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INDIRIZZO IN ODONTOSTOMATOLOGIA PREVENTIVA

XXVII CICLO

EFFECT OF A SUGAR-FREE CHEWING GUM CONTAINING MAGNOLIA BARK EXTRACT ON THE DEVELOPMENT OF CARIES LESIONS IN HEALTHY ADULT VOLUNTEERS: A RANDOMIZED CONTROLLED INTERVENTION TRIAL.

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CONTENTS:

ORIGINAL PAPERS

This thesis is based on the following four papers, which will be referred in the text by their Roman numerals:

I. Carta G, Cagetti MG, Cocco F, Sale S, Lingström P, Campus G: Distal risk factors and dental caries in Italian Adults -a cross-sectional survey. Submitted to *Clinical Oral Investigation.*

II. Carta G, Cagetti MG, Cocco F, Sale S, Lingström P, Campus G: Caries risk profiles in Italian adults using Cariogram and International Caries Detection and Assessment System (ICDAS). Submitted to *Brazilian Oral Research.*

III. Carta G, Cagetti MG, Cocco F, Sale S, Lingström P, Campus G: Effect of sugar-free chewing gum containing magnolia bark extract on interdental plaque pH in healthy adult volunteers: a Randomized Controlled Intervention Trial. Ready for submission.

IV. Carta G, Cagetti MG, Cocco F, Sale S, Lingström P, Campus G: Effect of a Sugar-Free Chewing Gum Containing Magnolia Bark Extract on different variables related to caries in healthy adult volunteers: a Randomized Controlled Intervention Trial. Ready for submission.

INTRODUCTION

Dental caries is the most common oral disease worldwide and it affects the majority of individuals in all age groups during their lifetime (Selwitz *et al*., 2007). It is a multifactorial, chronic and degenerative disease affecting dental hard tissues. Its aetiology is related to a triad of indispensable factors: cariogenic bacteria in dental plaque, fermentable carbohydrates intake and host defences, mainly represented by saliva flow (Keyes and Jordan, 1963). In addition, socioeconomic and behavioural factors influence the disease pattern (Petersen *et al.,* 2005). The multifactorial aetiology of dental caries requires a risk assessment that includes the largest number of different variables.

Caries risk assessment

Identifying risk in individuals or population is difficult when it comes to chronic diseases that are caused by multiple factors that develop over a long period of time. Previously, the caries risk factors, which attracted most interest, were factors associated with the local caries process itself, such as the consumption of sugar, plaque, hygiene regimen and the host. According to Burt (2005), these factors should be extended. He stated "we should broaden our view of risk to include social determinants of health and population health". Risk factors are often discussed in the literature and one of the most relevant questions is whether the factor is "causal" or "associated". The focus on the non-biological determinants of the disease (socio-behavioural and environmental factors) is essential (Holst *et al*., 2001). The patient's educational level should be also considered in assessing risk (Paulander *et al*., 2003). It is one of the most frequently used socioeconomic indicators: lower educational level has resulted statistically associated with greater severity of dental caries in adults (Costa *et al.,* 2012). Increased understanding of the aetiology of major oral diseases today gives a good basis for implementation of oral health programmes. Among the prediction models available, the caries risk software "Cariogram" has been developed and validated to illustrate graphically the chance of an individual of avoiding caries in the next future (Campus *et al*., 2011). The program takes into account interactions among individually-assessed risk factors and evaluates them in a weighted way. Caries risk assessment has moved from the mere addition of individual risk factors to an approach in which risk factors are weighted, based on the putative role they play in the aetiology of the disease.

Sugar consumption as a caries-promoting factor

Modern concepts regard caries as an interaction between genetic and environmental factors, where biological, social, behavioural and psychological factors are expressed in a highly complex interactive manner with the dental biofilm as the key element (Kalesinskas *et al*., 2014). Although nowadays it is generally believed that several microorganisms may contribute to disease, *mutans streptococci* (MS) are known to be associated with the development of dental caries lesions (Selwitz *et al*., 2007). In fact, cariogenic bacteria, recognized as the main etiological factor universally, adhere to tooth surfaces and produce acids through the metabolism of their carbohydrates. Sucrose is the main triggering factor for development of the bacterial biofilm, as depicted by Marsh *et al.* (2003). The consumption of fermentable carbohydrate-containing food has long been associated with the development of caries (Marshall, 2009). Moreover, intake frequency plays a major role, too (Lingström *et al*., 2003). The causal mechanism is related to the production of organic acids by different cariogenic microorganisms through the fermentation of dietary carbohydrates (Koo *et al*., 2013). When the biofilm is matured, the presence of sucrose and/or starch further promotes plaque cariogenicity by constantly keeping pH at 5 or even lower. The production of acids in the dental biofilm reduces the pH level, which may further lead to a demineralization of enamel and dentine (Bowen *et al*., 2011).

The role of saliva

Some host defence factors influence this complex interaction between acid-producing cariogenic bacteria and fermentable carbohydrate. Especially saliva, both in quantity and quality, plays a major role in caries prevention. The complex mix of salivary constituents provides an effective set of systems for lubricating and protecting the soft and hard tissues. Protection of soft tissues is afforded against desiccation, penetration, ulceration, and potential carcinogens by mucin and anti-proteases. A major protective function results from the salivary role in maintenance of the ecological balance in the oral cavity via: debridement/lavage, aggregation and reduced adherence by both immunological and non-immunological means, and direct antibacterial activity. Saliva also possesses anti-fungal and anti-viral systems. Saliva is effective in maintaining pH in the oral cavity, contributes to the regulation of plaque pH, and helps neutralize reflux acids in the esophagus. Salivary maintenance of tooth integrity is dependent on mechanical cleansing and fermentable carbohydrate clearance.

Caries prevention: use of chewing gum

Although it is difficult to control human behaviour, many caries-preventive measures have been designed, including the daily use of fluoride toothpastes and antiseptic mouth-rinses, water fluoridation, dental sealants, oral health educational programs as well as regular visits to dentist office. However, the recent epidemiological studies show the trend to global increase in dental caries that clearly indicate a need for development of new and effective prophylactic approaches (Marcenes *et al*., 2013; Bagramian *et al*., 2009).

It is known that the use of sugar-free chewing gum, as part of normal oral hygiene, contributes to prevent dental caries (Deshpande and Jadad, 2008). The higher flow rate of stimulated saliva promotes more rapid oral clearance of sugars and increase of its buffering capacity in order to neutralise the plaque pH and decrease the risk of caries development (Dodds *et al*., 2012). Besides salivary enhancement, several compounds are added to chewing gum in order to increase their caries-protective value. Polyols, such as sorbitol, a hexitol derived from glucose, and xylitol, a naturally occurring pentatol, are often used as sugar substitutes (Burt, 2006). Dozens of studies have demonstrated that habitual xylitol consumption decreases counts of *mutans Streptococci* (MS) and demonstrated its clinical benefits both as noncariogenic and cariostatic agents (Söderling, 2009). Several mechanisms can be proposed to be responsible for the MS decrease as follows: growth inhibition, a decrease in the amount of plaque, elevated pH in the mouth, a decrease in adhesive polysaccharides produced by MS and specific inhibition of stress proteins. However, in order to obtain these positive effects, both a high daily dose of xylitol as well as frequent intake have recently been suggested (Milgrom *et al*., 2006; Holgerson *et al*., 2007; Ly *et al*., 2008).

Magnolia Bark Extract

Natural extracts from plants are generally included as ingredients in the formulation of candies and chewing gums in order to increase their anticaries properties. Studies *in vitro* have shown that some of these extracts possess antibacterial activity against cariogenic and periodontopathic bacteria (Ho *et al*., 2001; Greenberg *et al*., 2007, 2008); however, the clinical effects still remain unproven (Wallace, 2004). Magnolia Bark Extract (MBE) is a plant extract obtained from the bark of magnolia that has been widely used in traditional Chinese medicine for 2,000 years (Watanabe *et al*., 1983). *Magnolia officinalis* has been used for the treatment of acute pain, diarrhea, coughs and urinary problems. The two main constituents of the bark of this medicinal plant are magnolol and honokiol. They are known to possess a variety of pharmacological properties, including therapeutic-related activities (Teng *et al*., 1990), central nervous system inhibition (Watanabe *et al*., 1983), anti-inflammatory effects (Wang *et al*., 1995), antimicrobial activity (Clark *et al*., 1981), antioxidative activity and free radical scavenging activity (Lo *et al*., 1994). Magnolol and honokiol have also been reported to inhibit the growth of *Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans, Capnocytophaga gingivalis* and *Veillonella disper in vitro* and reduce the dental caries values in rats (Chang *et al*., 1998; Ho *et al*., 2001; Greenberg *et al*., 2007). Consequently, the antimicrobial properties of MBE against cariogenic and plaque bacteria have showed promising results.

AIMS

The overall aim of this thesis was to collect knowledge about dental health in adults aged 30-45 years and evaluate different variables related to caries after a chewing period of one year. In more detail, the objectives of this thesis were:

- To evaluate the association between caries disease, scored using International Caries Detection and Assessment System (ICDAS), and distal caries risk factors.

- To examine the correlation among socio-behavioural factors, caries status and caries risk, calculated through Cariogram, in an adult population.

- To assess the effect of sugar-free chewing-gum containing magnolia bark extract and xylitol on plaque pH, on salivary concentration of oral bacteria and on caries development.

METHODS

Ethics approval

The present study was carried out in Sassari (Italy) under the supervision of the Italian WHO Collaborating Centre for Epidemiology and Community Dentistry and lasted from September 2012 to September 2014.

The study was designed as a randomized clinical trial, approved by the Ethics Committee of the University of Sassari (record n°1083/L of 23/07/2012) and registered (Protocol Registration Receipt NCT01588210) at http://www.clinicaltrial.gov .

Study design (paper I and II)

These studies were designed as a cross-sectional survey, carried out in Sassari (Sardinia, Italy), under the supervision of the WHO Collaborating Centre for Epidemiology and Community Dentistry of Milan (Italy). The survey lasted for 14 months, from September 2012 to October 2013. The study design was registered (2013_01_21_a) by the Sassari local office of the National Bioethics Committee.

Study population (paper I and II)

Data from the Italian National Institute for Statistics (http:// www.comuni-italiani.it/090/statistiche/redditic.html) for 2011 gave the number of 30-45 year-olds living in the town as 22,614. Power analysis was performed using G*Power 3.1.3 for Apple using logistic regression with an odds ratio of 1.5, an error probability of 0.04 and the total sample size was set at 432. A letter explaining the purpose of the study with an informed consent was mailed, in collaboration with the municipal electoral registry office, to 5% (1131 subjects) of the age considered population (30-45 years) living in Sassari. More then fifty percent, 577 subjects (51.02% acceptance rate) accepted to participate; 82 did not attend the examination and 15 did not fill in completely the questionnaire. Overall 480 subjects, 52.92% males and 47.08% females, (mean age± Standard Error 40.72±0.33) were examined.

Methods
Paper I

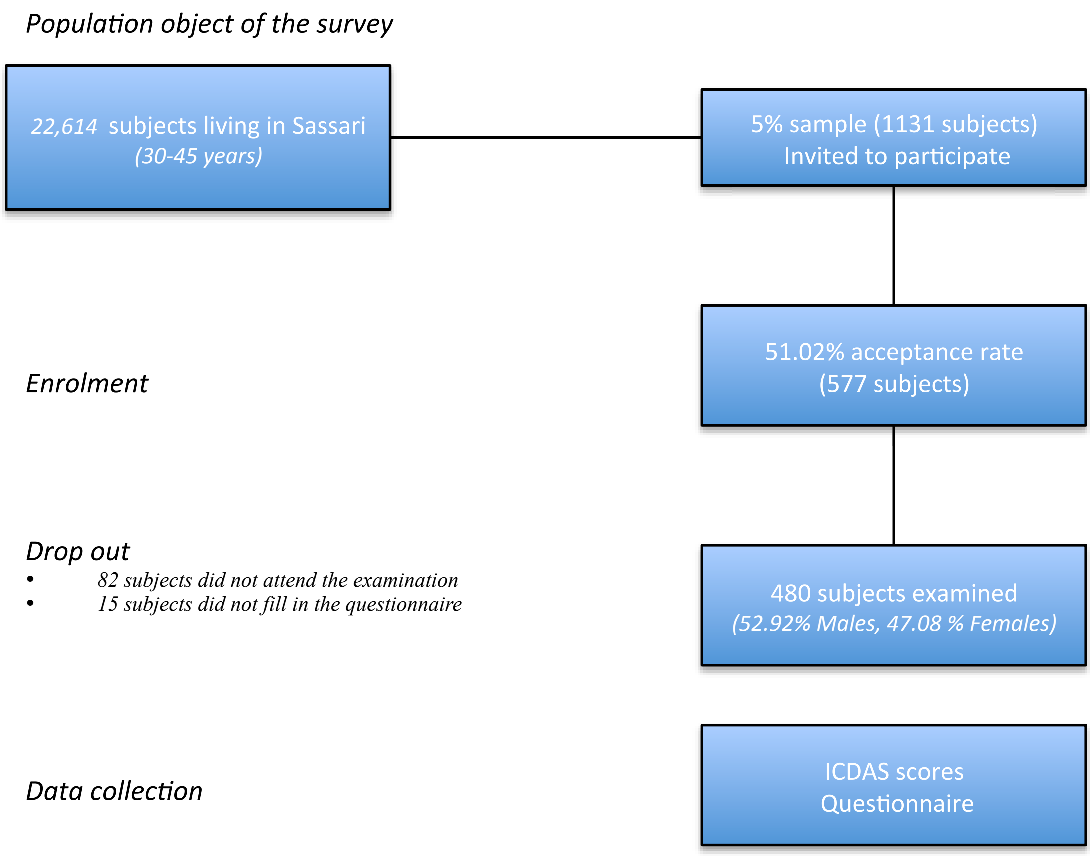
A benchmark examiner (GCampus) trained and calibrated one examiner (GCarta) that performed all dental screenings. Baseline training consisted in one-day (6 hours) theoretical course, followed by examination of 54 extracted teeth plus a session of 120 photographs of extracted teeth. Two days after the theoretical curse a clinical trial involving examination of fifty-five adults subjects was performed. The subjects were re-examined after 72 hours. Inter-examiner reliability with the "benchmarck" (GCampus) was evaluated using fixed-effects analysis of variance. Intra-examiner reproducibility was assessed as the percentage of agreement using Cohen's kappa statistic (Castiglia *et al.*, 2007). Good inter-examiner reliability was found for the merged ICDAS index 1, with no significant difference from benchmark values ($p = 0.15$) and a low mean square of error (0.47). Intra-examiner reliability was also high (Cohen's Kappa statistic = 0.88).
Subjects were examined in the Dental Institute of the University of Sassari, after a professional teeth cleaning, sitting on a dental chair, using a mouth mirror and a Community Periodontal Index probe (approved by the WHO) under optimal lighting; the teeth were dried if necessary. The ICDAS index allows registration of the decayed surfaces without bite-wing radiographs, and also the type of the filling, the teeth absents for caries or other reasons were registered (Honkala *et al.*, 2011).

Questionnaire (Construction and Development)

A simplified structured self-compiled questionnaire was submitted to the participants before the clinical examination. To guarantee the anonymous status, the questionnaire was printed on one side of a sheet, while the clinical chart was on the other side. The questionnaire was pre-tested for control of reliability and validity (14). It was highly structured and divided into three domains: (1) personal data (i.e. age, gender, educational level, employment categorization), (2) life-style behaviour (i.e. smoking and dietary habits), (3) oral health behaviour (i.e. tooth brushing, frequency of dental check-ups). The following distal variables were selected: gender, smoking habits, educational level and employment categories.

The figure n°1 displays the flow-chart of the survey.

Figure 1. Flow-chart of the survey (paper I).



Paper II

Questionnaire

A simplified structured self-compiled questionnaire together with the informed consent was submitted to the participants. The questionnaire was pre-tested for control of reliability and validity5. It was highly structured and divided into different domains: (1) personal data (*i.e.* age, gender, educational level, job categorization etc.), (2) life-style behaviour (*i.e.* smoking and dietary habits), (3) oral health behaviour (*i.e.* tooth brushing, use of fluoride, frequency of dental check-ups).

Caries assessments

One calibrated examiner (GCarta) performed all the dental screenings.

The Number of missing teeth due to caries was categorized as follow: no missing teeth, 1-2 missing teeth, 3-5 missing teeth and more than 5 missing teeth. The presence of fillings was classified in: no fillings, mainly tooth coloured restorations, mainly amalgam restorations and presence at least of one crown. Plaque index was assessed in the buccal surface using Silness and Löe's scale.

Microbiological evaluation

After the clinical examination, an evaluation of the mutans streptococci (MS) concentration in saliva was performed. No-stimulated whole saliva was collected over 150 s in sterile vials (Nunc, Kamstrup, Denmark). The samples were processed within 45 min after collection and the analysis was carried out using CRT BACTERIA (Ivoclar Vivadent AG, Liechtenstein). The presence of *mutans streptococci* over 10^5 CFU/ml suggests a high caries risk.

Caries risk assessment using Cariogram

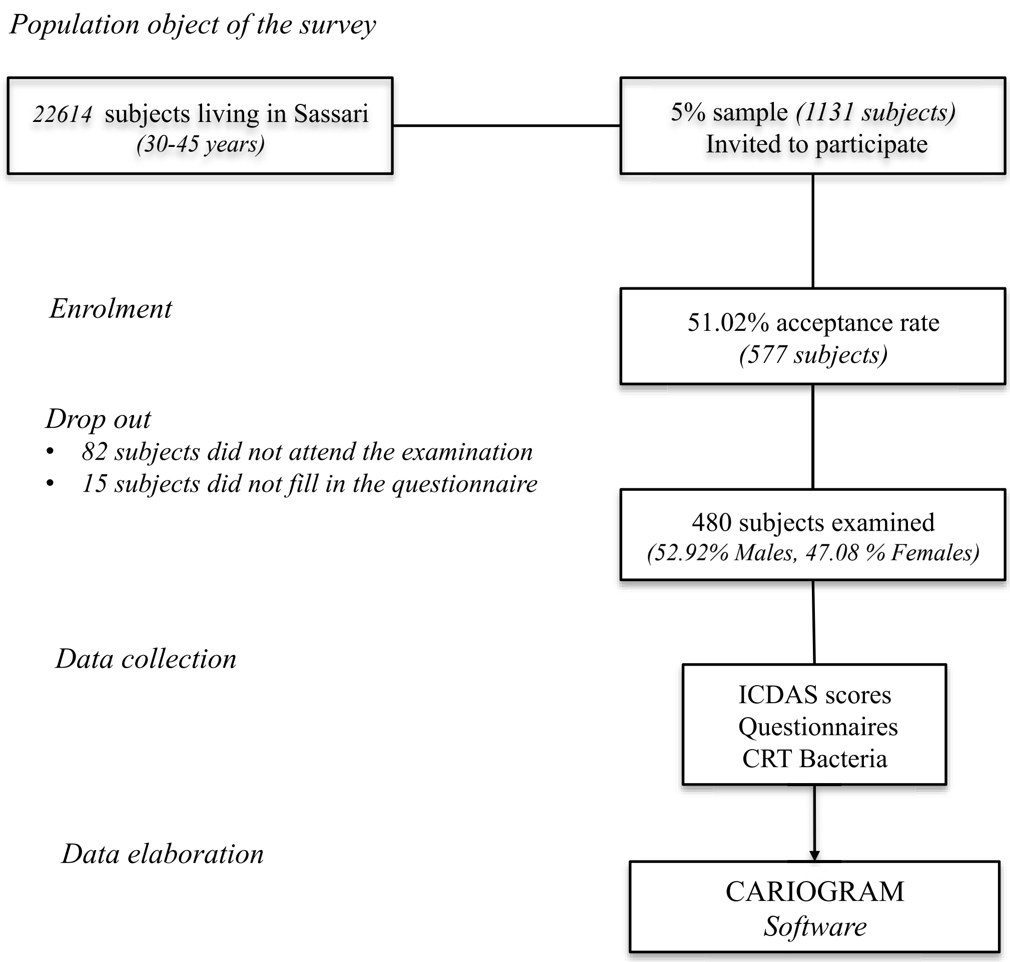
Information based on caries-related factors was collected and entered into the Cariogram. The following seven caries-related factors were included in the Cariogram software (Paulander *et al*., 2003):

1. Caries experience (from ICDAS index registered during the clinical examination);
2. Diet, contents (from questionnaires);

3.	Diet, frequency (from questionnaires);
4.	Salivary levels of *mutans streptococci* in saliva (from CRT Bacteria);
5.	Plaque amount and grade of oral hygiene (from clinical examination)
6.	Related general diseases (from questionnaires)
7.	Fluoridation programme (from questionnaires)

A score 1 in all cases was assigned for clinical judgment in order to give a standard value. The "Chance to avoid new caries in the future" was calculated and the subjects were divided in three groups: low risk (61-80%), medium risk (41-60%) and high risk (21-40%).

