ESTUDIO DE LA MICROBIOTA ORAL Y DE LAS COMPLICACIONES RESPIRATORIAS DE LA DISFAGIA OROFARÍNGEA: FISIOPATOLOGÍA, DIAGNÓSTICO Y TRATAMIENTO DE LOS FACTORES DE RIESGO DE LA DISFAGIA OROFARÍNGEA Y LA NEUMONÍA ASPIRATIVA EN PACIENTES DE EDAD AVANZADA

Línea de investigación: Alteraciones de la motilidad digestiva humana

Tesis doctoral presentada por Omar Ortega Fernández
para optar al grado de Doctor

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HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

1. La neumonía aspirativa en ancianos se produce por la aspiración de contenido orofaríngeo colonizado por patógenos respiratorios. Los principales factores de riesgo de la neumonía aspirativa en ancianos son: a) la disfagia orofaríngea con alteraciones de la seguridad de la deglución (aspiraciones); b) vulnerabilidad (malnutrición, fragilidad, comorbilidad y deterioro funcional); y c) mala higiene oral y colonización por patógenos respiratorios de la cavidad orofaríngea.

2. Los pacientes ancianos con disfagia orofaríngea presentan alta prevalencia de alteraciones de la eficacia y la seguridad de la deglución, lo que incrementa el riesgo de infecciones respiratorias y neumonía aspirativa. Estas alteraciones pueden ser diagnosticadas mediante métodos de exploración clínica (MECV-V) y exploraciones complementarias como la videofluoroscopia.

3. La mala higiene oral y las enfermedades periodontales y caries son muy prevalentes en los pacientes ancianos con disfagia orofaríngea y favorecen cambios en la microbiota oral que predisponen a la colonización oral por patógenos respiratorios. Es posible caracterizar la composición de la microbiota orofaríngea y nasal en pacientes ancianos con disfagia orofaríngea mediante técnicas de biología molecular como la *High Throughput Sequencing* (pirosecuenciación).

4. La carga bacteriana de la orofaringe de los ancianos es superior a la de sus fosas nasales. La cavidad orofaríngea de los pacientes ancianos con disfagia orofaríngea está colonizada por diversos patógenos respiratorios *(Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa, Escherichia coli)* potencialmente causantes de neumonía aspirativa en una proporción superior a la que presentan los ancianos sin disfagia.

5. La estimulación sensorial de las vía aferente de la deglución mediante nuevos tratamientos como la estimulación farmacológica (agonistas TRPV1) o la estimulación eléctrica transcutánea pueden mejorar la seguridad y eficacia de la deglución en pacientes ancianos con DO.

OBJETIVOS

1. Evaluar la prevalencia y fisiopatología de los principales factores de riesgo de infección respiratoria y neumonía aspirativa en diferentes fenotipos de pacientes ancianos (≥70 años) con disfagia orofaríngea:

1.1. Factores de la deglución
- Factores de riesgo de disfagia y aspiración orofaríngea.
- Prevalencia de signos clínicos y videofluoroscópicos de disfagia orofaríngea.
- Fisiopatología de las alteraciones respuesta motora orofaríngea en ancianos con disfagia.

1.2. Estado de salud del individuo
- Comorbilidad, estado funcional, fragilidad y estado nutricional.
- Higiene oral y prevalencia de enfermedades periodontales y caries.

1.3. Factores microbiológicos
- Colonización orofaríngea por patógenos respiratorios.
- Carga total microbiana de la orofaringe y la cavidad nasal.
- Composición de la microbiota orofaríngea y nasal.

2. Evaluar mediante estudio videofluoroscópico, el efecto sobre la deglución y la respuesta motora oroafaríngea de dos abordajes terapéuticos, fundamentados en el aumento del estímulo sensorial, en pacientes ancianos con disfagia orofaríngea:
- Estimulación de los canales TRPV1 de la orofaringe mediante un agonista natural administrado en el bolo alimentario.
- Estimulación sensorial de las vía aferente de la faringe y laringe mediante estímulo eléctrico transcutáneo.

RESULTADOS

CAPÍTULO I

ORAL HEALTH IN OLDER PATIENTS WITH OROPHARYNGEAL DYSPHAGIA

Omar Ortega, Carlos Parra, Silvia Zarcero, José Nart, Olga Sakwinska, Pere Clavé. Oral health in older patients with oropharyngeal dysphagia. *Age Aging* 2014; 43(1): 132-7.

ABSTRACT

Background: Oropharyngeal dysphagia (OD), aspiration and poor oral health status are potential risk factors in elderly patients with aspiration pneumonia (AP).

Aim: To assess the oral hygiene status and the prevalence of periodontal disease and dental caries in elderly patients with OD.

Patients & methods: Fifty elderly patients (79.7±6.64 years) with OD associated with ageing or neurological diseases and 15 elderly patients without OD (77.01±4.51 years) were enrolled in this observational-transversal study. OD and aspiration were evaluated by videofluoroscopy (VFS). Oral health was assessed by: a) the Simplified Oral Hygiene Index (OHI-S); b) a complete periodontal examination, assessing the periodontal pocket depth, clinical attachment loss and bleeding on probing to study periodontal diseases (periodontitis, gingivitis), and c) the presence of dental caries.

Results: 8/50 elderly patients with OD presented VFS signs of aspiration, half of them silent; 40/50, signs of penetration into laryngeal vestibule and 16/50, oropharyngeal residue. Prevalence of edentulism and caries was higher in patients with OD. Dentate older patients with OD (30/50) presented the following complications a) poor oral hygiene in 18 patients (OHI-S 3.1-6); b) gingivitis in 2 and periodontitis in 28 and c) caries in 16.

Conclusions: Older patients with OD presented polymorbidity and impaired health status, high prevalence of VFS signs of impaired safety of swallow and poor oral health status with high prevalence of periodontal diseases and caries. These patients are at great risk of developing AP. We recommend a policy of systematic oral health assessment in elderly patients with OD.

INTRODUCTION

The incidence and prevalence of aspiration pneumonia (AP) in the community are poorly defined. The pathogenesis of AP presumes the contribution of risk factors that alter swallowing function, causing oropharyngeal aspiration, bacterial colonization and impaired immunity. The risk of these factors increases with age, underlying diseases and polymorbidity. Surprisingly, in the clinical setting, oropharyngeal dysphagia (OD) and aspiration are not usually considered aetiological factors in older patients with pneumonia [**1**].

The quantity and type of microbiota in aspirate, greatly affected by oral health, contribute to the development of AP [**2**]. In healthy mouths, oral biofilm is colonized by commensal microflora which acts as a barrier against the colonization of respiratory pathogens. Poor oral health reduces these bacteria, allowing the growth of pathogenic populations and changing the balance from a majority of gram positive microorganisms in healthy mouths to mostly gram negative in patients with periodontitis and caries [**3**]. Studies have found that impaired oral health status and oral diseases are important risk factors for AP and independent predictors of mortality from pneumonia in the elderly [**2-4**]. A recent editorial called for systematic screening of OD and gram-negative bacterial colonization in polymorbid older patients to prevent AP [**5**]. The combined effect of comorbidities, frailty, decreased immunological status, OD and poor oral health can lead to AP, hospital readmissions and death [**6,7**].

Oral health of elderly dysphagic patients has not been systematically studied. The aim of this observational-transversal study was to assess the

oral hygiene status, the prevalence of oral diseases and oral hygiene habits, the health status and comorbidities in this population.

MATERIAL & METHODS

Patient Sample

Population consisted of elderly patients consecutively referred for swallowing evaluation. Main inclusion criteria were age ≥70 and history of swallowing difficulties associated with ageing and/or neurological diseases. A control group of elderly patients with similar age and without OD was also included.

Experimental Design

Health status and comorbidities were assessed by the Charlson Comorbidity Index [**8**]. The Eating Assessment Tool (EAT-10) [**9**], a questionnaire to evaluate severity dysphagia symptoms, was collected in all subjects. Patients with swallowing complaints were also evaluated by videofluoroscopy (VFS) [**10**].

Oral health was assessed by the evaluation of periodontal diseases, dental caries, oral hygiene status and oral health habits. All procedures were performed on the same day.

Dysphagia assessment by VFS

The technical process used for VFS recordings has been described elsewhere [10,11]. VFS signs of impaired safety were classified according to Penetration-Aspiration Scale [12].

Oral health assessment

Oral examinations were performed by two periodontists and included: a) number of teeth; b) oral hygiene; c) periodontal diseases; d) caries and e) oral health habits.

Oral Hygiene: We used the Simplified Oral Hygiene Index (OHI-S), composed of two indexes: the Debris Index (DI-S, dental plaque,) and the Calculus Index (CI-S, mineralized debris). The CI-S and DI-S values range from 0 to 3; the OHI-S values range from 0 to 6 [13] *(Fig. 1)*.

Periodontal Diseases: were assessed by introducing a periodontal probe along the soft tissue wall at the gingival sulcus/pocket. Diagnosis was made using an established protocol [**14**].

Caries: were assessed at each dental surface (4 surfaces for incisors and canines; 5 for premolars and molars). We measured the per-

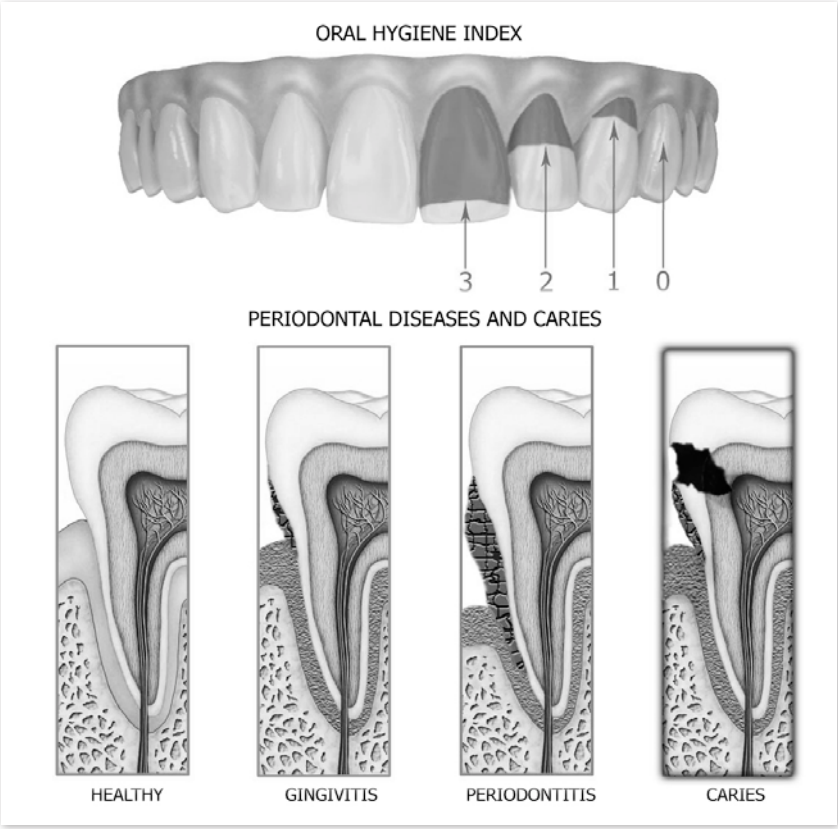

Figure 1. Descriptors for the evaluation of the Simplified Oral Hygiene Index, (OHI_S) periodontal disease and caries. The upper image shows the clinical criteria for the evaluation of the OHI-S. The numbers indicate the score for the debris or calculus index based on its accumulation (from 3 to 0 in each index). The lower image shows the clinical signs of different oral health status; from healthy gums with no inflammation or bone loss, gingivitis with gum inflammation, periodontitis with gum inflammation and alveolar bone loss and caries with tooth demineralization. Note the progressive loss of tissue and progressive biofilm accumulation according to the progression of the diseases.

centage of teeth with caries and surfaces affected [**15**].

Oral Hygiene Habits: a questionnaire was used to determine tooth brushing frequency, use of mouthwash, use of dentures and last visit to the dentist.

Data Management and Statistical Analysis

Qualitative data are presented as relative frequencies and analysed by the Chi-Squared Test and continuous data are presented as mean ± SD and compared with the Mann-Whitney U Test. Safety and efficacy of deglutition were assessed by prevalence of clinical or VFS signs. p values <0.05 were considered significant.

Conflicts of Interest

PC has served as consultant and received research funding from Nestlé Health Science. OS is employed by Nestec SA.

Declaration of Sources of Funding

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RESULTS

Demographics and health status

We studied 50 consecutive elderly patients with swallowing impairment between January and May 2011, (27 women) with a mean age of 79.7±6.64 years. OD was associated with stroke (29/50), ageing (16/50) and neurodegenerative diseases (5/50). The mean Charlson comorbidity score was 2.98±1.83 [**8**] and the mean EAT-10 score was 10.5±7.6. Mean number of drugs taken by patients was 7.56±2.97.

In addition, a group of 15 elderly controls (6 women) with a mean age of 77.09±4.51 years without OD was studied. The mean Charlson comorbidity score was 1.4±1.12 (p=0.0023 vs. OD) and the mean EAT-10 score was 0.23±0.6 (p<0.0001 vs. OD).

VFS signs of OD

VFS showed that prevalence of signs of impaired safety (29/50) and efficacy (38/50) of deglutition in the group with OD was very high. According to the Penetration-Aspiration Scale [**12**], 21/50 OD patients presented mild penetrations (scores 2-3); 15/50, severe penetrations into the laryngeal vestibule (scores 4-5), and 8/50, aspirations into the airway (scores 6-8). (*supplementary figure 1*). A total of 9/50 patients also presented chewing difficulties (*supplementary information*).

Oral health

OD Patients. 20/50 patients presented edentulism and all of them needed dentures to eat with. The following results are for dentate patients (30/50): a) mean number of teeth was 17±8.3; b) OHI-S results showed high prevalence of patients with poor oral hygiene (3.86±1.51) (*Fig. 2*); c) A total of 28 dentate patients presented periodontitis (6, mild; 11, moderate and 11, severe), 2 had gingivitis and none had healthy clinical oral status (p<0.0001). More than half (16) had caries with up to 23.05%±0.16 of dental pieces affected in each patient and 8.21%±5.14 of the surfaces of each tooth affected.

Only 33 patients reported cleaning their teeth or dentures at least once a day and only 11 patients had visited the dentist during the previous year.

Control Patients. Only 1/15 patient presented edentulism (p=0.0249 vs. OD) and mean number of teeth was 18.1±8.8. Among patients with teeth (14), OHI-S mean value was 3.25±1.35 (*Fig. 2*), 13 presented periodontitis and only 3, caries (p=0.0456 vs. OD) with a smaller percentage of teeth affected than patients with OD (7.63%±3.5, p=0.022). (*Supplementary table 1*).

DISCUSSION

Main results from this study show that older OD patients were at high risk of AP as they presented high prevalence of VFS signs of impaired safety of swallow, poor oral health status with high prevalence of

periodontal diseases and caries, and impaired health status with prevalent comorbidities and polymedication.

OD should be recognized as a major geriatric syndrome as its prevalence is very high in elderly patients and leads to multiple diseases and risk factors [8]. Studies on healthy elderly over 80 years found that ageing delayed and prolonged swallow response and increased oropharyngeal residue [16]. Impaired swallow response is caused by neurological diseases as well as the neurodegenerative process related to ageing [17], drugs with detrimental effects on consciousness or swallow response [18] and reduced tongue strength caused by sarcopenia [9]. In addition, many of our patients presented chewing difficulties. We have recently found that OD is an independent risk factor for the development of respiratory tract infections and community-acquired pneumonia in elderly patients [19,6]. Thus, the elderly, polymorbid, polymedicated and frail phenotype is at high risk for AP as they present high prevalence of oropharyngeal aspiration, impaired resistance to infections, and poor oral health, the three pathophysiological factors associated with AP [10]. Comorbidities and frailty are strongly related to impaired immunological status, periodontal diseases and oral colonisation by respiratory pathogens [7,8,10,18]. In addition, immune function in the oral cavity can be affected by oral residue after swallow and xerostomia [20].

The oral health status of our OD patients was very poor. Prevalence of edentulism, the final complication of periodontal disease, was found to be high-

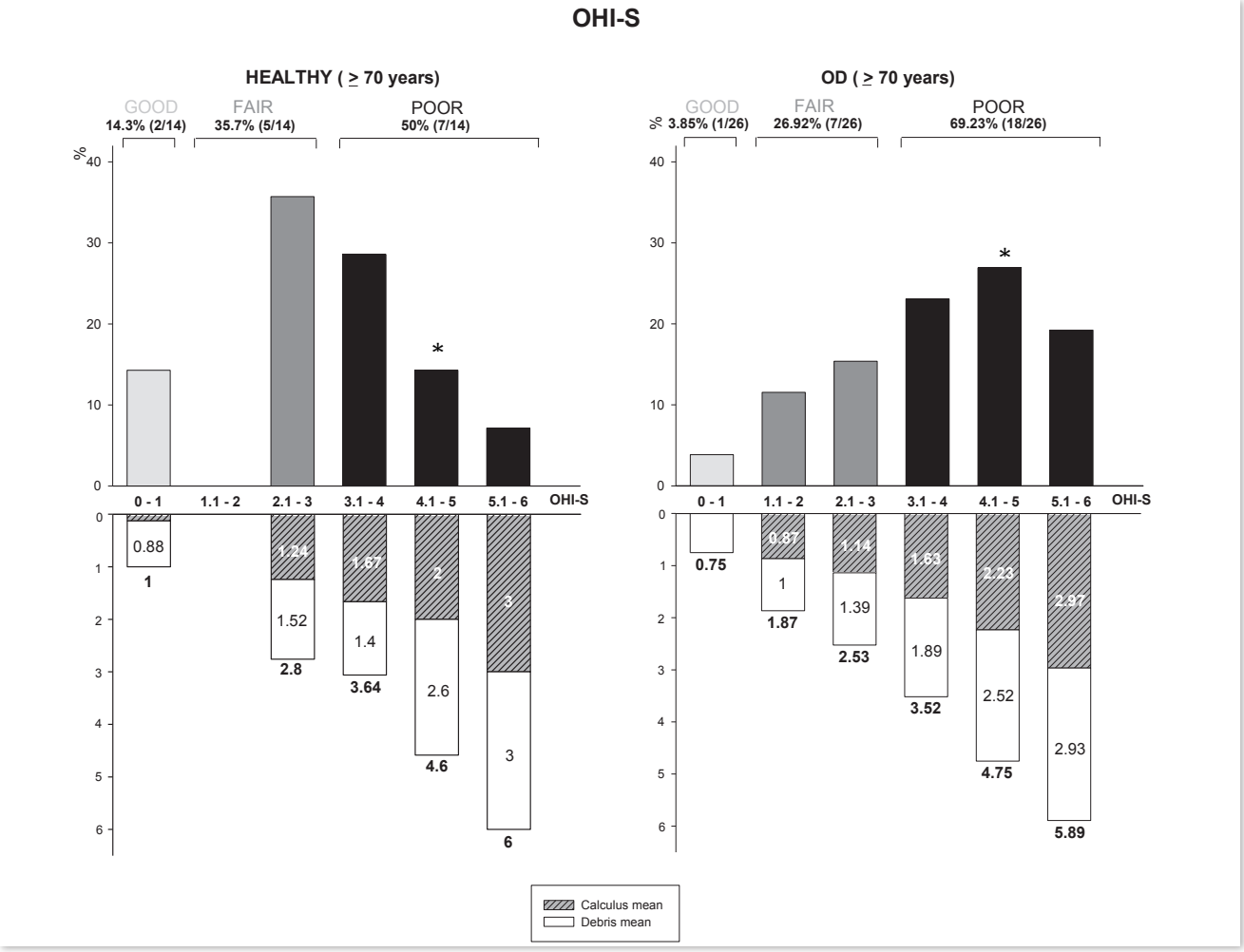

Figure 2. Results of the Simplified Oral Hygiene Index (OHI-S). Graphs showing the results of the OHI-S of healthy and OD valuable elderly patients. The upper part of the graphs shows the results of the index in three categories (good (0-1), fair (1.1-3) and poor (3.1-6)). On the lower part of the graph the OHI-S has been divided into its two components, debris and calculus. Mean results for the OHI-S and its components are shown. * *Indicates p<0.05.*

er (40%) in our OD patients compared to different studies on European senior citizens of similar ages [21, 22, 23] and to our control group. In addition OD patients had more caries than controls. Both groups of patients presented high OHI-S values caused by poor oral healthcare, periodontits and caries [13,24], slightly poorer in OD patients. In this process, the total number of bacteria is greatly increased, from 10^2–10^3 bacterial cells in a healthy gingival sulcus to 10^5–10^9 bacteria with periodontitis [3]. Periodontal pathogens may facilitate the colonization of the oral cavity by common pneumonia pathogens [25], and poor oral health has been associated with the appearance, severity and mortality of AP [2,26,27].

