to those of the other brackets considered in the study. The Victory and Equilibrium 2 brackets increased their releases at acid ph 2.5, and releases also appeared at ph 4.5 and 7 which were absent when new. Although these releases are far from the critical thresholds, they show that releases are present at all pH levels, whereas it was assumed that they would be absent at pH 7. In other studies, the amount of metal ion release of steel, nickel-free and titanium alloys was determined to assess cell viability and DNA damage (84-85). Twenty brackets and four tubes were incubated in a cell culture medium for 30 days. Of the three alloys tested the stainless-steel alloy showed the most toxicity with high releases of titanium, chromium, manganese and nickel. The results showed that, as the metal concentrations increase the DNA toxicity is proportionally higher. Chantarawaratiti et al. (36) compared the concentration of metal ion release with daily dietary intake. In all cases, the Cr and Fe release was lower than that recommended by the diet (35 mg and 8 mg, respectively). However, the ions released could accumulate in the cells of the gingival fibroblasts and induce DNA damage and an in vitro experiment cannot represent the entire corrosion process that occurs in the oral environment. Future studies could investigate the same process when orthopedic fixed appliances, for example Rapid Palatal Expander are used in growing patients (86-98).

Metal ion release is lower with respect to what is reported by previous studies because the bases of

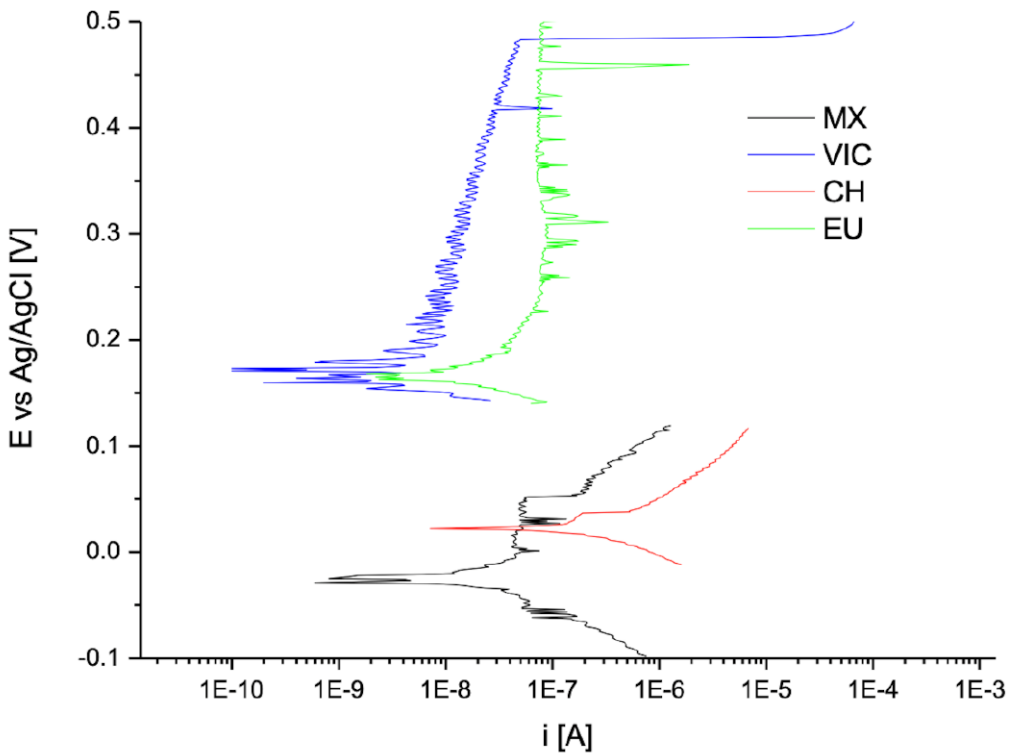

Fig. 6. *Dynamics potentia graphs performed on used brackets.*

brackets were resin-coated first.

It can be said that all brackets analyzed are safe and comply with the recommended safety standards, although the material best suited to the individual patient must be chosen clinically. If the patient is suspected of being sensitive to nickel or chromium, it is prudent not to use materials with critical releases of these elements.

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Dimensional stability of impression elastic materials: an in vitro study

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OBJECTIVE: The aim of this in-vitro study is to be able to provide useful information to implement adequate preservation of the impression before and during sending to the appropriate dental prosthetic laboratories, allowing good management of the materials used and the time spent in the chair.

MATERIALS AND METHODS: The study evaluated a total of 210 specimens, of which 168 alginate specimens and 42 silicone specimens, taking a total of 18,228 measurements. The dimensional stability was assessed by measuring linear dimensions and the weight at certain time points.

RESULTS: The effects within the sample group due to the time factor, the combination of time and alginate, the combination of time and environment are significantly important, both for Linear Dimension and for Weight. The effects of the factors between groups, "type of alginate" and "type of environment" are also significant. For silicones, the storage temperature and the type of mixing were not considered as factors that can influence their behavior. On the other hand, the combination of time and silicone is significant in the linear dimension, in fact the various types of silicone vary over time in different ways. Instead, the interaction between time and the type of conservation environment does not generate significant differences.

CONCLUSION: This first hypothesis has been refused, the data obtained show that the various brands of alginates on the market demonstrate different characteristics of stability as a function of time. The second hypothesis was confirmed by the data obtained, in fact the impressions made with silicones can be stored at room temperature in airtight containers even up to 30 days without excessive dimensional variations.

A dental impression material must be able to faithfully reproduce hard and soft tissues in their negative form, giving at the same time a stable result which don't risk to be deformed over time. This late aspect is important in order to guarantee the construction of precise dental artifacts or orthodontic appliances, with a good fitting in the oral cavity.

Especially in orthodontics, faithfulness and volume stability of impressions are very important.

For example, lingual orthodontic appliances are made by working set-up that are obtained after pouring of dental impression (1-15); due to fact that often specialized laboratories are far and dental impression could not be poured immediately, the stability over the time should be guaranteed (16-20).