Figure 2. Flow-chart of the survey (paper II).



Study design (paper III and IV)

The experimental design was a three-group, double-blinded, randomized controlled clinical trial (RCT).

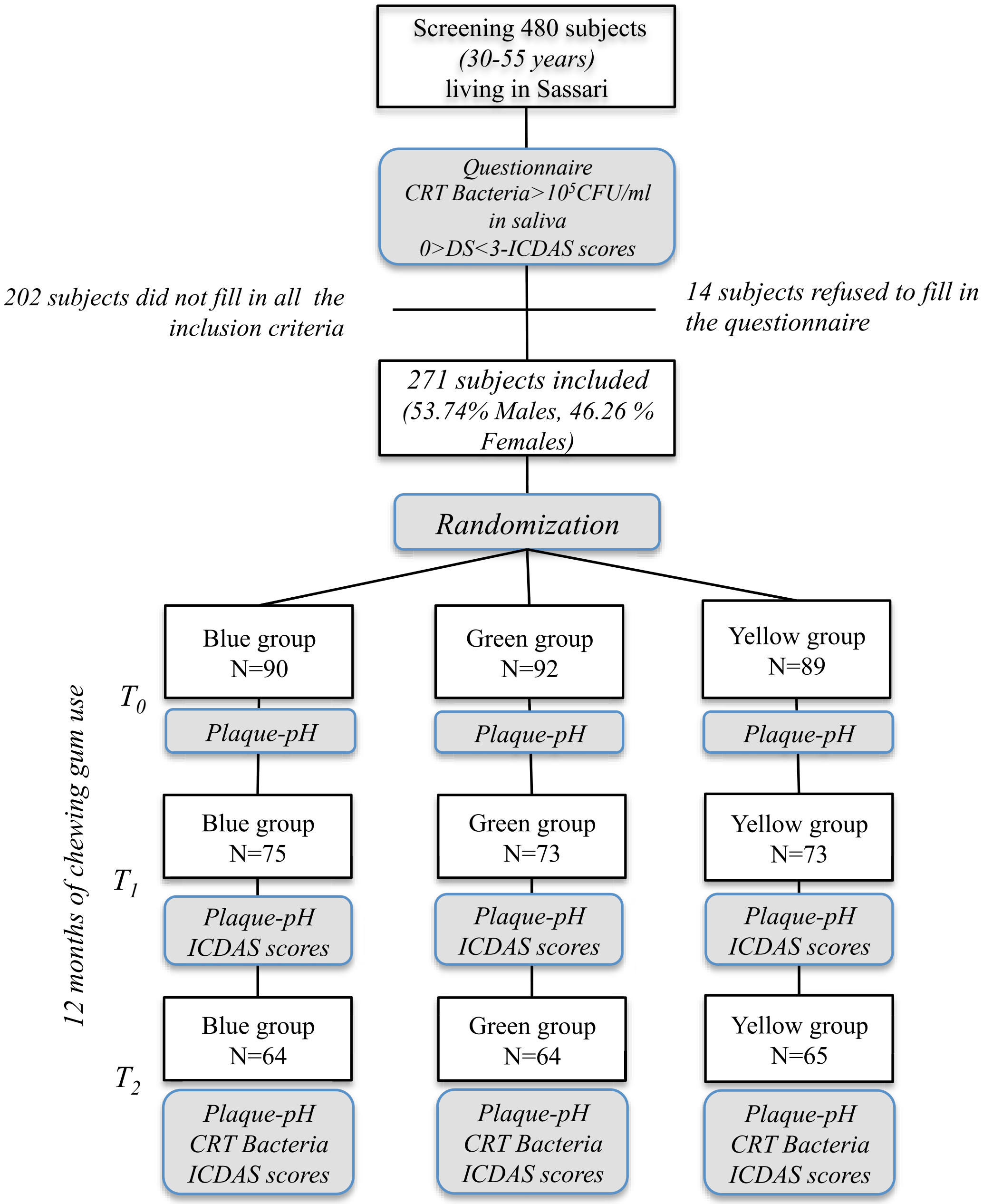
Study population (paper III and IV)

Data from the Italian National Institute for Statistics for 2011 gave the number of 30-45 year-olds living in the town as 22,614. Sample size for preliminary screening was performed trough G*Power 3.1.3 for Apple using logistic regression with an odds ratio of 1.5, an error probability of 0.04, the total sample was set at 312.

In order to get statistical comparison results, the number of subjects per group to be included in the analysis will be calculated. Considering a 35% difference among groups to be significant, and a 95% probability of obtaining a significant difference among groups at the 5% level, the resulting number of subjects per group was set 104.

With the collaboration of the municipal electoral registry office, a letter explaining the purpose of the study and the informed consent were distributed to 5% (1131 subjects) of the age group considered living in Sassari; 577 subjects (51.02% acceptance rate), aged between 30-45 years, accepted to participate were examined for conditions that would preclude participation. The flow-chart, displayed in the figure n°3, shows the design of the study.

Figure 3. Flow-chart of the study.



Methods (paper III and IV)

A simplified structured self-compiled questionnaire together with the informed consent was submitted to the participants before the clinical examinations. To guarantee the anonymous status, the questionnaire was printed on one side of a sheet, while the clinical chart was on the other side. The questionnaire was pre-tested for control of reliability and validity. It was highly structured and divided into different domains: (1) personal data (*i.e.* age, gender, educational level, job categorization etc.), (2) life-style behaviour (*i.e.* smoking and dietary habits), (3) oral health behaviour (*i.e.* tooth brushing, use of fluoride, frequency of dental check-ups) (Campus *et al*., 2011).

A benchmark examiner (GCampus) trained and calibrated one examiner (GCarta) that performed all dental screenings. Baseline training consisted in one-day (6 hours) theoretical course, followed by examination of 54 extracted teeth plus a session of 120 photographs of extracted teeth. Two days after the theoretical curse a clinical trial involving examination of fifty-five adults subjects was performed. The subjects were re-examined after 72 hours. Inter-examiner reliability with the "benchmarck" (GCampus) was evaluated using fixed-effects analysis of variance. Intra-examiner reproducibility was assessed as the percentage of agreement using Cohen's kappa statistic (Castiglia *et al*., 2007). Good inter-examiner reliability was found for the merged ICDAS index 1, with no significant difference from benchmark values ($p = 0.15$) and a low mean square of error (0.47). Intra-examiner reliability was also high (Cohen's Kappa statistic = 0.88). Subjects were examined sitting on an ordinary chair, using a mouth mirror and a Community Periodontal Index probe (approved by the WHO) under optimal lighting. The ICDAS index allows to register the surfaces decayed and also the level of caries (if initial or severe caries), the type of the fillings and the missing teeth for caries or for other reasons (Ismail *et al*., 2007).

The clinical examination was repeated at the end of the experimental period (12 months).

Inclusion criteria

The inclusion criteria for the recruitment of subjects are as follows:

1. Age 30-45;
2. Presence of a minimum of 12 natural teeth;
3. At least one surface cavitated caries lesion, but no more than three;
4. *Mutans streptococci* and *Lactobacilli* >10^5 CFU/ml saliva;
5. Systemically healthy as assessed by a medical questionnaire;
6. No use of antibiotics or participation in a clinical study in the previous 30 days;
7. No allergy to any of the ingredients of the study products;
8. No orthodontic banding or removable prosthesis;
9. Moderate gingivitis, no current periodontitis (no sites of probing pocket depth ≥5 mm or attachment loss of ≥2 mm, apart from gingival recession).
10. Absence of dysfunction of temporo-mandibular joint.

Subjects with a history of GI problems and with systemic disease that interfered with the oral ecosystem were excluded. The caries criterion was designed to include participants who are at risk of forming new lesions. In addition, those participants who were already consuming more than three pieces of sugar-free chewing gum a day were excluded. The elected participants agreed not to consume any other chewing gums than those supplied for the study.

All the participants were residents in an area with a low natural fluoride content in the drinking water, but they reported to use a fluoridated toothpaste on a regular basis.

Microbiological evaluation

Immediately after the clinical assessment, an evaluation of the *mutans streptococci* (MS) and *lactobacilli* concentration in saliva was performed. No-stimulated whole saliva was collected over 150 s in sterile vials (Nunc, Kamstrup, Denmark). The samples were transported to the Department of Microbiology of the University of Sassari and processed within 45 min after collection.

Mutans streptococci and *lactobacilli* counts in saliva were assessed and categorized using the dip-slide technique (CTR bacteria, Ivoclar Vivadent, Germany).

Subjects, who present a bacterial concentration >10^5 CFU/ml in their saliva sample (n=271), were invited to participate all baseline procedures and were enrolled. Then, subjects were randomly assigned to three groups (blue, green and yellow) and were blinded to group assignment, as were investigators. Block randomization ensured similar proportions of participants in each group. One using chewing gum containing MBE and xylitol, a group using chewing gum with the same content of xylitol but free of MBE and finally, a control group using a sugar-free chewing gum without MBE and xylitol.

Plaque-pH measurements

Interproximal-plaque pH of each subject was evaluated using pH indicator strips, which measure a pH value in the range of 4.0-7.0 (Spezialindikator, pH range 4.0-7.0; Merck, Darmstadt, Germany). The strip method determines changes in plaque pH, discriminating differences at the level of 0.2–0.5 pH units following a sugar (10%) challenge to the same extent as the micro-touch method (correlation coefficient 0.99). Strips are easy to use and suitable for a chair-side clinical use. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that could be more easily inserted into the interproximal space. The strip was held into the interdental space for 10 s, after which it was removed and its colour compared to the colour index scheme supplied by the manufacturer. The pH was determined to one decimal of the value.

For each subject, 3 measurements were carried out on 2 sites, between the 2nd premolar and the 1st molar right and left of the upper jaw. Measurements were performed before and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose. All measurements were carried out by the same examiner (GCarta). Plaque pH was assessed at baseline (t_0), after 6 (t_1) and 12 months (t_2) of chewing gum use.

Besides, each subject was instructed to chew 1 or 2 pellets for 5 min 3 times a day (2 in the morning, 2 after the midday meal and 1 in the afternoon). Daily gum packets were distributed on a weekly basis for the first 3 weeks, then every 2 months to promote and monitor compliance. The total daily intake of magnolol and honokiol in the group using chewing gum containing MBE and xylitol was 11.9 mg/day. The daily use of the three different chewing gums was carried out for 12 months. Three clinical evaluations for caries diagnosis were performed: one at baseline, a second after 6 months of chewing gum use and at the end of experimental period (after 12 months from baseline). A microbiological evaluation for cariogenic bacteria was performed at baseline and at the end of the chewing period.

Statistical methods

Data from the dental examination and questionnaire were entered in the FileMaker Pro 5.0 Runtime database and then exported to Excel® Microsoft spread sheet. All data were analysed using the software STATA® (v10.1 for Macintosh). For all statistical analysis, the statistical significance level was set at $\alpha=0.05$.

Paper I

Subjects were categorized according to maximum ICDAS score as the merged ICDAS (Braga *et al*., 2010) was calculated: ICDAS (0) as sound, ICDAS (1-2) initial caries stage referred to caries involving only the enamel with no evidence of dentine involved, ICDAS (3-4) moderate caries stage referred to caries involving breakdown of enamel or underlying dentine shadow and ICDAS (5-6) extensive stage decay with visible dentine involved. The number of missing teeth due to caries was recorded; the presence of fillings was categorized as follows: no fillings, mainly tooth coloured restoration, mainly amalgam restoration and presence at least of one crown.

The educational level was classified as: primary school, secondary school and university. The employment was categorized using the Italian National Institute for Statistics (http://www.comuni-italiani.it/090/statistiche/redditic.html). Smoking habits were coded following the WHO definition for tobacco user modified by the authors (Campus *et al*., 2011), as non-smoker and smoker that had started to smoke more than 3 years.

Descriptive statistics, cross-tabs and linear trend were calculated among caries severity (merged ICDAS) number of extracted teeth and number/type of restoration, and gender, smoking habit, educational level and employment categories. Next, multinomial logistic regression models were performed using as the dependent variable: merged ICDAS scores, numbers of missing teeth due to caries, and presence and type of fillings. The Akaike information criterion (AIC) was used to measure the goodness of fit of the statistical model. The possible modifying effects of covariates on the outcomes were tested by an interaction model (likelihood ratio test statistic). Multicollinearity might sometimes cause problems with regression results. This problem was solved using the DFBETA command in STATA, dropping the information that have too much influence on the regression line.

Paper II

Descriptive statistics and cross-tabs were calculated to investigate the relationship between dental health and different risk factors. The reference for each variable was set as follows: Gender (male), Caries status (ICDAS 5-6), Type of dental treatment (crown), Number of missing teeth due to caries (more than five), Educational level (middle school degree), Occupational status (unemployed), Smoking habits (yes), Frequency of toothbrushing (once a day), Frequency of dental check-ups (at pain).

A chi-square test was conducted for each variable and level of caries risk measured by Cariogram. Multinomial logistic regression was performed using Cariogram levels as the dependent variable.

The possible correlated variables were analysed using the principal component analysis (PCA) (Van Ooyen, 2001). The performances of Cariogram in predicting caries in the next future were evaluated by receiver operating characteristic (ROC) analysis.

Paper III

Descriptive statistics were performed about alimentary and oral hygiene habits of the sample. PH data of the 221 subjects (who finished the 6 months of the experimental period) were analysed for statistically significant differences using repeated measures of ANOVA.

Paper IV

Data on inter-proximal plaque pH at t_0, t_1 and t_2 were analysed for statistically significant differences using repeated measures of ANOVA. A chi-squared test for trend of odds was carried out for the bacterial concentrations for each group. Then, a chi-squared test was performed among the new proportion of bacterial counts of the three groups at t_2.

Furthermore, a non parametric test for trend for the levels of caries lesions was calculated across the three groups in the three times.

RESULTS

Paper I

Following ICDAS, the sample was split in sound subjects (36.46%), initial stage decay (5.63%), moderate stage (40.83) and at extensive stage decay (17.08%) (Table 1). More than 50% of the sample had no missing teeth due to caries (table n°2), less than 5% had no fillings, 33.29% had mainly tooth coloured fillings, 29.58% of the sample mainly amalgam fillings, finally 32.71% presented at least one crown (table n°3). Only 5.02% of the subjects had no caries experience *(data not in table)*.

More than one quarter of the subjects (26.67%) reported to have started smoking more than three years before the enrolment in the survey. According to the educational level, almost 50% of the subjects (47.29%) had a secondary school graduation and 34.79% had university degree. The sample was stratified following the employment categorization as follows: 16.31% unemployment, 24.28% housewife, 43.10% technician/clerk and finally 16.31% professional *(data not in table)*.

Table 1. Sample distribution (number and percentage in column) following caries severity (sound, initial stage, moderate stage and extensive stage) according to gender, smoking habits, educational level and employment categorization. Linear trend was calculated for each variable.

	Sound (ICDAS=0) *n° (%)*	Initial (ICDAS= 1/2) *n° (%)*	Moderate (ICDAS=3/4) *n° (%)*	Extensive (ICDAS>4) *n° (%)*
Total sample	175 (36.46)	27 (5.63)	196 (40.83)	82 (17.08)
Gender				
Males	99 (56.57)	17 (62.96)	97 (49.49)	41 (50.00)
Females	76 (43.43)	10 (37.04)	99 (50.51)	41 (50.00)
$\chi^2_{(3)}$=3.24 p=0.36 z=1.43 p=0.15				
Smoking habits				
Smoking	57 (32.57)	7 (36.93)	48 (24.49)	16 (19.52)
No smoking	118 (67.43)	20 (74.07)	148 (75.51)	66 (80.48)
$\chi^2_{(3)}$=5.75 p=0.11 z=2.37 p=0.02				
Educational level				
Primary school	28 (16.00)	3 (11.11)	34 (17.35)	21 (23.86)
Secondary school	90 (51.43)	10 (37.04)	94 (47.96)	33 (40.24)
University degree	57 (32.57)	14 (51.85)	68 (34.69)	28 (34.15)
$\chi^2_{(4)}$=8.43 p=0.21 z=-0.56 p=0.57				
Employment categorization				
Unemployed	25 (14.29)	7 (25.93)	31 (15.82)	15 (18.29)
Housewife	37 (21.14)	2 (7.41)	49 (25.00)	28 (34.15)
Technician/clerk	85 (48.57)	11 (40.73)	78 (39.79)	33 (40.24)
Professional	28 (16.00)	7 (25.93)	38 (19.39)	6 (7.32)
$\chi^2_{(9)}$=18.02 p=0.03 z=-1.98 p=0.05				

Tables n°1, 2 and 3 report the association between the distal variables (gender, smoking habits, educational level and employment categorization) and the caries severity (merged ICDAS scores, number of missing teeth due to caries disease and number and type of fillings). A statistical linear trend (z=2.37 p=0.02) was observed between ICDAS score and smoking habits as the percentage of smokers increases as the caries severity stages increase (table n°1). Caries severity was statistically significantly associated with employment categorization (z=1.98 p=0.05): the number of subjects that reported to have a skilled were significantly higher in those with an extensive stage of the disease. The number of missing teeth due to caries disease (table n°2) was statistically significant associated to gender (p=0.04), smoking habit (p<0.01) and educational level (p<0.01). Both smoking habits and educational level showed a statistically significant trend respect to the presence of missing teeth due to caries (p<0.01 and p=0.04, respectively).

Table 2. Sample distribution (number and percentage in column) following presence of missing teeth due to caries according to gender, smoking habits, educational level and employment categorization. Linear trend was calculated for each variable.

	No missing teeth *n° (%)*	1/2 missing teeth *n° (%)*	3/5 missing teeth *n° (%)*	>5 missing teeth *n° (%)*
Total sample	250 (52.08)	115 (23.96)	80 (16.67)	35 (7.29)
Gender				
Males	143 (57.20)	58 (50.43)	32 (40.00)	21 (60.00)
Females	107 (42.80)	57 (49.57)	48 (60.00)	14 (40.00)
$\chi^2_{(3)}$=8.18 p=0.04 z= 0.10 p=0.91				
Smoking habits				
Smoking	48 (19.20)	30 (26.08)	30 (38.50)	20 (56.14)
No smoking	202 (80.80)	85 (73.91)	50 (62.50)	15 (43.86)
$\chi^2_{(3)}$=28.57 p<0.01 z=3.21 p<0.01				
Educational level				
Primary school	34 (13.60)	16 (13.91)	25 (31.25)	11 (31.43)
Secondary school	107 (42.80)	69 (60.00)	33 (41.25)	18 (51.44)
University degree	109 (43.60)	30 (26.09)	22 (27.50)	6 (17.43)
$\chi^2_{(6)}$=33.30 p<0.01 z=2.08 p=0.04				
Employment categorization				
Unemployed	46 (18.40)	16 (13.91)	13 (16.25)	4 (11.43)
Housewife	57 (22.80)	32 (27.83)	17 (21.25)	10 (28.57)
Technician/clerk	106 (42.40)	49 (42.61)	40 (50.00)	18 (51.43)
Professional	41 (16.40)	18 (15.65)	10 (12.50)	3 (8.57)
$\chi^2_{(9)}$=3.00 p=0.96 z= 0.91 p=0.36				

The presence and/or the type of fillings (table n°3) were statistically significantly associated with gender (p=0.01) and educational level (p=0.02). No statistical significant linear trends were observed among presence and/or type of fillings and distal risk factors evaluated. Ordinal logistic regressions were carried out to study in deep the association between distal factors and caries data (table n°4). In the first model caries severity (ICDAS score) was used as dependent variable; smokers had a lower risk to have caries to an extensive stage (OR=1.40 95%CI=1.03–2.23 p<0.01). An effect modifier between smoking habit and educational level was observed as smoking was highly represented in the lower educational group (data not in table). The second model was run using the number of missing teeth due to caries as dependent; a protective role was played by the higher educational level -university degree (OR=0.69 95%CI=0.42–0.83 p<0.01). The presence and/or the type of fillings were the dependent variable of the final logistic model: the highest educational level, university degree, acted as a protective factor (OR=0.88 95%CI=0.74 –1.01 p=0.05). The number of missing teeth was also statistically positively associated with the number of crowns observed in the sample (OR=1.08 95%CI=1.03–1.14 p<0.01), with the highest educational level (university degree) as a protective factor (OR=0.56 95%CI=0.43–0.71 p<0.01). Moreover a high percentage of subjects (30.42%) showed missing teeth and no crowns.