The DI-S index measures the amount of the soft and fresh part of the biofilm while the CI-S describes the mineralized debris [28]. Our results suggest that daily tooth-brushing would decrease OHI-S by 50% in OD elderly patients and controls (*Fig.2*). A recent review found a preventive effect of mechanical oral hygiene on pneumonia and respiratory tract infection in elderly people in hospitals and nursing homes [29] and we can hypothesize that this effect would be greater among patients with OD with a higher risk for aspiration. Recently, the World Health Organization has recommended improving the oral health of the elderly. Furthermore, the US Centers for Disease Control recognizes aspiration of oropharyngeal organisms as an important aetiological route for the development of AP in elderly patients and recommends the implementation of "comprehensive hygiene programs" [15]. We suggest two targets to reduce the risk of AP among frail elderly patients and the consequent high morbidity and mortality: a) early screening, identification and treatment of patients with OD and aspiration using clinical methods applicable in all medical centres [11,19]; and b) routine assessment of oral health, improvement of oral hygiene, and appropriate treatment of oral diseases. In addition, due to the high prevalence of edentulism, chewing assessment could help to select the most appropriate dietary adjustment for these patients.

"PLEASE NOTE: Further details from introduction, methods and results have been placed as supplementary material supporting this document.

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ON-LINE SUPPLEMENTARY MATERIAL

INTRODUCTION

AP: A recent 10-year review found a 93.5% increase in the number of hospitalized elderly patients diagnosed with AP while other types of pneumonia in the elderly decreased [1].

We have recently found that OD is a highly prevalent and independent risk factor in most older patients with community-acquired pneumonia (CAP) [**17,18**]. Other studies found that oropharyngeal aspiration was associated with a 5.6-7-fold increase in risk for pneumonia [2] and that OD was a major risk factor for readmission for pneumonia in frail elderly patients [**6**].

Oral health: The health status of older patients is often affected by concomitant chronic diseases which increase the risk of mortality. [**8**]. Diabetes contributes to, and is negatively impacted by, poor oral health [3]. Furthermore, periodontal pathogens can induce inflammation not only locally but at distant sites [4] and increase the risk of cardiovascular disease [5].

METHODS

Oral examinations: The assessment of the OHI-S, periodontal diseases and caries was only performed in dentate patients. Oral examinations were conducted with the aid of mouth mirrors (Proclínic SA, Hospitalet del Llobregat, Spain) and periodontal probes (PCP-UNC 15®, Hu-Friedy, Chicago, IL, USA). The clinical examination included measurements of clinical attachment loss (CAL), probing pocket depth (PPD), bleeding on probing (BOP), and tooth mobility. We explored two buccal sites per tooth (mesio-buccal and mid-buccal), excluding third molars.
Oral hygiene habits questionnaire was answered by 39 patients.

EAT-10: The EAT-10 (Eating Assessment Tool) [**9**] is a symptom-specific questionnaire for dysphagia. It is composed of 10 questions which evaluate specific OD symptoms. The maximum score per question is 4 points; patients who score more than 3 points are at risk of OD and further assessment is needed. This questionnaire was given to all the patients as an OD screening.

VFS performance: patients were studied during the deglutition of series of 5 mL, 10 mL and 20 mL boluses with nectar, liquid, and pudding viscosity using a previously described algorithm for VFS studies [**7**]. Liquid viscosity was obtained by mixing 1:1 mineral water and the X-ray contrast Gastrografin (Berlimed SA, Madrid, Spain) at room temperature; nectar viscosity, by adding 2.4g of thickener Resource ThickenUp Clear (Nestlé Healthcare Nutrition, Vevey, Switzerland) to liquid solution, and pudding by adding 5.4 g of the thickener [**9**]. VFS signs of impaired safety or efficacy of deglutition were diagnosed according to accepted definitions [**10**,6].

RESULTS

Chewing capacity: 20/50 patients with OD used dentures. These were edentulous patients that used complete dentures to eat with. Up to 9/50 patients needed dietary adjustments for impaired chewing. Up to 38/50 patients with OD presented alterations in oral phase. Taken together, these results suggest that prevalence of chewing difficulties among our patients with OD was high.

Diseases: Patients with OD presented many comorbidities (n=50): 43 presented cardiovascular diseases (37, arterial hypertension; 16, arrhythmia; 6, ischemic heart disease); 41, central nervous system; 26, metabolic; 23, musculoskeletal; 21, respiratory; 20, uro-genital; 19, gastrointestinal; 19, endocrinal (14, diabetes mellitus II); 13, ophthalmologic; 10, otorhinolaryngological; 9, hepatic and 4, skin diseases.

Medication: patients with OD (n=50) took several drugs for cardiovascular (43) and nervous system (38) diseases, thrombosis (26), gastro-oesophageal reflux disease (proton pump inhibitors) (40), and diabetes (8, oral hypoglycaemic and 2, insulin). In addition, 17 patients took sedatives and 18 patients received medication for depression; 6, for epilepsy; 4, for Parkinson; 1, for dizziness and 1, for dementia with potential effects on swallow response.

Penetration-aspiration severity: Penetration into the laryngeal vestibule during the pharyngeal phase (Rosenbek level 3-5) was the most prevalent cause of unsafe deglutition and was observed in 24/44 of patients when swallowing liquid boluses according to our VFS protocols [11]. Increasing viscosity to nectar reduced prevalence to 12/50, and further increase to pudding viscosity reduced prevalence to 5/50 (p=0.002). Aspirations (Rosenbek 6-8) were present in 6/44 patients during liquid series, 2/50 patients at nectar and 1/50 patient during pudding viscosity (p<0.05) [**11**]. However, oral residue was present in 5/44 patients during liquid series, 5/50 patients during nectar series and 11/50 patients during pudding series. Pharyngeal residue was observed in 6/44 patients during liquid series and 11/50 patients during both nectar and pudding viscosity series.

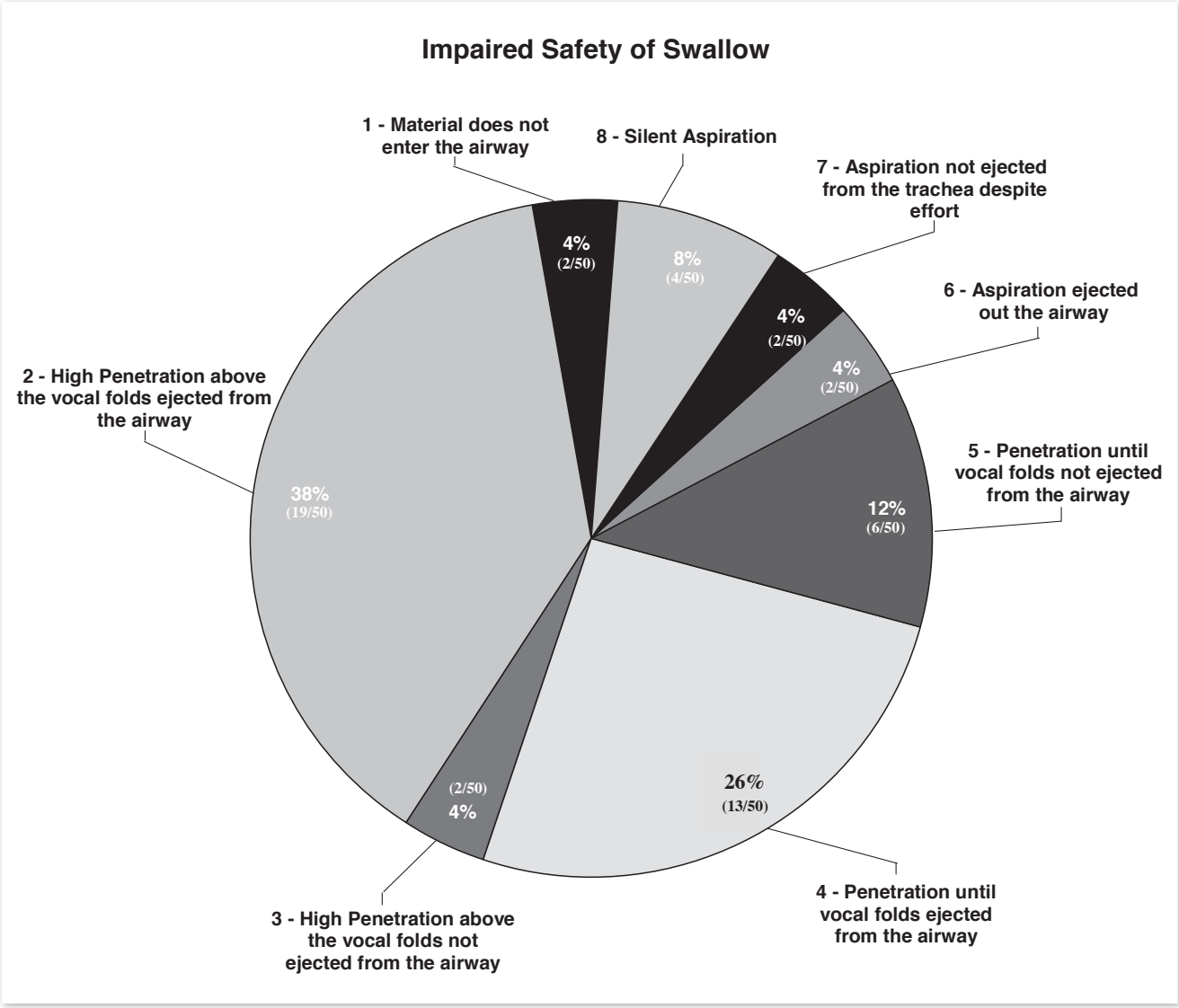

Figure 1. Safety of swallow among older patients with oropharyngeal dysphagia. Prevalence of patients with several levels of videofluoroscopic signs of impaired safety of swallow according to the Rosenbek Penetration-Aspiration Scale [11].

	OD Patients	Controls	*p*-value
N	50	15	
Age	79.7±6.64	77.09±4.51	0.1922
Edentulism	40% (20/50)	6.7% (1/15)	**0.0249**
nº teeth (dentate patients)	17±8.3	18.1±8.8	0.5123
OHI-S	3.86±1.5	3.25±1.35	0.2869
Oral Health Status			
Health	0% (0/30)	7.14%(1/14)	0.4375
Gingivitis	6.67% (2/30)	0% (0/14)	1
Periodontitis	93.3% (28/30)	92.86% (13/14)	1
MP	20% (6/30)	50% (7/14)	0.0821
MOP	36.7% (11/30)	21.43% (3/14)	0.3133
SP	36.7% (11/30)	21.43% (3/14)	0.3133
Caries	59.3% (16/27)	21.43% (3/14)	**0.0456**
% Teeth	23.05±0.16	7.63±3.5	**0.022**
% Surface (CI)	8.2±5.13	2.71±2.44	0.1032
Clinical assessment			
Charlson	2.98±1.83	1.4±1.12	**0.0023**
EAT-10	10.5±7.6	0.23±0.6	**<0.0001**
EAT-10 ≥3	84% (42/50)	0% (0/30)	**<0.0001**

Table 1. Oral health. Oral health status between elderly patients with OD and elderly controls (MP: mild periodontitis; MOP: moderate periodontitis; SP: severe periodontitis). OHI-S and prevalence of periodontal diseases and caries results are based on the dentate patients.

Additional references from the supplementary introduction and not in the main text (not in bold)

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APPENDIX I. ORAL HEALTH ISSUES

ABSTRACT

Oral health and hygiene status is of key importance in older patients with oropharyngeal dysphagia (OD) as it has been related with the development of respiratory infections and aspiration pneumonia. Poor oral hygiene is associated with the development of a gram negative microbiota and colonization by respiratory pathogens. In oral surfaces microbial biofilm develops naturally just after toothbrushing. If there is a lack of oral hygiene, this biofilm matures and microbial succession progress starts giving the chance to more pathogenic genera and species to colonize the oral cavity.

There are several methods to evaluate oral hygiene as the simplified oral hygiene index (OHI-S) and several methods to classify oral diseases such as the different grades of periodontal diseases. This annex to chapter I of the doctoral thesis explains the methodology used in this first chapter to assess and describe oral healh and hygiene status of our target population as well as the description of several oral diseases studied during this first part of the thesis. In addition, at the end of the document there are several examples with real pictures of patients with different oral pathologies.

ORAL HEALTH

Oral cavity and especially dentition, is of key importance for mastication and deglutition and oral components are designed to prepare food to be swallowed during the oral preparatory phase of swallowing. Elderly patients with OD generally have poor oral health and hygiene status as we have previously shown. This appendix describes the methods to study oral health status and periodontal diseases used in chapter I and includes several pictures and examples form real patients.

1. Oral hygiene index and oral biofilms

In chapter 1 we measured oral hygiene with the simplified oral-hygiene index developed by Greene and Vermillion (1). This index is a composed measurement of the accumulation of dental plaque and calculus in 6 different teeth and is helpful to evaluate dental covering or oral biofilm of subjects assessed. We have to differentiate between debris (soft foreign matter attached to the teeth; mucin, bacteria and food) and calculus (mineralized debris made composed of deposits of inorganic salts). Each index (calculus and debris) is evaluated independently according to the amount of deposits found in the target tooth. 3 teeth examined in the maxillary arch and 3 in the mandibular arch. We finally calculate the OHI-S by adding the results of the debris and calculus index (Figure 1).

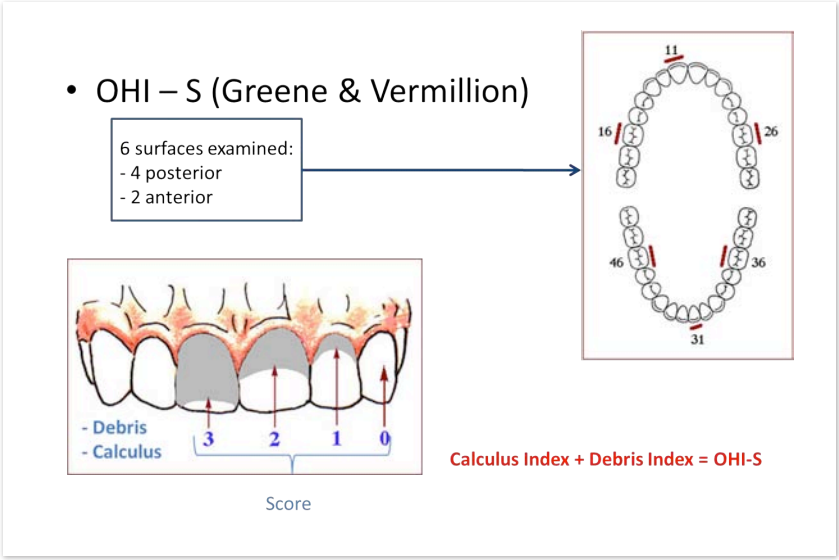


Figure 1. Simplified oral hygiene index (OHI-S). Teeth examined in the OHI-S and scores given depending on tooth coverage of debris and calculus (1).

1.1 Biofilm components

Dental plaque: has been defined as a "structured, resilient, yellow-grayish substance that adheres tenaciously to the intraoral hard surfaces, including removable and fixed restorations" (2). Dental plaque is broadly classified as supragingival or subgingival on the basis of its position on the tooth surface towards the gingival margin (3). It is mainly composed by microorganism containing more than 500 different microbial phylotipes and 1011 bacteria per gram of dental plaque (wet weight) (4-6). Dental plaque microbiota is organized in biofilm communities that are very similar to other biofilms found in nature. It has a heterogeneous structure with open fluid-filled channels that facilitate the diffusion of nutrients to the colonies through water channels (7-9). Attached microcolonies are embedded in an extracellular matrix formed of organic and inorganic materials through which the channels run. The extracellular matrix confers a special environment that protects and makes oral bacteria interact with other biofilm microbial populations forming complex metabolic interactions. These conditions make oral dental plaque microbiota very different from the free-floating bacteria (planktonic state) living suspended in saliva or crevicular fluid (3). Main organic components of dental plaque are polysaccharides, proteins, glycoproteins, lipids and DNA (10). Main inorganic components are calcium and phosphorus with traces of sodium, potassium and fluoride. Dental plaque can be easily removed by proper toothbrushing.

Calculus: is the product of mineralization of dental plaque and is generally covered by a layer of unmineralized plaque. As the proportion of mineral content of dental plaque increases, calcification occurs forming dental calculus. It normally appears in regions were mineral components are more available like next to dentition areas that are close to salivary ducts. Like in dental plaque, we can find supragingival or subgingival dental calculus. To eliminate calculus properly a professional oral hygiene should be performed (3).

1.2 Biofilm formation

The formation of the oral biofilm is normally classified in three different phases: 1) formation of the pellicle on the tooth surface; 2) initial adhesion/ attachment of bacteria; 3) colonization / plaque maturation.

1) **Pellicle formation:** the so-called "acquired pellicle" covers all the surfaces of the oral cavity, including hard and soft tissues. It is a layer of organic material containing more than 180 peptides, proteins and glycoproteins that can act as adhesion site for bacteria (11-13). It is formed in few minutes/hours.
2) **Adhesion:** shortly after pellicle formation there are some bacteria that have adhesins in their cell surfaces that can interact with receptors present on the surface of the pellicle. Only a low percentage of oral bacteria can perform this union and that is the reason why these organisms are generally the most abundant found in immature biofilms. During the first 4 - 8 hours, from 60 to 80% of the bacteria present in the primary biofilm are from the genus *Streptococcus* (Gram positive) (14;15). In addition bacteria from the genus *Actinomyces* and *Veillonella* are common inhabitant of the primary oral biofilm (Table 1) (4;16). All of them are defined as "primary colonizers" and bring new opportunities to other bacterial genus to further colonize the biofilm by providing new binding sites for adhesion and by changing biofilm environment due to metabolic byproducts.