The same considerations could be applied both for clear aligners therapy, in which the reproduction of the anatomy teeth should be as precise as possible to ensure optimal fitting (21) and therefore aesthetics of clear aligners, (22-24) good predictability of what

Key words: Impression elastics materials; dimensional stability; in-vitro study

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planned (25-30) and to plan miniscrew insertion where an optimal matching between digital models and CBCT must be ensured (da Lombardo 2010 a Maino 2020) (31-42). However, a good stability over time should be expected generally also to obtain good measurement of archform (43-45), diameters of arches (46,47) and for building some common orthodontic appliances like rapid maxillary expanders (48-53), gnathological appliances (54-62), indirect bonding procedures (63-69) or production of fixed retainers (70, 71).

Impression materials placed on the market must be conformed to * ISO-UNI and ANSI /ADA.

Based on their chemical and physical characteristics, impression materials are usually classified into:

1. Non-elastic or rigid impression plasters, zinc oxide paste - eugenol, thermoplastic pastes, impression waxes.
2. Elastic impression plasters: hydrocolloids (agar-agar, alginates) or elastomers (polysulphides, polyethers and polyvinylsiloxanes).
3. Duplication materials.

Considering the elastic and non-elastic materials, the substantial difference between the two categories is that the elastic ones can be temporarily deformed during the impression's extraction from patient's mouth, regaining the original shape.

Dimensional stability indicates the ability of an impression to maintain its shape and remain unchanged from a volumetric point of view after removal from the oral cavity, in normal environmental conditions, without progressive retraction phenomena over time.

If we consider Nicholls' definition of "*dimensional stability*" as "the ability (of a material) to maintain accuracy over time and "distortion" can be defined as a "relative movement of a single point or group of points with respect to initial reference positions such that the permanent deformation is evident. "

It is affected by the type of setting reaction, the coefficient of thermal expansion, which is provided for elastomeric materials in specification No. 19 of the ADA (in the order of 0.2%), and the volumes of the material used (72).

Generally irreversible hydrocolloids show a sharp contraction during the conversion phase from sol to insoluble gel. This shrinkage of the material occurs mainly towards the center of mass or in the mass of the material where there is the maximum thickness (73). For example, the palatal area on the imprint is reproduced smaller with respect to the corresponding area of oral cavity.

The nature of the composition of the alginates, the conditions and the storage time before pouring are the three fundamental parameters that influence the dimensional stability of the impression (74,75).

Furthermore, at a clinical level, when the impression material is firmly bonded to the tray, the contraction of the material occurs towards the tray, causing an increase in the diameter of the structures as they are subject to centrifugal forces (76). The materials undergo a contraction due to syneresis and water evaporation. The amount of contraction caused by syneresis is generally greater than that caused by evaporation.

The American Dental Association recommends that all dental impressions be disinfected and rendered harmless before passing them on to other people who will have to work on them. Before 1991, the only recommended procedure was to rinse the fingerprints under running water.

This simple operation already reduces the bacteria present on the surface of the impression of about 90% (77). Later, however, guidelines were introduced that precisely provide for washing, disinfection and rinsing of impressions.

This in vitro study aims to evaluate the dimensional stability over time of the elastic impression materials most commonly used in orthodontic clinical practice, analyzing some variables (mixing techniques of the material, environmental factors, time factor) which, referring to data of literature, appear to influence their behavior. The aim of this work is to be able to provide useful information to implement adequate preservation of the impression before and during sending to the appropriate dental prosthetic laboratories, allowing good management of the materials used and the time spent in the chair.

MATERIALS AND METHODS

The vitro study proposed has the purpose of directly

evaluate the dimensional stability of elastic impression materials stored under different environmental conditions, with interest for the impression materials used in the orthodontic field.

- Irreversible hydrocolloids: variables of the type of alginate, the mixing technique, the storage times, the storage temperature, the use of spray disinfectant were considered.
- Synthetic elastomers: variables of the type of silicone, storage times, use of spray disinfectant were considered.

The study evaluated a total of 210 specimens, of which 168 alginate specimens and 42 silicone specimens, taking a total of 18,228 measurements (17,136 linear measurements and 1,092 weight measurements).

In our experimentation, specimen 1BA of the ISO 527-2: 1997 standard was used (Fig. 1A, 1B).

The ISO 527-2: 1997 standard which contemplates the use of the type of specimen identified by us, would suggest the use of handlebar-shaped specimens of type 1A or 1B shaped with a straight axis.

We investigated 3 parameters:

- b2 = 10 ± 0.5mm width of the ends
- b1 = 5 ± 0.5 mm width of the narrow part
- h ≥ 2 mm (thickness)

The thickness h was measured at the center of the ends of each sample. These values were used as a reference to determine the sample sizes.

The dimensional stability was assessed by measuring the three linear dimensions mentioned above and the weight at certain time points. Regarding irreversible water-based hydrocolloids, as well as synthetic elastomers, 5 times were considered.

T0: initial moment, after the sample has been taken as an imprint of the specimen, rinsed in distilled water at room temperature and eventually subjected to disinfection. The measurements taken at T0 were considered as reference for evaluating the dimensional variation over time.

- T1: detection at 1 hour.
- T2: 24-hour survey.
- T3: survey at 120 hours - 5 days.
- T4: survey at 168 hours - 7 days.

In addition to the 1-hour and 24-hour measurements, which are the time intervals generally evaluated in the protocols for the preservation of alginates, the 120-hour measurements have been introduced (to evaluate long-life alginates with long dimensional stability), and the measurements at 168 hours (as a limit comparison period with respect to the new materials defined as substitutes for alginates and with respect to silicones).