Table 3. Sample distribution (number and percentage in column) following presence of fillings and/or type according to gender, smoking habits, educational level and employment categorization. Linear trend was calculated for each variable.

	No restoration *n° (%)*	Mainly tooth-coloured *n° (%)*	Mainly amalgam *n° (%)*	At least one crown *n° (%)*
Total sample	21 (4.38)	161 (33.54)	140 (29.17)	158 (32.92)
Gender				
Males	11 (52.38)	81 (50.31)	91 (65.00)	71 (34.94)
Females	10 (47.62)	80 (49.69)	49 (35.00)	87 (55.06)
$\chi^2_{(3)}$=12.68 p=0.01 z= 0.91 p=0.36				
Smoking habits				
Smoking	3 (14.29)	42 (26.09)	34 (24.59)	49 (31.01)
No smoking	18 (85.71)	119 (73.91)	106 (75.41)	109 (68.99)
$\chi^2_{(3)}$=3.61 p=0.31 z= 1.47 p=0.14				
Educational level				
Primary school	3 (14.28)	23 (14.29)	24 (17.14)	36 (22.78)
Secondary school	14 (66.67)	80 (49.69)	69 (49.29)	64 (40.51)
University degree	4 (19.05)	58 (36.02)	47 (33.57)	58 (36.71)
$\chi^2_{(6)}$=16.68 p=0.02 z= -0.33 p=0.74				
Employment categorization				
Unemployed	1 (4.76)	24 (14.91)	24 (17.14)	29 (18.35)
Housewife	2 (9.53)	41 (25.47)	32 (22.86)	41 (25.95)
Technician/clerk	15 (71.43)	67 (41.61)	61 (43.57)	64 (40.51)
Professional	3 (14.28)	29 (18.01)	23 (16.43)	24 (15.19)
$\chi^2_{(9)}$=9.36 p=0.42 z=- 1.64 p=0.10				

Table 4. Ordinal logistic regression estimation

Caries severity

Number of observations =480   Log Likelihood= -568.86 p=0.01

Covariates	OR (SE)	p-value	95% CI
Gender (*ref. females*)	1.17 (0.21)	0.35	0.84 – 1.66
Smoking (*ref. smokers*)	0.60 (0.11)	<0.01	0.41 – 0.88
Educational level (*ref. university degree*)	0.96 (0.12)	0.74	0.74 – 1.23
Employment categorization (*ref. professional*)	0.84 (0.00)	0.08	0.69 – 1.02

Missing teeth due to caries

Number of observations =480   Log Likelihood= -561.15 p<0.01

Covariates	OR (SE)	p-value	95% CI
Gender (*ref. females*)	1.14 (0.21)	0.45	0.81 – 1.61
Smoking (*ref. smokers*)	0.89 (0.12)	0.48	0.69 – 1.16
Educational level (*ref. university degree*)	0.69 (0.10)	<0.01	0.42 – 0.83
Employment categorization (*ref. professional*)	1.12 (0.12)	0.24	0.92 – 1.38

Presence of filling and type

Number of observations =480   Log Likelihood= -587.42 p<0.01

Covariates	OR (SE)	p-value	95% CI
Gender (*ref. females*)	1.14 (0.20)	0.43	0.82 – 1.60
Smoking (*ref. smokers*)	1.25 (0.24)	0.24	0.86 – 1.82
Educational level (*ref. university degree*)	0.88 (0.08)	0.05	0.74 – 1.01
Employment categorization (*ref. professional*)	0.98 (0.13)	0.90	0.77 – 1.27

Paper II

The study population consisted of 480 subjects (mean age 40.73, SE±0.33), 52.92% males and 47.08% females. The caries prevalence in the sample was 63.54%. Considering ICDAS scores, 5.62% of the sample had, at least, an initial caries (ICDAS=1-2), whereas 40.83% of the sample presented, at least, one moderate decay (ICDAS=3-4), and 17.08% a severe one (ICDAS=5-6). About dental treatments, 4.39% of the sample did not have any sign of dental treatment, 33.31% had, at least, one tooth coloured restoration, 29.58% had an amalgam restoration and 32.72% presented, at least, one crown of different materials.

Almost half of the sample (47.92%) had, at least, one missing tooth due to caries. The mean number of sound teeth registered in the sample was 24.83 (SE±0.16). Each subject presented 3.66 (SE±0.14) tooth coloured restorations, 2.96 (SE±0.13) amalgam restorations and 1.58 (SE±0.14) crowns. *(data not in table).*

Regarding questionnaires data analysis, 17.92% had middle school graduation, most of the subjects (47.29%) had high school graduation and a quite high percentage of subjects (34.79%) had university degree. 60.42% of the sample brushed their teeth three times a day. Only 26.7% of the subjects were smokers and most of them have been smoking for more than 5 years.

Relating to caries risk, the majority of the sample (78.12%) presented a medium caries risk (41-60% of chance to avoid new caries lesions in the future), 18.75% had a high caries risk (<41% of chance to avoid new caries lesions in the future) and 3.12% had a low caries risk (>60% of chance to avoid new caries lesions in the future).

Table n°5 shows a chi-square test between the level of caries risk, calculated through Cariogram software, and background variables. A statically significant correlation ($p<0.01$) was evident between the caries risk and ICDAS score. The level of caries risk was also correlated in a statically significant way with the number of missing teeth due to caries ($p=0.01$), the Educational level ($p=0.04$), the Smoking habit ($p=0.01$), the Frequency of toothbrushing ($p=0.02$) and the Frequency of dental check-ups ($p<0.01$).

Table 5. Sample distribution following sample's caries risk level (low, medium and high) according to caries status, number of missing teeth due to caries, educational level, smoking habits, frequency of toothbrushing and of dental check-ups.

Caries status

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Caries-free	11(2.29)	156(32.50)	8(1.66)
Initial caries (ICDAS 1-2)	1(0.21)	24(5.00)	2(0.42)
Moderate caries (ICDAS 3-4)	3(0.62)	148(30.83)	45(9.37)
Severe caries (ICDAS 5-6)	0(0.00)	47(9.79)	35(7.29)

$\chi^2_{(6)}=65.13$ $p<0.01$

Number of missing teeth due to caries

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
No-missing teeth	13(2.71)	201(41.87)	36(7.50)
1-2 missing teeth	2(0.42)	95(19.79)	18(3.75)
3-5 missing teeth	0(0.00)	54(11.25)	26(5.42)
>5 missing teeth	0(0.00)	25(5.21)	10(2.08)

$\chi^2_{(6)}=22.54$ $p=0.01$

Educational level

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Middle school degree	0(0.00)	67(13.96)	19(3.96)
High school degree	5(1.04)	185(38.54)	37(7.71)
University degree	10(2.08)	123(25.62)	34(7.08)

$\chi^2_{(4)}=9.91$ $p=0.04$

Smoking habits

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
No-smokers	15(3.12)	280(58.33)	57(11.87)
Smokers	0(0.00)	95(19.79)	33(6.87)

$\chi^2_{(2)}=10.40$ $p=0.01$

Frequency of toothbrushing

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Once a day	0(0.00)	5(1.25)	0(0.00)
Twice a day	2(0.41)	133(27.71)	48(10.00)
3 times a day	13(2.71)	236(49.16)	41(8.54)
4 times a day	0(0.00)	1(0.21)	0(0.00)

$\chi^2_{(6)}=14.47$ $p=0.02$

Frequency of dental check-ups

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Each 6 month	5(1.25)	46(8.54)	4(0.83)
Once a year	7(1.46)	132(27.50)	24(5.00)
Once 2 years	0(0.00)	101(21.04)	23(4.79)
At pain	3(0.62)	96(20.00)	39(8.12)

$\chi^2_{(6)}=25.04$ $p<0.01$

A multinomial logistic regression model was run for caries risk factors (table n°6) and Cariogram score was used as dependent variable. The presence of caries lesions at ICDAS level 5-6 and the presence of more than 5 missing teeth were statistically associated with Cariogram score (OR= 2.36 95%CI=1.83–3.03 and OR=1.43 95%CI=1.13 –1.82, respectively).

Table 6. Multinomial logistic regression estimates for significantly associated caries risk covariates to Cariogram scores.

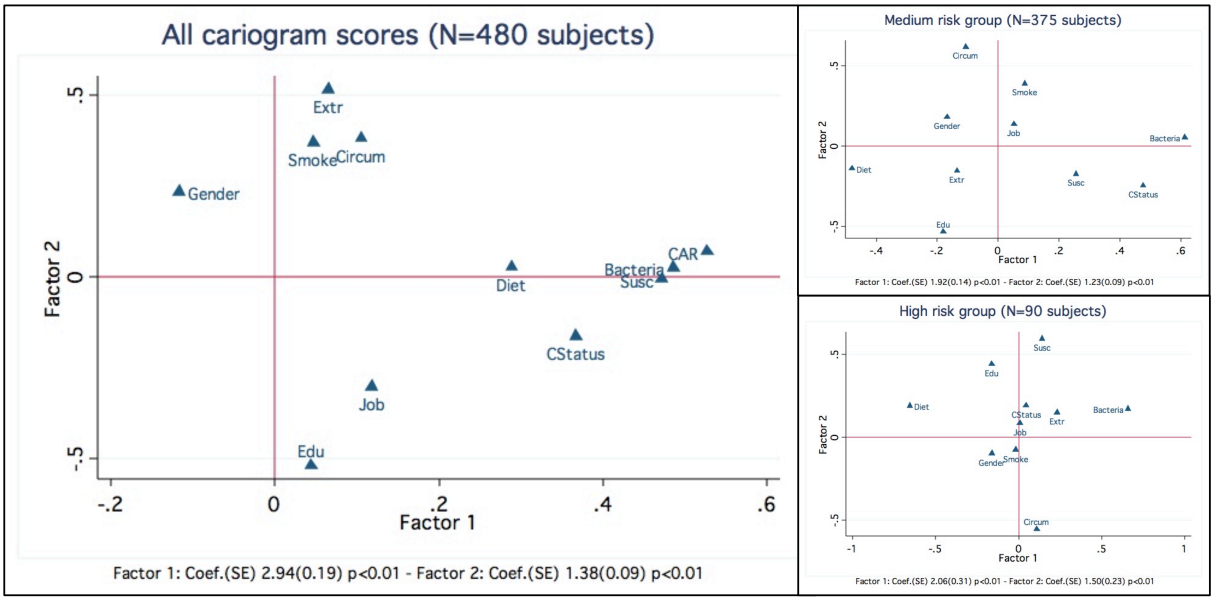
Number of observations =480 Log Likelihood= -248.33 p<0.01

Covariates	OR (SE)	p-value	95% CI
Caries status *(ICDAS 5-6)*	2.36 (0.30)	<0.01	1.83 – 3.03
Number of missing teeth *(>5 missing teeth)*	1.43 (0.17)	<0.01	1.13 – 1.82
Smoking habits *(Yes)*	1.28 (0.16)	0.36	0.76 – 2.14
Dental check-ups *(At pain)*	1.28 (0.16)	0.04	1.00 – 1.65
Tooth brushing frequency *(3 times a day)*	0.64 (0.15)	0.06	0.40 – 1.01

Principal Component Analysis (PCA) was performed on the data set, first on the total sample and in the two main groups stratified by Cariogram scores (caries medium-risk group and high-risk group); the low caries risk group was too small and PCA was not performed. In all PCA analysis the first two eigenvalues, obtained from distance matrix between groups, collectively account for more than 66% of the total variance (86.13%;

59.35 and 26.78%, respectively in the total sample; 52.47% and 21.15% respectively and 66.30% in the high-risk group 42.68% and 23.62% two eigenvalues respectively). Figure n°4 displays the Orthogonal Rotation (varimax) of the first two principal coordinates in the total sample and in the two main groups stratified by Cariogram scores. In the total sample, the Cariogram score, the Bacteria and the Susceptibility sectors from Cariogram tend to form a separate cluster with a high goodness of fit (63.74); the Number of missing teeth due to caries, Smoking habits and Circumstances from Cariogram tend to form another cluster with a high goodness of fit (61.82); however, the Level of education, the Gender and the Job categorization were clearly separated from the other variables. Severe caries status (ICDAS 5-6), Number of missing teeth due to caries more than 5, unemployed, smokers and male tend to form a cluster with a medium high (0.50–0.75) goodness of fit in the high-risk group. In the medium risk-group no clear cluster was identified, all variables stand separated one from each other. Sensitivity and specificity between ICDAS scores and Cariogram measured by ROC analysis were 0.85 and 0.87, respectively, so the gain in certainty was 1.71, while the area under the ROC curve was 0.92 *(data not table).*

Figure 4. Principal Component Analysis. Orthogonal rotation (varimax).



Legend: Extr= > 5 extracted teeth due to caries; Gender=male; Smoke =smoking habits; Circum=circumstances in Cariogram analysis, Diet= diet frequency and Diet content; Job= occupational status (unemployed); *Edu= level of education (middle school degree); Bacteria= salivary mutans streptococci; Susc= susceptibility in Cariogram analysis; CAR= level of caries risk calculated through Cariogram; CStatus= caries status using ICDAS.*

Paper III

There were no reports of taste disturbances or any other side effects in the magnolia and control groups. The sample was composed of 80.45% smokers and of 19.55% no-smokers. 63.80% of the sample reported to brush their teeth three times a day, then 33.03% twice a day. According to dietary habit, 73.06% were used to snacking between meals, other hand only 43.64% was used to drink soft drinks.

A total of 221 subjects (58.47% males and 41.53% females) completed the six-month period; data on plaque pH were calculated on 75 subjects in the magnolia group, 73 in the xylitol group and 73 in the control group.

Table n°7 shows the changes in plaque pH among the three groups after 6 months chewing gum use. It is evident that there is a statically significant difference between the plaque pH measurements at the baseline and the pH measurements rerun after 6 months. Compared with the baseline, chewing either xylitol gum or MBE gum or sugar-free gum significantly increased the interproximal plaque pH in the green and blue group (p<0.01) and in the yellow group (p=0.02).

Table 7. Results of repeated measures of ANOVA on inter-proximal plaque pH after 6 months of chewing gum use.

GREEN GROUP	**Min pH** ***Mean (SE)***	**Max pH** ***Mean (SE)***	**pH fall** ***Mean (SE)***
T_0	5.43(0.05)	6.95(0.02)	1.52(0.06)
T_1	5.60(0.07)	6.99(0.01)	1.44(0.06)
Repeated measures of ANOVA	*F=2.48 p<0.01*	*F=2.58 p<0.01*	*F=2.95 p<0.01*
Huynh-Feldt epsilon:	*1.00*	*1.00*	*1.00*
Greenhouse-Geisser epsilon:	*1.00*	*1.00*	*1.00*
Box's conservative epsilon:	*1.00*	*1.00*	*1.00*

BLUE GROUP	**Min pH** ***Mean (SE)***	**Max pH** ***Mean (SE)***	**pH fall** ***Mean (SE)***
T_0	5.48(0.05)	6.90(0.04)	1.48(0.06)
T_1	5.63(0.06)	6.95(0.03)	1.38(0.06)
Repeated measures of ANOVA	*F=5.40 p<0.01*	*F=2.61 p<0.01*	*F=10.09 p<0.01*
Huynh-Feldt epsilon:	*1.00*	*1.00*	*1.00*
Greenhouse-Geisser epsilon:	*1.00*	*1.00*	*1.00*
Box's conservative epsilon:	*1.00*	*1.00*	*1.00*

YELLOW GROUP	**Min pH** ***Mean (SE)***	**Max pH** ***Mean (SE)***	**pH fall** ***Mean (SE)***
T_0	5.52(0.06)	6.90(0.03)	1.41(0.06)
T_1	5.54(0.05)	6.93(0.03)	1.48(0.06)
Repeated measures of ANOVA	*F=2.00 p=0.01*	*F=2.45 p<0.01*	*F=2.40 p=0.02*
Huynh-Feldt epsilon:	*1.00*	*1.00*	*1.00*
Greenhouse-Geisser epsilon:	*1.00*	*1.00*	*1.00*
Box's conservative epsilon:	*1.00*	*1.00*	*1.00*

Paper IV

The experimental period was finished by 193 subjects (71.22% of the initial sample). Data on plaque pH were calculated on subjects in 64 subjects in the blue group, 64 in the green group and 65 in the yellow group, and are displayed in the table n°8.

Table 8. Results of repeated measures of ANOVA on inter-proximal plaque pH after 12 months of chewing gum use.

GREEN GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.34(0.05)	6.99(0.01)	1.63(0.06)
T_1	5.46(0.06)	6.99(0.01)	1.53(0.06)
T_2	5.6(0.06)	6.99(0.01)	1.39(0.06)
Repeated measures of ANOVA	**p<0.01 F=4.71**	**p<0.01 F=2.25**	**p<0.01 F=4.74**
Huynh-Feldt epsilon:	0.87	-	0.86
Greenhouse-Geisser epsilon:	0.83	-	0.83
Box's conservative epsilon:	0.50	0.50	0.50

BLUE GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.38(0.05)	6.87(0.04)	1.48(0.06)
T_1	5.62(0.06)	6.97(0.02)	1.36(0.06)
T_2	5.60(0.06)	6.95(0.03)	1.34(0.06)
Repeated measures of ANOVA	**p<0.01 F=4.40**	**p<0.01 F=3.36**	**p<0.01 F=4.16**
Huynh-Feldt epsilon:	0.80	0.72	0.92
Greenhouse-Geisser epsilon:	0.77	0.70	0.88
Box's conservative epsilon:	0.50	0.50	0.50

YELLOW GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.45(0.06)	6.88(0.03)	1.43(0.07)
T_1	5.48(0.05)	6.95(0.02)	1.47(0.06)
T_2	5.49(0.06)	6.99(0.01)	1.49(0.06)
Repeated measures of ANOVA	**p<0.01 F=7.35**	**p<0.01 F=2.33**	**p<0.01 F=4.74**
Huynh-Feldt epsilon:	1.05	0.86	1.05
Greenhouse-Geisser epsilon:	0.99	0.83	0.99
Box's conservative epsilon:	0.50	0.50	0.50

Table n°9 shows the distribution of subjects on bacterial concentration in saliva at the beginning and at the end of chewing period. While at the baseline (T_0) the total sample presented a bacterial concentration $>10^5$ CFU/ ml saliva, at the end of the experimental period, it is evident the reduction of bacterial count, especially in the green and blue group ($p<0.01$), and in the yellow group ($p=0.01$).

Table 9. Sample distribution according to concentration of oral bacteria at the beginning and at the end of the experimental period.

GREEN GROUP	**CRT Bacteria** *>10^5 CFU/ ml saliva*	**CRT Bacteria** *<10^5 CFU/ ml saliva*
T_0	100%	0
T_2	48.65%	51.35%
$\chi^2_{(1)}$ for trend of odds=25.22		***p<0.01***

BLUE GROUP	**CRT Bacteria** *>10^5 CFU/ml saliva*	**CRT Bacteria** *<10^5 CFU/ ml saliva*
T_0	100%	0
T_2	70.27%	29.73%
$\chi^2_{(1)}$ for trend of odds=39.54		***p<0.01***

YELLOW GROUP	**CRT Bacteria** *>10^5 CFU/ml saliva*	**CRT Bacteria** *<10^5 CFU/ml saliva*
T_0	100%	0
T_2	69.44%	30.56%
$\chi^2_{(1)}$ for trend of odds=12.80		***p=0.01***

Considering the new bacterial counts in T_2, a chi-squared pearson test, displayed in table n°10, shows a statically significant difference between the three groups ($p<0.01$).

Table 10. A chi-squared pearson test and sample distribution at T_2 of bacterial concentrations according to the different group membership.

GROUP	**CRT Bacteria** *$>10^5$ CFU/ml saliva*	**CRT Bacteria** *$<10^5$ CFU/ml saliva*	**Total**
Green	48.65%	51.35%	100%
Blue	29.73%	70.27%	100%
Yellow	69.44%	30.56%	100%
$\chi^2_{(1)}=31.38$			***p<0.01***

The non parametric test for trend across the three groups, showed in the table n°11, highlights a significant statistical trend for the moderate caries across all the three groups ($p>|z|<0.05$). The overall decrease of caries lesions was due to the dental treatments carried out by 47.47% of the sample (as shown in table n°12).

Table 11. Non parametric test for trend of caries lesions across the three groups.