Initially, at a distance of the surface of ≈50 nm, the main forces that play a role in bacterial-pellicle adhesion are van der Waals attractive forces and electrostatic repulsive forces. These initial attachments are reversible and it has been determined that from 10 - 50 ligand-receptor interactions are needed to attain irreversible binding of a bacterial cell to the pellicle (17). After initial adhesion, bacteria are irreversibly attached to the teeth pellicle by means of spe-

cific adhesins on the bacterial cell surface (normally proteins) and complementary receptors (proteins, glycoproteins, or polysaccharides) in the acquired pellicle. The ability to bind to specific surface receptors will determine the type of bacteria that will colonize the acquired pellicle surfaces. After primary colonization, the process of co-adhesion or co-aggregation will occur as new receptors for attachment by other bacteria will bring the opportunity to the development of microcolonies, and if biofilms remains stable (no oral hygiene), to a mature biofilm (18;19). At least 18 oral bacterial genus have shown some form of co-aggregation (20). But there are some bacteria that are of key importance in this process of secondary colonization. *Fusobacterium nucleatum* is the most abundant gram negative specie in healthy sites, and its number highly increases in periodontally diseased sites (21). It acts as a co-aggregation bridge between non-co-aggregating bacteria because it can co-aggregate with all species of early and late colonizers (19;22-24). These late colonizing bacteria are called the "secondary colonizers" and the most common ones are from the genus *Prevotella, Fusobacterium, Treponema, Porphyromonas and Campilobacter* (Table 1). Between primary and secondary colonization a shift in the type of microbiota is produced; bacterial composition turns from a majority of gram positive bacteria in primary colonization to a majority of gram negative bacteria in secondary colonization and biofilm maturation (19;25). In addition, an increase of biodiversity is produced from early colonization (low diversity) to late colonization (high diversity) (19). Every new microbe that is attached offers a new surface to co-aggregate to other microorganisms. Each microorganism can co-aggregate with specific microbial partners, establishing a complex microbial network (Figure 2) (3).

Primary colonizers	**Secondary colonizers**
Streptococcus gordonii	*Campylobacter gracilis*
Streptococcus intermedius	*Campylobacter rectus*
Streptococcus mitis	*Campylobacter showae*
Streptococcus oralis	*Eubacterium nodatum*
Streptococcus sanguinis	*Aggregatibacter actinomycetemcomitans* serotype b
Actinomyces gerencseriae	*Fusobacterium nucleatum* spp *nucleatum*
Actinomyces israelii	*Fusobacterium nucleatum* spp *vincentii*
Actinomyces naeslundii	*Fusobacterium nucleatum* spp *polymorphum*
Actinomyces oris	*Fusobacterium periodonticum*
Aggregatibacter actinomycetemcomitans serotype a	*Parvimonas micra*
Capnocytophaga gingivalis	*Prevotella intermedia*
Capnocytophaga ochracea	*Prevotella loescheii*
Capnocytophaga sputigena	*Prevotella nigrescens*
Eikenella corrodens	*Streptococcus constellatus*
Actinomyces odontolyticus	*Tannerella forsythia*
Veillonella parvula	*Porphyromonas gingivalis*
	Treponema denticola

Table 1. Most prevalent primary and secondary colonizers of oral biofilm. *Adapted from Carranza's Clinical Periodontology (3).*

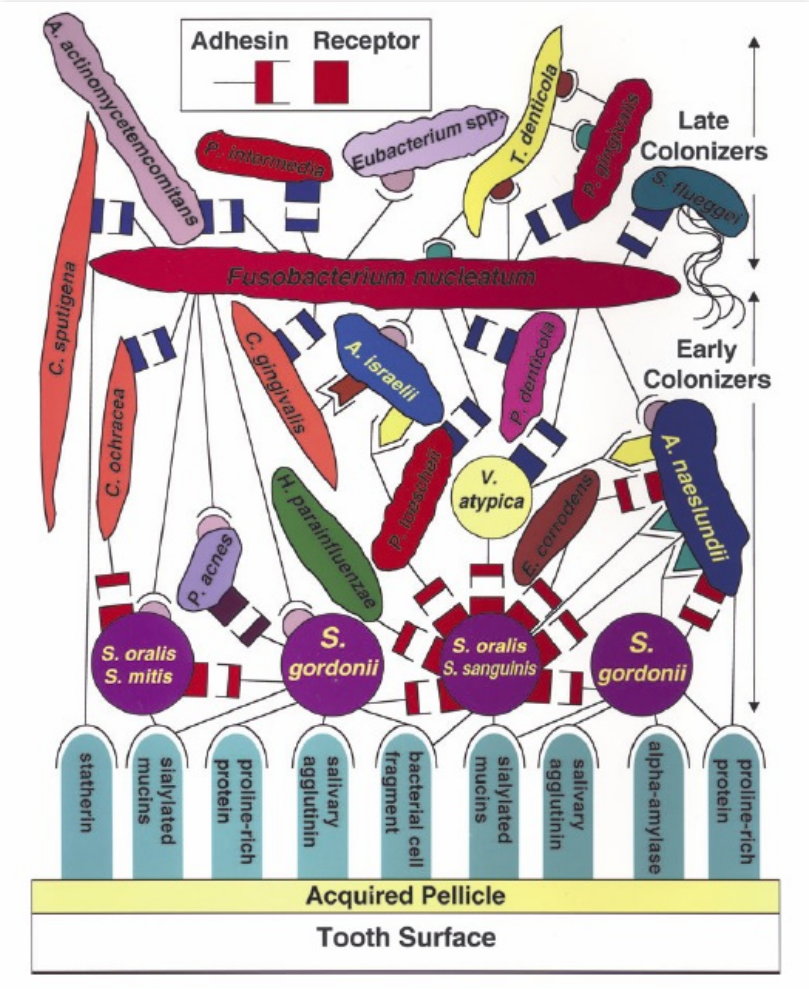


Figure 2. Oral bacterial colonization and dental plaque development. Oral microbial succession model showing recognition of salivary pellicle receptors by primary colonizing bacteria, and co-aggregations between early colonizers, Fusobacteria and secondary colonizers of the tooth surface. *From Kolenbrander 1993, 2006 (18;19).*

2. Oral diseases

In our study (Chapter I) we assessed oral diseases with specialized probes and by using previously defined classifications. Periodontal diseases were assessed with a periodontal probe and a classification shown in Table 2 by the Centers of Disease Control and Prevention (CDC) (26). Caries was determined by direct observation with the help of a dentist mirror and a probe.

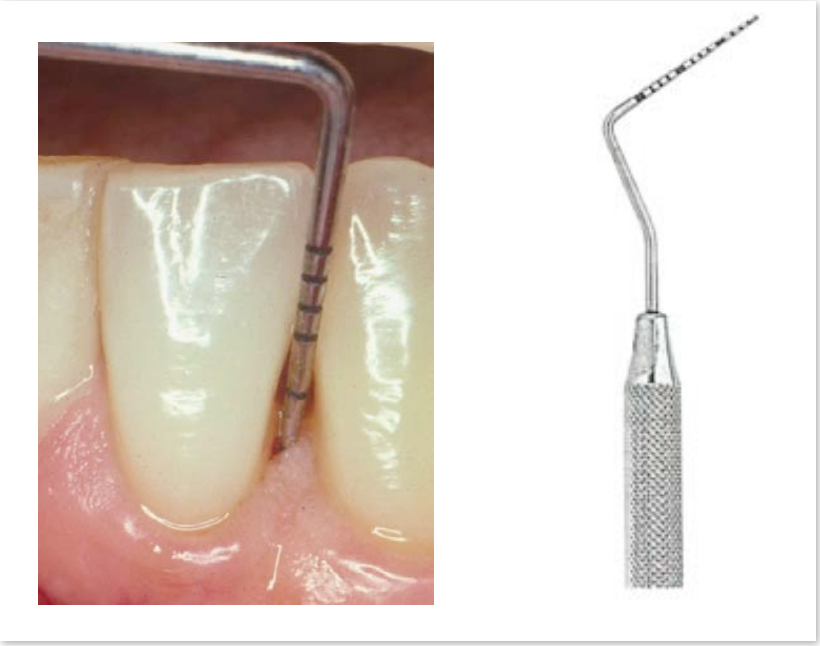


Figure 3. Periodontal probing. Periodontal probing process (left) and a periodontal probe (right). The primary aim of this probe is to measure pocket depths around teeth in order to establish the state of health of the periodontium. The head of the probe is marked in millimeters for accuracy and readability.

DISEASE CATHEGORY	CLINICAL DEFINITIONS		
	Clinical Attachment Loss (CAL)		Periodontal Depth/Inflammation
Severe Periodontitis	≥2 interproximal sites with CAL ≥6mm (not on same tooth)	and	≥2 interproximal sites with PD ≥5mm on same teeth as CAL + evidence of clinical inflammation.
Moderate Periodontitis	≥2 interproximal sites with CAL ≥4mm (not on same tooth)	and	≥2 interproximal sites with PD ≥5mm (not on same tooth) + evidence of clinical inflammation.
Mild Periodontitis	≥2 interproximal sites with CAL ≥2mm (not on same tooth)	or	≥2 interproximal sites with PD = 4 mm + evidence of clinical inflammation.
Gingivitis	No evidence of CAL	and	Clinical evidence of gingival inflammation
Clinical Health	No evidence of CAL	and	No evidence of inflammation

Table 2. Periodontal disease severity classification used in chapter I. Modified form "Clinical Case Definition Proposed by the CDC (Centers of Disease Control and Prevention) Working Group for use in Population-Based Surveillance of Periodontitis (CDC – AAP Working Group)" (26).

2.1 Gingivitis

Is referred to the inflammatory process of the gingival tissue that surrounds the teeth due to the presence of microbial biofilm (3;27). This biofilm is formed through the lack of good oral hygiene practices. Generally, optimal plaque control leads to the complete resolution of this early gingival inflammation (28). The prevalence of gingivitis increases with age (28).

Main clinical signs of gingivitis are the following (3):

1. Redness and sponginess of the gingival tissue.
2. Bleeding on provocation.
3. Changes in contour.
4. Presence of calculus or plaque with no radiographic evidence of crestal bone loss.

Gingival inflammation is mediated by the release of microbial products that cause damages to the epithelial and connective tissue and intercellular constituents (collagenase, hyaluronidase, protease, chondroitin sulfatase, endotoxin). These products will induce the production of vasoactive substances (prostaglandin E2, interferon, tumor necrosis factor, and interleukin-1) by activation of monocytes and macrophages that will alter the properties of gingival tissue (29-31).

2.2 Periodontal disease

Is referred to the inflammatory process of the gingival tissue and the loss of tooth attachment by the destruction of the periodontal ligament and alveolar bone. Gingivitis is the prior status of periodontal disease. During the process there is a migration of the crevicular epithelium that forms the periodontal pockets. The process ends up with the progressive loss of teeth. Periodontal disease is the primary cause of tooth loss in adults (3;32).

Main clinical findings in patients with Periodontitis are the following (3).

1. Supragingival and subgingival plaque and calculus
2. Gingival swelling, redness, and loss of gingival stippling
3. Altered gingival margins (e.g., rolled, flattened, cratered papillae, recessions)
4. Pocket formation
5. Bleeding on probing
6. Attachment loss (angular or horizontally)
7. Bone loss
8. Root furcation involvement (exposure)
9. Increased tooth mobility
10. Change in tooth position
11. Tooth loss

Biofilm accumulation, like in gingivitis, is considered the primary causing agent of periodontal disease (33). In patients with healthy gingiva, oral cav-

ity contains mostly few facultative gram-positive microorganisms, mostly coccoid cells and straight rods (34;35). Diseased gingiva is associated with anaerobic and microaerophilic gram-negative flora with increased numbers of spirochetes and motile rods and with specific periodontopathogens like *Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola (also known as red complex)* (34-37). There are additional microorganisms that are thought to be involved in the process including *Aggregatibacter actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans), Prevotella intermedia, Campylobacter rectus, and Fusobacterium nucleatum* (37). In patients with poor oral hygiene, the inflammatory progress will develop and eventually result in the loss of attachment of dentition (38).

Periodontopathogens influence the pro-inflammatory immune response of the host by increasing concentrations of pro-inflammatory mediators (39). Moreover, some periodontopathogens are able to produce proteases that affect directly tissue stability and host immune response (40). These bacteria can colonize the intercellular space under exfoliating epithelial cells and can be found also deeper between epithelial cells as well as accumulating on the basement lamina. Some of them can traverse the basement lamina and invade the subepithelial connective tissue (41).

2.3 Edentulism

Is defined as the loss of teeth and can be partial or total (no teeth). Is mainly caused by periodontal disease and is more common in the older population. Edentulism negatively affects patients' chewing and speaking, esthetics, and self-esteem. A study found that 45% of people wearing dentures had oral lesions caused by their denture (43). Although having no teeth, regular and proper dental care is very important for edentulous patients to maintain good oral health and hygiene status and to avoid bacterial colonization (32).

2.4 Caries

Dental caries comprises the progressive disruption of tooth structure caused by acids as a product of bacterial metabolism in the presence of sugars. If caries is not treated, it can cause tooth loss and progression into the pulp of the tooth that may cause pain, dental abscess, bacteremia, facial/pharyngeal infection, septicemia, and rarely cavernous sinus thrombosis (32).

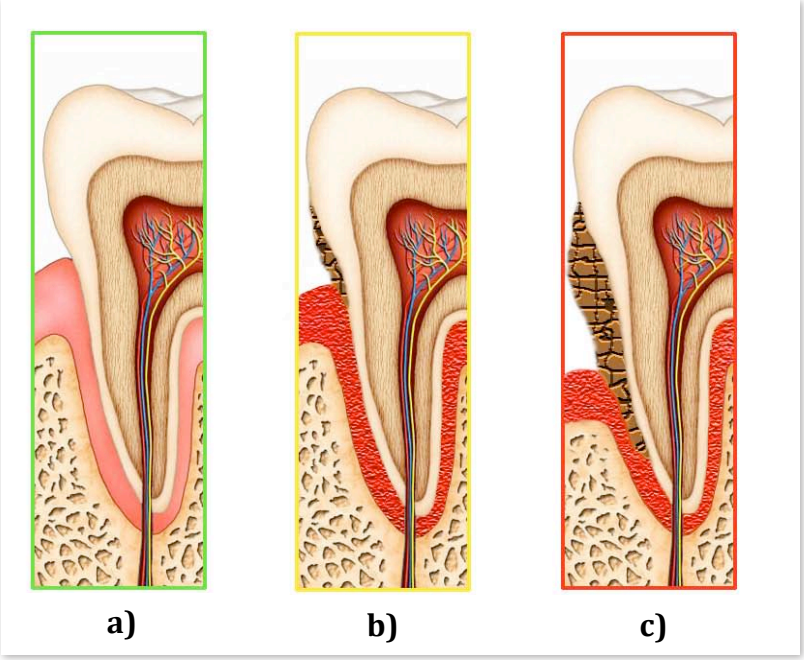


Figure 4. Evolution of periodontal disease. a) Healthy periodontium with no inflammation (gingivitis) and or bone loss (periodontitis); b) periodontal inflammation (gingivitis), there is still no bone loss; c) Periodontitis with inflammation and bone loss. *Adapted from Ortega O 2014 (42).*

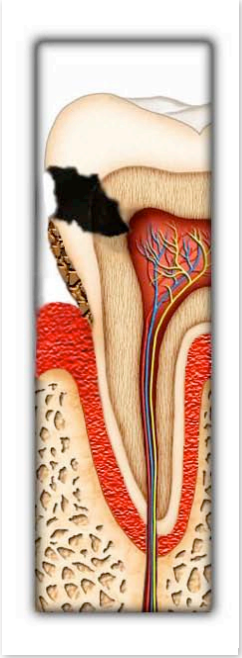


Figure 5. Caries. Disruption of tooth structure. *Adapted from Ortega O 2014 (42).*

2.5 Examples of oral diseases with real patients. Images courtesy of Universitat Internacional de Catalunya, Odontology, department of Periodontology.

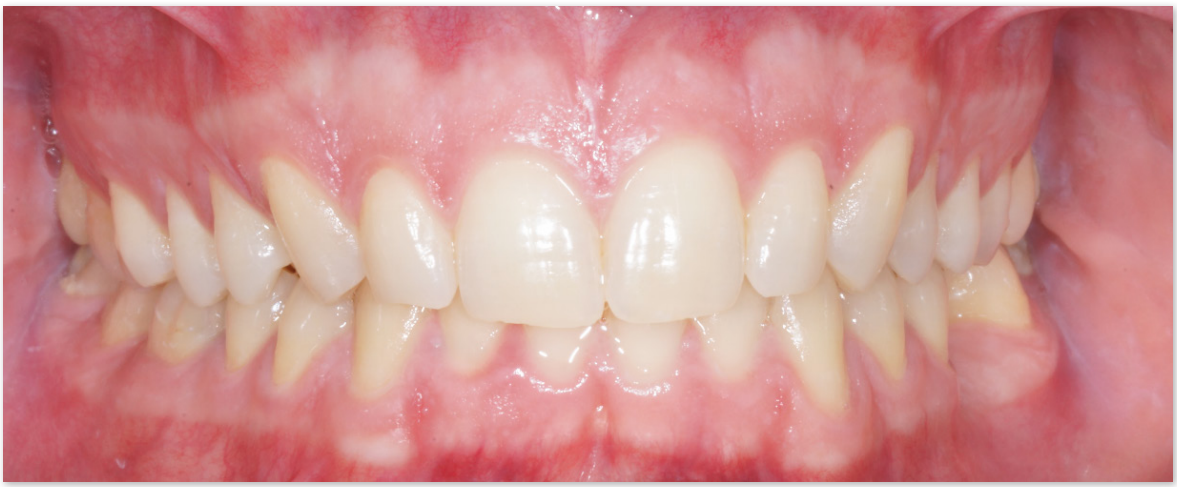


Figure 6. Healthy oral cavity with no inflammation of the gums and accumulation of dental plaque and/or calculus.