Linear measurements of each sample were carried out by a single operator (GG) using a mechanical contact measurement method by means

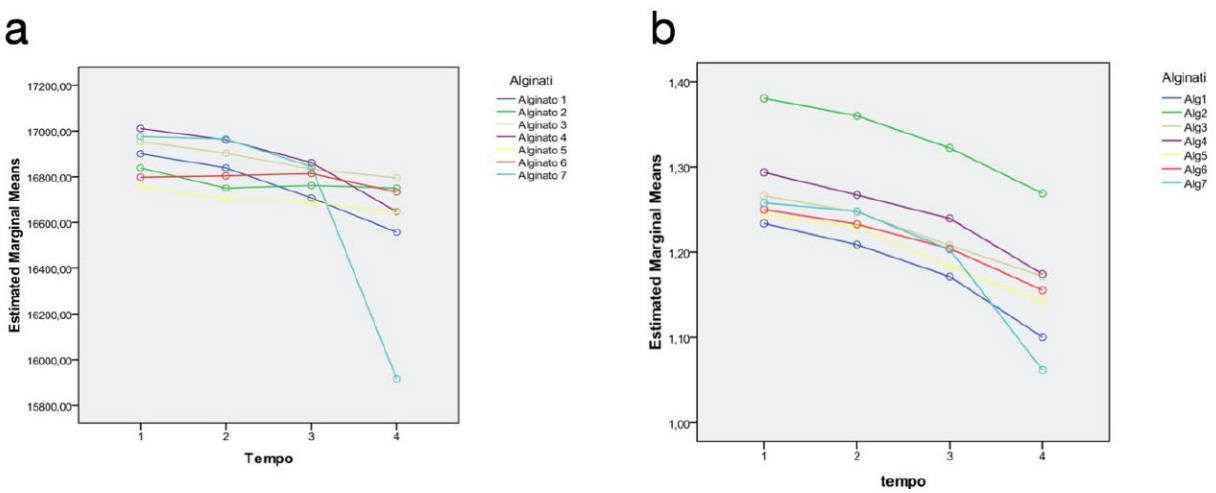

Fig. 1. *Comparisons between the different Alginates for the variation of linear size (A) and weight (B) over time.*

of a micrometer or digital centesimal palmer (11) for outdoor whose measurement range is 0 - 25 mm.

In order to evaluate the reproducibility of the measurement method of linear dimensions used in the study, the measurements of some samples by an external observer were repeated in a random manner. The comparison between the average values of the measurements made by the two operators for the 3 dimensions taken into consideration was not statistically significant, confirming the reproducibility of the measurement method (78).

Alginates

The null hypotheses underlying the research for alginates were that it does not exist difference in dimensional stability over time between the best-known irreversible hydrocolloids from imprints on the market under different storage conditions, regardless of the mixing technique used and that the use of the spray disinfectant produces effects similar on dimensional stability in various types of materials. 168 impressions of the ISO UNI EN ISO 527-2: 1997 specimen were produced, for each of the 7 alginates evaluated, 24 samples were made (Table I).

The impressions were divided into 4 groups in based on mixing technique and storage temperature, and in two more groups depending on whether disinfection was applied. 84 specimens were produced by manual mixing and another 84 by mixing mechanics. The samples thus obtained were all stored in a humid environment relative to 100%, half of each group was stored at temperature environment (25 ° C ± 2 ° C) and half at a temperature of 3 ° C (± 2 ° C), half of the disinfection and the other half was not disinfected. The protocol for the preparation of the specimens takes up aspects of protocols already implemented in literature (79).

The choice to store the specimens in airtight sachets came from a study (80) in which the sample stored in airtight bags was more stable than that stored directly in the refrigerator or at room temperature, this was due to the less volume of air present in the hermetic sachets, so there was a saturation shorter and faster. Furthermore, the presence of 100% humidity is the best condition conservation indicated in other studies.

Silicones

The null hypothesis regarding silicone materials assumes that silicones have one better long-term dimensional stability than hydrocolloid based materials aqueous, that the use of the spray disinfectant does not alter its dimensional stability. 42 impressions of the ISO UNI EN ISO 527-2: 1997 specimen were produced, for each of the 7 silicones evaluated, 6

Table I. *Water-based irreversible hydrocolloids and silicones tested*

Water based alginate	**Productor**	**Setting time**
Type I: Hidrogum 5	Zhermack Spa	Extra fast 1’50’’
Type II: Algeniux fast	Major Spa	Fast set 2’30’’
Type III: Orthoprint	Zhermack Spa	Extra fast 1’50’’
Type IV: Alginkid	Major Spa	Extra fast 1’50’’
Type VII: Cavex Orthotrace	Cavex Leone	Fast set 2’30’’
Type V: Alginiplus Tropical	Major Spa	Fast set 2’00’’
Type VI: Neocolloid	Zhermack Spa	Normal set 3’30’’
Silicones	**Productor**	**Setting time**
Type IV: Elite HD+ putty	Zhermack Spa	Normal set
Type III: Elite HD+ light	Zhermack Spa	Fast set
Type V: Hidrorise putty	Zhermack Spa	Normal set
Type I: Hidrorise light	Zhermack Spa	Fast set
Type II: Freealgin	Zhermack Spa	Fast set
Type VI: Orthogum	Zhermack Spa	Fast set
Type VII: Occlufast rock	Zhermack Spa	Fast set

samples were made (Table I).

The protocol for the preparation of the silicone specimens provided for the mixing of material according to the technique of each type of silicone. Once the data collection procedures were completed, the sample was stored according to parameters of the "disinfected / non-disinfected group" to which it belongs.

Statistical analysis

On the measurement of the Linear Dimension (Linear measurements B2, B1 and S were added together to obtain a single linear dimension whose variation over time was evaluated) and the Weight, a General Linear Model - Repeated Measures was carried out and used to analyze the samples.

For what concerned alginates, the effects of the following variables were evaluated:

- "Type of alginate";
- "Type of Environment";
- "Type of Mixing" over time (from T0 to T3).
- For what concerned silicones, the effects of the following variables were evaluated:
- "Type of silicone";
- "Type of Environment" over time (from T0 to T5).

The data provided was in no way related to the commercial name of the product. Comparisons were made with Sidak's post-hoc test. All comparisons with a $p < 0.05$ were considered statistically significant therefore indicative of variations or modifications. SPSS Software ver.17 was used for all analyzes.

RESULTS

The effects within the sample group due to the time factor, the combination of time and alginate, the combination of time and environment are significantly important, both for Linear Dimension and for Weight. The effects of the factors between groups, "type of alginate" and "type of environment" are also significant. The "type of mixing" does not appear to have significant effects on the Linear Dimension variations at the different time points, while about the Weight variations it is a significant factor.

Alginates

For the linear dimension and for the weight, the Pairwise Comparisons test carried out on the 4 time points underwent significant variations. To have an exact evaluation of the variations undergone by the materials in the time intervals from T0 to T2 and from T0 to T3, the percentage differences with respect to the initial value were calculated, both for the linear dimension and for the weight.