	ICDAS 1-2 *n(SE)*	**ICDAS 3-4** *n(SE)*	**ICDAS 5-6** *n(SE)*
Green Group			
T_0	28(4.88)	55(5.29)	5(1.91)
T_1	18(4.67)	40(6.15)	5(1.91)
T_2	15(4.40)	39(6.24)	4(2.11)
	Z=-0.79 **p**>|z|=0.43	**Z**=-2.15 **p**>|z |=0.03	**Z**=0.00 **p**>|z |=1.00
Blue Group			
T_0	20(5.47)	60(5.24)	2(1.39)
T_1	20(5.47)	38(5.64)	0
T_2	20(5.47)	34(5.80)	0
	Z=0.00 **p**>|z |=1.00	**Z**=-3.19 **p**>|z |<0.01	**Z**=-1.74 **p**>|z |=0.08
Yellow Group			
T_0	16(4.17)	57(6.15)	5(2.10)
T_1	11(3.15)	43(6.70)	4(1.91)
T_2	11(3.15)	36(6.57)	4(1.91)
	Z=-0.81 **p**>|z |=0.41	**Z**=-2.63 **p**>|z |=0.01	**Z**=-0.36 **p**>|z |=0.72

Table 12. Sample distribution among the three groups, according to the execution or not of dental treatments during the experimental period.

GROUP	**No dental treatments** (**%** *of subjects*)	**Dental treatments** (**%** *of subjects*)
Green	59.46	40.54
Blue	48.65	51.35
Yellow	50.00	50.00
Total	52.73%	47.27%
$\chi^2_{(2)}$=3.08		***p=0.21***

DISCUSSION

In spite of the importance of epidemiological studies in adults, only few studies on the caries situation of Italian adults had been published (Calderara *et al*., 1992; Senna *et al*., 2005; Campus *et al*., 2011), reporting a high caries figure. In the present survey almost the totality of the sample showed caries experience (paper I). In more than half of the sample a carious lesion was recorded. In Italy, oral health policies are mainly focused on children and the oral health care services for adults are limited. Dental services are one of the weaknesses of the Italian national health system, therefore, an accurate assessment of caries risk would allow a better use of limited resources. Cariogram might represent a helpful tool to assess caries risk before the development of disease (Campus *et al*., 2012). A simplified Cariogram, with the exclusion of two risk factors, may be used in clinical practice, when a full inclusion of risk factors is not achievable (Lee *et al*., 2013). Few information exists on the performance of Cariogram in adult individuals, so the aim of the paper II was to describe the relation among caries status, background variables and caries risk calculated using Cariogram. More than two-thirds of the sample showed a medium risk and most of the remaining sample, a high risk of future disease development. These findings showed a statically significant correlation with the Caries status and the number of missing teeth due to caries, measured using ICDAS criteria. Two distal factors, smoking and employment categorization, resulted statistically significant associated to caries figures measured using merged ICDAS index (paper I). Smoking is a risk factor for general health as well as for oral health, ranging from the staining of teeth to oral cancer. In heavy smokers (>10 cigarettes/day), a higher caries prevalence respect to no-smokers was reported (Campus *et al*., 2011). Among background variables, educational level, smoking habit, frequency of toothbrushing and frequency of dental check-ups showed a statically significant association with Cariogram risk groups (paper II). In particular, the educational level is one of the most frequently used socioeconomic indicators: lower educational level has been statistically associated with greater prevalence of dental caries (Aranibar *et al*., 2014). In this perspective, the present investigation confirms that the most prevalent oral disease, dental caries, is influenced by distal risk factors, such as a low level of education and employment, variables that are also strongly related each other (Laaksonen *et al*., 2005).

The finding of a statistical significant association among Cariogram score and socioeconomic and behavioural variables is quite new and interesting, since it highlights that even if the Cariogram model includes proximal risk factors for caries almost exclusively, distal risk factors, such as smoking and educational level, are strongly correlated with the risk assessed through the Cariogram (Costa *et al.,* 2012). According to the results (paper II), it is also possible to suppose that people who have no job, can have more caries lesions and can lose more teeth due to caries, not having the economic resources to receive a dental treatment, as in Italy the public dental services for adults are very limited and dental care have to be paid directly by the patient.

The papers I and II have the typical limitation of a cross-sectional survey. A limit could be related to the non-generalizability of the results. Although the number of participants was high, it was not possible to elucidate whether the answers to the questionnaire items were not affected by biases. Furthermore, the studied population belonged to the medium-low socio-economic group, living in an area with a low mean income per capita (Bratthall *et al*., 2005). It is possible to suppose that in a population with different socio-economic and behavioural variables, the distinct risk factors may have a different influence on the caries disease.

In the papers III and IV, 271 subjects, presenting a high caries risk, were enrolled in a clinical trial in order to evaluate the effect of sugar-free chewing gum on plaque pH, on bacterial concentration in saliva and on the development of caries lesions after a chewing period of a year. The main finding of the present RCT was that the use of chewing gum for a year improves the oral status, controlling the plaque acidogenicity and cariogenicity.

Previous studies support some results of this study. The most recent systematic reviews have concluded xylitol- and polyol-containing chewing gums and/or candies and lozenges have caries-preventive effects when used routinely, and should be included in the armamentarium for caries prevention, noting that the effect may be primarily due to saliva stimulation (Deshpande and Jadad, 2008; Fontana *et al*., 2012). Shu *et al.* (2007), who also studied the effect of two kinds of gums on dental plaque pH, observed that compared with the baseline, chewing either tea polyphenol gum or sugar-free gum could increase the plaque pH recovery due to the sucrose rinsing challenge and it could maintain the dental plaque pH above the resting value. Thabuis *et al.* (2013) in their study also compared two different kinds of chewing gums and concluded that sugar-free chewing gum sweetened with either maltitol or xylitol can similarly reduce plaque acidogenicity compared to gum base through a decrease in oral bacteria presence. These results are due to the essential role of saliva, which is particularly stimulated through chewing gum use. Salivary buffers could reserve the low pH by neutralizing the acids produced by cariogenic organism. The American Dental Association's (ADA, 2011) meta-analysis of polyol studies that recorded caries in permanent teeth indicated that there was a statistically significant reduction in caries with the use of sucrose-free polyol gums compared with no gum (PF = 39%).

However, is possible to underline some limits of the study design. First of all, the study population belonged to an age range, in which the habit to chew daily chewing gum is not common, so it was difficult to find immediately a complete compliance. Secondly, adults aged more than 40 years were frequently bearer of removable prosthesis or crowns, and also presented TMJ disorders. It is established that chewing more than 3 hours a day would cause TMD symptoms of arthralgia and myofascial pain (Correia *et al*., 2014). All these aspects had been considered during the enrolment, which lasted about a year due to the difficulty to find subjects presenting all the inclusion criteria and the willingness to participate in the study. On the third hand, it was uneasy to obligate subjects not to go to dentist for a year, after becoming aware of the presence of caries at the first clinical examination.

An important issue, therefore, was whether or not the selected study group was representative for the general population of that age group.

The observed absence of new caries lesions after a year could be ascribed to salivary stimulation throughout the chewing process, the lack of sucrose and the inability of bacteria to metabolize polyols into acids, as depicted by Mickenautsch *et al.* (2007). It is already evident that in a moderate caries population practicing normal oral hygiene, including the use of fluoride dentifrices, an after-meal gum-chewing regimen can significantly reduce the rate of caries development (Szöke *et al*., 2005). Therefore, it has a great importance, taking into consideration all heavy expenses for dental treatment, which in many countries, as in Italy, are not covered (or covered only in part) by governmental health care programs. Although the components of the chewing gum, used by the three different groups, are still unknown, it is already possible to support the use of sugar-free chewing gum as part of normal oral hygiene to prevent dental caries.

CONCLUSIONS

The main conclusions from this thesis are that:

- Smoking habits and employment categorization were distal variables statistically significant associated with caries status in Italian adults, while educational level was associated to the number of missing teeth due to caries.

- The Cariogram model was able to identify the caries-related factors in an adult population. A direct association among the risk categories from Cariogram, the caries status and some socio-behavioural variables was verify.

- A continuative use of chewing gum, promoting the flow of saliva and controlling plaque acidogenicity, provides an effective means for the prevention of caries disease.

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Distal risk factors associated to caries disease in adults -a cross-sectional survey.

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Running title
Caries experience and distal risk factors in adults

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ABSTRACT

Objectives: To evaluate in a sample of Italian adults (30-45 years) the association between caries disease, scored using International Caries Detection and Assessment System (ICDAS), and distal caries risk factors, both unalterable and immutable in a short period of time.

Materials and Methods: 480 subjects, with a mean age of 40.72±0.31 (SE), 52.86% males and 47.12% females, were examined using the ICDAS index codifying each subject with the highest ICDAS score. A simplified structured self-compiled questionnaire was submitted and data regarding distal variables were collected. Multinomial logistic regression models were performed using as the dependent variable: the merged ICDAS (), the numbers of missing teeth due to caries and presence and type of fillings.

Results: Following ICDAS, the sample was split in sound (36.46%), initial stage decay (5.63%), moderate stage (40.83) and extensive stage decay (17.08%). Smokers had a lower risk to have caries to an extensive stage (OR=1.40 $_{95\%}$CI=1.03–2.23 $p<0.01$). A high number of missing teeth were positively statistically significant associated with smoking (OR=2.41 $_{95\%}$CI=1.64–3.55 $p<0.01$), conversely a protective rule was played by the higher educational level (OR=0.69 $_{95\%}$CI=0.42–0.83 $p<0.01$).

Conclusion: Smoking habits and employment categorization were distal variables statistically significant associated with caries status in Italian adults, while educational level was associated to the number of missing teeth due to caries.

Key Words

Adults, caries experience, educational level, epidemiology, gender, ICDAS, smoking habits

INTRODUCTION

Risks to health do not occur in isolation. The sequence leading to the development of a disease may embrace both proximal and distal factors – the proximal ones act directly or almost directly to cause of a disease, while the distal ones work further back in the causal chain and act via a number of intermediary causes (1,2). Risk factors that lead to development of caries lesions in an individual on a particular extent are likely to have their origin in a multifactor chain of events that probably have begun years previously. It is essential that the whole causal chain need to be considered in the assessment of risks involved in caries development, including unalterable distal risk factors, as gender, race, educational level in adultoodh, and immutable in a short period distal risk factors as smoking habit or occupational status (3).

Although children are the primary recipients of caries preventive programs in Western countries, dental caries is still one of the primary causes of tooth loss among adults (4-6). Epidemiological studies on adults reported a decrease in caries experience during the final decades of the 20th century and the start of the 21st in several countries such as Australia, Brazil, Norway, Sweden and USA (5-10), but in the 2000s stagnation has been described (11). Only few studies in Italian adults on caries figures had been published (12-14), all these papers reported high caries prevalence data.

Understanding the risk factors of dental caries in adults, including in the analysis a range of proximal factors and/or distal that might be immutable for short and/or long period of time, could provide evidence for the formulation of new policies designed to reduce social and economic inequalities (1).

Cariogenic bacteria act directly as the main proximal factor with mutans streptococci playing a major role (15). The diet rich in fermentable sugars is another crucial proximal factor in caries development. Moreover socioeconomic and behavioural factors also influence the disease pattern (14-17). The relationship between Socio-Economic-Status (SES) indicators and health is likely to be related to the distal nature of the measure of SES used *i.e.* educational level or income. The relationship between SES and caries among adults is not as strong as is found for children. However, there are relatively few studies on this topic (18,19). Among the socioeconomic indicators, the educational level is one of the most frequently used: lower educational level has been statistically associated with greater prevalence of dental caries (18).

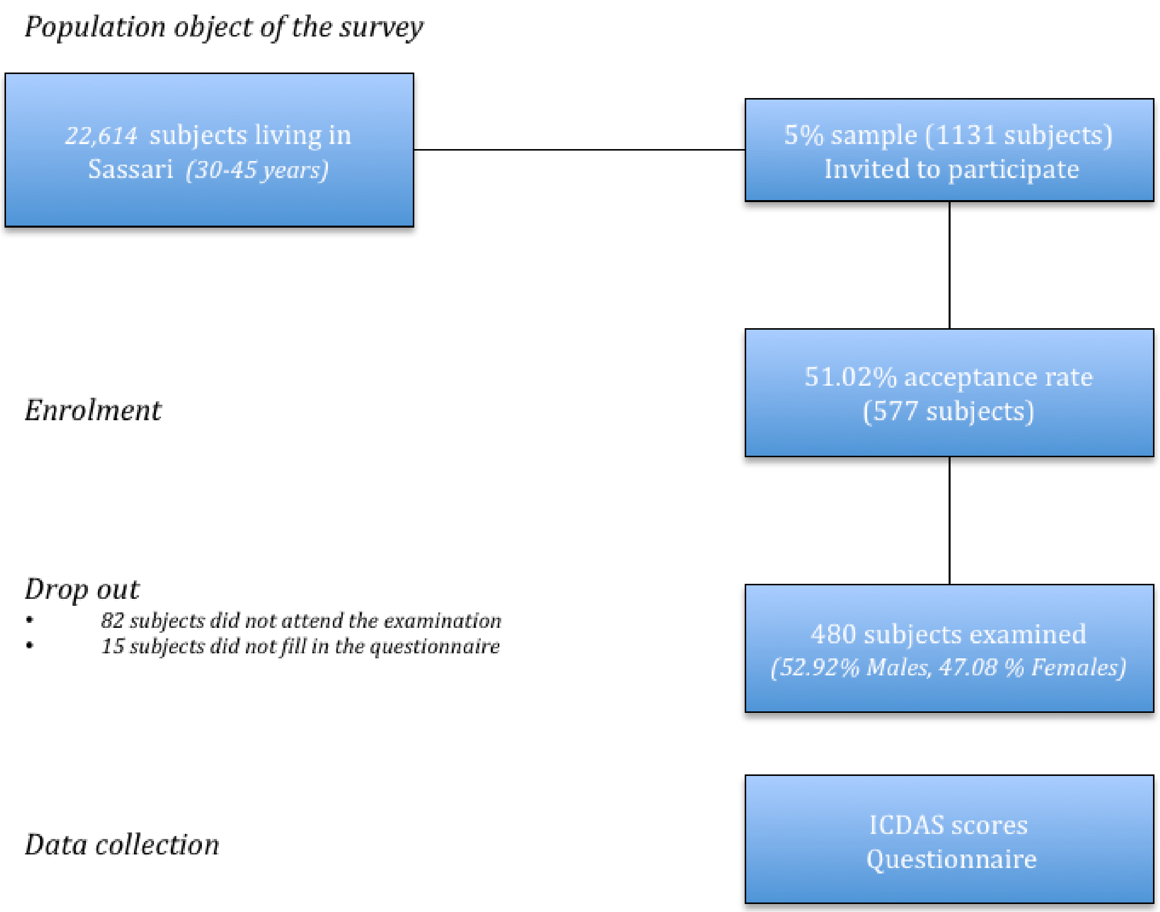
The aim of this cross-sectional survey was to establish whether caries severity scored using the ICDAS index, is associated with some distal and immutable for short and/or long period of time variables in Italian (Sardinian) adults aged 30-45 years.

MATERIALS AND METHODS

Subjects and Study Design

The present survey was carried out in Sassari (Sardinia, Italy), under the supervision of the WHO Collaborating Centre for Epidemiology and Community Dentistry of Milan (Italy) and lasted for 14 months, from September 2012 to October 2013. The study design was registered (2013_01_21_a) by the Sassari local office of the National Bioethics Committee. Data from the Italian National Institute for Statistics (20) for 2011 gave the number of 30-45 year-olds living in the town as 22,614. Power analysis was performed using G*Power 3.1.3 for Apple using logistic regression with an odds ratio of 1.5, an error probability of 0.04 and the total sample size was set at 432. A letter explaining the purpose of the study with an informed consent was mailed, in collaboration with the municipal electoral registry office, to 5% (1131 subjects) of the age considered population (30-45 years) living in Sassari. More then fifty percent, 577 subjects (51.02% acceptance rate) accepted to participate; 82 did not attend the examination and 15 did not fill in completely the questionnaire. Overall 480 subjects, 52.92% males and 47.08% females, (mean age± Standard Error 40.72±0.33) were examined

Figure 1. Flow chart of the design of the survey.



Caries registration

A benchmark examiner (GCampus) trained and calibrated one examiner (GCarta) that performed all dental screenings. Baseline training consisted in one-day (6 hours) theoretical course, followed by examination of 54 extracted teeth plus a session of 120 photographs of extracted teeth. Two days after the theoretical curse a clinical trial involving examination of fifty-five adults subjects was performed. The subjects were re-examined after 72 hours. Inter-examiner reliability with the "benchmarck" (GCampus) was evaluated using fixed-effects analysis of variance. Intra-examiner reproducibility was assessed as the percentage of agreement using Cohen's kappa statistic (21). Good inter-examiner reliability was found for the merged ICDAS index 1, with no significant difference from benchmark values ($p = 0.15$) and a low mean square of error (0.47). Intra-examiner reliability was also high (Cohen's Kappa statistic = 0.88).

Subjects were examined in the Dental Institute of the University of Sassari, after a professional teeth cleaning, sitting on a dental chair, using a mouth mirror and a Community Periodontal Index probe (approved by the WHO) under optimal lighting; the teeth were dried if necessary. The ICDAS index allows registration of the decayed surfaces without bite-wing radiographs, and also the type of the filling, the teeth absents for caries or other reasons were registered (22).

Questionnaire (Construction and Development)

A simplified structured self-compiled questionnaire was submitted to the participants before the clinical examination. To guarantee the anonymous status, the questionnaire was printed on one side of a sheet, while the clinical chart was on the other side. The questionnaire was pre-tested for control of reliability and validity (14). It was highly structured and divided into three domains: (1) personal data (*i.e.* age, gender, educational level, employment categorization), (2) life-style behaviour (*i.e.* smoking and dietary habits), (3) oral health behaviour (*i.e.* tooth brushing, frequency of dental check-ups). The following distal variables were selected: gender, smoking habits, educational level and employment categories.

Statistical Analyses

Data from the dental examination and questionnaire were entered in the FileMaker Pro 9.0 Runtime database and then exported to Excel® Microsoft spread sheet.

Subjects were categorized according to maximum ICDAS score as the merged ICDAS was calculated: ICDAS (0) as sound, ICDAS (1-2) initial caries stage referred to caries involving only the enamel with no evidence of dentine involved, ICDAS (4-6) moderate caries stage referred to caries involving breakdown of enamel or underlying dentine shadow and ICDAS (5-6) extensive stage decay with visible dentine involved. The number of missing teeth due to caries was recorded; the presence of fillings was categorized as follows: no fillings, mainly tooth coloured restoration, mainly amalgam restoration and presence at least of one crown.

The educational level was classified as: primary school, secondary school and university. The employment was categorized using the Italian National Institute for Statistics (20). Smoking habits were coded following the WHO definition for tobacco user modified by the authors (14), as non-smoker and smoker that had started to smoke more than 3 years.

Descriptive statistics, cross-tabs and linear trend were calculated among caries severity (merged ICDAS) number of extracted teeth and number/type of restoration, and gender, smoking habit, educational level and employment categories. Next, multinomial logistic regression models were performed using as the dependent variable: merged ICDAS scores, numbers of missing teeth due to caries, and presence and type of fillings. The Akaike information criterion (AIC) was used to measure the goodness of fit of the statistical model. The possible modifying effects of covariates on the outcomes were tested by an interaction model (likelihood ratio test statistic). Multicollinearity might sometimes cause problems with regression results. This problem was solved using the DFBETA command in STATA, dropping the information that have too much influence on the regression line. Anyway, after the data elaboration, no statistical significant multicollinearity was observed and so it was decided to report findings without outliers (24). All data were analysed using the software STATA® (Mac version 10.1). For all analyses, the statistical significance level was set at α=0.05.

RESULTS

Following ICDAS, the sample was split in sound subjects (36.46%), initial stage decay (5.63%), moderate stage (40.83) and at extensive stage decay (17.08%) (Table 1). More than 50% of the sample had no missing teeth due to caries (Table 2), less than 5% had no fillings, 33.29% had mainly tooth coloured fillings, 29.58% of the sample mainly amalgam fillings, finally 32.71% presented at least one crown (Table 3). Only 5.02% of the subjects had no caries experience (*data not in table*).

More than one quarter of the subjects (26.67%) reported to have started smoking more than three years before the enrolment in the survey. According to the educational level, almost 50% of the subjects (47.29%) had a secondary school graduation and 34.79% had university degree. The sample was stratified following the employment categorization as follows: 16.31% unemployment, 24.28% housewife, 43.10% technician/clerk and finally 16.31% professional (*data not in table*).