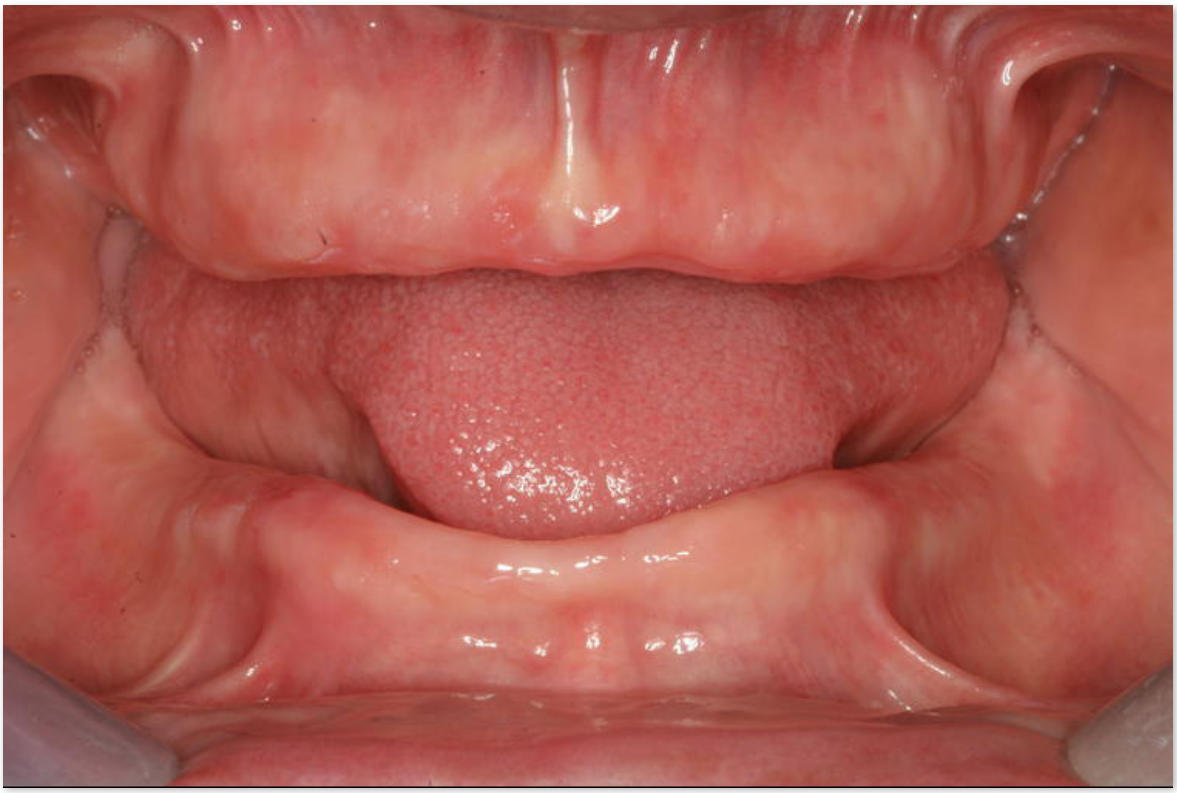


Figure 7. Edentulous patient. *From Foundation for Oral Rehabilitation.*

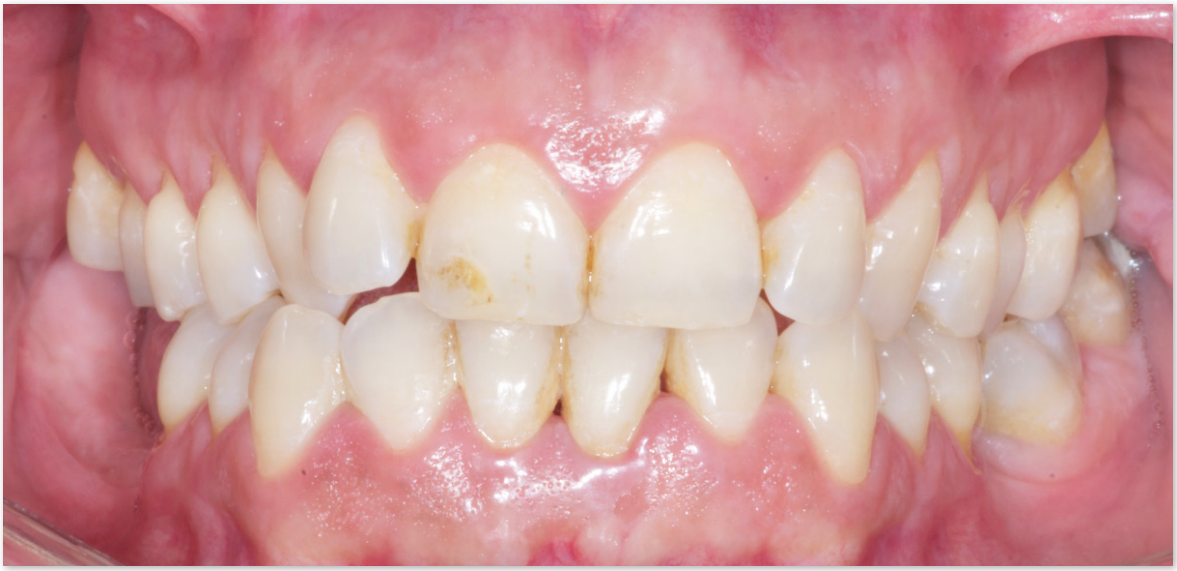


Figure 8. Gingivitis with gingival inflammation and accumulation of dental plaque and calculus.

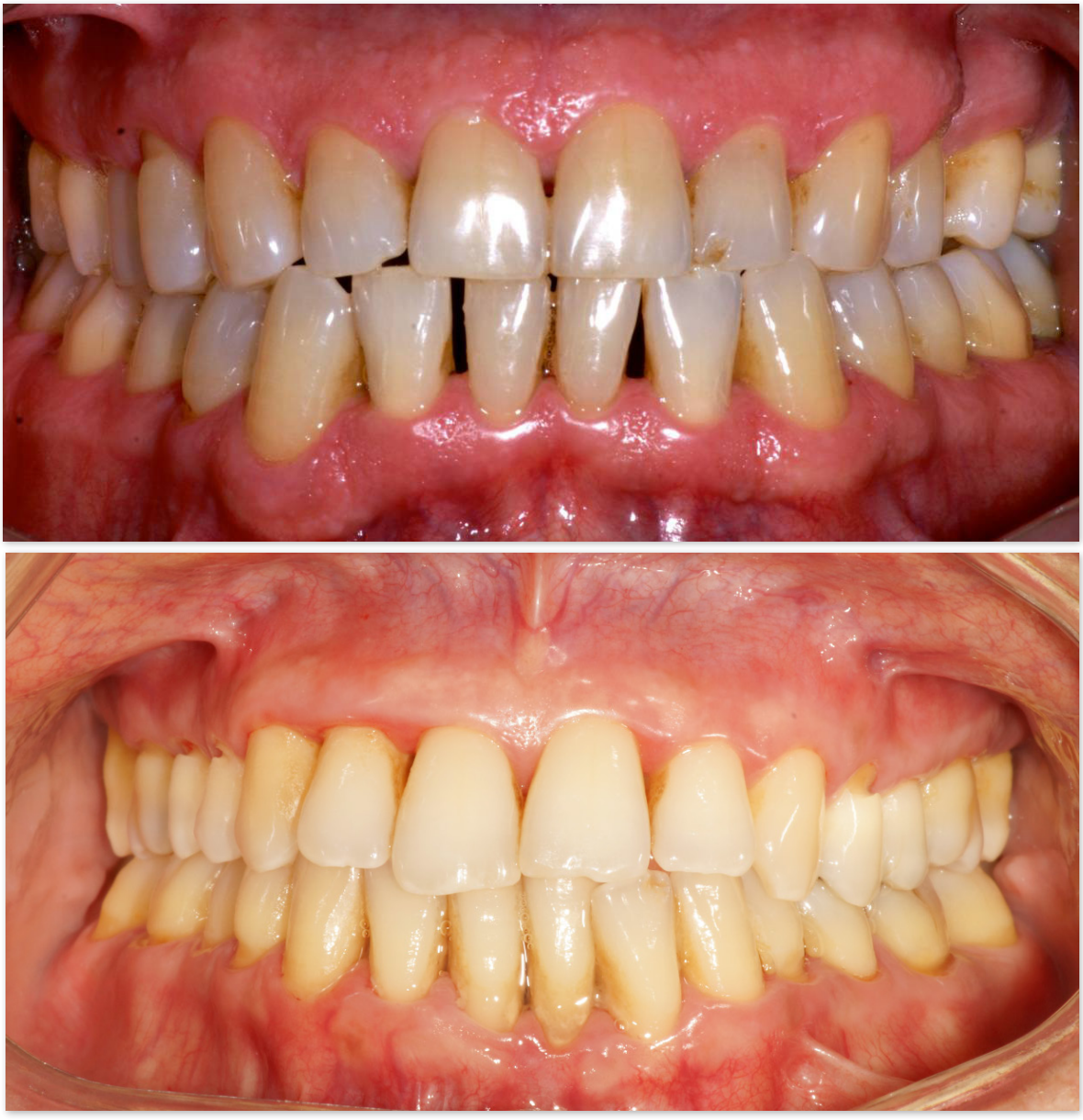


Figure 9. Generalized chronic periodontitis with inflammation, altered gingival margins and accumulation of plaque and calculus.

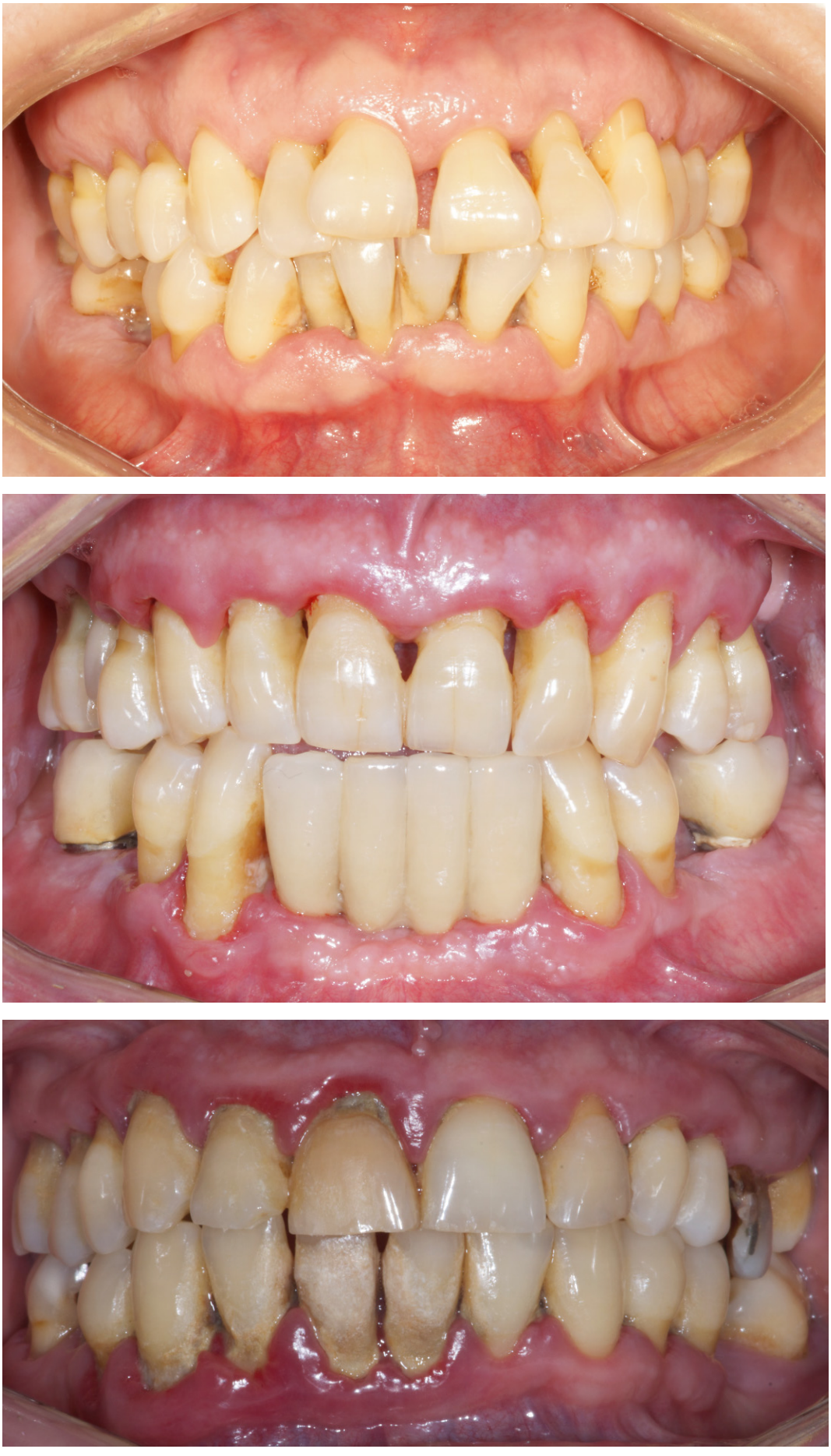
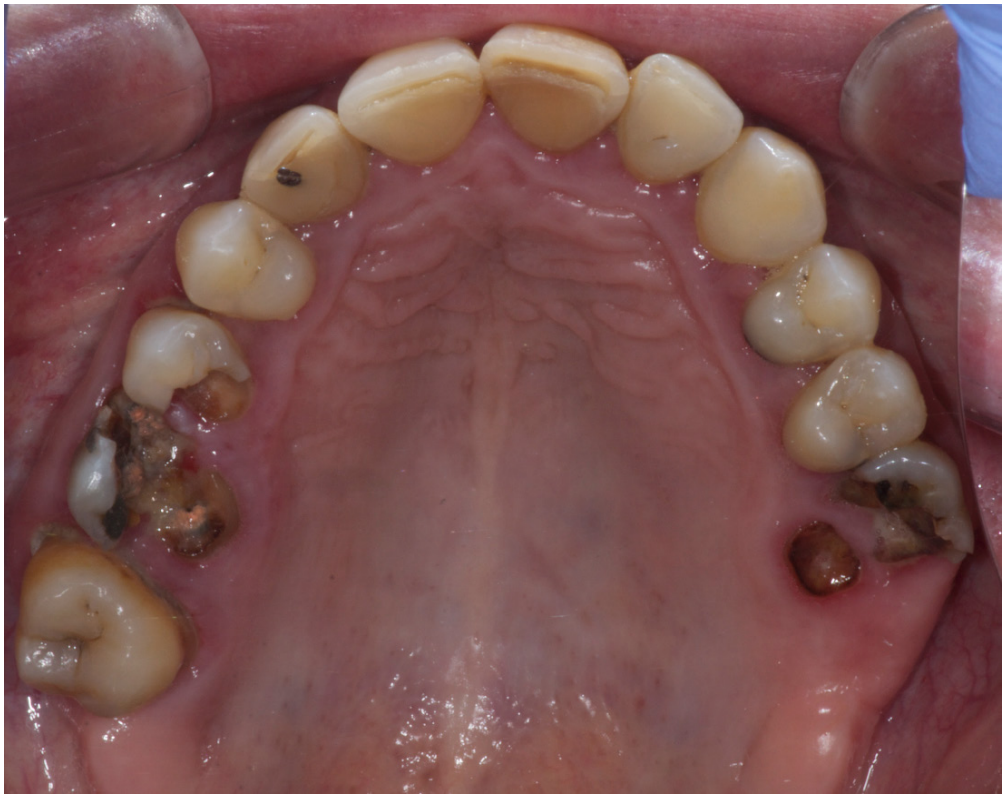


Figure 10. Severe chronic periodontitis with high inflammation, altered gingival margins and accumulation of plaque and calculus.



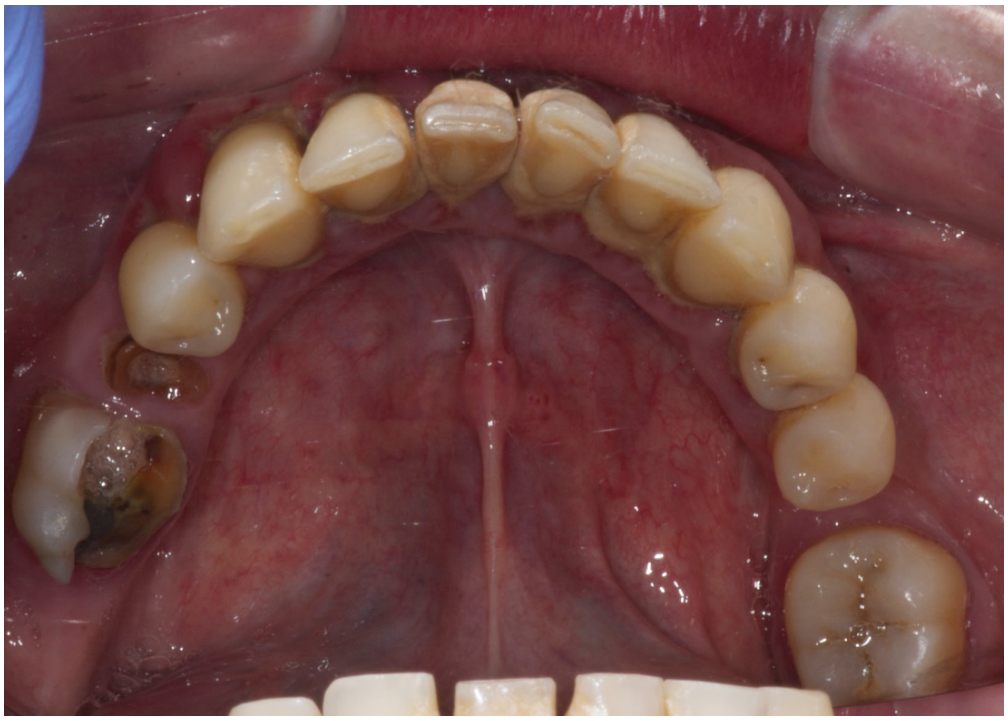


Figure 11. Active caries with high disruption of tooth structure.

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CAPÍTULO 2

High prevalence of colonization of oral cavity by respiratory pathogens in frail older patients with oropharyngeal dysphagia

Omar Ortega, Olga Sakwinska, Séverine Combremont, Bernard Berger, Julien Sauser, Carlos Parra, Silvia Zarcero, José Nart, Silvia Carrión, Pere Clavé. High prevalence of colonization of oral cavity by respiratory pathogens in frail older patients with oropharyngeal dysphagia. *Neurogastroenterol Motil* 2015; 27(12):1804-16.

ABSTRACT

Background: Aspiration pneumonia (AP) is caused by dysfunctional swallowing resulting in aspiration of material colonized by respiratory pathogens. The aim of this study was to assess and compare the swallowing physiology, health status, oral health status and oral/nasal microbiota in frail older patients with oropharyngeal dysphagia (OD) and a control group.

Methods: We studied 47 frail older (>70yr) patients (FOP) with OD by videofluoroscopy (17 with acute pneumonia -APN-, 15 with prior pneumonia–PNP- and 15 without) and 14 older controls without OD (H). Oral/nasal colonization by five respiratory pathogens was evaluated by qPCR while commensal microbiota composition was assessed by pyrosequencing.

Key Results: a) FOP with OD presented similar co-morbidities, poor functionality, polymedication, and prevalent videofluoroscopic signs of impaired safety of swallow (33.3-61.5%). However, patients with OD-APN also presented malnutrition, delayed laryngeal vestibule closure (409.23±115.6ms; p <0.05) and silent aspirations (15.6%). b) Oral health was poor in all groups, 90% presented periodontitis and 72%, caries. c) Total bacterial load was similar in all groups but higher in the oropharynx (>10^8CFU/mL) than in the nose (<10^6CFU/mL) (p <0.0001). Colonization by respiratory pathogens was very high: 93% in OD patients (p <0.05 vs. H); 93% in OD-PNP (p <0.05 vs. H); 88% in OD-APN (p =0.07 vs. H), and lower in controls (67%).

Conclusions & Inferences: FOP with OD had impaired health status, poor oral health, high oral bacterial load and prevalence of oral colonization by respiratory pathogens and VFS signs of impaired safety of swallow, and were therefore at risk for contracting AP.

INTRODUCTION

Aspiration pneumonia (AP) has been defined as pneumonia contracted by a patient with oropharyngeal dysphagia (OD) who aspirates colonized oropharyngeal material [1,2]. AP occurs in older people with swallowing disorders [1]. A review on older patients hospitalized for pneumonia found a 93.5% increase in AP, while other types of pneumonia decreased [3], and the proportion of admissions due to AP among all admissions due to pneumonia increased gradually with age [4]. Both results have been attributed to increased prevalence of OD among older people. We recently found OD was an independent risk factor for lower respiratory tract infections [5], community-acquired pneumonia (CAP) in older patients [6], and pneumonia in frail older patients (FOP) [7]. In addition, OD is an indicator of severity and mortality in older patients with CAP. One-year mortality for patients with pneumonia and OD reached 40% and 55% for patients above 70 and 80 years respectively [6,7]. In nursing home residents with OD, AP occurs in 43%-50% during the first year, with a mortality of up to 45% [8]. Finally, in a prospective study we found that OD was a risk factor for readmission for pneumonia, bronchoaspirations and AP in older patients from an acute geriatric unit and that nearly 5% of all hospital readmissions and 80% of hospital readmissions for AP were attributable to OD, indicating the relevance of OD in terms of healthcare resource consumption [9].

OD is a symptom of swallowing disorder recognized by the World Health Organization (WHO) with specific ICD codes (787.2, R13) [10]. It is underdiagnosed despite its prevalence among the older population: 23% in independently-living older people, 55% in hospitalized older patients, and 56% to 78% in institutionalized older people [11-13]. Impaired safety (penetrations and aspirations) in FOP is related to delayed laryngeal vestibule closure (LVC) and caused by impaired neural swallow response due to neurogenic diseases and aging [14].