After 24 hours (T2):

- The alginate that has undergone the least variation in size linear is type VI alginate (positive change

Table II. *Dimensional variation (%) of alginate samples at 24h days and at 5 days*

Silicones	Maximum linear dimensional change after 24 hours (%)	Maximum linear dimensional change after 5 days (%)	Maximum weight change after 24h (%)	Maximum weight change after 5 days (%)
Type IV: Elite HD+ putty	- 0.07	- 0.01	- 0.03	- 0.02
Type III: Elite HD+ light	+ 0.20	+ 0.19	- 1.63	- 1.63
Type V: Hidrorise putty	- 1.17	- 0.90	- 1.25	- 1.54
Type I: Hidrorise light	+ 0.17	+ 0.51	- 0.44	- 0.27
Type II: Freealgin	- 0.18	- 0.71	- 0.11	- 0.33
Type VI: Orthogum	- 0.18	- 0.00	- 1.03	- 0.60
Type VII: occlufast rock	+ 0.13	+ 0.12	- 0.73	- 0.82

- expansion) followed by type II (negative change - contraction) and type V (negative change - contraction);
- For weight, the smallest variation was found in Alginate type VI followed by type IV and type II (all negative variations - weight loss).
- After 5 days (T3):
- The linear dimension that has undergone the least negative variation is that of type II alginate, followed by type VI and type V;
- For Weight, the smallest variation was found in alginate type III, followed by type VI and type II together with type V.

The most stable alginates appear to be VI, II and V. The profiles of the seven Alginates in relation to the variation undergone over time by the Linear Dimension and the Weight, show a certain stability up to time T2 and a more consistent variation from time T2 to time T3, for alginate VII (Fig. 1A, 1B).

Comparing the behavior over time between the alginates analyzed (Post Hoc Test), some statistically significant differences are found both for the linear dimension and for the weight (only the significant ones are reported) (Table II).

Silicones

For silicones, the storage temperature and the type of mixing were not considered as factors that can influence their behavior. On the other hand, the combination of time and silicone is significant in the linear dimension, in fact the various types of silicone vary over time in different ways. Instead, the interaction between time and the type of conservation environment does not generate significant differences.

As for the Weight, the variations over time of the different types of silicone are not different. The interaction between time and the type of storage environment is significant, but the significance could be due to the different weight of the two groups ("non-disinfected environment" group and "disinfected environment" group) rather than to different variations over time.

- The percentage variations in Linear Dimension and Weight between T0-T3 and between T0-T5 for each silicone show that after 5 days (T3):
- Silicone that has undergone the least variation of the linear dimension is 4 (negative change - contraction) followed by 7 (positive change - dilation) and from 1.

Table III. *Dimensional variation (%) of silicone samples at 5 days and 30 days*

Water-based alginate	Maximum linear dimensional change after 5 days (%)	Maximum linear dimensional change after 30 days (%)	Maximum weight change after 24h (%)	Maximum weight change after 5 days (%)
Type I: Hidrogum 5	- 1.15	- 2.03	- 5.11	- 10.92
Type II: Algeniux fast	- 0.21	- 0.29	- 4.28	- 8.20
Type III: Orthoprint	- 0.68	- 0.91	- 4.55	- 7.45
Type IV: Alginkid	- 0.87	- 2.13	- 4.15	- 9.14
Type VII: Cavex Orthotrace	- 0.78	- 6.27	- 4.43	- 15.74
Type V: Alginiplus Tropical	- 0.40	- 0.38	- 3.70	- 7.58
Type VI: Neocolloid	+ 0.09	- 0.67	- 4.87	- 8.20

- About the Weight, the smallest variation was found in silicone 4 followed by 2, followed by 1.
- After 30 days (T5):
- The linear dimension that has undergone the least variation is that of silicone 6 followed by 4, followed by 7.
- Regarding Weight, the least variation is found in silicone 4, followed by 1, followed by 2.

The profiles of the seven Silicones for the linear dimension show a relative stability up to time T3 and important variations in the following times; while for the Weight there is a stability common to all types. It could be concluded that for the linear dimension silicone 4 is the most stable over time, but analyzing the results (Fig. 2A, 2B) we find a linear dimensional variation of the same at time T4 (7 days) and then a return almost to the initial size. For the weight, on the other hand, it remains stable always. Figures above show considerable weight stability over time for all silicones, but different initial weights of the materials. As for the linear dimension, the significance resulting in the "Post Hoc Test" among the different silicones mainly concern silicone 4, which differs from almost all the others. (Table III).

DISCUSSION

Dimensional stability of impression elastic materials is a key point in contemporary dentistry since the accuracy and efficacy of some orthodontic appliances like fixed appliances (81-84) or the precision of connection between fixture and crown in implant prosthesis depends on the precision of the impression (85-89). Due to the profound differences in protocol between this study and what is present in the literature, it is very difficult to make an especially quantitative comparison between the results obtained in the research presented here and the other studies. We will therefore limit ourselves to a comparison of the results in terms of the behavior of the tested materials.

About the irreversible hydrocolloids analyzed in the study in question, the results of the statistical analysis carried out show how the dimensional variation, both linear and weight, occurs over time in a different way for each brand of alginate, and alginates assume a different behavior at the various time points and in the various environmental combinations (temperature / disinfectant).

The mixing technique, manual or mechanical, on the other hand, does not influence the variation of the linear dimension but only that of the weight; the weight of the samples changes differently in the seven types of alginate based on the mixing technique used.