Tables 1, 2 and 3 report the association between the distal variables (gender, smoking habits, educational level and employment categorization) and the caries severity (merged ICDAS scores, number of missing teeth due to caries disease and number and type of fillings). A statistical linear trend ($z=2.37$ $p=0.02$) was observed between ICDAS score and smoking habits as the percentage of smokers increases as the caries severity stages increase (Table 1).

Table 1. Sample distribution (number and percentage in column) following caries severity (sound, initial stage, moderate stage and extensive stage) according to gender, smoking habits, educational level and employment categorization.. Linear trend was calculated for each variable.

	Sound (ICDAS=0) *n° (%)*	Initial (ICDAS= 1/2) *n° (%)*	Moderate (ICDAS=3/4) *n° (%)*	Extensive (ICDAS>4) *n° (%)*
Total sample	175 (36.46)	27 (5.63)	196 (40.83)	82 (17.08)
Gender				
Males	99 (56.57)	17 (62.96)	97 (49.49)	41 (50.00)
Females	76 (43.43)	10 (37.04)	99 (50.51)	41 (50.00)
z=1.43 p=0.15				
Smoking habits				
Smoking	57 (32.57)	7 (25.93)	48 (24.49)	16 (19.52)
No smoking	118 (67.43)	20 (74.07)	148 (75.51)	66 (80.48)
z=2.37 p=0.02				
Educational level				
Primary school	28 (16.00)	3 (11.11)	34 (17.35)	21 (23.86)
Secondary school	90 (51.43)	10 (37.04)	94 (47.96)	33 (40.24)
University degree	57 (32.57)	14 (51.85)	68 (34.69)	28 (34.15)
z=-0.56 p=0.57				
Employment categorization				
Unemployed	25 (14.29)	7 (25.93)	31 (15.82)	15 (18.29)
Housewife	37 (21.14)	2 (7.41)	49 (25.00)	28 (34.15)
Technician/clerk	85 (48.57)	11 (40.73)	78 (39.79)	33 (40.24)
Professional	28 (16.00)	7 (25.93)	38 (19.39)	6 (7.32)
z=-1.98 p=0.05				

Table 2. Sample distribution (number and percentage in column) following presence of missing teeth due to caries according to gender, smoking habits, educational level and employment categorization. Linear trend was calculated for each variable.

	No missing teeth *n° (%)*	1/2 missing teeth *n° (%)*	3/5 missing teeth *n° (%)*	>5 missing teeth *n° (%)*
Total sample	250 (52.08)	115 (23.96)	80 (16.67)	35 (7.29)
Gender				
Males	143 (57.20)	58 (50.43)	32 (40.00)	21 (60.00)
Females	107 (42.80)	57 (49.57)	48 (60.00)	14 (40.00)
z= 0.10 p=0.91				
Smoking habits				
Smoking	48 (19.20)	30 (26.08)	30 (38.50)	20 (56.14)
No smoking	202 (80.80)	85 (73.91)	50 (62.50)	15 (43.86)
z=3.21 p<0.01				
Educational level				
Primary school	34 (13.60)	16 (13.91)	25 (31.25)	11 (31.43)
Secondary school	107 (42.80)	69 (60.00)	33 (41.25)	18 (51.44)
University degree	109 (43.60)	30 (26.09)	22 (27.50)	6 (17.43)
z=2.08 p=0.04				
Employment categorization				
Unemployed	46 (18.40)	16 (13.91)	13 (16.25)	4 (11.43)
Housewife	57 (22.80)	32 (27.83)	17 (21.25)	10 (28.57)
Technician/clerk	106 (42.40)	49 (42.61)	40 (50.00)	18 (51.43)
Professional	41 (16.40)	18 (15.65)	10 (12.50)	3 (8.57)
z= 0.91 p=0.36				

Caries severity was statistically significantly associated with employment categorization (z=1.98 p=0.05): the number of subjects that reported to have a skilled were significantly higher in those with an extensive stage of the disease. The number of missing teeth due to caries disease (Table 2) was statistically significant associated to gender (p=0.04), smoking habit (p<0.01) and educational level (p<0.01). Both smoking habits and educational level showed a statistically significant trend respect to the presence of missing teeth due to caries (p<0.01 and p=0.04, respectively).

The presence and/or the type of fillings (Table 3) were statistically significantly associated with gender (p=0.01) and educational level (p=0.02). No statistical significant linear trends were observed among presence and/or type of fillings and distal risk factors evaluated.

Table 3. Sample distribution (number and percentage in column) following presence of fillings and/or type according to gender, smoking habits, educational level and employment categorization. Linear trend was calculated for each variable.

	No restoration *n° (%)*	Mainly tooth-coloured *n° (%)*	Mainly amalgam *n° (%)*	At least one crown *n° (%)*
Total sample	21 (4.38)	161 (33.54)	140 (29.17)	158 (32.92)
Gender				
Males	11 (52.38)	81 (50.31)	91 (65.00)	71 (34.94)
Females	10 (47.62)	80 (49.69)	49 (35.00)	87 (55.06)
z= 0.91 p=0.36				
Smoking habits				
Smoking	3 (14.29)	42 (26.09)	34 (24.59)	49 (31.01)
No smoking	18 (85.71)	119 (73.91)	106 (75.41)	109 (68.99)
z= 1.47 p=0.14				
Educational level				
Primary school	3 (14.28)	23 (14.29)	24 (17.14)	36 (22.78)
Secondary school	14 (66.67)	80 (49.69)	69 (49.29)	64 (40.51)
University degree	4 (19.05)	58 (36.02)	47 (33.57)	58 (36.71)
z= -0.33 p=0.74				
Employment categorization				
Unemployed	1 (4.76)	24 (14.91)	24 (17.14)	29 (18.35)
Housewife	2 (9.53)	41 (25.47)	32 (22.86)	41 (25.95)
Technician/clerk	15 (71.43)	67 (41.61)	61 (43.57)	64 (40.51)
Professional	3 (14.28)	29 (18.01)	23 (16.43)	24 (15.19)
z=- 1.64 p=0.10				

Ordinal logistic regressions were carried out to study in deep the association between distal factors and caries data (Table 4). In the first model caries severity (ICDAS score) was used as dependent variable; smokers had a lower risk to have caries to an extensive stage (OR=1.40 $_{95\%}$CI=1.03–2.23 p<0.01). An effect modifier between smoking habit and educational level was observed as smoking was highly represented in the lower educational group (*data not in table*). The second model was run using the number of missing teeth due to caries as dependent; a protective role was played by the higher educational level -university degree (OR=0.69 $_{95\%}$CI=0.42–0.83 p<0.01). The presence and/or the type of fillings were the dependent variable of the final logistic model: the highest educational level, university degree, acted as a protective factor (OR=0.88 $_{95\%}$CI=0.74 –1.01 p=0.05). The number of missing teeth was also statistically positively associated with the number of crowns observed in the sample (OR=1.08 $_{95\%}$CI=1.03–1.14 p<0.01), with the highest educational level (university degree) as a protective factor (OR=0.56 $_{95\%}$CI=0.43–0.71 p<0.01). Moreover a high percentage of subjects (30.42%) showed missing teeth and no crowns (*data not in table*).

Table 4. Ordinal logistic regression estimation

Caries severity

Number of observations =480 Log Likelihood= -568.86 p=0.01

Covariates	OR (SE)	p-value	95% CI
Gender (*ref. females*)	1.17 (0.21)	0.35	0.84 – 1.66
Smoking (*ref. smokers*)	0.60 (0.11)	<0.01	0.41 – 0.88
Educational level (*ref. university degree*)	0.96 (0.12)	0.74	0.74 – 1.23
Employment categorization (*ref. professional*)	0.84 (0.00)	0.08	0.69 – 1.02

Missing teeth due to caries

Number of observations =480 Log Likelihood= -561.15 p<0.01

Covariates	OR (SE)	p-value	95% CI
Gender (*ref. females*)	1.14 (0.21)	0.45	0.81 – 1.61
Smoking (*ref. smokers*)	0.89 (0.12)	0.48	0.69 – 1.16
Educational level (*ref. university degree*)	0.69 (0.10)	<0.01	0.42 – 0.83
Employment categorization (*ref. professional*)	1.12 (0.12)	0.24	0.92 – 1.38

Presence of filling and type

Number of observations =480 Log Likelihood= -587.42 p<0.01

Covariates	OR (SE)	p-value	95% CI
Gender (*ref. females*)	1.14 (0.20)	0.43	0.82 – 1.60
Smoking (*ref. smokers*)	1.25 (0.24)	0.24	0.86 – 1.82
Educational level (*ref. university degree*)	0.88 (0.08)	0.05	0.74 – 1.01
Employment categorization (*ref. professional*)	0.98 (0.13)	0.90	0.77 – 1.27

DISCUSSION

The present cross-sectional survey had verified that caries severity, scored using the ICDAS index, and the number of missing teeth due to caries are associated with some distal and immutable for short and/or long period of time factors in a population of Italian adults. Two of the four distal factors investigated, smoking habits and employment categorization, were statistically significant associated with caries figures and always smoking and educational level were associated with teeth lost.

In spite of the importance of epidemiological studies in adults, only few studies on the caries situation of Italian adults had been published (12-14), reporting a high caries figure. In the present survey almost the totality of the sample showed caries experience. In more than half of the sample a carious lesion was recorded.. Previous reports on similar study groups (4-9) observed a decrease in caries experience among adults and elderly starting from the 1970s, but in the 2000s stagnation was observed (11).

In Italy, oral health policies are mainly focused on children and the oral health care services for adults are limited. Dental services are one of the weaknesses of the Italian national health system. The primary dental health service is based on private health care providers; thus, oral care is mainly financed by direct payment or, to a lesser extent, through private insurance schemes (30).

As mentioned above two distal factors, smoking and employment categorization, resulted statistically significant associated to caries figures measured using merged ICDAS index. Smoking is a risk factor for general health as well as for oral health, ranging from the staining of teeth to oral cancer. In heavy smokers (>10 cigarettes/day), a higher caries prevalence respect to no-smokers was reported (14). In the present sample smoking habit resulted statistically related with caries severity both in bivariate and multivariate statistics; nevertheless data from this survey show an association between caries severity and smoke opposite to what might be expected: the number of smokers decreases with increasing the severity of dental caries. Smoke seems to plays as protective factor. One possible explanation for this result could be related to the small number of smokers examined (less than one fifth of the sample). In addition, smoking has been evaluated only in terms of time, assessing whether the subject smoked at least 3 years before the examination and not in terms of number of cigarettes smoked per day, a factor that could explain a result in contradiction with the literature data. A different trend was observed between smoking habit and number of missing teeth due to caries. Data show that smokers have a higher number of missing teeth due to caries. This finding is in line with data from young Finnish smokers, that were found to be more likely to lose teeth than no smokers (31). Smoking is associated with poor health behaviour, and it might also reflect a negative attitude towards the preservation of natural teeth, tooth extraction can be considered as a form of treatment.

In the present survey an association between the employment categorization and the caries severity was found: subjects that reported to have a better employment (professional) showed significantly lower extensive stage of the disease, showing that a more skilled employment plays a protective effect on the severity of caries.

The level of education is an individual-level indicator that has a strong correlation with health. The knowledge, personal and social skills provided through education may better endow individuals to access, maintain and improve their own health [(16]). Educational level is a distal and immutable variable as it remains stable after the school years and during adulthood and may thus have a constant impact on health. Evidence of the association between dental caries in adults and socioeconomic indicators showed that educational level is one of the most frequently used variable: the lower the educational level the greater the severity of dental caries (18). In the present survey, the education level was strongly associated with the number of missing teeth. Data from the literature show that among dentate individuals, there is a larger number of remaining teeth in people with a higher level of education than in those of same age group with a lower level of education (28,29). In the present survey, subjects with the higher educational level (university degree) showed lower number of missing teeth due to caries than subjects with a lower degree of schooling. Education level was not associated with the caries severity and presence and/or type of fillings. This finding was confirmed by a previous study in a Swedish population, showing no significant differences between individuals with different schooling regarding fillings presence (29). Despite the different welfare regarding public dental services between Italy and Sweden, similar results were obtained. In Italy the public dental services for adults are very limited and dental care have to be paid directly by the patient; people with a low educational level often have also a less remunerative employment and more difficulties to pay for care.. Gender is considered a key demographic indicator and it is related to oral health (7). Nevertheless, in the present survey gender was not associated to any clinical variable considered (caries severity, missing teeth and presence and type of filling). This result is consistent with the finding of other studies (32) where the two genders showed no statistically significant differences in teeth loss, but in contrast with others (33).

Proximal and distal risk factors related to several chronic diseases are common to most oral diseases, than a common approach regarding risk factor is desirable for an effective preventive approach (1). In this perspective, the present investigation confirms that the most prevalent oral disease, dental caries, is influenced by distal risk factors, such as a low level of education and employment, variables that are also strongly related each other (27).

The present study has the typical limitation of a cross-sectional survey. Although the number of participants was high, it was not possible to elucidate whether the answers to the questionnaire items were not affected by biases.

In conclusion, the findings of the present investigation provide evidence of an association between distal and immutable for short and/or long period of time variables and dental caries in adults.

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FC was involved in the data collection and analysis

SS was involved in the data acquisition

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Conflict of interest. The authors declare that they have no competing interests.

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II

Caries-risk profiles in Italian adults using Cariogram and ICDAS.

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ABSTRACT

The aim of this study is to examine the correlation among socio-behavioural factors, caries status and caries risk, calculated through Cariogram, in an adult population. 480 subjects (mean age 40.73, SE ± 0.33) randomly selected from the municipal electoral registry, consented to participate in the survey. Subjects were examined and the ICDAS index was registered. A highly structured questionnaire was submitted to investigate: (1) personal data (i.e. age, gender, educational level, job categorization etc.), (2) life-style behaviour (i.e. smoking and dietary habits), (3) oral health behaviour (i.e. tooth brushing, use of fluoride, dental check-ups' frequency). An evaluation of the mutans streptococci concentration in saliva was also performed. Information on caries-related factors was entered into the Cariogram, in order to generate for each subject an individual caries risk profile. Multinomial logistic regression was performed using Cariogram levels as the dependent variable. The possible correlated variables were analysed using the principal component analysis (PCA). Considering ICDAS scores, 5.62% of the sample had at least an initial caries lesion (ICDAS=1-2), whereas 40.83% of the sample presented, at least, one moderate decay (ICDAS=3-4), and 17.08% a severe one (ICDAS=5-6). Caries lesion at ICDAS level 5-6 and more than 5 missing teeth were statistically associated with Cariogram scores (OR= 2.36 95%CI=1.83–3.03 and OR=1.43 $_{95\%}$CI=1.13 –1.82, respectively). Results suggest that the Cariogram model was able to identify the caries-related factors in an adult population. A direct association among the risk categories from Cariogram, the caries status and some socio-behavioural variables was verify.

INTRODUCTION

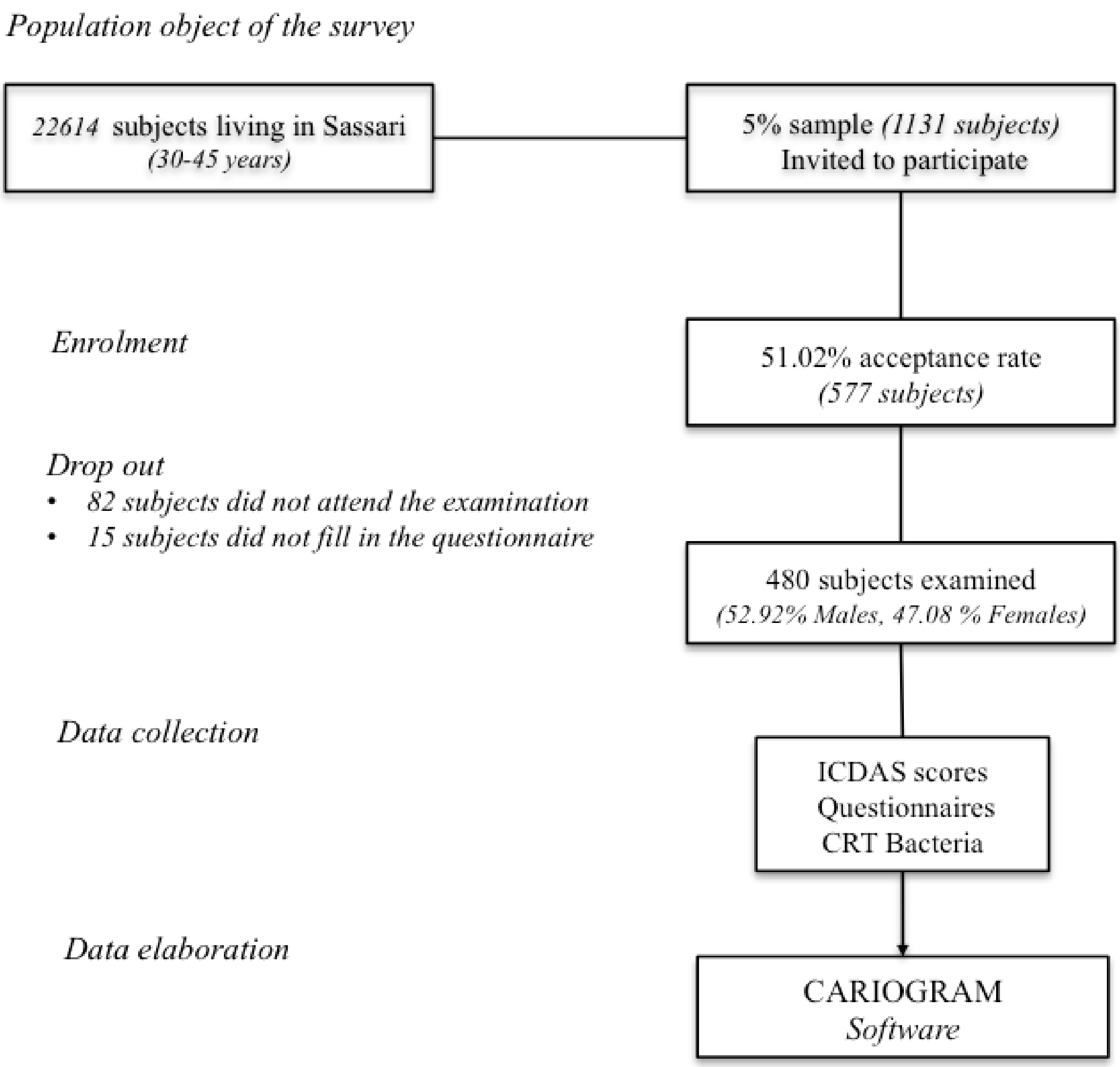
Although children are the primary recipients of caries-prevention programs in the Western world, dental caries is still one of the primary causes of tooth loss among adults.[1-3] Among the clinical risk factors, mutans streptococci play an important role in the development of dental caries. However, the focus on the non-biological determinants of the disease (socio-behavioural and environmental factors) is essential.[4] Lower educational-level has resulted statistically associated with greater severity of dental caries in adults.[5,6] Among the prediction models available, the software "Cariogram" has been developed and validated to illustrate graphically the chance of an individual of avoiding caries in the next future.[7,8]
Common outcomes used in studying the epidemiology of dental caries, such as DMFT and DMFS indices may be insufficient for investigating the risk factors that lead to specific patterns of decay.[9] The International Caries Detection Assessment System (ICDAS) based on best available evidence for detecting early and later stage caries severity, should lead to the acquisition of better quality information in order to support decision-making at both individual and public health levels.[10]
The aim of this study was to examine the correlation among socio-behavioural factors, caries status and caries risk, calculated through Cariogram, in an adult population.

METHODS

Study design
The present study was carried out in Sassari (Sardinia, Italy) and lasted 14 months, from September 2012 to October 2013. The study design was registered (2013_01_21_a) by the Sassari local office of the National Bioethics Committee.
Data from the Italian National Institute for Statistics for 2011 gave the number of 35-45 year-olds in the town of Sassari as 22,614. Power analysis was performed using G*Power 3.1.3 for Apple using logistic regression, with an odds ratio of 1.5, an error probability of 0.04 and the total sample size was set at 432. A letter explaining the purpose of the study and the informed consent were distributed to 5% (1131 subjects) of the age group considered living in Sassari through the collaboration of the municipal electoral registry

office; 577 subjects (51.02% acceptance rate) accepted to participate; 82 did not attend the examination and 15 did not fill in completely the questionnaire (figure 1).