The pathophysiology of AP involves three key elements: OD with impaired safety of swallow and aspirations; frailty and impaired health and immunological status, and poor oral health and hygiene with bacterial colonization [15-21]. The oral cavity contains a complex microbial ecosystem of commensal and pathogenic bacteria dependent on oral health and hygiene. We found that older patients with OD had poor oral health with high prevalence of periodontitis and caries [15], signifying high risk for AP in patients with impaired safety of swallow [16-18]. Interventions treating oral health and hygiene reduced the incidence of respiratory infections and pneumonia in older patients suggesting AP can be prevented with adequate identification and care [19-21].

Oral microbiota of dysphagic patients is not well described in the literature so we designed and developed a proof of concept study to assess and compare the health status of the patients, the mechanisms of oropharyngeal dysfunction, the oral health and hygiene status, oral and nasal microbiota and colonization by respiratory pathogens. The aim of the study was to better understand the pathophysiology of AP in different phenotypes of frail older patients with OD in order to develop therapeutic strategies against colonizing respiratory pathogens in the future.

MATERIAL & METHODS

Study population

Study population included 61 older patients, 70 years of age or more admitted prospectively to the Gastrointestinal Physiology Unit of the Hospital de Mataró between April 2011 and January 2012. We recruited three phenotypes of frail older patients with OD and one control group of persons without OD: **Group 1 (OD-PNP)**: 15 patients with dysphagia who had had prior pneumonia, recruited from the outpatient dysphagia clinic; **Group 2 (OD)** 15 patients with dysphagia, without pneumonia or history of previous pneumonia, recruited from the same place; **Group 3 (OD-APN)**: 17 dysphagic patients with acute pneumonia (PN) that were recruited from the emergency department on hospital admission before antibiotic treatment; and **Group 4 (H):** 14 older control patients without OD, randomly selected from a primary care database in Mataró (Barcelona, Spain) [13]. Inclusion criteria were age greater or equal to 70 years of age, confirmed OD by clinical assessment and VFS (Group 1, 2 and 3), frail condition established according to Fried criteria (Group 1, 2, 3) and confirmed absence of OD by clinical assessment using the Volume-Viscosity Swallow Test (V-VST), in Group 4, older controls [11,22]. Exclusion criteria were antibiotic intake during the previous month, patients with severe pulmonary disorders needing oxygen, allergy to any medication or iodinated contrast media, and surgery in the past 3 months. All participants were informed about the study and gave their written consent. The study protocol (10.33 NRC) was approved by the Institutional Ethics Review Board of the Hospital de Mataró with the committee code 03/11 and was conducted following the principles and rules of the Declaration of Helsinki.

Experimental Design

A prospective, observational, transversal study was designed to assess oral health, swallowing function, nasal and oral microbiota and the oral and general health status of older patients. The study consisted of two visits performed on two consecutive days and included the following procedures:

a) **Health status and comorbidities.** A geriatric assessment was carried out by a multidiscipli-

nary team and included: (i) demographic data, (ii) frailty with the Fried criteria [23], iii) comorbidities according to the Charlson Comorbidity Index [24], (iv) functional capacity according to the Barthel Index [25], (v) nutritional status with the validated Spanish version of the Mini Nutritional Assessment short form (MNA-sf) [26], and body mass index (BMI) , vi) muscular force (measured with a TKK 5001-Grip A hand dynamometer. Takei Scientific Instruments, Japan), measuring maximal grip strength in kilograms with the dominant hand [9], and vii) medication.

b) **Diagnosis of pneumonia.** We have described the criteria for the diagnosis of pneumonia in older patients previously [6,7] and is based on clinical findings, laboratory data and infiltrations verified by X-ray. Pneumonia Severity Index (PSI) was also determined in each patient [27]. Presence or absence of prior pneumonia was determined by reviewing the clinical history of the previous 5 years.

c) **Assessment of OD.**

d) c.1) Clinical assessment. All patients were examined with the V-VST, a validated and accurate clinical assessment tool that uses three viscosity series (nectar-like, thin liquid and spoon-thick) and three volumes (5, 10 and 20 mL) to assess clinical signs of impaired efficacy and safety of swallow combined with pulsoximetry to detect silent aspirations [22,28,29]. We used a xanthan gum-based thickener (Resource ThickenUp Clear, Nestlé Healthcare NutritionHealth Science, Vevey, Switzerland).

c.2) Videofluoroscopy. Methodology used for VFS has been described previously [14,22,28]. Briefly, patients were studied during swallowing in lateral projection which included the oral cavity, pharynx, larynx, and cervical esophagus. Videofluoroscopic recordings were obtained by using a Super XT-20 Toshiba Intensifier (Toshiba Medical Systems Europe, Zoetermeer, the Netherlands) and recorded at 25 frames/s using a Panasonic AG DVX-100B video camera (Matsushita Electric Industrial Co, Osaka, Japan). Signs of impaired efficacy of deglutition were diagnosed according to accepted definitions [28,29] and signs of impaired safety were diagnosed by using the Penetration-Aspiration Scale (PAS) [30]. Quantitative measurements of oropharyngeal swallow response (timing of swallow response) were obtained during 5mL swallows using Swallowing Observer software (Image and Physiology SL, Barcelona, Spain) [29].

e) **Oral health assessment.** Oral health and hygiene were assessed by two dentists and included number of teeth and dental caries, simplified Oral Hygiene Index (OHI-S) [31] and assessment of periodontal diseases according to the Centers of Disease Control and Prevention Working Group [32]. Dental evaluations (OHI-S and oral diseases) were made on dentate patients [15].

f) **Sampling of oral/nasal microbiota.** Samples were collected from eight locations of the upper respiratory-alimentary tract: the nasal cavity, nasopharynx, oropharynx, tongue, sputum, saliva, dental plaque and oral rinsing of 10mL sterile water (Figure 1) for each person on two visits, one on admission (V1) and one 24h later (V2). Samples were taken early in the morning before breakfast and tooth brushing. Patients with OD-APN had only one swab collection on admission prior to antibiotic administration (V1). We used specially-designed sterile swabs for each location (Copan 480CE, 482C, SL solution and Amies transport medium, COPAN Brescia, Italy). We obtained dental plaque samples from the mesial buccal surface of the first molars using a sterile Gracey curette 11/12 (LM-dental, Finland) and stored them in Amies transport medium (COPAN Brescia, Italy). Afterwards, samples were frozen at -80ºC for posterior analysis of oral microbiota.

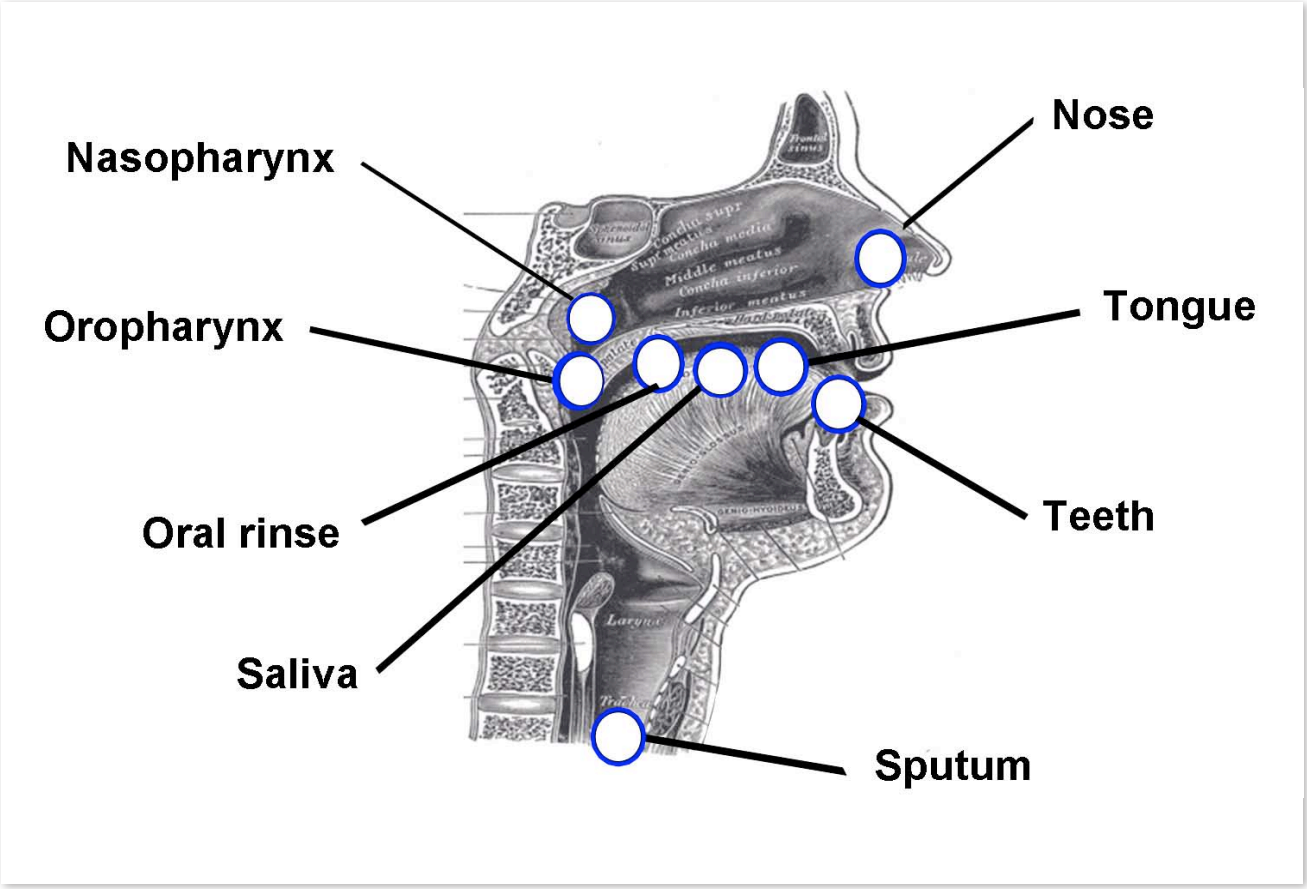

Figure 1. Anatomical sampling locations.

Analytical procedures

All analytical procedures were done blind. Extraction of total DNA was carried out using QiaAmp DNA mini kit.

a) **qPCR.** The quantification of five frequent AP pathogens (*Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli*), as well as total bacterial load was performed by qPCR. For *P. aeruginosa, S. aureus,* and *E. coli*, a commercial kit was used and the manufacturer's instructions were followed (PrimerDesign, UK). For the detection of *S. pneumoniae* and *H. influenzae*, primers targeting the genes lytA and hpd, respectively, were used [33]. For total bacterial load, the assay described by Nadkarni et al 2002 was used [34]. The pathogen loads are expressed as Colony Forming Unit (CFU) per mL of sample. For each qPCR assay, detection limit was slightly different and varied from $5x10^3$ CFU/mL to $3x10^4$ CFU/mL for specific pathogens, and $5x10^4$ CFU/mL for total bacterial load.

b) **Pyrosequencing.** The variable region V4-V5-V6 (456) of the 16S rRNA gene was used to characterize the microbiota by the barcoded-primer approach to multiplex pyrosequencing as previously described [35] and detailed in Supplementary Methods.

Data Management and Statistical Analysis

a) **Clinical data.** Qualitative data are presented as relative and absolute frequencies and analyzed by the Fisher's exact test and the chi-square test for multiple comparisons; continuous data are presented as mean±standard deviation (SD) and compared with the non-parametric Mann-Whitney U-test or Kruskal-Wallis test for multiple comparisons with the Dunns post-test or with the parametric t-test or 1-way ANOVA for multiple comparisons with the

Bonferroni's post-test. To assess normality we used the D'Agostino and Pearson omnibus normality test. Statistical significance was accepted if *p*-values were less than 0.05. Statistical analysis was performed using GRAPHPAD PRISM 5 (San Diego, CA, USA).

b) Total bacterial load. The values of total bacterial load were averaged over all visits and replicates to get a single total bacterial load value for each person. Comparisons were made in order to see if there was at least one group different from the others for each anatomical location. The same methodology was applied to compare the locations for each group. After checking the assumptions of normality and homogeneity of variances, either ANOVA was performed on the natural log-transformed data (nose, oral rinse, saliva, sputum) or Kruskal-Wallis test (nasopharynx, sputum, teeth, tongue).

c) Colonization by respiratory pathogens. Qualitative analysis was carried out with the proportion detectable being the outcome variable. Due to the sparseness of data at the individual pathogen level, the proportion detectable was aggregated over all the five measured pathogens. Thus a binary variable was created with a value of 1 if a subject had detectable pathogen load for at least one of the five pathogens at either of the two visits, and otherwise 0. This binary was computed over the two visits because the pathogen colonization was very similar at each visit. This proportion was then compared across the four patient groups and for each anatomical location. For statistical testing, a logistic regression with logit link was fitted to group, location and their interaction as covariates. Log-odds-ratios were then estimated in comparison to the healthy older group, along with unadjusted *p*-values. In addition, adjusted *p*-values for multiple comparisons, using the single step procedure, were calculated to maintain a familywise error rate of 5% for each location.

d) **We have developed a scale of colonization intensity for five pathogens.** The data were sparse and the detection limit of the qPCR assay varied among the five pathogens. First, for each pathogen, scaled pathogen load was obtained as the ratio of the pathogen load to the corresponding limit of detection. Individual level data consisted of all five scaled pathogen loads. Means of these five scaled loads were used after log-transformation, to obtain the overall Composite Pathogen Load (CPL) on the log-scale. Undetectable values were marked as <1. When all individual scaled pathogen loads were <1, CPL was not considered. Thus, CPL is a composite measure of colonization intensity by all five pathogens in each sample and patient group. A linear mixed model was used with patient group, anatomical location and their interaction as fixed effects and persons as random effects to adjust for correlation between measurements.

e) **Correlation between visits.** For agreement between the results from two visits for detectability of any pathogen, Kappa coefficient between the two measurements was computed for each location. For CPL data, correlation coefficients were computed for each location and by patient group.

RESULTS

Patient demographics and health status

Group 1 (OD-PNP) consisted of 15 patients (82.5±6.21 years; 9 men) with dysphagia and prior pneumonia; **Group 2 (OD)**, 15 patients (80.7±5.43 years; 9 men) with dysphagia and no prior episodes of pneumonia; **Group 3 (OD-APN)**, 17 patients with dysphagia (81.8±8.15 years; 9 men) hospitalized for an episode of acute pneumonia but not yet on antibiotics and **Group 4 (H),** a control group of 14 older persons (76±2.17; 9 men) without OD. General features of patients are described in Table 1. Patients with OD were older,

presented more comorbidities, poorer functionality and nutritional status, less force and higher medication intake than controls. When a multiple comparison for every item was applied, we found the patients with poorest conditions were those with acute pneumonia (OD-APN, Table 1). Etiological diagnosis of pneumonia in Group 3 was determined in 7/17 patients. *Streptococcus pneumoniae* (2), *Haemophilus influenzae* (2), methicillin-resistant (MR) *Staphylococcus aureus* (1), co-infection of MR *Staphylococcus aureus* and *Pseudomonas aeruginosa* (1) and *Moraxella catarrhalis* (1) were found as etiologic agents in these patients. Among the entire group, 12 patients presented CAP and 5 were patients admitted from nursing homes. Mean PSI score was 3.63±0.81 suggesting a low to moderate risk of mortality among these patients with APN.

	G1 OD-PNP	G2 OD	G3 OD-APN	G4 H	*p*-value
Subjects	15	15	17	14	
Sex (♂)	60% (9)	60% (9)	52.9% (9)	64.3% (9)	ns
Age	82.5±6.21	80.7±5.43	81.8±8.15	76±2.17*	**0.0247**
Dysphagia Cause					
Aging (%)	33.3 (5)	33.3 (5)	70.6(12)	-	
Stroke (%)	53.3 (8)	53.3 (8)	17.6 (3)	-	ns
NDD (%)	13.3 (2)	13.3 (2)	11.8 (2)	-	
Charlson	2.87±1.46	3±1.5	2.87±2.1	1.14±0.9*#	**0.0055**
0 (%)	6.67 (1)	6.67 (1)	6.25 (1)	14.3 (2)	
1-2 (%)	26.7 (4)	20 (3)	37.5 (6)	78.6 (11)	ns
3-4 (%)	53.3 (8)	53.3 (8)	37.5 (6)	7.14 (1)	
≥5 (%)	13.3 (2)	20 (3)	18.8 (3)	0	
Barthel	82.7±21	85.3±24.5	83.9±17.1	100*†	**0.0039**
MNA-sf	10.5±2.6	10.9±2.3	9.7±2.7	12.6±1.7†	**0.018**
Malnourished (0-7) (%)	13.3 (2)	13.3 (2)	21.4 (3)	0	
At risk (8-11) (%)	46.7 (7)	26.7 (4)	50 (7)	35.7 (5)	ns
Well-nourished (12-14) (%)	40 (6)	60 (9)	28.6 (4)	64.3 (9)	
BMI (Kg/m²)	28.1±4.7	25.9±4.6	25.6±4.8	29.4±3.4	ns
Hand grip (kg)	18.6±9.8	18.93±7.7	17.3±8.5	29.1±7.5**#††	**0.0014**
Drugs/patient	8.8±3.9	7.93±3.7	9.76±4.2	5.23±2.2†††	**0.0018**

Table 1. Health Status. Demographical, clinical and nutritional characteristics of the study groups. OD-PNP: Patients with oropharyngeal dysphagia and prior pneumonia; OD: Patients with oropharyngeal dysphagia; OD-APN: Patients with acute pneumonia and oropharyngeal dysphagia; H: Healthy older persons. When comparing groups with multiple comparisons, statistical significance is shown by using several symbols for every group comparison: * *p*-value <0.05, and ** *p*-value <0.01 for a comparison against Group 1 (OD-PNP); # *p*-value <0.05, and ## *p*-value <0.01 for a comparison against Group 2 (OD); † *p*-value <0.05, †† *p*-value <0.01, and ††† *p*-value <0.001 for a comparison against Group 3 (OD-APN).

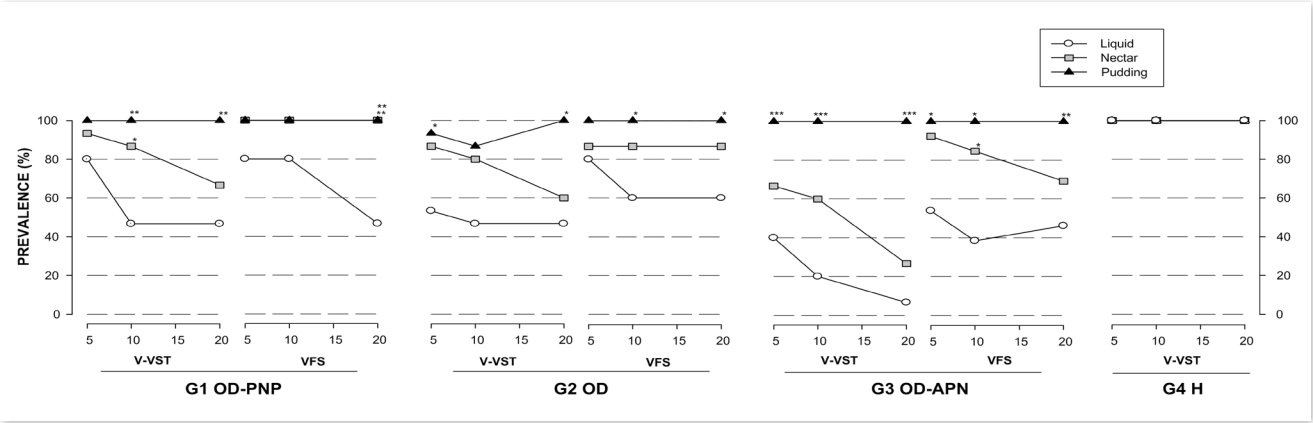

Figure 2. Prevalence of patients with safe swallow according to each bolus volume and viscosity in V-VST and VFS studies in the study population. Safety of swallow was expressed as percentage of patients that could swallow without clinical or VFS signs of impaired safety. OD-PNP: Patients with oropharyngeal dysphagia and prior pneumonia; OD: Patients with oropharyngeal dysphagia; OD-APN: Patients with acute pneumonia and oropharyngeal dysphagia; H: Healthy older persons. *p<0.05, **p<0.01, ***p<0.001 vs. thin liquid.