The analysis of the variation undergone in the different storage conditions seems to highlight a certain stability of the materials at the temperature of 3 ° C in no-disinfected environment, both for Linear Dimension and for Weight. These results agree with Roydhouse et al. (90) and in line with those of Dahl et

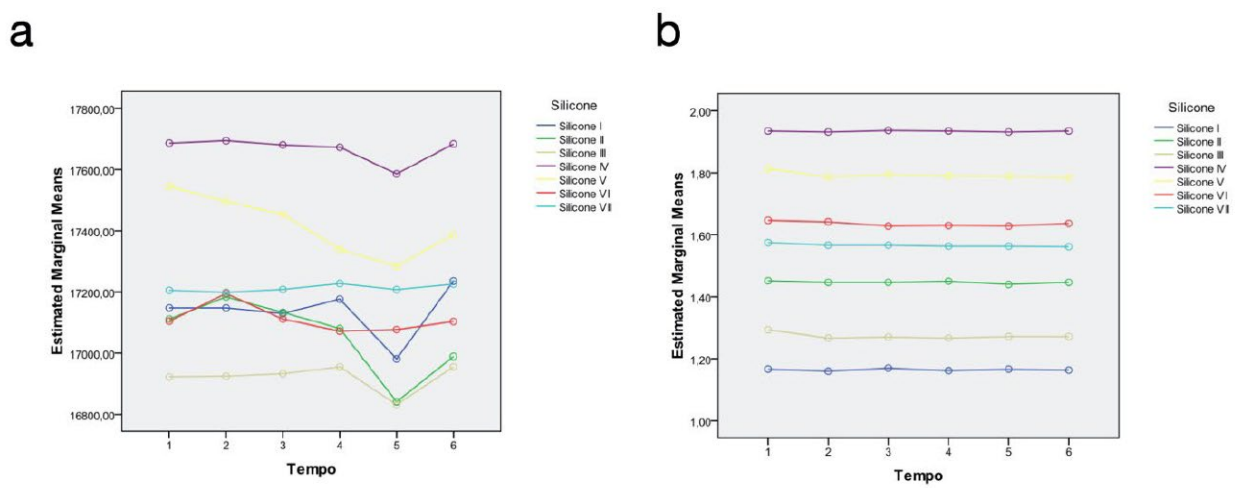

Fig. 2. *Comparisons between the different silicones for the variation of linear size (A) and weight (B) over time*

al. (91) Storage in the fridge at 8 ± 2 ° C can be done for 6 hours (92). This is probably related to the ability of alginate to retain water at low levels temperatures and this makes the material more dimensionally stable. Todd et al. support the role of water in dimensional variations to the various storage temperatures (93). The conservation conditions adopted in their study of 2013 greatly affected the volume of water in the footprints in alginate. The hot conditions (46°C) caused a significant expansion of the water content (considering that the coefficient of thermal expansion of water pure is 0.0001-0.0004 for every 1°C in the range 10°C - 50°C).

The most critical time for all types of alginates, is about the dimensional variation that the weight, was after 24 hours from the realization of the sample. At 24 hours, the only alginate that showed the least variation dimensional and the only positive (expansion) was type VI Neocolloid, while the others all suffered a contraction.

The linear contraction continues over time, showing a certain dimensional stability up to T2 and a more consistent variation from T2 to T3 (5 days). For the values found, we can define the alginates analyzed as stable at 24 hours and in agreement with the cut-offs adopted in the literature (94) and with the first published ADA specification.

The long-term storage of alginates involves not only the drawback of the loss of stability but also a microbiological drawback. On the surface of some samples of the study conducted here, the formations of Ife of fungi, visible to the naked eye, were observed.

The data from the statistical analysis conducted on synthetic elastomers show how the different silicones have undergone different dimensional variations based on the type, but in any case, not influenced by the disinfected environment / non-disinfected environment factor.

Variations in linear dimensions have a critical moment at seven days, from the fifth day to the seventh day the samples seem to undergo a linear contraction that falls within 30 days; instead the weight undergoes a variation within the first hour and afterwards it remains constant over time.

As already specified, for the weight the interaction between time and the type of storage environment is significant, but the significance could be due to the different weight of the two groups ("non-disinfected environment" group and "disinfected environment" group) rather than to different variations over time. The disinfected samples of all silicones show a heavier weight than the corresponding non-disinfected ones, this can lead to the assumption that the disinfectant is absorbed and maintained by the piece for the duration of the test.

In the period from 24 hours to 5 days there is a slight linear dimensional variation, but not significant, between the different environments, with greater expansion in the disinfected environment, while the variation in weight, in the same time interval and in the disinfected environment, it assumes a parallel behavior but quantitatively there is a greater contraction of the disinfected samples which in T0 had a greater weight.

The dimensional variations of silicones found in the study we conducted correspond to the values attributed in the literature to Standard No.19 ANSI / ADA – Dental Elastomeric Impression Materials: 2004 and the ISO 4823: 1992 standard which they consider clinically acceptable dimensional variations of less than 0.5% after 24 hours and not higher than 1.5% after 24 hours. Furthermore, the results of this study do not differ from the data of the most recent literature (95-105), where impression silicones are described as stable materials in as they do not undergo variations of more than 1.5%, even if stored in environmental situations with unfavorable thermal conditions.

The work proposed here is an in vitro study that allows us to formulate statistical conclusions that may have implications in the clinical setting. From the results we have obtained, we can recommend some precautions to be implemented for the conservation of the impressions based on the material used.

The study was initiated assuming two null hypotheses. The first null hypothesis regarding alginates hypothesized (1) that there is no difference in dimensional stability over time between the best known irreversible hydrocolloids from impressions on the market under different storage conditions (2), regardless of the mixing technique used and the use of spray disinfectant produces similar effects on

dimensional stability in various types of materials. This first hypothesis has been refuted, the data obtained show that the various brands of alginates on the market demonstrate different characteristics of stability as a function of time, even if all are better preserved in conditions of low temperatures not disinfected in an environment with relative humidity at 100%. The disinfected environment creates a situation of better stability at room temperature, while at low temperatures the execution or otherwise of the disinfection procedure does not produce statistically significant differences.

The second null hypothesis regarding silicone materials hypothesized that (1) silicones have better long-term dimensional stability than water-based hydrocolloid materials (2), that the use of spray disinfectant does not alter dimensional stability. This hypothesis was confirmed by the data obtained, in fact the impressions made with silicones can be stored at room temperature in airtight containers even up to 30 days without excessive dimensional variations and the execution of a spray disinfection procedure does not cause statistically significant changes in dimensional stability.

Authorship contributions

i) Conceptualization of the study and data: GG; ii) drafting of manuscript and review: PM and ME.

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Interbracket distance differences between different lingual systems

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OBJECTIVE: The aim of the study was to analyze and compare the interbracket distance in crowded arches of different lingual appliances.

MATERIALS AND METHODS: The stereolithographic models of the same patient with 4 different lingual appliances were used to analyze the interbracket distance. 3Shape software was used to analyze the interbracket distance for each arch segment and by means of a mathematical formula the force expressed by the different appliances in the different arches segments was calculated.