Figure 1. Flow-chart of the survey



Questionnaire

A simplified structured self-compiled questionnaire together with the informed consent was submitted to the participants. The questionnaire was pre-tested for control of reliability and validity[5]. It was highly structured and divided into different domains: (1) personal data (*i.e.* age, gender, educational level, job categorization etc.), (2) life-style behaviour (*i.e.* smoking and dietary habits), (3) oral health behaviour (*i.e.* tooth brushing, use of fluoride, frequency of dental check-ups).

Caries assessments

One calibrated examiner (GiC) performed all the dental screenings. Intra- and inter-examiner reliability was assessed before the beginning of the survey by examining and re-examining (after 72 h) fifty-five subsequent study participants. Inter-examiner reliability was evaluated using fixed-effects analysis of variance in comparison with benchmark values (GC). Intra-examiner reproducibility was assessed as the percentage of agreement using Cohen's kappa statistic.[11] Good inter-examiner reliability was found for the merged ICDAS score 1-2, 3-4 and 5-6, with no significant difference from benchmark values (p=0.15) and a low mean square of error (0.47). Intra-examiner reliability was also high, Cohen's Kappa statistic=0.88. Subjects were examined sitting on an ordinary chair, using a mouth mirror and a Community Periodontal Index probe under optimal lighting. Subjects were categorized according to maximum ICDAS score.

The Number of missing teeth due to caries was categorized as follow: no missing teeth, 1-2 missing teeth, 3-5 missing teeth and more than 5 missing teeth. The presence of fillings was classified in: no fillings, mainly tooth coloured restorations, mainly amalgam restorations and presence at least of one crown. Plaque index was assessed in the buccal surface using Silness and Löe's scale.

Microbiological evaluation

After the clinical examination, an evaluation of the mutans streptococci (MS) concentration in saliva was performed. No-stimulated whole saliva was collected over 150 s in sterile vials (Nunc, Kamstrup, Denmark). The samples were processed within 45 min after collection and the analysis was carried out using CRT BACTERIA (Ivoclar Vivadent AG, Liechtenstein). The presence of mutans streptococci over 10^5 CFU/ml suggests a high caries risk.

Caries risk assessment using Cariogram

Information based on caries-related factors was collected and entered into the Cariogram[5]. The following seven caries-related factors were included in the Cariogram software:[5]

1. Caries experience (from ICDAS index registered during the clinical examination);
2. Diet, contents (from questionnaires);
3. Diet, frequency (from questionnaires);
4. Salivary levels of mutans streptococci in saliva (from CRT Bacteria);
5. Plaque amount and grade of oral hygiene (from clinical examination)

6. Related general diseases (from questionnaires)
7. Fluoridation programme (from questionnaires)

A score 1 in all cases was assigned for clinical judgment in order to give a standard value.

The "Chance to avoid new caries in the future" was calculated and the subjects were divided in three groups: low risk (61-80%), medium risk (41-60%) and high risk (21-40%).

Statistical analyses

Descriptive statistics and cross-tabs were calculated to investigate the relationship between dental health and different risk factors. The reference for each variable was set as follows: Gender (male), Caries status (ICDAS 5-6), Type of dental treatment (crown), Number of missing teeth due to caries (more than five), Educational level (middle school degree), Occupational status (unemployed), Smoking habits (yes), Frequency of toothbrushing (once a day), Frequency of dental check-ups (at pain).

A chi-square test was conducted for each variable and level of caries risk measured by Cariogram. Multinomial logistic regression was performed using Cariogram levels as the dependent variable. The possible correlated variables were analysed using the principal component analysis (PCA).[11] The performances of Cariogram in predicting caries in the next future were evaluated by receiver operating characteristic (ROC) analysis.[18] p values<0.05 were considered as statistically significant. All data were analysed using the software STATA® (v10.1 for Macintosh).

RESULTS

The study population consisted of 480 subjects (mean age 40.73, SE±0.33), 52.92% males and 47.08% females. The caries prevalence in the sample was 63.54%. Considering ICDAS scores, 5.62% of the sample had, at least, an initial caries (ICDAS=1-2), whereas 40.83% of the sample presented, at least, one moderate decay (ICDAS=3-4), and 17.08% a severe one (ICDAS=5-6). About dental treatments, 4.39% of the sample did not have any sign of dental treatment, 33.31% had, at least, one tooth coloured restoration, 29.58% had an amalgam restoration and 32.72% presented, at least, one crown of different materials. Almost half of the sample (47.92%) had, at least, one missing tooth due to caries. The mean number of sound teeth registered in the sample was 24.83 (SE±0.16). Each subject presented 3.66 (SE±0.14) tooth coloured restorations, 2.96 (SE±0.13) amalgam restorations and 1.58 (SE±0.14) crowns. *(data not in table).*

Regarding questionnaires data analysis, 17.92% had middle school graduation, most of the subjects (47.29%) had high school graduation and a quite high percentage of subjects (34.79%) had university degree. 60.42% of the sample brushed their teeth three times a day. Only 26.7% of the subjects were smokers and most of them have been smoking for more than 5 years.

Relating to caries risk, the majority of the sample (78.12%) presented a medium caries risk (41-60% of chance to avoid new caries lesions in the future), 18.75% had a high caries risk (<41% of chance to avoid new caries lesions in the future) and 3.12% had a low caries risk (>60% of chance to avoid new caries lesions in the future).

Table 1 shows a chi-square test between the level of caries risk, calculated through Cariogram software, and background variables. A statically significant correlation (p<0.01) was evident between the caries risk and ICDAS score. The level of caries risk was also correlated in a statically significant way with the number of missing teeth due to caries (p=0.01), the Educational level (p=0.04), the Smoking habit (p=0.01), the Frequency of toothbrushing (p=0.02) and the Frequency of dental check-ups (p<0.01).

Table 1. Sample distribution following sample's caries risk level (low, medium and high) according to gender, caries status, educational level, occupational status and smoking habits.

<u>Gender</u>

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Males	8(1.66)	192(40.00)	54(11.25)
Females	7(1.46)	183(38.12)	36(7.50)

$\chi^2_{(2)}=2.26$ $p=0.32$

<u>Caries status</u>

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Caries-free	11(2.29)	156(32.50)	8(1.66)
Initial caries (ICDAS 1-2)	1(0.21)	24(5.00)	2(0.42)
Moderate caries (ICDAS 3-4)	3(0.62)	148(30.83)	45(9.37)
Severe caries (ICDAS 5-6)	0(0.00)	47(9.79)	35(7.29)

$\chi^2_{(6)}=65.13$ $p<0.01$

<u>Type of dental treatments</u>

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
No-treatments	0(0.00)	18(3.75)	3(0.62)
Tooth-coloured restorations	3(0.62)	126(26.25)	31(64.58)
Amalgam restorations	6(1.25)	112(23.33)	24(5.00)
Crowns	6(1.25)	119(24.79)	32(8.00)

$\chi^2_{(6)}=3.27$ $p=0.77$

<u>Number of missing teeth due to caries</u>

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
No-missing teeth	13(2.71)	201(41.87)	36(7.50)
1-2 missing teeth	2(0.42)	95(19.79)	18(3.75)
3-5 missing teeth	0(0.00)	54(11.25)	26(5.42)
>5 missing teeth	0(0.00)	25(5.21)	10(2.08)

$\chi^2_{(6)}=22.54$ $p=0.01$

<u>Educational level</u>

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Middle school degree	0(0.00)	67(13.96)	19(3.96)
High school degree	5(1.04)	185(38.54)	37(7.71)
University degree	10(2.08)	123(25.62)	34(7.08)

$\chi^2_{(4)}=9.91$ $p=0.04$

<u>Occupational status</u>

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Unemployed	1(0.21)	59(12.29)	18(3.75)
Housewife	6(1.25)	85(17.71)	25(5.21)
Technician/clerk	4(0.83)	168(35.00)	35(7.29)
Professional	4(0.83)	63(13.12)	12(2.50)

$\chi^2_{(6)}=7.15$ $p=0.31$

Smoking habits

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
No-smokers	15(3.12)	280(58.33)	57(11.87)
Smokers	0(0.00)	95(19.79)	33(6.87)

$\chi^2_{(2)}=10.40$ $p=0.01$

Frequency of toothbrushing

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Once a day	0(0.00)	5(1.25)	0(0.00)
Twice a day	2(0.41)	133(27.71)	48(10.00)
3 times a day	13(2.71)	236(49.16)	41(8.54)
4 times a day	0(0.00)	1(0.21)	0(0.00)

$\chi^2_{(6)}=14.47$ $p=0.02$

Frequency of dental check-ups

	Low Caries Risk *n° (%)*	Medium Caries Risk *n° (%)*	High Caries Risk *n° (%)*
Each 6 month	5(1.25)	46(8.54)	4(0.83)
Once a year	7(1.46)	132(27.50)	24(5.00)
Once 2 years	0(0.00)	101(21.04)	23(4.79)
At pain	3(0.62)	96(20.00)	39(8.12)

$\chi^2_{(6)}=25.04$ $p<0.01$

A multinomial logistic regression model was run for caries risk factors (table 2) and Cariogram score was used as dependent variable. The presence of caries lesions at ICDAS level 5-6 and the presence of more than 5 missing teeth were statistically associated with Cariogram score (OR= 2.36 $_{95\%}$CI=1.83–3.03 and OR=1.43 $_{95\%}$CI=1.13 –1.82, respectively).

Table 2. Multinomial logistic regression estimates for significantly associated caries risk covariates to Cariogram scores.

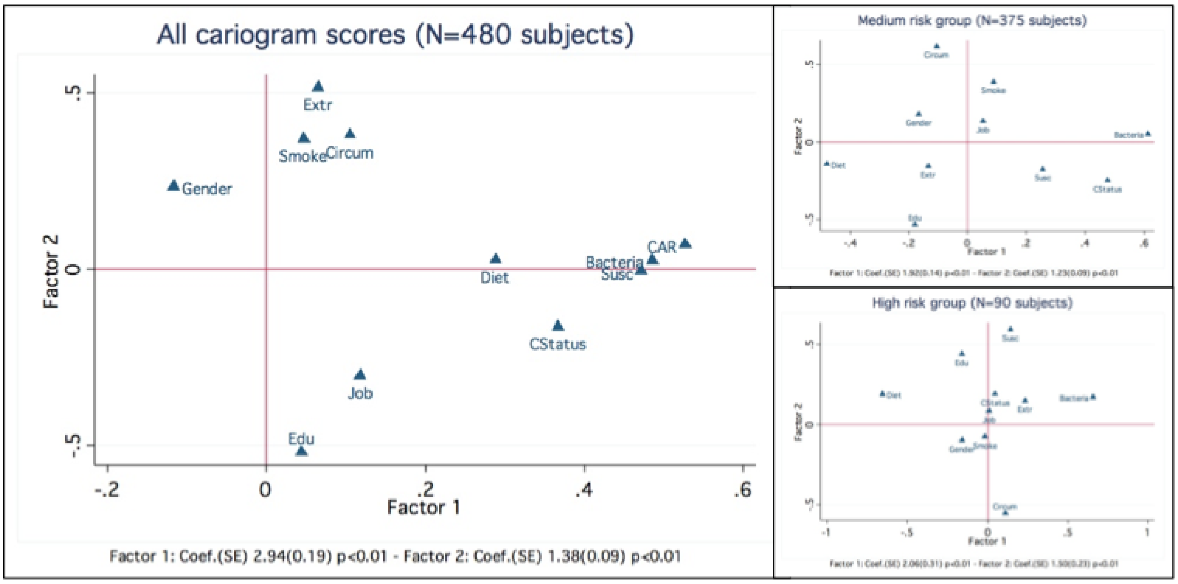
Number of observations =480   Log Likelihood= -248.33   p<0.01

Covariates	OR (SE)	p-value	95% CI
Caries status *(ICDAS 5-6)*	2.36 (0.30)	<0.01	1.83 – 3.03
Number of missing teeth *(>5 missing teeth)*	1.43 (0.17)	<0.01	1.13 – 1.82
Smoking habits *(Yes)*	1.28 (0.16)	0.36	0.76 – 2.14
Dental check-ups *(At pain)*	1.28 (0.16)	0.04	1.00 – 1.65
Tooth brushing frequency *(3 times a day)*	0.64 (0.15)	0.06	0.40 – 1.01

Principal Component Analysis (PCA) was performed on the data set, first on the total sample and in the two main groups stratified by Cariogram scores (caries medium-risk group and high-risk group); the low caries risk group was too small and PCA was not performed. In all PCA analysis the first two eigenvalues, obtained from distance matrix between groups, collectively account for more than 66% of the total variance (86.13%; 59.35 and 26.78%, respectively in the total sample; 52.47% and 21.15% respectively and 66.30% in the high-risk group 42.68% and 23.62% two eigenvalues respectively). Figure 2 displays the Orthogonal Rotation (varimax) of the first two principal coordinates in the total sample and in the two main groups stratified by Cariogram scores. In the total sample, the Cariogram score, the Bacteria and the Susceptibility sectors from Cariogram tend to form a separate cluster with a high goodness of fit (63.74); the Number of missing teeth due to caries, Smoking habits and Circumstances from Cariogram tend to form another cluster with a high goodness of fit (61.82); however, the Level of education, the Gender and the Job categorization were clearly separated from the other variables. Severe caries status (ICDAS 5-6), Number of missing teeth due to caries more than 5, unemployed, smokers and male tend to form a cluster with a medium high (0.50–0.75) goodness of fit in the high-risk group. In the medium risk-group no clear cluster was identified, all variables stand separated one from each other. Sensitivity and specificity between ICDAS scores and

Cariogram measured by ROC analysis were 0.85 and 0.87, respectively, so the gain in certainty was 1.71, while the area under the ROC curve was 0.92 (*data not table*).

Figure 2. Principal Component Analysis. Orthogonal rotation (varimax).



Legend: Extr= > 5 extracted teeth due to caries; Gender=male; Smoke =smoking habits; Circum=circumstances in Cariogram analysis, Diet= diet frequency and Diet content; Job= occupational status (unemployed); Edu= level of education (middle school degree); Bacteria= salivary mutans streptococci; Susc= susceptibility in Cariogram analysis; CAR= level of caries risk calculated through Cariogram; CStatus= caries status using ICDAS.

DISCUSSION

Since caries distribution is remarkably uneven, an accurate assessment of caries risk would allow a better use of limited resources.[13,14] Cariogram might represent a helpful tool to assess caries risk before the development of disease.[7] A simplified Cariogram, with the exclusion of two risk factors, may be used in clinical practice, when a full inclusion of risk factors is not achievable.[15] Few information exists on the performance of Cariogram in adult individuals.[14-16,18], so the aim of this paper was to describe the relation among caries status, background variables and caries risk calculated using Cariogram. More than two-thirds of the sample showed a medium risk and most of the remaining sample, a high risk of future disease development. These findings showed a statically significant correlation with the Caries status and the number of missing teeth due to caries, measured using ICDAS criteria. Similar results were reported in Spanish young adults with a statistically significant association between caries risk profile determined by Cariogram and past caries experience.[14] Otherwise, in a high-caries adult population, different results were found: Cariogram risk categories did not fit with caries experience;[19] a possible explanation might be linked to the hypothesis that a high-caries population surpasses the ability of Cariogram to properly elucidate the association between caries risk and caries experience. Nevertheless, a direct association between the categorized outcomes of the Cariograms and caries status was found in a group of Saudi adults with several dental restorations.[16] Caries still remains an important reason for the loss of teeth,[1,2,13] and the results of this paper noted the correlation between this variable and the caries risk profile, measured by Cariogram model; moreover the multinomial logistic regression outcome underlines an association between Cariogram score and the higher ICDAS score and also the higher Number of missing teeth.

Among background variables, Educational level, Smoking habit, Frequency of toothbrushing and Frequency of dental check-ups showed a statically significant association with Cariogram risk groups. In particular, the educational level is one of the most frequently used socioeconomic indicators: lower educational level has been statistically associated with greater prevalence of dental caries.[18] The finding of a statistical significant association among Cariogram score and socioeconomic and behavioural variables is quite new and interesting, since it highlights that even if the Cariogram model includes proximal risk factors for caries almost exclusively, distal risk factors, such as smoking and educational level, are strongly correlated with the risk assessed through the Cariogram.[6]

PCA analysis supports the concept that caries risk is divisible into patterns attributable to distinct risk factors that work in accord: in the total sample two different clusters might be noted; the first one centred on Cariogram score with two Cariogram sectors, Susceptibility (fluoride program, saliva secretion and buffer capacity) and Bacteria (plaque amount and MS), while the second noteworthy finding is the association of a high Number of missing teeth due to caries, Smoking habits and Circumstances (caries experience and related diseases) which further supports the involvement of environmental risk factors on caries pattern. The interpretation of the PCA on Cariogram high risk group is that people with a severe Caries status (ICDAS 5-6), high Number of missing teeth due to caries, unemployed, smokers and male are strictly associated to create a distinct disease pattern. Socioeconomic factors often have a heavy impact on the biological factors, influencing variables as the diet or the oral hygiene habits.[13, 20] Therefore, it is possible to suppose that people who have no job, can have more caries lesions and can lose more teeth due to caries, not having the economic resources to receive a dental treatment, as in Italy the public dental services for adults are very limited and dental care have to be paid directly by the patient.

This study benefited from several strengths, as the large sample of participants, the surface-level caries assessment making the different modelling caries patterns possible. A limit could be related to the non-generalizability of the results. The studied population belonged to the medium-low socio-economic group, living in an area with a low mean income per capita.[20] It is possible to suppose that in a population with different socio-economic and behavioural variables, the distinct risk factors may have a different influence on the caries disease.

In conclusion the Cariogram model was able to identify the caries-related factors in an adult population. A direct association among the risk categories from Cariogram, the caries status and some socio-behavioural variables was verify.

Availability of supporting data

The authors are ready to put supporting data available if request.

Competing interests

The authors declare that they have no competing interests.

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III

Effect of sugar-free chewing gum containing magnolia bark extract on interdental plaque pH in healthy adult volunteers: a preliminary randomized controlled intervention trial

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ABSTRACT

Aim: The effect of magnolia bark extract (MBE) on interdental plaque pH administered daily through sugar-free chewing gum was evaluated. The study was performed with healthy adult volunteers as a randomized double-blind intervention
trial.

Methods: 271 Subjects, aged 30-55 years and with at least one surface cavitated caries lesion were enrolled and divided into three groups (blue, yellow and green): one using chewing gum containing MBE and xylitol, a group using chewing gum with only xylitol and finally, a control group using sugar-free chewing gum without MBE and xylitol. Each subject was instructed to chew 1 or 2 pellets for 5 min 3 times a day for 12 months. Plaque pH was assessed using the strip method after a mouth rinse with 10% sucrose at baseline (T_0) and after 6 months (T_1) of chewing gum use. Measures of plaque pH will be also performed after 12 months of chewing gum use. Data were analysed for statistically significant differences using repeated measures of ANOVA.

Results: 168 subjects completed the six-months use of chewing gum. Data on interdental plaque pH were calculated on 73 subjects in the yellow group, 75 in the blue group and 73 in the green one. Subjects from green and blue groups ($p<0.01$) and yellow group ($p=0.02$) showed a plaque acidogenicity at T_1 significantly lower compared to baseline.

Conclusion: Currently, six-month use of two varieties of chewing gum, whose contents are still unknown, showed beneficial effects on oral health, controlling plaque acidogenicity to reduce caries risk for two groups.

INTRODUCTION

Dental caries is a multifactorial, chronic and degenerative disease, which affects the majority of individuals in all age groups during their lifetime (Petersen *et al.,* 2005). Its etiology is related to a triad of indispensable factors: cariogenic bacteria in dental plaque, fermentable carbohydrates intake and host defences mainly represented by saliva flow. In addition, socioeconomic and behavioural factors influence the disease pattern (Keyes and Jordan, 1963; Selwitz *et al*., 2007).