Dysphagia assessment

a) **Clinical signs.** V-VST. Signs of impaired safety (cough, oxygen desaturation or voice change) were found in 53.3% patients with OD-PNP, 86.7% with OD and 93% patients with OD-APN (p<0.05 vs. OD-PNP). Prevalence of safe swallows is shown in Figure 2. The control group did not present any signs of impaired efficacy or safety of OD during V-VST (Figure 2).

b) **VFS signs of OD.** Videofluoroscopic study showed high prevalence of VFS signs of impaired efficacy and safety of swallow among the three groups of patients with OD (Figure 2). Oropharyngeal residue in the mouth, vallecula or pyriform sinus was found in 60% of patients with OD-PNP and OD and 61.5% of patients with OD-APN. Only 53.3%, 69.2% and 46.15% of patients with OD-PNP, OD and OD-APN respectively completed thin liquid series safely. Impaired safety (PAS>2) was observed in 46.7%, 33.3% and 61.5% of patients with OD-PNP, OD and OD-APN respectively and mean PAS was 2.73±1.3, 2.6±1.6, and 3.85±2.3 among groups. Aspirations were observed in 15.4% patients with OD-APN and all were silent (level 8 PAS). Increasing bolus viscosity in all groups of patients with OD greatly improved safety of swallow with maximal effect at spoon-thick viscosity. Using this viscosity, 100% of all patients from the three groups with OD were able to complete the series safely (p <0.001 G1 and G3; and p<0.05 G2 vs. thin liquid).

c) **Oropharyngeal swallow response.** LVC time was delayed in the three groups of patients with OD, patients with OD-PNP taking 338.7±108.9ms , patients with OD, 317.33±63.19ms and patients with OD-APN, even longer with 409.2±115.6 ms; p<0.05 vs both groups.

Oral health and hygiene

Oral health and hygiene status were found to be similar and very poor in all groups, with high prevalence of periodontal diseases and caries and high accumulation of plaque and/or calculus (Table 2). We found a slightly healthier status in the control group but without significant differences (Table 2).

The following results are based on dentate patients. OHI-S data showed high prevalence of patients with poor oral hygiene and very few with good oral hygiene status (OHI-S value between 0-1). More than 50% of this poor OHI-S in all groups was caused by accumulation of soft dental plaque that can be easily removed by tooth brushing (Table 2). Prevalence of periodontitis (>87%) and caries (>72%) was very high in all groups of patients with OD. Only 40% of OD-PNP, nearly 25% of OD

and OD-APN and 55.6% healthy patients had visited the dentist during the previous year.

Oral microbiota

Microbiota composition

Microbiota composition was evaluated in samples taken from four anatomical locations on the first visit. 245'410 good quality sequencing reads were obtained, with a median of 1569 reads per sample. As seen on rarefaction plots, this sequencing depth permitted making robust conclusions regarding the diversity measures, even though the plateau in terms of detection of all bacterial taxa was not reached (*Supplementary Figure 1*). Microbiota composition showed high inter-individual variability; however nasopharynx microbiota was markedly different from oral (from oral rinse and saliva) in all groups (Figure 3).

Microbiota of nasopharynx was dominated by *Moraxella*, *Corynebacterium*, *Staphylococcus*, *Streptococcus* and *Alloiococcus*, while saliva and oral rinse were quite similar to each other and contained predominantly *Veionella*, *Neisseria*, *Prevotella*, *Porphyromonas*, *Haemophilus*, and streptococci. The differences among nasopharynx and oral samples were highly significant (permanova performed on unifrac distances, unweighted Pseudo-F = 10.9, $p < 0.001$; weighted, Pseudo-F = 13.8, $p < 0.001$). As many as 87 out of 113 identified Operational Taxonomic Units (OTUs) differed significantly between the anatomical locations (not shown). Nasopharyngeal microbiota was much less diverse than that in oral rinse and saliva (*Supplementary Figure 2 and 3*). The differences among patient groups were less pronounced (Figure 3). Control group showed higher microbiota diversity, significant for saliva ($p = 0.03$, *Supplementary Table 1*). Microbiota composition showed no significant differences among patient groups (performed on unifrac distances, unweighted and weighted $p > 0.05$); the same was true for individual bacterial taxa (data not shown).

	G1 OD-PNP	G2 OD	G3 OD-APN	G4 H	P-value
Subjects	14	13	11	13	
Edentulism (%)	35.7 (5)	15.4 (2)	27.3 (3)	7.7 (1)	ns
Number of teeth	16.22±8.6	18.6±10.1	18.11±6.8	21.17±6.2	ns
OHI-S	3.13±1.5	3.4±1.1	3.26±1.5	2.54±1.3	ns
0 – 1 (good) (%)	11.1 (1)	0	14.3 (1)	16.7 (2)	ns
1.1 – 3 (fair) (%)	33.3 (3)	45.5 (5)	14.3 (1)	58.3 (7)	
3.1 – 6 (poor) (%)	55.6 (5)	54.5 (6)	71.4 (5)	25 (3)	
Plaque (%)	60.25	58.49	55.86	68.68	ns
Calculus (%)	39.75	41.51	44.14	31.32	ns
Oral diseases Healthy (%)	11.11 (1)	9.09 (1)	0	8.33 (1)	ns
Gingivitis (%)	0	0	0	0	ns
Periodontitis (%)	88.9 (8)	90.9 (10)	87.5 (7)	91.7 (11)	ns
Caries (%)	77.8 (7)	72.7 (8)	85.71 (6)	50 (6)	ns
Oral habits (persons)	5	11	10	9	
Tooth brushing (≥1/day) (%)	60 (3)	63.63 (7)	60 (6)	88.9 (8)	ns
Denture cleaning (≥1/day) (%)	40 (2)	45.5 (5)	30 (3)	33.3 (3)	ns
Last visit dentist (≤1 yr) (%)	40 (2)	27.27 (3)	20 (2)	55.6 (5)	ns

Table 2. Oral hygiene and health of the study groups. OD-PNP: patients with oropharyngeal dysphagia and prior pneumonia; OD: patients with oropharyngeal dysphagia; OD-APN: patients with acute pneumonia and oropharyngeal dysphagia; H: healthy older persons. When comparing groups with multiple comparisons, statistical significance is shown by using several symbols for every group comparison: ***** P-value <0.05 for a comparison against Group 1 (OD-PNP); **#** P-value <0.05 for a comparison against Group 2 (OD); **†** P-value <0.05 for a comparison against Group 3 (OD-APN). Double symbol indicates a P-value <0.01.

Bacterial load

The total bacterial load (TBL), which reflects the amount of global colonization by both commensals and pathogens, showed no significant differences among patient groups (ns, Figure 4). There was, however, a clear distinction between the nasal and oral locations (Figure 4 and *Supplementary Table 2*). The least colonized location was the nasopharynx with bacterial loads in the range of 4-5 logs, there were slightly higher loads in the nose and the oropharynx, and the highest loads of up to 8 logs were in the remaining anatomical locations: tongue, teeth, saliva, and oral rinse. The TBL of sputum was similar to oral localizations in all groups (Figure 4).

Prevalence of colonization by respiratory pathogens

The prevalence of colonization by respiratory pathogens was the lowest among the control group (Table 3) and the difference between this group (H) and other patient groups with OD was significant for four anatomical locations when the unadjusted *p*-values were considered. Most patients were colonized by at least one of the five respiratory pathogens in any of the investigated locations (Table 3).

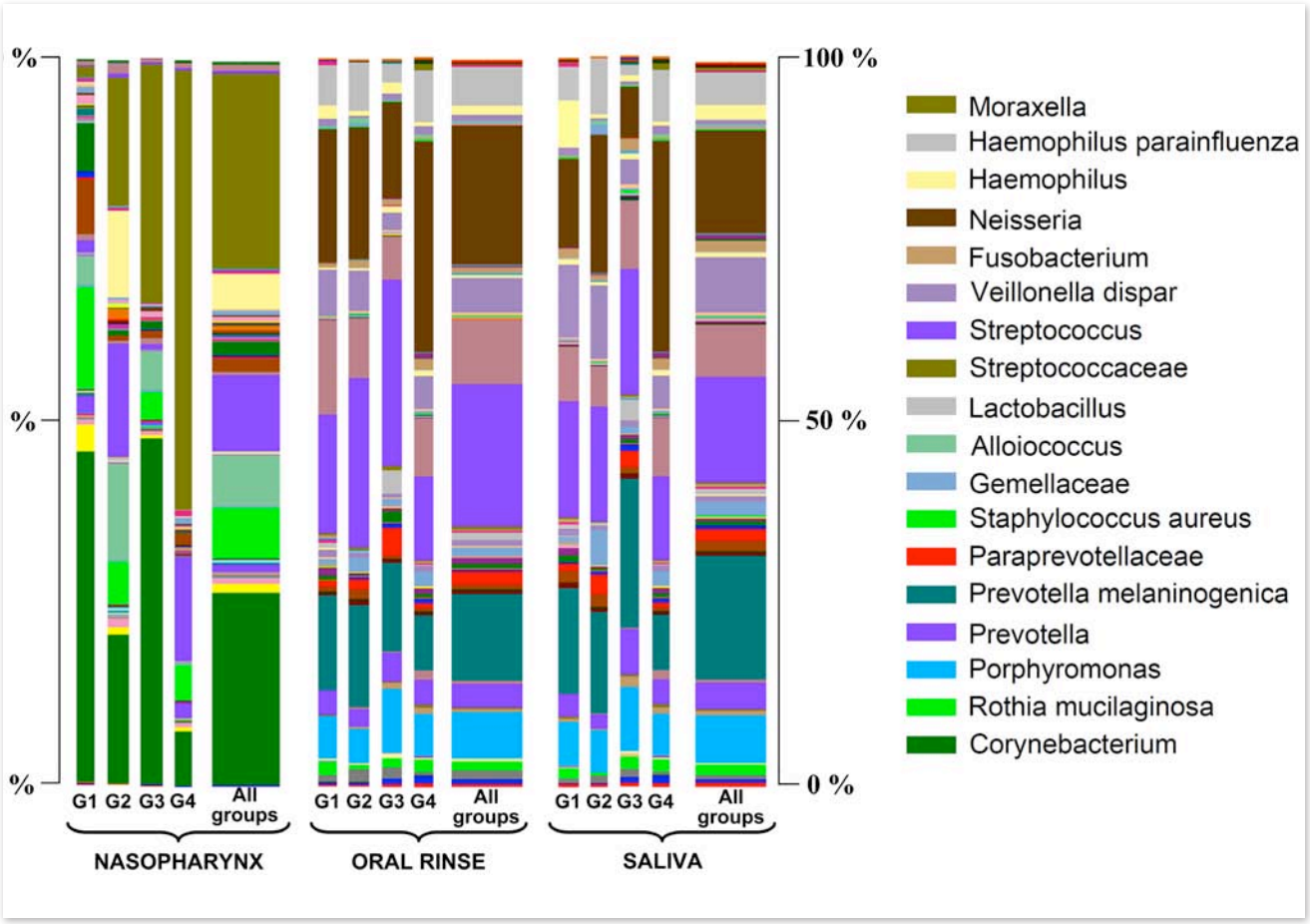

Figure 3. Microbiota composition of the investigated anatomical locations and groups of patients. The relative abundance of the OTUs is shown. The graph shows the most prevalent microbial populations colonizing nasopharynx and two oral locations. OD-PNP: patients with oropharyngeal dysphagia and prior pneumonia; OD: patients with oropharyngeal dysphagia; OD-APN: patients with acute pneumonia and oropharyngeal dysphagia; H: healthy older persons. The legend only names taxa with over 1% relative abundance. *Full legend is provided as supplementary material (Supplementary Figure 4).*

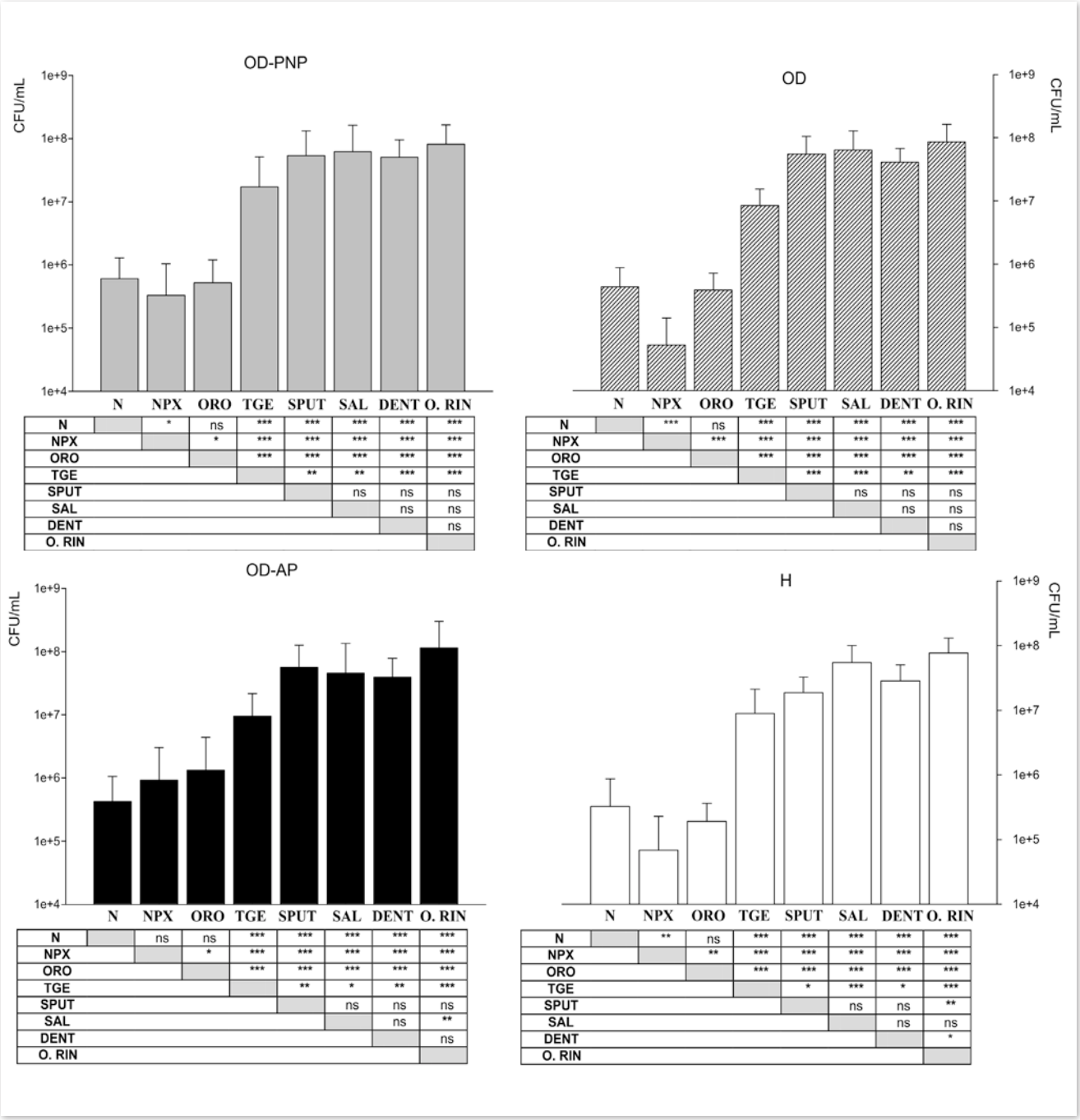

Figure 4. Total bacterial load for each location by group. N: nasal cavity; NPX: nasopharynx; ORO: oropharynx; TGE: tongue; SPUT: Sputum; SAL: saliva; DENT: dental surface; O. RIN: oral rinse. Multiple comparisons for anatomical locations are indicated in the table below for each group. ns: not significant; * p<0.05; ** p<0.01; *** p<0.001.

It is important to note that in patients from Group 3 (OD-APN), qPCR analysis confirmed the presence of 6/7 respiratory pathogens already detected as causative agents of the pneumonia using classical microbiological methods. The only pathogen that was not detected by qPCR (*M. catarrhalis*) was one not analyzed in our study. In addition, 4/6 pathogens detected by qPCR were found in oral samples.

Pathogen load

Quantitative measurement of colonization by all 5 respiratory pathogens determined by the CPL is shown in Figure 5. At most anatomical locations, healthy older persons had the lowest pathogen load, and OD-APN the highest, while the two other patient groups showed intermediate loads. These differences

were significant for oral rinse, saliva and tongue (Figure 5). The differences among the patient groups were greatest in oral rinse, particularly between healthy persons and OD-APN which was significant after adjustment for multiple comparisons (Figure 5).

Correlation between the two visits

High reproducibility of measurements between the two visits was observed, with Kappa values 0.70 – 0.91 for the qualitative measure of proportion of patients colonized by any respiratory pathogen, and correlation coefficients 0.79 – 0.98 for the Composite Pathogen Load.

DISCUSSION

This study provides further evidence to confirm our hypothesis that there are three key factors involved in AP in the frail older population: a) impaired health status with many comorbidities, reduced functionality, weakness, polymedication and impaired nutritional status; b) OD with impaired safety of swallow and aspirations; and c) oral dysbiosis with oral colonization by respiratory pathogens [15,36,37]. Our study suggests that oral colonization by respiratory pathogens is a key factor for pneumonia in older patients. This further supports the view that OD and aspiration are much more common in older patients with pneumonia than once believed, suggesting that most of these pneumonias are caused by aspiration [36,38].

OD patients from the three groups presented impaired health status compared to the control group. A major morbidity in older patients with OD is malnutrition which compromises recovery in many diseases and increases morbi-mortality, prolonged length of hospital stay and higher treatment costs [39]. Impaired health status, malnutrition and low functionality are known to be associated with impaired immunity, increased risk of infections, pneumonia and increased mortality [7,40,41]. In earlier studies, we found that older patients with OD and patients with malnutrition presented increased mortal-

Location	Group	Proportion	*p*-value
Nasal cavity	OD-PNP	0.60	0.23
	OD	0.53	0.36
	OD-APN	0.53	0.36
	H	0.47	
Nasopharynx	OD-PNP	0.13	0.50
	OD	0.33	0.10
	OD-APN	0.41	**0.05**
	H	0.13	
Oropharynx	OD-PNP	0.27	0.09
	OD	0.20	0.15
	OD-APN	0.41	**0.02**
	H	0.07	
Oral rinse	OD-PNP	0.60	0.23
	OD	0.80	**0.03**
	OD-APN	0.62	0.19
	H	0.47	
Saliva	OD-PNP	0.53	0.23
	OD	0.73	**0.04**
	OD-APN	0.56	0.18
	H	0.40	
Sputum	OD-PNP	0.73	0.22
	OD	0.87	0.06
	OD-APN	0.60	0.50
	H	0.60	
Teeth (dental plaque)	OD-PNP	0.29	0.45
	OD	0.20	0.67
	OD-APN	0.41	0.20
	H	0.27	
Tongue	OD-PNP	0.33	0.50
	OD	0.40	0.35
	OD-APN	0.47	0.22
	H	0.33	
Any Location	OD-PNP	0.93	**0.04**
	OD	0.93	**0.04**
	OD-APN	0.88	0.07
	H	0.67	

Table 3. Proportion of patients colonized by respiratory pathogens at tested locations. OD-PNP: patients with OD and prior pneumonia; OD: patients with oropharyngeal dysphagia; OD-APN: patients with OD and acute pneumonia; H: healthy older persons. P-values are for comparisons of a given group with healthy older persons. Significant values are shown in bold and values just short of significance are underlined.

ty rates, the poorest outcome being for patients with both conditions with a one-year mortality of 65.8% [39]. In the present study we have further confirmed these clinical characteristics in FOP with OD, especially OD-APN [6], clearly showing their high vulnerability to acute diseases.