RESULTS: Incognito, eBrace and Harmony reported an average interbracket distance of less than 6 mm, while STb system showed an average values of 7 mm.

CONCLUSION: The interbracket distance, which have an influence on the system stiffness and rotational control of the teeth, is significantly greater in STb appliances compared to eBrace, Incognito and Harmony appliances.

The lingual bracket slot has a width sufficient to guarantee tip control but not excessive in order not to reduce excessively the interbracket distance, already reduced in lingual orthodontics. The archwire length is greater on the vestibular side and this difference is more evident in some teeth such as the lower anterior ones where the arch length on the lingual side is markedly decreased as the radius of curvature has decreased (Fig. 1).

In the posterior teeth, however, the interbracket distance on the buccal and lingual side is very similar. On the other hand, it is increased from the lingual side only in the space between the canine and the maxillary premolar. The archwire strength is inversely proportional to the cube of the interbracket distance and this means that for small reductions in the distance between two brackets, the force transmitted to the elements will increase considerably.

Recent studies have shown that strength can be up to 7.88 times greater on the lingual side when the interbracket distance is reduced, particularly when brackets with increased mesio-distal dimensions are used. (1)

By using a full-thickness square wire in a square slot it is possible to transmit the same information but exerting much less force. The increased elasticity of a wire also allows for easier insertion and easier clinical management. This concept is even more valid and important in lingual orthodontics where the interbracket spaces are reduced, it is more difficult to engage the wires and we need to use wires that exert lighter forces. The biomechanical features of lingual appliance are really important since this kinds of appliances became popular in the last years . Even if some teeth are missing or implant or prosthesis are inserted (2-4) or there is no compliance (5), lingual orthodontics appliance can treat the most common malocclusions in a effective way (6).

Keywords: crowded arches, brackets, interbracket distance

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However, lingual biomechanics is more complicated respect to the labial one especially in complex cases (extractions, open bite, impactions) (7, 8) when a perfect 3D control of the teeth is required (5) or a perfect muscular balance is missing (9) . The most important biomechanical difference between labial and lingual approach regards interbrackets distance and wire stiffness.

The aim of the present study is to analyze and compare the interbracket distance in crowded arches of different lingual appliances.

MATERIALS AND METHODS

The stereolithographic models of the same patient with 4 different lingual appliances were used to analyze the interbracket distance (Fig. 2). 3Shape software was used to analyze the interbracket distance for each arch segment and by means of a mathematical formula the force expressed by the different appliances in the different arches segments was calculated.

The upper and lower precision impressions and the construction bite were taken and sent to the four manufacturers of the selected lingual appliances.

We also scanned the impressions with a digital scanner (Smart Open Technology) and obtained the stereolithographic model of the single and occluded dental arches. To perform the comparative analysis between the different lingual systems, the following lingual appliances were selected: i) eBrace; ii) Harmony; iii) Incognito; and iv) STb. Due to overcrowding, the 4 manufacturers have provided for the bandage of the bracket on element 4.3 at a later time (Fig. 3).

The interbracket distance was calculated for each segment of the 4 arches scanned by 3Shape software (Fig. 4). Three measurements were taken for each interbracket distance. The mean and standard deviation were calculated for each measured distance.

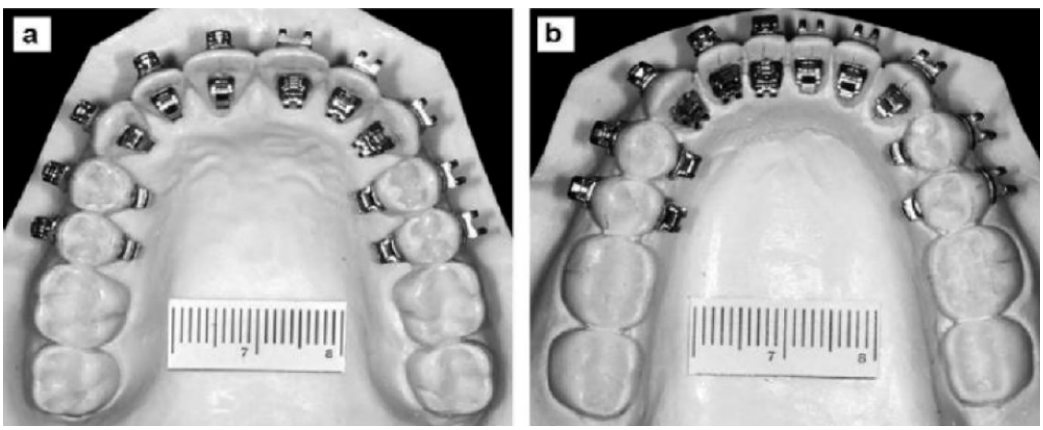

Fig. 1. *Dynamometer Instron 4467*

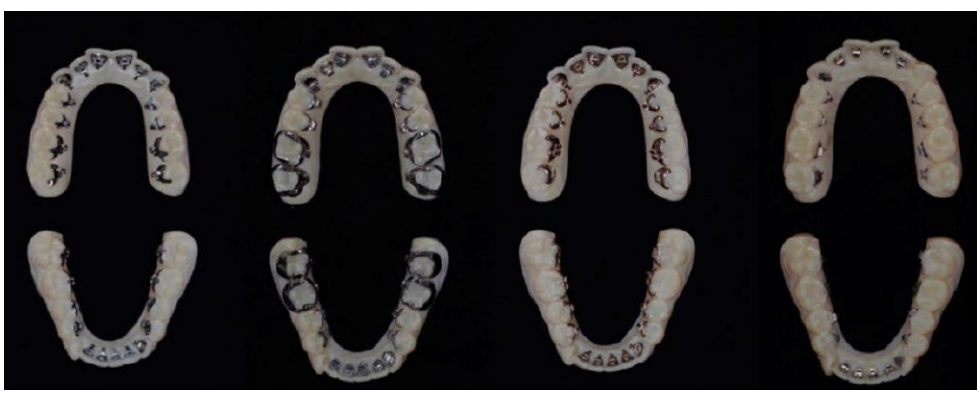

Fig. 2. *Brackets on the support*

Knowing the arcs used for the yarn sequence of each selected system, knowing the modulus of elasticity of the material, calculated the moment of inertia, using the formula for elastic yarns:

$$F = 192\ I\ EY / L3$$

and the formula for superelastic yarns:

$$F = 16\ IEU / LD$$

$$U = 12\ DYm / L2$$

It was possible to derive the force generated by the different systems on the individual dental elements in relation to the different interbracket distance and the material section of the wire used.