Cariogenic bacteria, universally recognized as the main etiological factor, adhere to tooth surfaces and produce acids through the metabolism of their carbohydrates (Marshall, 2009; Lingström *et al*., 2003). The production of acids in the dental biofilm reduces the pH level, which may further lead to a demineralization of enamel and dentine. Among the host defence factors, saliva, both in quantity and quality, plays a major role in caries prevention, facilitating the clearance of fermentable carbohydrates as well as the return of plaque pH back to its original level after a previous acidogenic challenge. In order to enhance saliva protection properties, sugar-free chewing gum is recommended (Burt, 2006). Stimulated saliva through chewing gum helps to provide a number of dental benefits: first, the higher flow rate promotes more rapid oral clearance of sugars; second, the high pH and buffering capacity of the stimulated saliva help to neutralise plaque pH after a sugar challenge; and, lastly, studies have shown enhanced remineralisation of early caries-like lesions (Dodds, 2012). Besides salivary enhancement, several compounds are added to these products in order to increase their caries-protective value. Polyols, such as sorbitol, a hexitol derived from glucose, and xylitol, a naturally occurring pentatol, are often used as sugar substitutes (Burt, 2006). Several studies have demonstrated clinical benefits of xylitol both as non-cariogenic and cariostatic agent (Edgar, 1998). However, in order to obtain these positive effects, both a high daily dose of xylitol as well as frequent intake have been suggested (Milgrom *et al*., 2006; Holgerson *et al*., 2007; Thabuis *et al*., 2013). Söderling *et al.* (1989) showed that, among 19- to 35-year-olds, consumption of 10.9 g xylitol/day for 14 days resulted in reductions of plaque and salivary *S. mutans*, as well as a decrease in the amount of plaque by 29.4%, and enhanced resistance to pH drops induced by sucrose rinse.

Natural extracts from plants are also included as ingredients in the formulation of candies and chewing gums in order to increase their anticaries properties. Magnolia bark extract (MBE) is obtained from the bark of magnolia. Its two main constituents (magnolol and honokiol) have been reported to inhibit the growth of periodontopathic microorganisms, *Porphyromonas gingivalis, Prevotella gingivalis, Actinobacillus actinomycetemcomitans, Capnocytophaga gingivalis*, and *Veillonella disper,* suggesting a potential therapeutic use as a safe oral antiseptic for the prevention and the treatment of periodontal disease (Chang *et al*., 1998; Ho *et al*., 2001). Furthermore, MBE and its two main constituents demonstrated a strong germ-kill effect against bacteria responsible for halitosis and also against Streptococcus mutans, bacteria involved in dental caries formation (Greenberg *et al*., 2007).

The hypothesis of this study is that the acidogenicity of interproximal plaque-pH will decrease compared to baseline after 6 months of daily use of sugar-free magnolia bark extract (MBE) chewing gum use. To validate this hypothesis a randomized double-blind clinical trial was designed and performed in adults aged 30-55 years.

METHODS

Ethics approval

The present study was carried out in Sassari (Italy) under the supervision of the Italian WHO Collaborating Centre for Epidemiology and Community Dentistry and lasted from September 2012 to September 2014.

The study was designed as a randomized clinical trial, approved by the Ethics Committee of the University of Sassari (n°1083/L, 23/07/2012) and registered (Protocol Registration Receipt NCT01588210) at http://www.clinicaltrial.gov.

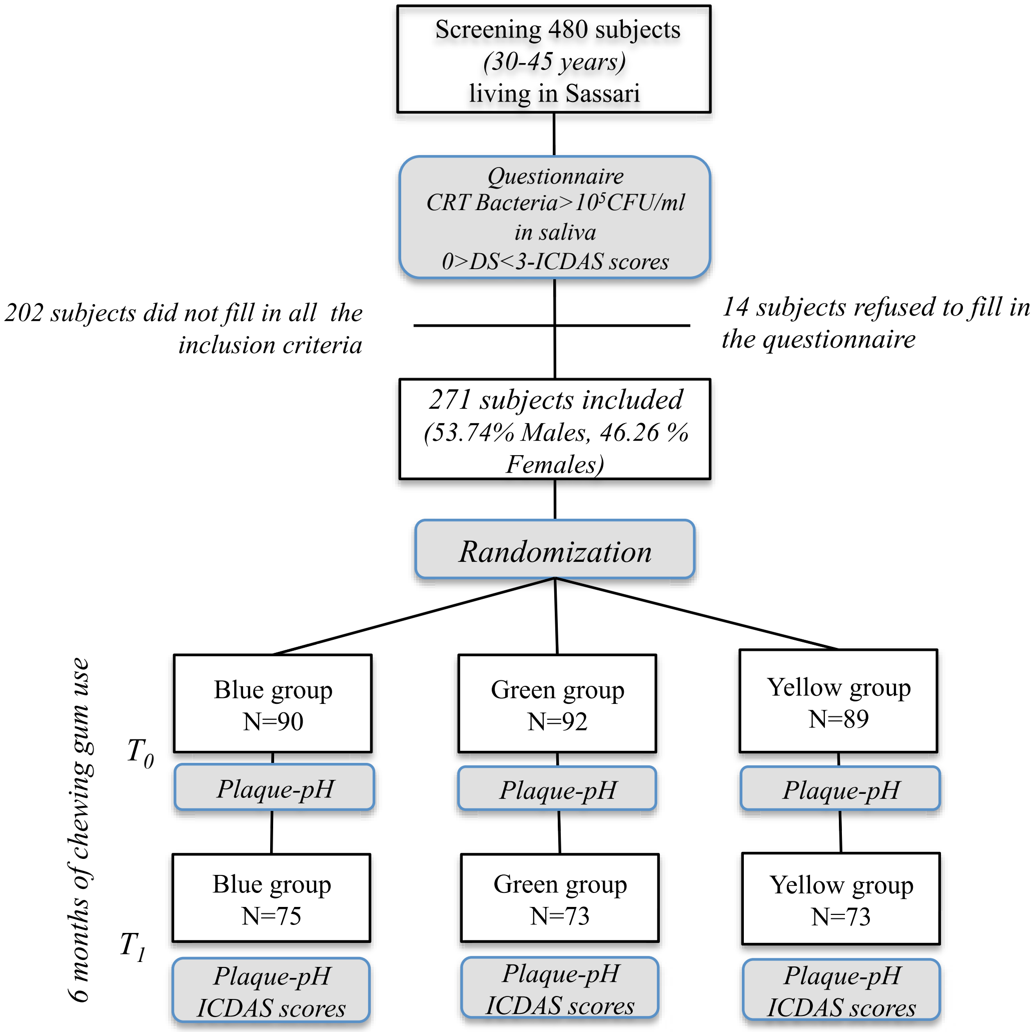
Study population

Data from the Italian National Institute for Statistics for 2011 gave the number of 30-45 year-olds living in the town as 22,614. Sample size for preliminary screening was performed trough G*Power 3.1.3 for Apple using logistic regression with an odds ratio of 1.5, an error probability of 0.04, the total sample was set at 312.

In order to get statistical comparison results, the number of subjects per group to be included in the analysis will be calculated. Considering a 35% difference among groups to be significant, and a 95% probability of obtaining a significant difference among groups at the 5% level, the resulting number of subjects per group was set 104.

With the collaboration of the municipal electoral registry office, a letter explaining the purpose of the study and the informed consent were distributed to 5% (1131 subjects) of the age group considered living in Sassari; 577 subjects (51.02% acceptance rate) accepted to participate were examined for conditions that would preclude participation. The flow-chart, displayed in the figure n°1, shows the design of the study.

Figure 1. Flow chart of the study design



Inclusion criteria

1. Age 30-45;
2. Presence of a minimum of 12 natural teeth;
3. At least one surface cavitated caries lesion, but no more than three;
4. Mutans streptococci/mL concentration in saliva >10^5 CFU;
5. Systemically healthy as assessed by a medical questionnaire;
6. No use of antibiotics or participation in a clinical study in the previous 30 days;

7. No allergy to any of the ingredients of the study products (xylitol or maltitol);
8. No orthodontic banding or removable prosthesis;
9. Moderate gingivitis, no current periodontitis (no sites of probing pocket depth ≥5 mm or attachment loss of ≥2 mm, apart from gingival recession).

Subjects with a history of GI problems and with systemic disease that interfered with the oral ecosystem were excluded. The caries criterion was designed to include participants who are at risk of forming new lesions. In addition, those participants who were already consuming more than three pieces of sugar-free chewing gum a day were excluded. The elected participants agreed not to consume any other chewing gums than those supplied for the study.

All the participants were residents in an area with a low natural fluoride content in the drinking water, but they reported to use a fluoridated toothpaste on a regular basis.

Design and procedures

The experimental design was a three-group, double-blinded, randomized controlled clinical trial (RCT).

A simplified structured self-compiled questionnaire together with the informed consent was submitted to the participants before the clinical examinations. To guarantee the anonymous status, the questionnaire was printed on one side of a sheet, while the clinical chart was on the other side. The questionnaire was pre-tested for control of reliability and validity. It was highly structured and divided into different domains: (1) personal data (i.e. age, gender, educational level, job categorization etc.), (2) life-style behaviour (i.e. smoking and dietary habits), (3) oral health behaviour (i.e. tooth brushing, use of fluoride, frequency of dental check-ups) (Campus *et al*., 2011).

Screening saliva samples were taken for *mutans streptococci* enumeration from potential subjects who met the inclusion criteria (n=320). Subjects with $>10^4$ CFU/mL in their saliva sample (n=278) were invited to participate all baseline procedures and were enrolled. Then, subjects were randomly assigned to three groups (blue, green and yellow) and were blinded to group assignment, as were investigators. Block randomization ensured similar proportions of participants in each group. One using chewing gum containing MBE and xylitol, a group using chewing gum with the same content of xylitol but free of MBE and finally, a control group using a sugar-free chewing gum without MBE and xylitol.

Daily gum packets were distributed on a weekly basis for the first 3 weeks, then every 2 months to promote and monitor compliance. The total daily intake of magnolol and honokiol in the group using chewing gum containing MBE and xylitol was 11.9 mg/day. The daily use of the three different chewing gums was carried out for 12 months.

Caries assessments

One calibrated examiner (GiC) performed all the dental screenings. Intra- and inter-examiner reliability was assessed before the beginning of the survey by examining and re-examining (after 72 h) fifty-five subsequent study participants. Inter-examiner reliability was evaluated using fixed-effects analysis of variance in comparison with benchmark values (GC). Intra-examiner reproducibility was assessed as the percentage of agreement using Cohen's kappa statistic (Castiglia *et al*., 2007). Good inter-examiner reliability was found for the merged ICDAS score 1-2, 3-4 and 5-6, with no significant difference from benchmark values ($p = 0.15$) and a low mean square of error (0.47). Intra-examiner reliability was also high, Cohen's Kappa statistic = 0.88. Subjects were examined sitting on an ordinary chair, using a mouth mirror and a Community Periodontal Index probe (approved by the WHO) under optimal lighting. The ICDAS index allows to register the surfaces decayed and also the level of caries (if initial or severe caries), the type of the fillings and the missing teeth for caries or for other reasons. (Ismail *et al*., 2007). Subjects were categorized according to maximum ICDAS score as follows: ICDAS (0) as caries free; ICDAS (1-2) initial caries level referred to caries involving only the enamel with no evidence of dentine involved, ICDAS (3-4) moderate caries level referred to caries involving enamel and partially dentine, ICDAS (5-6) severe destructive caries.

The Number of missing teeth due to caries was categorized as follow: no missing teeth, 1-2 missing teeth, 3-5 missing teeth and > 5 missing teeth. The presence of fillings was classified in: no fillings, mainly tooth coloured restorations, mainly amalgam restorations and presence at least of one crown.

Plaque index was assessed in the buccal surface of six index teeth (1.6 / 1.1 / 2.4 / 3.6 / 4.1 / 4.4) using Silness and Löe's scale, as follow: "0" indicating no plaque; 1 plaque adhering to the free gingival margin and adjacent area of the tooth; "2" moderate accumulation of soft deposits in the gingival pocket or on the tooth gingival margin; and "3" abundance of soft deposits in the gingival pocket and/or on the tooth gingival margins.

Plaque-pH measurements

Interproximal-plaque pH of each subject was evaluated using pH indicator strips, which measure a pH value in the range of 4.0-7.0 (Spezialindikator, pH range 4.0-7.0; Merck, Darmstadt, Germany). The strip method determines changes in plaque pH, discriminating differences at the level of 0.2-0.5 pH units following a sugar (10%) challenge to the same extent as the micro-touch method (correlation coefficient 0.99). Strips are easy to use and suitable for a chair-side clinical use. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that could be more easily inserted into the interproximal space. The strip was held into the interdental space for 10 s, after which it was removed and its color compared to the color index scheme supplied by the manufacturer. The pH was determined to one decimal of the value.

For each subject, 3 measurements were carried out on 2 sites, between the 2nd premolar and the 1st molar right and left of the upper jaw. Measurements were performed before and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose. All measurements were carried out by the same examiner (GiC). Plaque pH was assessed at baseline (t_0) and after 6 months (t_1) of chewing gum use.

Statistical analysis

Descriptive statistics about some variables on the sample were calculated.
PH data of the 221 subjects (who finished the 6 months of the experimental period) were analysed for statistically significant differences using repeated measures of ANOVA.
All data were analysed using the software STATA® (v10.1 for Macintosh). For all statistical analysis, the statistical significance level was set at $\alpha=0.05$.

RESULTS

There were no reports of taste disturbances or any other side effects in the magnolia and control groups. Table n°1 shows the distribution of the sample according to the sex (53.74% males, 46.26% females) among the three groups. The sample was composed of 80.45% smokers and of 19.55% no-smokers. 63.80% of the sample reported to brush their teeth three times a day, then 33.03% twice a day. According to dietary habit, 73.06% were used to snacking between meals, other hand only 43.64% was used to drink soft drinks.

Table 1. Sample distribution according to sex among the three groups.

GROUP	Males N(%)	Females N(%)	Total N(%)
Green	48 (52.17)	44(47.83)	92(100)
Blue	50(55.56)	40(44.44)	90(100)
Yellow	48(53.93)	41(46.07)	89(100)
Total	*146(53.74)*	*125(46.26)*	*271 (100)*
$\chi^2_{(2)}$**=1.35**			***p=0.51***

Table n°2 highlights that the majority of subjects had a medium level of education (48.71% with secondary school) and that there were not differences of sample distribution between the three groups.

Table 2. Sample distribution according to the level of education among the three groups.

GROUP	Primary school N(%)	Secondary school N(%)	University degree N(%)
Green	20 (21.74)	44(47.83)	28(30.43)
Blue	21(23.33)	45(50.00)	24(26.67)
Yellow	20(22.47)	43(48.31)	26(29.21)
Total	*61(22.51)*	*132(48.71)*	*78(28.78)*
$\chi^2_{(4)}$**=0.33**			***p=0.1***

A total of 221 subjects (58.74% males and 41.53% females) completed the six-month period (figure n°1); data on plaque pH were calculated on 75 subjects in the magnolia group, 73 in the xylitol group and 73 in the control group.

Table n°3 shows the changes in plaque pH among the three groups after 6 months chewing gum use. It is evident that there is a statically significant difference between the plaque pH measurements at the baseline and the pH measurements rerun after 6 months. Compared with the baseline, chewing either xylitol gum or MBE gum or sugar-free gum significantly increased the interproximal plaque pH in the green and blue group ($p<0.01$) and in the yellow group ($p=0.02$).

Table 3. Results of repeated measures of ANOVA on inter-proximal plaque pH after 6 months of chewing gum use.

GREEN GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.43(0.05)	6.95(0.02)	1.52(0.06)
T_1	5.60(0.07)	6.99(0.01)	1.44(0.06)
Repeated measures of ANOVA	***F=2.48 p<0.01***	***F=2.58 p<0.01***	***F=2.95 p<0.01***
Huynh-Feldt epsilon:	*1.00*	*1.00*	*1.00*
Greenhouse-Geisser epsilon:	*1.00*	*1.00*	*1.00*
Box's conservative epsilon:	*1.00*	*1.00*	*1.00*

BLUE GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.48(0.05)	6.90(0.04)	1.48(0.06)
T_1	5.63(0.06)	6.95(0.03)	1.38(0.06)
Repeated measures ofANOVA	***F=5.40 p<0.01***	***F=2.61 p<0.01***	***F=10.09 p<0.01***
Huynh-Feldt epsilon:	*1.00*	*1.00*	*1.00*
Greenhouse-Geisser epsilon:	*1.00*	*1.00*	*1.00*
Box's conservative epsilon:	*1.00*	*1.00*	*1.00*

YELLOW GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.52(0.06)	6.90(0.03)	1.41(0.06)
T_1	5.54(0.05)	6.93(0.03)	1.48(0.06)
Repeated measures ofANOVA	***F=2.00 p=0.01***	***F=2.45 p<0.01***	***F=2.40 p=0.02***
Huynh-Feldt epsilon:	*1.00*	*1.00*	*1.00*
Greenhouse-Geisser epsilon:	*1.00*	*1.00*	*1.00*
Box's conservative epsilon:	*1.00*	*1.00*	*1.00*

DISCUSSION AND CONCLUSION

The hypothesis of this study is confirmed by the results: sugar-free chewing gums reduce plaque acidogenicity. Shu *et al*. (2007), who also studied the effect of two kinds of gums on dental plaque pH, observed that compared with the baseline, chewing either tea polyphenol gum or sugar-free gum could increase the plaque pH recovery due to the sucrose rinsing challenge and it could maintain the dental plaque pH above the resting value. Thabuis *et al.* (2013) in their study also compared two different kinds of chewing gums and concluded that sugar-free chewing gum sweetened with either maltitol or xylitol can similarly reduce plaque acidogenicity compared to gum base through a decrease in oral bacteria presence. These results are due to the essential role of saliva, which is particularly stimulated through chewing gum use. Salivary buffers could reserve the low pH by neutralizing the acids produced by cariogenic organism.

It needs to consider that the results of this study are preliminary forasmuch as the complete experimental period lasts 12 months. Nevertheless, it is already possible to underline some limits of the study design. First of all, the study population belonged to an age range, in which the habit to chew daily chewing gum is not common, so it was difficult to find immediately a complete compliance. Secondly, adults aged more than 40 years were frequently bearer of removable prosthesis or crowns with temporary cement, and also presented TMJ disorders. All these aspects had been considered during the enrolment. On the third hand, it was difficult to obligate subjects not to go to dentist for a year, after knowing the presence of caries at the first clinical examination. In fact, in the next study the improvement of plaque pH and of the microbiological evaluation will be correlated with the dental treatments carried out during the experimental period and the caries status in the last clinical examination.

However, there is already a consistent evidence to support the use of sugar-free chewing gum as part of normal oral hygiene to prevent dental caries (Deshpande *et al*., 2008). The observed caries reduction can be ascribed to salivary stimulation throughout the chewing process, the lack of sucrose and the inability of bacteria to metabolize polyols into acids (Mickenautsch *et* *al*., 2007). If MBE really affects the MS level could be found at the end of experimental period when microbiological evaluation will be repeated.

Consequently, sugar-free chewing gum controlling plaque acidogenicity, used immediately after meals, has a caries-reducing effect.

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IV

Effect of a Sugar-Free Chewing Gum Containing Magnolia Bark Extract on different variables related to caries in healthy adult volunteers: a Randomized Controlled Intervention Trial

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ABSTRACT

Aim: The effect of magnolia bark extract (MBE) on the development of caries lesions administered daily through sugar-free chewing gum was evaluated. A randomized double-blind intervention trial was performed.

Methods: 480 subjects (mean age±40.72) were examined using ICDAS index. A self-compiled questionnaire was submitted. 271 subjects, presenting inclusion criteria, were enrolled and divided into three groups (blue, yellow and green): one using chewing gum containing MBE and xylitol, another group using chewing gum with xylitol but free of MBE and finally, a control group using a sugar-free chewing gum. Each subject was instructed to chew one pellet for 5 min 3 times a day for 12 months. Plaque-pH using the strip method, a clinical examination using ICDAS scores and a microbiological evaluation (CRT Bacteria) were assessed at baseline (T_0), after 6 months (T_1) and 12 months (T_2) of chewing gum use. Data were analysed for statistically significant differences using repeated measures of ANOVA.

Results: Data on 193 subjects were calculated and the total sample showed a plaque acidogenicity and salivary concentration of bacteria at T_2 significantly lower compared to baseline.

Conclusion: Twelve-month use of sugar-free chewing gum showed beneficial effects on oral health, controlling plaque acidogenicity to reduce caries risk.