The V-VST showed many signs of impaired safety in the OD groups, especially in OD and OD-APN groups. Despite the high diagnostic sensitivity and specificity of the V-VST for clinical signs of impaired safety of swallow [28], we confirmed our clinical findings with VFS.

We found prevalent VFS signs of impaired efficacy (mainly residue) and safety of swallow in the OD groups, particularly OD-APN. Oropharyngeal residue caused by weak tongue propulsion [29] was found in 60% (G1 and G2) and 61.5% (G3) of OD patients, causing deficient oropharyngeal clearance, which can be aggravated by reduced salivation (associated with ageing, medication and dehydration) [18]. Effective salivary flow and swallowing are needed to clear gram-negative bacteria from the oral and pharyngeal cavities [42]. Reduction in mechanical clearance, together with poor oral hygiene and impaired immunity, could contribute to oral and pharyngeal colonization by opportunistic or potential respiratory pathogens and gram-negative bacteria [43].

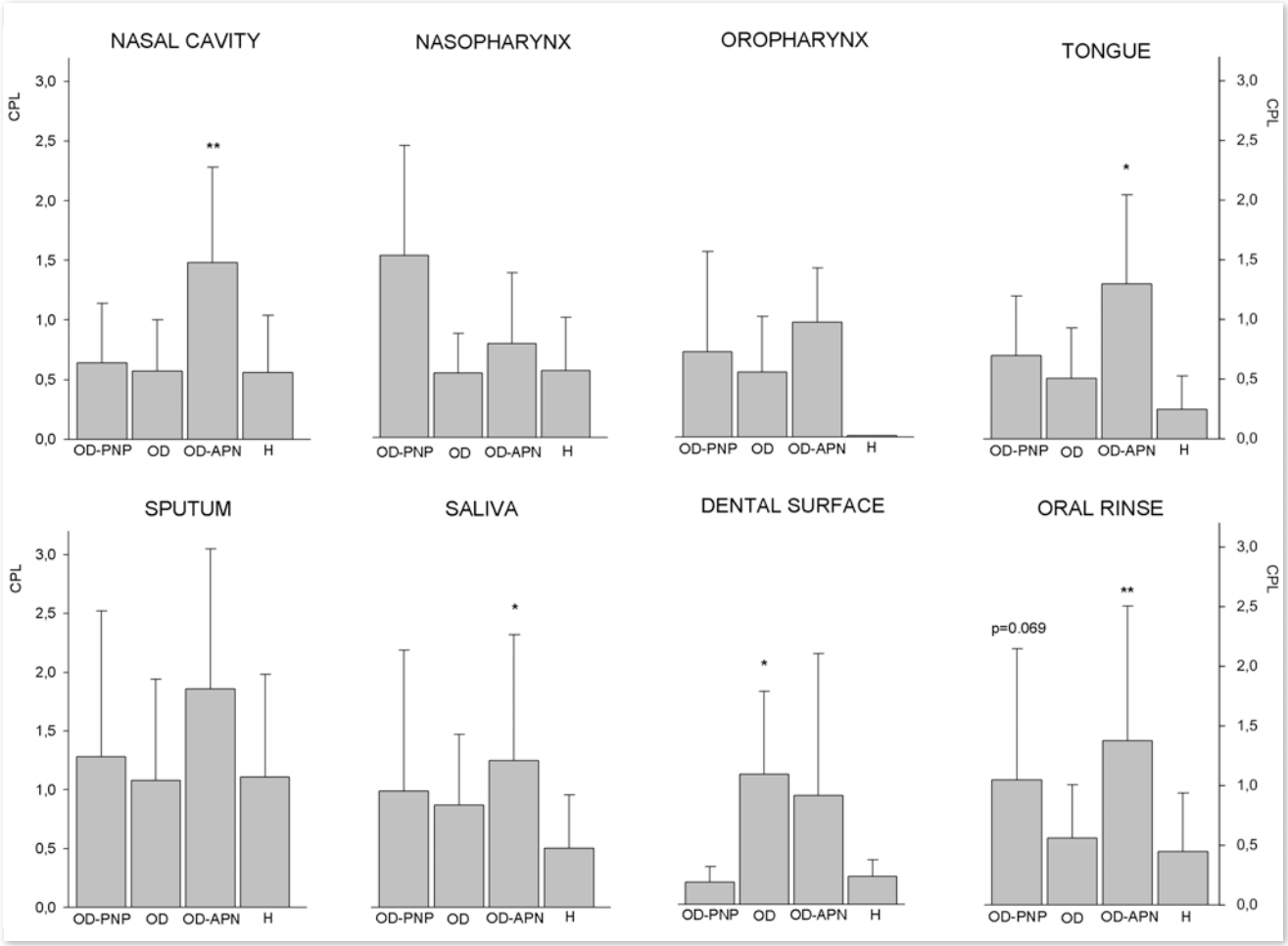

Figure 5. Pathogen load measured with the CPL in all groups and locations. OD-PNP: patients with oropharyngeal dysphagia and prior pneumonia; OD: patients with oropharyngeal dysphagia; OD-APN: patients with acute pneumonia and oropharyngeal dysphagia; H: healthy older persons. P-values are for comparison of a given group with healthy older persons.*P<0.05; **P<0.01.

In this study, we also confirmed that OD-APN patients had severely impaired swallow and airway protection mechanisms. We observed VFS signs of impaired safety in 46.7% (OD-PNP), 33.3% (OD) and 61.5% (OD-APN) of our patients, showing that aspiration is a common finding in older patients with pneumonia [6,7]. We previously found that prevalence of OD in patients

with CAP was 91.7% and delayed laryngeal vestibule closure (LVC) was the main mechanism of impaired airway protection in this and that article [6]. LVC was seriously delayed in all OD groups in our study, especially OD-APN, increasing risk of aspirations. In addition, all the aspirations presented by OD-APN were silent (15.4%), indicating probable impaired cough reflex. A recent study on older patients with CAP showed similar results with a prevalence of silent aspirations of 16.7% [6]. The high prevalence of OD and risk factors among older patients in this study suggests that a high percentage of CAPs in older persons are caused by oropharyngeal aspirations. In addition, 4/7 pathogens that were found to be the etiological cause of pneumonia in 7/17 patients from group OD-APN on admission, were then found in oral samples of the qPCR analysis. We thus propose that the main etiological mechanism that leads to pneumonia in FOP is aspiration rather than inhalation. If true, prevention and treatment of pneumonia in FOP need revising. Finally, our VFS studies show that increasing bolus viscosity can avoid aspirations; up to 100% of all groups with OD presented safe swallow when using spoon-thick viscosity. Thus, OD in these patients with pneumonia can be diagnosed by clinical methods and/or complementary explorations, and aspirations effectively avoided by using thickeners at an appropriate level of viscosity.

Many studies have reported that the presence of caries, periodontal disease and dental plaque are associated with the appearance, severity and mortality of AP in older patients and a relationship has been established between oral colonization and respiratory pathogens [2,16,18,38,44-47]. Periodontal pathogens may facilitate the colonization of the oral cavity by pneumonia pathogens [47]. Periodontal disease and poor oral hygiene result in a higher concentration of gram-negative and anaerobic oral pathogens in saliva [48]. Oral biofilm acts as a reservoir for respiratory pathogens, which appear in the oral cavity before colonizing the lungs in patients with AP [18,49]. We previously found a high prevalence of periodontal diseases (93%) and caries (53%) and high accumulation of dental plaque and calculus measured with the OHI-S in older patients with OD [15]. In our study, we found that all older patients presented similar poor oral health and hygiene, slightly better in the control group. Patients with OD-APN, however, presented the highest rates of colonization and bacterial load by respiratory pathogens, thus indicating that factors such as poor nutritional and health status also contribute to oral dysbiosis and colonization by respiratory pathogens. In consequence, oral health and hygiene assessment should be performed as a part of dysphagia assessment in older patients and oral hygiene recommendations should be provided to them and their caregivers. Appropriate mechanical toothbrushing and the use of antiseptic mouthwashes like chlorhexidine will reduce bacterial colonization and improve oral health and hygiene.

Colonization by respiratory pathogens in all the groups reached 90% when any of the five pathogens and all anatomical locations were considered. Despite similarly poor oral health, control group were clearly less colonized, both in terms of prevalence and pathogen load (CPL). A study with independently-living older people (mean age 68.6±4.6y) showed that prevalence of opportunistic respiratory pathogens in oral samples detected by culture techniques was 13.6% and that patients colonized were older than not colonized patients [50]. The higher prevalence of colonization in our study is most likely due to the use of more sensitive and specific methodology and the inclusion of older frail patients with OD.

Remarkably, oral colonization by respiratory pathogens was more prevalent than nasal or nasopharyngeal colonization, normally considered the prime sites of colonization by respiratory pathogens. We hypothesize that high prevalence of oral colonization is related to poor oral hygiene, low clearance of pathogens through impaired salivary function and poor nutritional/immunological status.

Despite higher colonization by respiratory pathogens seen in dysphagic patients, total bacterial load was similar among groups. This suggests that impaired functional status of dysphagic patients had more impact on respiratory pathogens than it did on commensal microbiota. The finding that microbiota composition was similar among patient groups further supports this notion. Interestingly, microbiota composition and total bacterial load were very different in nasopharynx compared to oral locations. These results agree with previous studies comparing oral and nasal microbiota of healthy individuals [51-54].

Oral colonization by respiratory pathogens together with impaired health status and impaired safety of swallow make patients very vulnerable to respiratory infections and AP. Specific interventions treating oral health and hygiene in these patients are needed as well as treatment against oral respiratory pathogens to restore healthy microbiota. One systematic review recommended that "oral health care consisting of tooth brushing after each meal, cleaning dentures once a day, and regular professional oral health care, as the best intervention to reduce the incidence of aspiration pneumonia" [21]. It is important to note that more than 50% OHI-S composition found in all groups was caused by the accumulation of dental plaque, which is a soft bacterial biofilm easily removable by correct tooth brushing. In a review, *Sjogren et al* found a preventive effect of mechanical oral hygiene on pneumonia and respiratory tract infection in hospitalized and institutionalized older people [19]. The importance of good oral care evaluations and practices has been stated in several publications that have demanded the participation of nurses and/or dentists to improve and manage older patients' mouths and to advise and educate relatives and carers about oral hygiene [55]. Several studies, including randomized clinical trials, have shown that improving oral hygiene reduces the incidence of pneumonia [19,20,56-58] and would prevent 10% of deaths from pneumonia in older nursing-home residents [19]. This indicates the great importance of mouth care in older patients at risk of AP. Recently, the WHO has recommended strategies to enhance oral health in older people. In addition, the US Centers for Disease Control and Prevention recognizes aspiration of microorganisms as an important etiological route for the development of AP in older patients and recommends "comprehensive hygiene programs" [59]. However, oral healthcare in older patients is still not properly managed or standardized in many hospitals and nursing homes [60]. We recently found that OD is a very prevalent and relevant risk factor associated with hospital readmission for pneumonia in the elderly; the incidence rate of hospital readmission for pneumonia was 3.67 readmissions per 100 person-years in individuals without OD and 6.7 in those with OD [9]. Therefore, as we are currently doing in our hospital, we suggest systematically screening for dysphagia, MN and oral health and hygiene in frail older patients to prevent complications; and we recommend that multidisciplinary dysphagia teams include a dental care professional.

In summary, in this study we have found three main elements contributing to the pathophysiology of AP in older people: first, oral dysbiosis and colonization by respiratory pathogens; second, OD and impaired safety of swallow with aspirations, and third a frail or vulnerable patient with impaired nutritional status and altered immunity. Today, with simple and economic tools, we are able to treat these three main elements to avoid AP by teaching oral hygiene to improve oral health status and avoid colonization, rheologically adapting fluids and solid foods to prevent aspirations and supplementing dietary intake to manage malnutrition and improve nutritional status.

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ON-LINE SUPPLEMENTARY MATERIAL

SUPPLEMENTARY INFORMATION

Sample preparation and sequencing

Total DNA was extracted with QiaAamp DNA mini kit, following the manufacturer's instructions.

Partial 16S rRNA gene sequences were amplified from the samples using the barcoded-primer approach to multiplex pyrosequencing. Primers were designed as previously proposed [1,2,3], primer 1061R: *CTATGCGCCTTGCCAGCCCGCTCAG*GC**CRRCACGAGCTGACGAC**; primer BSF517/E517F: *CGTATCGCCTCCCTCGCGCCATCAG*NNNNNNNNAG**GCCAGCAGCCGCGGTAA**, where the adapter sequences for Roche 454 FLX Titanium sequencing are *in italics*; NNNNNNNN designate the sample-specific eight-base barcodes used to tag each PCR product; underline indicates linkers, and **bold** corresponds to broadly conserved 16S ribosomal RNA gene regions.

PCR reaction were carried out in a total volume of 50 µl with 10µl of DNA extract, 1x Expand High Fidelity ^{PLUS} buffer 1 (Roche Applied Science, Basel, Switzerland), 50 µM of each dNTP (Roche Applied Science, Basel, Switzerland), 40 pmol of each primer (Microsynth, Balgach, Switzerland), 5 U Expand High Fidelity ^{PLUS} Enzyme mix (Roche Applied Science, Basel, Switzerland). The PCR parameters were 94°C for 2 min, 30 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, followed by 72°C for 7 min. After checking the amplification of the correct band, the PCR products were purified and quantified. After equimolar pooling, the amplicons were sequenced on Roche 454 GS-FLX-Titanium Sequencer.

Processing of the sequencing data

Raw sequence data were analyzed using a blend of Mothur v.1.33.0 [4] and QIIME v.1.8 [5] software packages. Pyrosequencing reads were denoised with the mothur implementation of PyroNoise [6] according to the 454 SOP [7]. Chimeras were identified using usearch61 in QIIME [8]. Then, the sequences were trimmed as described in the mothur 454 SOP in order to keep sequences overlapping the same 16S region. Subsequent analytical steps were performed in QIIME. OTUs de novo picking at 97% identity was performed using uclust [9]. Taxonomy assignment of OTU representative sequences used the RDP Classifier with confidence threshold of 0.6 [10] on the Greengenes reference database v.13.8 [11]. The same sequences were aligned using PyNast [12] on the Greengenes core reference alignment [13]. The resulting multiple alignments was then filtered and used to build a phylogenetic tree with FastTree [14]. After quality filtering [15], phylogenetic distances between all samples were computed as UniFrac distances [16]. Diversity analyses based on the Unifrac distances were performed in QIIME. Permanova is a non-parametric multivariate anaylsis of variance and it was performed according to Anderson MJ using QIIME package [17].

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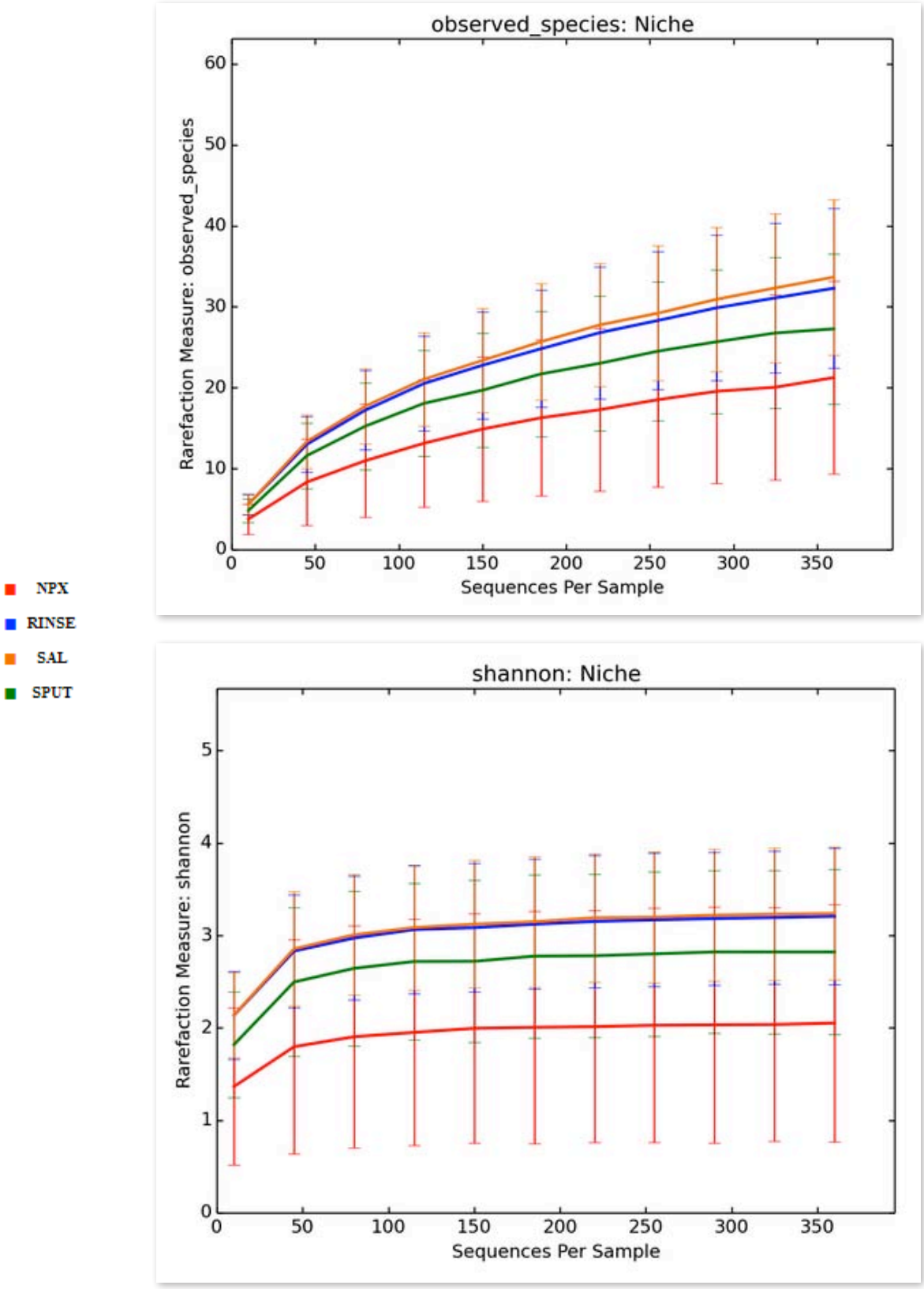
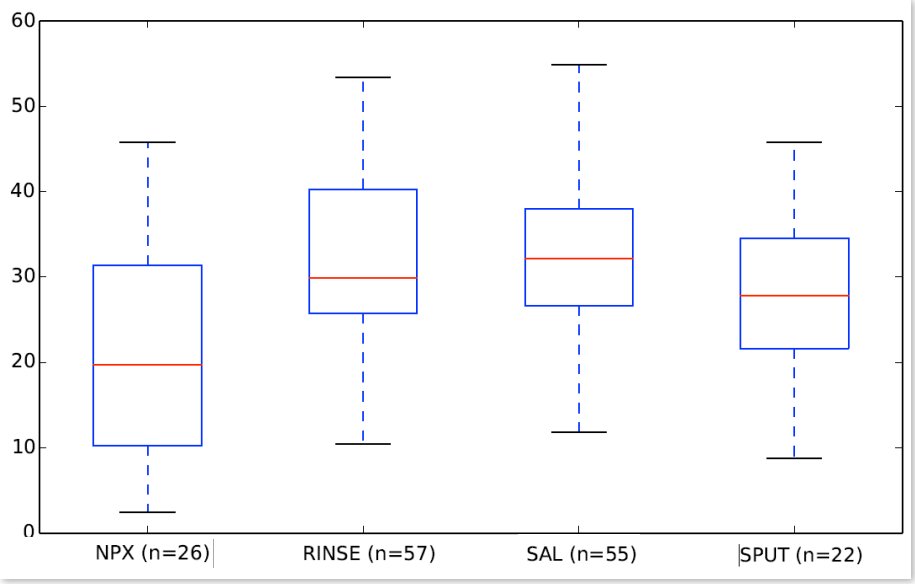
SUPPLEMENTARY FIGURES AND TABLES