U is the coefficient of proportionality between three characteristics of a superelastic alloy wire at the point of transition to the martensitic phase. It is a characteristic of the alloy that does not depend on the

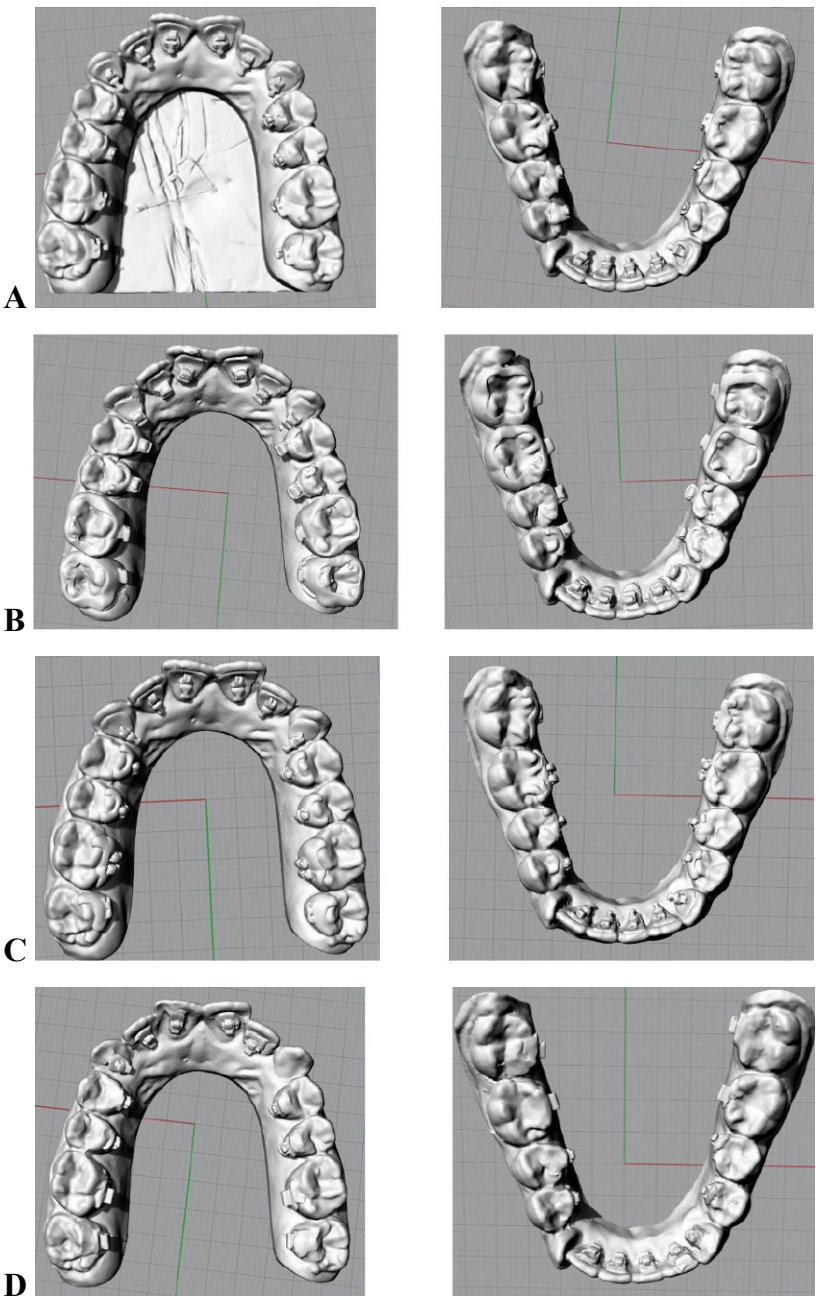

Fig. 3. *Different arch wires used*

section of the wire or on the interbrackets distance.

It is obtained experimentally by applying the formula:

$$u = 12\ ZY / L2$$

Where Z is the diameter of the wire (round); Y is the deflection value in the horizontal plane at which the transition to the martensitic phase occurs (identified with the point β in the graph force-deflection); and L is the interbrackets distance.

The three parameters (L, Y and Z) are in constant relationship with each other, and this one

proportionality constant is u: coefficient of proportionality between the martensitic transformation point of a wire of known diameter and the square of the interbrackets distance. It is a pure number.

Constructing a graph that relates Y and L2 for a superelastic wire of known diameter, u represents the slope of the line.

Sample: (SS) 0.018 = 0.46mm

F = 192 I E Y / L3

I = π D4 / 64 = (3.14 x 0.464) / 64 = 0.0021 Emin = 17x104 megapascals

Emax = 20x104 megapascals

YA = 0.5mm YB = 1mm YC = 1.5mm

L = distance between brackets

With Emin = 17x104 megapascals YA = 0,5mm: -> F = 34272 / L3

With Emin = 17x104 megapascals YB = 1mm: -> F = 68544 / L3

With Emin = 17x104 megapascals YC = 1,5mm: -> F = 102816 / L3

With Emax = 20x104 megapascals YA = 0,5mm: -> F = 40320 / L3

With Emax = 20x104 megapascals YB = 1mm: -> F = 80640 / L3

With Emax = 20x104 megapascals YC = 1,5mm: -> F = 120960 / L3

Below is the mathematical formula applied to superelastic yarns:

SUPERELASTIC THREADS: (NiTi)

F = 16 I E U / L D

I = π D4 / 64

E = 3.8 x 104 megapascals U = 12 DY / L2

Y = 1mm

For 0.016 -> F = 10115 / L3 For 0.014 -> F = 5879.85 / L3

.014 = 0.358mm

Statistical analysis

The comparison for the interbracket distance (expressed in mm) between the different manufacturers evaluated was carried out on the average of the values of each group (four, corresponding to the different brands). A one-way ANOVA analysis of variance was performed, followed by post-hoc Holm-Sidak test. Data were expressed as mean ± SE of 72-75 measurements per group. Data and graphs were obtained using GraphPad Prism 6.0 statistical software.