INTRODUCTION

Dental caries continues to be one of the most prevalent human diseases, despite the availability of various prophylactic means. Modern concepts regard caries as an interaction between genetic and environmental factors, where biological, social, behavioural and psychological factors are expressed in a highly complex interactive manner with the dental biofilm as the key element (Kalesinskas *et al*., 2014). Sucrose is the main triggering factor for development of the bacterial biofilm, as depicted by Marsh *et al.* (2003). Therefore, the major critical element is the dietary intake of fermentable carbohydrates, in particular sucrose, that is frequently found in high concentrations in sweets, biscuits, snacks, sweet drinks, etc. When the biofilm is matured, then the presence of sucrose and/or starch further promotes plaque cariogenicity by constantly keeping pH at 5 or even lower (Koo *et al*., 2013; Bowen *et al*., 2011). Some host factors, especially saliva, influence this complex interaction between acid-producing cariogenic bacteria and fermentable carbohydrate. Although it is difficult to control human behaviour, many caries-preventive measures have been designed, including the daily use of fluoride toothpastes and antiseptic mouth-rinses, water fluoridation, dental sealants, oral health educational programs as well as regular visits to dentist office. However, the recent epidemiological studies show the trend to global increase in dental caries that clearly indicate a need for development of new and effective prophylactic approaches (Marcenes *et al*., 2013; Bagramian *et al*., 2009). The use of sugar-free chewing gum, as part of normal oral hygiene, contributes to prevent dental caries (Deshpande and Jadad, 2008). The higher flow rate of stimulated saliva promotes more rapid oral clearance of sugars and increase of its buffering capacity in order to neutralise the plaque pH and decrease the risk of caries development (Dodds *et al.,* 2012). Otherwise, such persistent acidic environment within the biofilm results in demineralization of tooth enamel, then this long-term process leads to cavitation (Featherstone, 2008). Importantly, the acidic conditions within biofilm favor the growth of more acid-tolerant bacteria such as mutans streptococci and lactobacilli (Paes Leme *et al*., 2006). It is widely known that the consumption of xylitol can lead to less plaque and less MS bound to plaque (Söderling, 2009). This study intends to assess the effect of sugar-free chewing-gum containing magnolia bark extract and xylitol on plaque pH and MS salivary concentration. Another hypothesis of this double-blind randomized clinical trial is to evaluate the effect of chewing gum on caries development, according to the fact that caries reduction is usually attributed to growth inhibition of MS (Marsh *et al*., 2009).

METHODS

Ethics approval

The present study was carried out in Sassari (Italy) under the supervision of the Italian WHO Collaborating Centre for Epidemiology and Community Dentistry and lasted from September 2012 to September 2014.

The study was designed as a randomized clinical trial, approved by the Ethics Committee of the University of Sassari (verbale n°1083/L in data 23/07/2012) and registered (Protocol Registration Receipt NCT01588210) at http://www.clinicaltrial.gov.

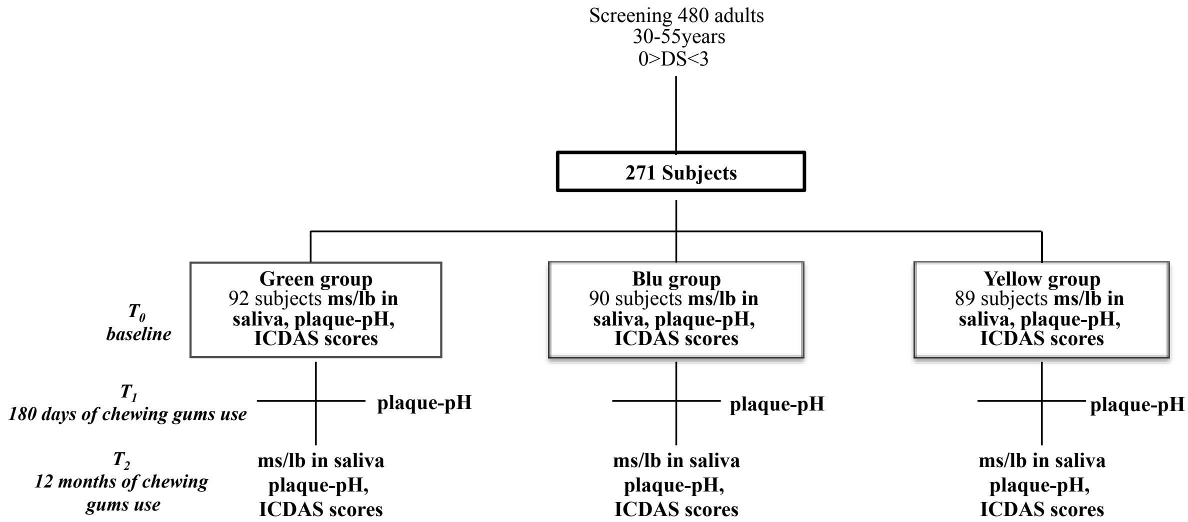
Study population

Data from the Italian National Institute for Statistics for 2011 gave the number of 30-45 year-olds living in the town as 22,614. Sample size for preliminary screening was performed trough G*Power 3.1.3 for Apple using logistic regression with an odds ratio of 1.5, an error probability of 0.04, the total sample was set at 312.

In order to get statistical comparison results, the number of subjects per group to be included in the analysis will be calculated. Considering a 35% difference among groups to be significant, and a 95% probability of obtaining a significant difference among groups at the 5% level, the resulting number of subjects per group was set 104.

With the collaboration of the municipal electoral registry office, a letter explaining the purpose of the study and the informed consent were distributed to 5% (1131 subjects) of the age group considered living in Sassari; 577 subjects (51.02% acceptance rate), aged between 30-45 years, accepted to participate were examined for conditions that would preclude participation. The flow-chart, displayed in the figure n°1, shows the design of the study.

Figure 1. Flow-chart of the study.



A simplified structured self-compiled questionnaire together with the informed consent was submitted to the participants before the clinical examinations. To guarantee the anonymous status, the questionnaire was printed on one side of a sheet, while the clinical chart was on the other side. The questionnaire was pre-tested for control of reliability and validity. It was highly structured and divided into different domains: (1) personal data (*i.e.* age, gender, educational level, job categorization etc.), (2) life-style behaviour (*i.e.* smoking and dietary habits), (3) oral health behaviour (*i.e.* tooth brushing, use of fluoride, frequency of dental check-ups) (Campus *et al*., 2011).

A benchmark examiner (GCampus) trained and calibrated one examiner (GCarta) that performed all dental screenings. Baseline training consisted in one-day (6 hours) theoretical course, followed by examination of 54 extracted teeth plus a session of 120 photographs of extracted teeth. Two days after the theoretical curse a clinical trial involving examination of fifty-five adults subjects was performed. The subjects were re-examined after 72 hours. Inter-examiner reliability with the "benchmarck" (GCampus) was evaluated using fixed-effects analysis of variance. Intra-examiner reproducibility was assessed as the percentage of agreement using Cohen's kappa statistic (Castiglia *et al*., 2007). Good inter-examiner reliability was found for the merged ICDAS index 1, with no significant difference from benchmark values ($p = 0.15$) and a low mean square of error (0.47). Intra-examiner reliability was also high (Cohen's Kappa statistic = 0.88). Subjects were examined sitting on an ordinary chair, using a mouth mirror and a Community Periodontal Index probe (approved by the WHO) under optimal lighting. The ICDAS index allows to register the surfaces decayed and also the level of caries (if initial or severe caries), the type of the fillings and the missing teeth for caries or for other reasons (Ismail *et al*., 2007).

The clinical examination was repeated at the end of the experimental period (12 months).

Inclusion criteria

The inclusion criteria for the recruitment of subjects are as follows:

1. Age 30-55;
2. Presence of a minimum of 12 natural teeth;
3. At least one surface cavitated caries lesion, but no more than three;
4. *Mutans streptococci* and *Lactobacilli* $>10^5$ CFU/ml saliva;
5. Systemically healthy as assessed by a medical questionnaire;
6. No use of antibiotics or participation in a clinical study in the previous 30 days;
7. No allergy to any of the ingredients of the study products;
8. No orthodontic banding or removable prosthesis;
9. Moderate gingivitis, no current periodontitis (no sites of probing pocket depth ≥ 5 mm or attachment loss of ≥ 2 mm, apart from gingival recession).
10. Absence of dysfunction of temporo-mandibular joint

Subjects with a history of GI problems and with systemic disease that interfered with the oral ecosystem were excluded. The caries criterion was designed to include participants who are at risk of forming new lesions. In addition, those participants who were already consuming more than three pieces of sugar-free chewing gum a day were excluded. The elected participants agreed not to consume any other chewing gums than those supplied for the study.

All the participants were residents in an area with a low natural fluoride content in the drinking water, but they reported to use a fluoridated toothpaste on a regular basis.

Microbiological evaluation

Immediately after the clinical assessment, an evaluation of the *mutans streptococci* (MS) and *lactobacilli* concentration in saliva was performed. No-stimulated whole saliva was collected over 150 s in sterile vials (Nunc, Kamstrup, Denmark). The samples were transported to the Department of Microbiology of the University of Sassari and processed within 45 min after collection.

Mutans streptococci and *lactobacilli* counts in saliva were assessed and categorized using the dip-slide technique (CTR bacteria, Ivoclar Vivadent, Germany).

Subjects, who present a bacterial concentration $>10^5$ CFU/ml in their saliva sample (n=271), were invited to participate all baseline procedures and were enrolled. Then, subjects were randomly assigned to three groups (blue, green and yellow) and were blinded to group assignment, as were investigators. Block randomization ensured similar proportions of participants in each group. One using chewing gum containing MBE and xylitol, a group using chewing gum with the same content of xylitol but free of MBE and finally, a control group using a sugar-free chewing gum without MBE and xylitol.

Plaque-pH measurements

Interproximal-plaque pH of each subject was evaluated using pH indicator strips, which measure a pH value in the range of 4.0-7.0 (Spezialindikator, pH range 4.0-7.0; Merck, Darmstadt, Germany). The strip method determines changes in plaque pH, discriminating differences at the level of 0.2–0.5 pH units following a sugar (10%) challenge to the same extent as the micro-touch method (correlation coefficient 0.99). Strips are easy to use and suitable for a chair-side clinical use. Each strip was cut into 4 pieces (approx. 2 mm in width) in order to get a strip that could be more easily inserted into the interproximal space. The strip was held into the interdental space for 10 s, after which it was removed and its color compared to the color index scheme supplied by the manufacturer. The pH was determined to one decimal of the value.

For each subject, 3 measurements were carried out on 2 sites, between the 2^{nd} premolar and the 1^{st} molar right and left of the upper jaw. Measurements were performed before and at 2, 5, 10, 15, 20 and 30 min after a mouth rinse with 10% sucrose. All measurements were carried out by the same examiner (GCarta). Plaque pH was assessed at baseline (t_0), after 6 (t_1) and 12 months (t_2) of chewing gum use.

Besides, each subject was instructed to chew 1 or 2 pellets for 5 min 3 times a day (2 in the morning, 2 after the midday meal and 1 in the afternoon). Daily gum packets were distributed on a weekly basis for the first 3 weeks, then every 2 months to promote and monitor compliance. The total daily intake of magnolol and honokiol in the group using chewing gum containing MBE and xylitol was 11.9 mg/day. The daily use of the three different chewing gums was carried out for 12 months. Three clinical evaluations for caries diagnosis were performed: one at baseline, a second after 6 months of chewing gum use and at the end of experimental period (after 12 months from baseline). A microbiological evaluation for cariogenic bacteria was performed at baseline and at the end of the chewing period.

Statistical analysis

Data on inter-proximal plaque pH at t_0, t_1 and t_2 were analysed for statistically significant differences using repeated measures of ANOVA. A chi-squared test for trend of odds was carried out for the bacterial concentrations for each group. Then, a chi-squared test was performed among the new proportion of bacterial counts of the three groups at t_2.

Furthermore, a non parametric test for trend for the levels of caries lesions was calculated across the three groups in the three times.

All data were analysed using the software STATA® (v10.1 for Macintosh). For all statistical analysis, the statistical significance level was set at $\alpha=0.05$.

RESULTS

The experimental period was finished by 193 subjects (71.22% of the initial sample). Data on plaque pH were calculated on subjects in 64 subjects in the blue group, 64 in the green group and 65 in the yellow group, and are displayed in the table n°1.

Table 1. Results of repeated measures of ANOVA on inter-proximal plaque pH after 12 months of chewing gum use.

GREEN GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.34(0.05)	6.99(0.01)	1.63(0.06)
T_1	5.46(0.06)	6.99(0.01)	1.53(0.06)
T_2	5.6(0.06)	6.99(0.01)	1.39(0.06)
Repeated measures of ANOVA	**p<0.01 F=4.71**	**p<0.01 F=2.25**	**p<0.01 F=4.74**
Huynh-Feldt epsilon:	**0.87**	**-**	**0.86**
Greenhouse-Geisser epsilon:	**0.83**	**-**	**0.83**
Box's conservative epsilon:	**0.50**	**0.50**	**0.50**

BLUE GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.38(0.05)	6.87(0.04)	1.48(0.06)
T_1	5.62(0.06)	6.97(0.02)	1.36(0.06)
T_2	5.60(0.06)	6.95(0.03)	1.34(0.06)
Repeated measures of ANOVA	**p<0.01 F=4.40**	**p<0.01 F=3.36**	**p<0.01 F=4.16**
Huynh-Feldt epsilon:	**0.80**	**0.72**	**0.92**
Greenhouse-Geisser epsilon:	**0.77**	**0.70**	**0.88**
Box's conservative epsilon:	**0.50**	**0.50**	**0.50**

YELLOW GROUP	**Min pH** *Mean (SE)*	**Max pH** *Mean (SE)*	**pH fall** *Mean (SE)*
T_0	5.45(0.06)	6.88(0.03)	1.43(0.07)
T_1	5.48(0.05)	6.95(0.02)	1.47(0.06)
T_2	5.49(0.06)	6.99(0.01)	1.49(0.06)
Repeated measures of ANOVA	**p<0.01 F=7.35**	**p<0.01 F=2.33**	**p<0.01 F=4.74**
Huynh-Feldt epsilon:	**1.05**	**0.86**	**1.05**
Greenhouse-Geisser epsilon:	**0.99**	**0.83**	**0.99**
Box's conservative epsilon:	**0.50**	**0.50**	**0.50**

Table n°2 shows the distribution of subjects on bacterial concentration in saliva at the beginning and at the end of chewing period. While at the baseline (T_0) the total sample presented a bacterial concentration >10^5 CFU/ ml saliva, at the end of the experimental period, it is evident the reduction of bacterial count, especially in the green and blue group (p<0.01), and in the yellow group (p=0.01).

Table 2. Sample distribution according to concentration of oral bacteria at the beginning and at the end of the experimental period.

GREEN GROUP		**CRT Bacteria** *>10^5 CFU/ ml saliva*	**CRT Bacteria** *<10^5 CFU/ ml saliva*
	T_0	100%	0
	T_2	48.65%	51.35%
$\chi^2_{(1)}$ for trend of odds=25.22			***p<0.01***

BLUE GROUP		**CRT Bacteria** *>10^5 CFU/ml saliva*	**CRT Bacteria** *<10^5 CFU/ ml saliva*
	T_0	100%	0
	T_2	70.27%	29.73%
$\chi^2_{(1)}$ for trend of odds=39.54			***p<0.01***

YELLOW GROUP		**CRT Bacteria** *>10^5 CFU/ml saliva*	**CRT Bacteria** *<10^5 CFU/ml saliva*
	T_0	100%	0
	T_2	69.44%	30.56%
$\chi^2_{(1)}$ for trend of odds=12.80			***p=0.01***

Considering the new bacterial counts in T_2, a chi-squared pearson test, displayed in table n°3, shows a statically significant difference between the three groups (p<0.01).

Table 3. A chi-squared pearson test and sample distribution at T_2 of bacterial concentrations according to the different group membership.

GROUP	**CRT Bacteria** *>10^5 CFU/ml saliva*	**CRT Bacteria** *<10^5 CFU/ml saliva*	**Total**
Green	48.65%	51.35%	100%
Blue	29.73%	70.27%	100%
Yellow	69.44%	30.56%	100%
$\chi^2_{(1)}$=31.38			***p<0.01***

The non parametric test for trend across the three groups, showed in the table n°4, highlights a positive trend for the moderate caries across all the three groups (p>|z |<0.01). The overall decrease of caries lesions was due to the dental treatments carried out by 47.47% of the sample (as shown in table n°5).

Table 4. Non parametric test for trend of caries lesions across the three groups.

	ICDAS 1-2 *n(SE)*	**ICDAS 3-4** *n(SE)*	**ICDAS 5-6** *n(SE)*
Green Group			
T_0	28(4.88)	55(5.29)	5(1.91)
T_1	18(4.67)	40(6.15)	5(1.91)
T_2	15(4.40)	39(6.24)	4(2.11)
	Z=-0.79 **p**>|z|=0.43	**Z**=-2.15 **p**>|z |=0.03	**Z**=0.00 **p**>|z |=1.00
Blue Group			
T_0	20(5.47)	60(5.24)	2(1.39)
T_1	20(5.47)	38(5.64)	0
T_2	20(5.47)	34(5.80)	0
	Z=0.00 **p**>|z |=1.00	**Z**=-3.19 **p**>|z |<0.01	**Z**=-1.74 **p**>|z |=0.08
Yellow Group			
T_0	16(4.17)	57(6.15)	5(2.10)
T_1	11(3.15)	43(6.70)	4(1.91)
T_2	11(3.15)	36(6.57)	4(1.91)
	Z=-0.81 **p**>|z |=0.41	**Z**=-2.63 **p**>|z |=0.01	**Z**=-0.36 **p**>|z |=0.72

Table 5. Sample distribution among the three groups, according to the execution or not of dental treatments during the experimental period.

	No dental treatments (*% of subjects*)	**Dental treatments** (*% of subjects*)
Green group	59.46	40.54
Blue group	48.65	51.35
Yellow group	50.00	50.00
Total	52.73%	47.27%
$\chi^2_{(2)}=3.08$		$p=0.21$

DISCUSSION AND CONCLUSION

The main finding of the study was that the use of chewing gum for a year improves the oral status, controlling the plaque acidogenicity and cariogenicity.

Previous studies support some results of this study. The most recent systematic reviews have concluded xylitol- and polyol-containing chewing gums and/or candies and lozenges have caries-preventive effects when used routinely, and should be included in the armamentarium for caries prevention, noting that the effect may be primarily due to saliva stimulation (Deshpande and Jadad, 2008; Fontana *et al*., 2012). Shu *et al.* (2007), who also studied the effect of two kinds of gums on dental plaque pH, observed that compared with the baseline, chewing either tea polyphenol gum or sugar-free gum could increase the plaque pH recovery due to the sucrose rinsing challenge and it could maintain the dental plaque pH above the resting value. Thabuis *et al.* (2013) in their study also compared two different kinds of chewing gums and concluded that sugar-free chewing gum sweetened with either maltitol or xylitol can similarly reduce plaque acidogenicity compared to gum base through a decrease in oral bacteria presence. These results are due to the essential role of saliva, which is particularly stimulated through chewing gum use. Salivary buffers could reserve the low pH by neutralizing the acids produced by cariogenic organism. The American Dental Association's (ADA, 2011) meta-analysis of polyol studies that recorded caries in permanent teeth indicated that there was a statistically significant reduction in caries with the use of sucrose-free polyol gums compared with no gum (PF = 39%).

However, t is possible to underline some limits of the study design. First of all, the study population belonged to an age range, in which the habit to chew daily chewing gum is not common, so it was difficult to find immediately a complete compliance. Secondly, adults aged more than 40 years were frequently bearer of removable prosthesis or crowns, and also presented TMJ disorders. It is established that chewing more than 3 hours a day would cause TMD symptoms of arthralgia and myofascial pain (Correia *et al*., 2014). All these aspects had been considered during the enrolment, which lasted about a year due to the difficulty to find subjects presenting all the inclusion criteria and the willingness to participate in the study. On the third hand, it was uneasy to obligate subjects not to go to dentist for a year, after becoming aware of the presence of caries at the first clinical examination.

An important issue, therefore, was whether or not the selected study group was representative for the general population of that age group.

The observed absence of new caries lesions after a year could be ascribed to salivary stimulation throughout the chewing process, the lack of sucrose and the inability of bacteria to metabolize polyols into acids, as depicted by Mickenautsch *et al.* (2007). It is already evident that in a moderate caries population practicing normal oral hygiene, including the use of fluoride dentifrices, an after-meal gum-chewing regimen can significantly reduce the rate of caries development (Szöke *et al*., 2005).

Therefore, it has a great importance, taking into consideration all heavy expenses for dental treatment, which in many countries, as in Italy, are not covered (or covered only in part) by governmental health care programs. There is consistent evidence to support the use of xylitol- and sorbitol-containing chewing gum as part of normal oral hygiene to prevent dental caries (Deshpande and Jadad, 2008).

In conclusion, a continuative use of chewing gum, promoting the flow of saliva and controlling plaque acidogenicity, provides an effective means for the prevention of caries disease.

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