The comparison for the interbracket distance (expressed in mm) between the different manufacturers for each segment was carried out through a two-way

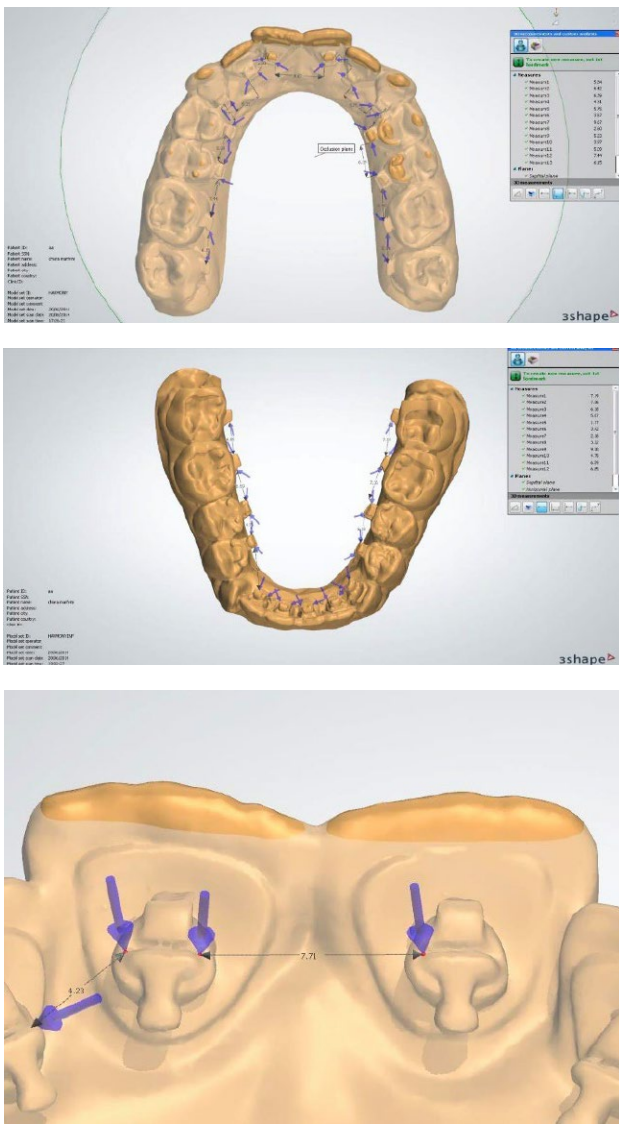

Fig. 4. *Load deflection test*

ANOVA, followed by the post-hoc Holm-Sidak test. Data and graphs were obtained using GraphPad Prism 6.0 statistical software.

RESULTS

The data analysis showed that the interbracket distance is significantly lower for the Incognito, E-brace and Harmony manufacturers, compared to STb, with a $p < 0.05$ for all three comparisons (Table I).

Incognito, eBrace and Harmony reported an average interbracket distance of less than 6 mm, while STb system showed an average values of 7 mm.

DISCUSSION

The force expressed by the orthodontic wires is inversely proportional to the cube of the interbracket distance and in lingual orthodontics this data is of fundamental importance and reminds us that even for small reductions in the interbracket distance there will be a significant increase in the force applied to the dental elements (10-13).

For this reason, during the early stages of orthodontic treatment, in the lingual technique there is a need to use superelastic arches and very light forces.

The distance between the point of application of the force and the center of resistance of the tooth is different in vestibular and lingual orthodontics and this affects the magnitude of the moment that is created and the consequent movement of the tooth (14, 15).

The choice of the type (material) of wire and the presence of folds on the arch affects its characteristics of rigidity and elasticity.

As the wire cross-section increases, the resistance to deflection always increases. SS steel arches always express greater forces than TMA and NiTi wires (16-20).

Table I. *Comparison between different appliance*s

Appliances comparison	P-Value
Incognito vs. eBrace	0.9922
Incognito vs. Harmony	0.9922
Incognito vs. STb	0.0386
eBrace vs. Harmony	0.9922
eBrace vs. STb	0.0460
Harmony vs. STb	0.0337

Orthodontic treatment can be divided into three phases-initial alignment, space closure and final detailing. The orthodontists seem to have the most difficulty with lingual appliances during initial tooth alignment, space closure final detailing. During the space closure the use of skeletal anchorage especially on the palatal area (21, 22) can solve a lot of critical situations but during the aligment and finishing phases critical situations can happen. This may be the direct result of using arch wires that are too stiff and thus not compensating for the decreased lingual interbracket distance in the anterior of the arches. Both of these phases of treatment require arch wire flexibility sufficient to achieve full bracket engagement.

Orthodontists routinely look at the degree of crowding when selecting an arch wire during initial alignment. When tooth alignment discrepancies are used as the criterion for wire selection, practitioners using lingual appliances must choose wires with less stiffness than they would normally use with labial appliances in order to deliver similar forces to the teeth.

Orthodontists who have routinely used rigid wires for detailing with labial appliances must now realize that using the same wire for anterior detailing with lingual appliances is inappropriate. Heavy wires used with lingual appliances during final detailing will not deliver physiologic forces to the teeth due to the decreased interbracket distance. Thus, the need to modify archwire selection during final detailing seems as reasonable and necessary as it does during initial alignment.

In particular, a reduced interbracket distance leads to an increase in the rigidity of the system, but at the same time also an increase in the control of rotations and tip. For this reason it is necessary to find the right interbracket distance to correctly balance these aspects during an orthodontic treatment. Therefore, the fact that one system has an average interbracket distance of more than 1 mm compared to the others has both positive and negative aspects. These aspects must be taken into consideration by the clinician when selecting the archwire sequence (23). Despite the reduced inter brackets distance, it has been demonstrated fixed lingual appliances are really effective (24-32) and their predictability could be

higher than aesthetic removable appliances (33-48).

The present study highlighted that the interbracket distance have an influence on stiffness of the system and rotational control; and the interbracket distance is significantly greater in STb appliances compared to eBrace, Incognito and Harmony appliances.

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