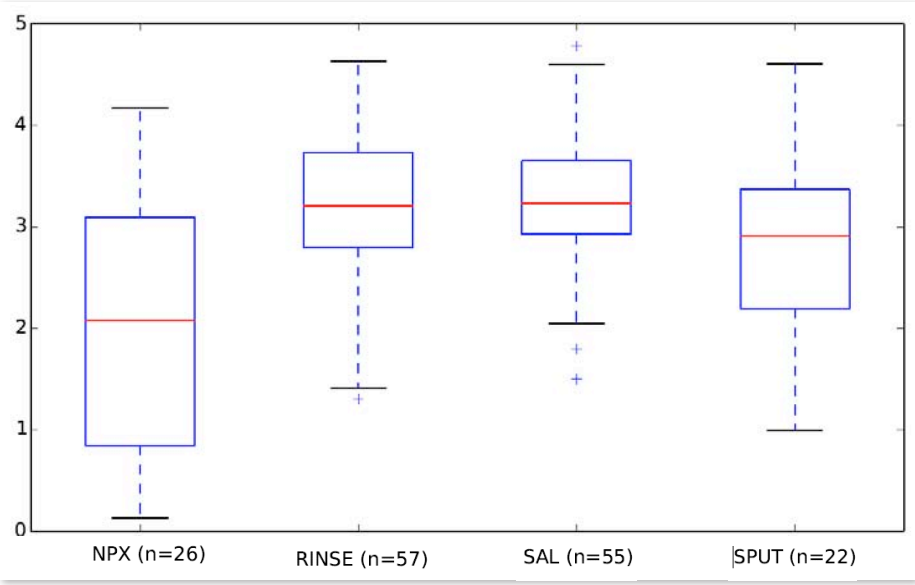


Supplementary Figure 1. Rarefaction plots. Number of observed OTUs (upper graph); and Shannon diversity index (lower graph). Shannon diversity Index is used to characterize species diversity in a community. Shannon's index accounts for both abundance and evenness of the species present. NPX: nasopharynx; RINSE: oral rinse; SAL: saliva; SPUT: sputum.



NPX		*	*	Ns
RINSE			ns	Ns
SAL				Ns
SPUT				

Supplementary Figure 2. Number of observed species as a function of anatomical niche in all groups of patients. Anatomical locations which are significantly different ($p<0.05$) are indicated in the table. NPX: nasopharynx; RINSE: oral rinse; SAL: saliva; SPUT: sputum.



NPX		*	*	Ns
RINSE			ns	Ns
SAL				Ns
SPUT				

Supplementary Figure 3. Shannon diversity index for different niches in all groups of patients. Anatomical locations which are significantly different ($p<0.05$) are indicated in the table. Shannon Diversity Index is used to characterize species diversity in a community. NPX: nasopharynx; RINSE: oral rinse; SAL: saliva; SPUT: sputum.

Group 1	Group 2	Group 1 mean	Group 2 mean	Group 1 SD	Group 2 SD	*p*-value
NPX. OD	NPX. OD-APN	24.4	17.9	10.5	10.7	0.494
NPX. OD	NPX. H	24.4	20.4	10.5	15.02	0.785
NPX. OD-PNP	NPX. OD	29.7	24.4	8.7	10.51	0.704
NPX. OD-PNP	NPX. OD-APN	29.7	17.9	8.7	10.7	0.209
NPX. OD-PNP	NPX. H.	29.7	20.4	8.7	15.02	0.537
NPX. H	NPX. OD-APN	20.4	17.9	15.0	10.7	0.788
OR. OD	OR. H	29.7	37.4	7.2	10.6	0.178
OR. OD-APN	OR. OD	33.0	29.7	11.3	7.212	0.532
OR. OD-APN	OR. H	33.0	37.4	11.3	10.6	0.472
OR. OD-PNP	OR. OD	29.2	29.7	7.4	7.212	0.905
OR. OD-PNP	OR. OD-APN	29.2	33.0	7.4	11.34	0.475
OR. OD-PNP	OR. H.	29.2	37.4	7.4	10.6	0.125
SAL. OD-APN	SAL. OD	35.5	28.3	11.1	5.16	0.218
SAL. OD-PNP	SAL. OD	31.4	28.3	6.7	5.16	0.429
SAL. OD-PNP	SAL.OD-APN	31.4	35.5	6.7	11.07	0.457
SAL. OD-PNP	SAL. H.	31.4	37.6	6.7	9.573	0.217
SAL. H	SAL.OD	37.6	28.3	9.6	5.16	**0.039**
SAL. H	SAL. OD-APN	37.6	35.5	9.6	11.07	0.712

Supplementary Table 1. Microbiota diversity. Number of observed species among patient groups in nasopharynx (NPX), oral rinse (OR) and saliva (SAL).

Supplementary Figure 4. Full legend of OTU of Figure 3 (Microbiota composition of the investigated anatomical locations and groups of patients).

k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__Actinomyces;s__
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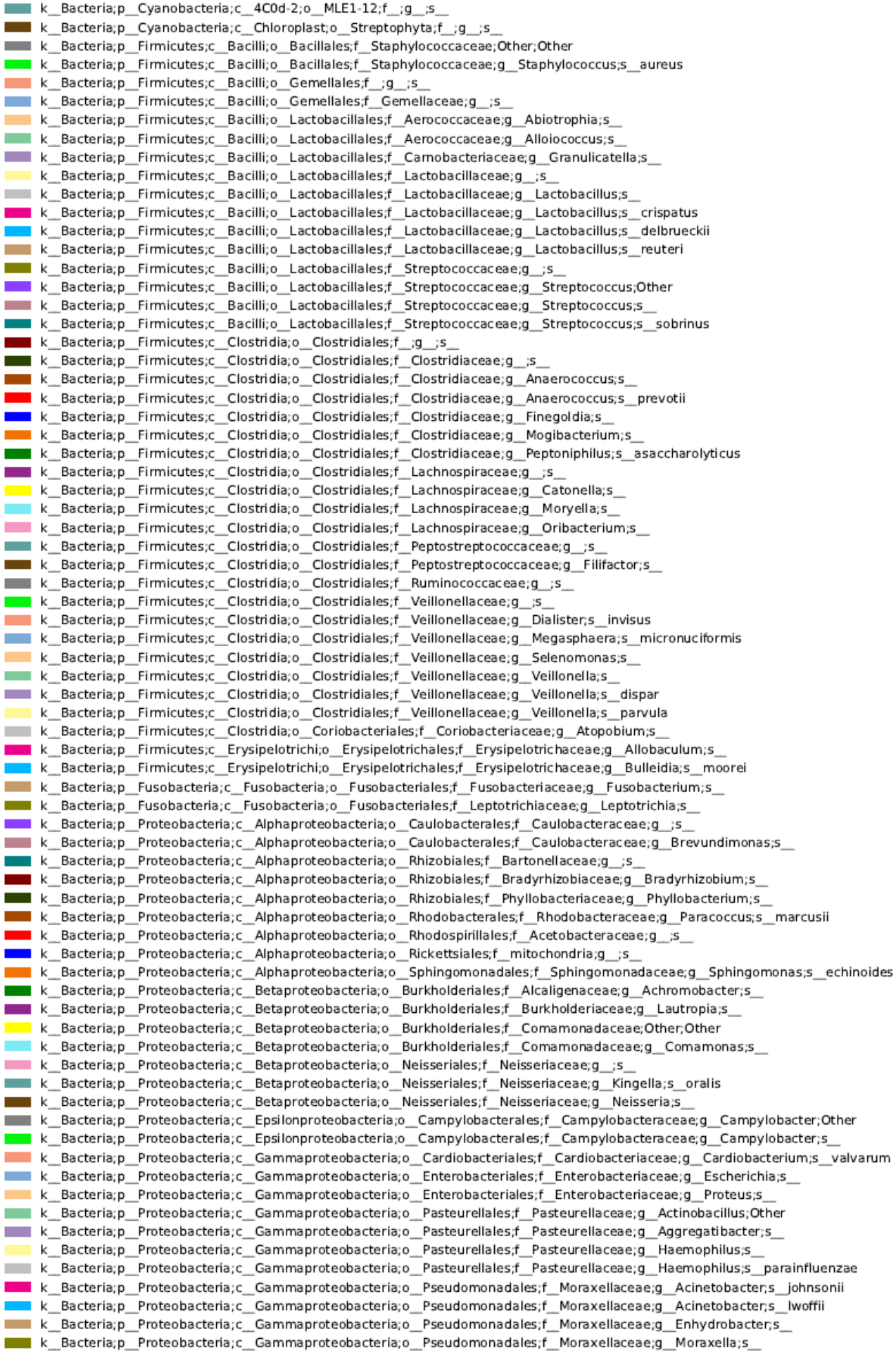
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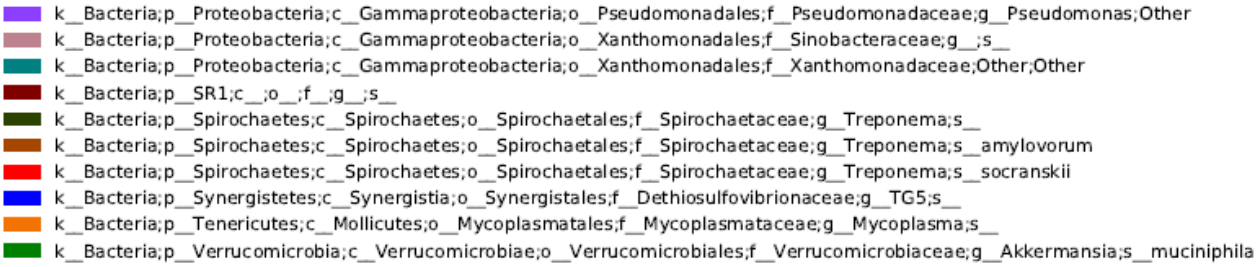


group (ratios)	estimate	lower	upper	p.value	pvalue.ss
OD−APN					
Nose/Teeth	0.007	0.001	0.040	0.000	0.000
Nasopharynx/Teeth	0.004	0.001	0.029	0.000	0.000
Oropharynx/Teeth	0.018	0.003	0.109	0.000	0.000
Oral rinse/Teeth	2.035	0.333	12.444	0.173	1.000
Saliva/Teeth	0.477	0.078	2.918	0.156	0.999
Sputum/Teeth	0.931	0.148	5.872	0.893	1.000
Tongue/Teeth	0.168	0.028	0.997	0.001	0.050
Nasopharynx/Nose	0.636	0.094	4.318	0.411	1.000
Oropharynx/Nose	2.670	0.437	16.331	0.060	0.960
Oral rinse/Nose	303.503	49.613	1856.179	0.000	0.000
Saliva/Nose	71.160	11.632	435.201	0.000	0.000
Sputum/Nose	138.867	22.011	875.882	0.000	0.000
Tongue/Nose	24.997	4.201	148.709	0.000	0.000
Oropharynx/Nasopharynx	4.199	0.603	29.258	0.010	0.517
Oral rinse/Nasopharynx	477.273	68.481	3325.436	0.000	0.000
Saliva/Nasopharynx	111.902	16.056	779.685	0.000	0.000
Sputum/Nasopharynx	218.375	30.444	1565.989	0.000	0.000
Tongue/Nasopharynx	39.309	5.787	266.922	0.000	0.000
Oral rinse/Oropharynx	113.659	18.080	714.312	0.000	0.000
Saliva/Oropharynx	26.649	4.239	167.478	0.000	0.000
Sputum/Oropharynx	52.004	8.025	336.913	0.000	0.000
Tongue/Oropharynx	9.361	1.530	57.251	0.000	0.002
Saliva/Oral rinse	0.234	0.037	1.474	0.006	0.375
Sputum/Oral rinse	0.458	0.071	2.964	0.146	0.999
Tongue/Oral rinse	0.082	0.013	0.504	0.000	0.000
Sputum/Saliva	1.951	0.301	12.643	0.214	1.000
Tongue/Saliva	0.351	0.057	2.148	0.045	0.921
Tongue/Sputum	0.180	0.029	1.135	0.001	0.106
OD					
Nose/Teeth	0.009	0.001	0.062	0.000	0.000
Nasopharynx/Teeth	0.001	0.000	0.007	0.000	0.000
Oropharynx/Teeth	0.009	0.001	0.060	0.000	0.000
Oral rinse/Teeth	1.825	0.273	12.183	0.271	1.000
Saliva/Teeth	1.559	0.234	10.408	0.416	1.000
Sputum/Teeth	1.269	0.190	8.468	0.663	1.000
Tongue/Teeth	0.161	0.024	1.076	0.001	0.077
Nasopharynx/Nose	0.113	0.017	0.755	0.000	0.007
Oropharynx/Nose	0.964	0.144	6.437	0.947	1.000
Oral rinse/Nose	196.565	29.439	1312.106	0.000	0.000
Saliva/Nose	167.934	25.151	1120.994	0.000	0.000
Sputum/Nose	136.620	20.461	911.966	0.000	0.000
Tongue/Nose	17.360	2.600	115.881	0.000	0.000
Oropharynx/Nasopharynx	8.527	1.277	56.922	0.000	0.010
Oral rinse/Nasopharynx	1738.351	260.350	11603.821	0.000	0.000
Saliva/Nasopharynx	1485.155	222.430	9913.689	0.000	0.000
Sputum/Nasopharynx	1208.223	180.954	8065.119	0.000	0.000
Tongue/Nasopharynx	153.526	22.993	1024.815	0.000	0.000
Oral rinse/Oropharynx	203.854	30.531	1360.764	0.000	0.000
Saliva/Oropharynx	174.162	26.084	1162.565	0.000	0.000
Sputum/Oropharynx	141.687	21.220	945.785	0.000	0.000
Tongue/Oropharynx	18.004	2.696	120.179	0.000	0.000
Saliva/Oral rinse	0.854	0.128	5.703	0.773	1.000
Sputum/Oral rinse	0.695	0.104	4.640	0.505	1.000
Tongue/Oral rinse	0.088	0.013	0.590	0.000	0.001
Sputum/Saliva	0.814	0.122	5.430	0.705	1.000
Tongue/Saliva	0.103	0.015	0.690	0.000	0.004
Tongue/Sputum	0.127	0.019	0.848	0.000	0.017

Supplementary Table 2.
Estimated ratios between all locations for the 4 groups of patients.

group (ratios)	estimate	lower	upper	p.value	pvalue.ss
OD-PNP					
Nose/Teeth	0.011	0.002	0.073	0.000	0.000
Nasopharynx/Teeth	0.003	0.000	0.019	0.000	0.000
Oropharynx/Teeth	0.008	0.001	0.058	0.000	0.000
Oral rinse/Teeth	1.786	0.259	12.327	0.297	1.000
Saliva/Teeth	0.838	0.121	5.787	0.751	1.000
Sputum/Teeth	0.660	0.096	4.553	0.454	1.000
Tongue/Teeth	0.127	0.018	0.875	0.000	0.022
Nasopharynx/Nose	0.250	0.036	1.728	0.013	0.586
Oropharynx/Nose	0.790	0.118	5.274	0.666	1.000
Oral rinse/Nose	168.040	25.167	1121.697	0.000	0.000
Saliva/Nose	78.895	11.816	526.640	0.000	0.000
Sputum/Nose	62.065	9.295	414.295	0.000	0.000
Tongue/Nose	11.924	1.786	79.593	0.000	0.001
Oropharynx/Nasopharynx	3.157	0.457	21.795	0.039	0.895
Oral rinse/Nasopharynx	671.435	97.236	4635.118	0.000	0.000
Saliva/Nasopharynx	315.240	45.653	2176.201	0.000	0.000
Sputum/Nasopharynx	247.992	35.914	1711.964	0.000	0.000
Tongue/Nasopharynx	47.643	6.900	328.897	0.000	0.000
Oral rinse/Oropharynx	212.668	31.851	1419.600	0.000	0.000
Saliva/Oropharynx	99.848	14.954	666.506	0.000	0.000
Sputum/Oropharynx	78.548	11.764	524.324	0.000	0.000
Tongue/Oropharynx	15.090	2.260	100.731	0.000	0.000
Saliva/Oral rinse	0.470	0.070	3.134	0.166	1.000
Sputum/Oral rinse	0.369	0.055	2.465	0.069	0.974
Tongue/Oral rinse	0.071	0.011	0.474	0.000	0.000
Sputum/Saliva	0.787	0.118	5.251	0.660	1.000
Tongue/Saliva	0.151	0.023	1.009	0.001	0.053
Tongue/Sputum	0.192	0.029	1.282	0.003	0.195
H					
Nose/Teeth	0.009	0.001	0.057	0.000	0.000
Nasopharynx/Teeth	0.002	0.000	0.011	0.000	0.000
Oropharynx/Teeth	0.009	0.001	0.060	0.000	0.000
Oral rinse/Teeth	3.839	0.575	25.626	0.014	0.611
Saliva/Teeth	2.398	0.359	16.010	0.110	0.996
Sputum/Teeth	0.870	0.130	5.809	0.799	1.000
Tongue/Teeth	0.290	0.043	1.938	0.024	0.774
Nasopharynx/Nose	0.182	0.025	1.303	0.003	0.201
Oropharynx/Nose	1.044	0.156	6.966	0.938	1.000
Oral rinse/Nose	448.557	67.180	2994.204	0.000	0.000
Saliva/Nose	280.233	41.970	1870.609	0.000	0.000
Sputum/Nose	101.676	15.228	678.709	0.000	0.000
Tongue/Nose	33.920	5.080	226.420	0.000	0.000
Oropharynx/Nasopharynx	5.742	0.800	41.174	0.002	0.164
Oral rinse/Nasopharynx	2467.899	344.050	17697.573	0.000	0.000
Saliva/Nasopharynx	1541.804	214.943	11056.443	0.000	0.000
Sputum/Nasopharynx	559.409	77.987	4011.584	0.000	0.000
Tongue/Nasopharynx	186.621	26.017	1338.280	0.000	0.000
Oral rinse/Oropharynx	429.821	64.374	2869.134	0.000	0.000
Saliva/Oropharynx	268.528	40.217	1792.473	0.000	0.000
Sputum/Oropharynx	97.429	14.592	650.359	0.000	0.000
Tongue/Oropharynx	32.503	4.868	216.962	0.000	0.000
Saliva/Oral rinse	0.625	0.094	4.170	0.389	1.000
Sputum/Oral rinse	0.227	0.034	1.513	0.007	0.395
Tongue/Oral rinse	0.076	0.011	0.505	0.000	0.000
Sputum/Saliva	0.363	0.054	2.422	0.064	0.967
Tongue/Saliva	0.121	0.018	0.808	0.000	0.012
Tongue/Sputum	0.334	0.050	2.227	0.045	0.920

ADDITIONAL RESULTS

1. Proportion of patients colonized

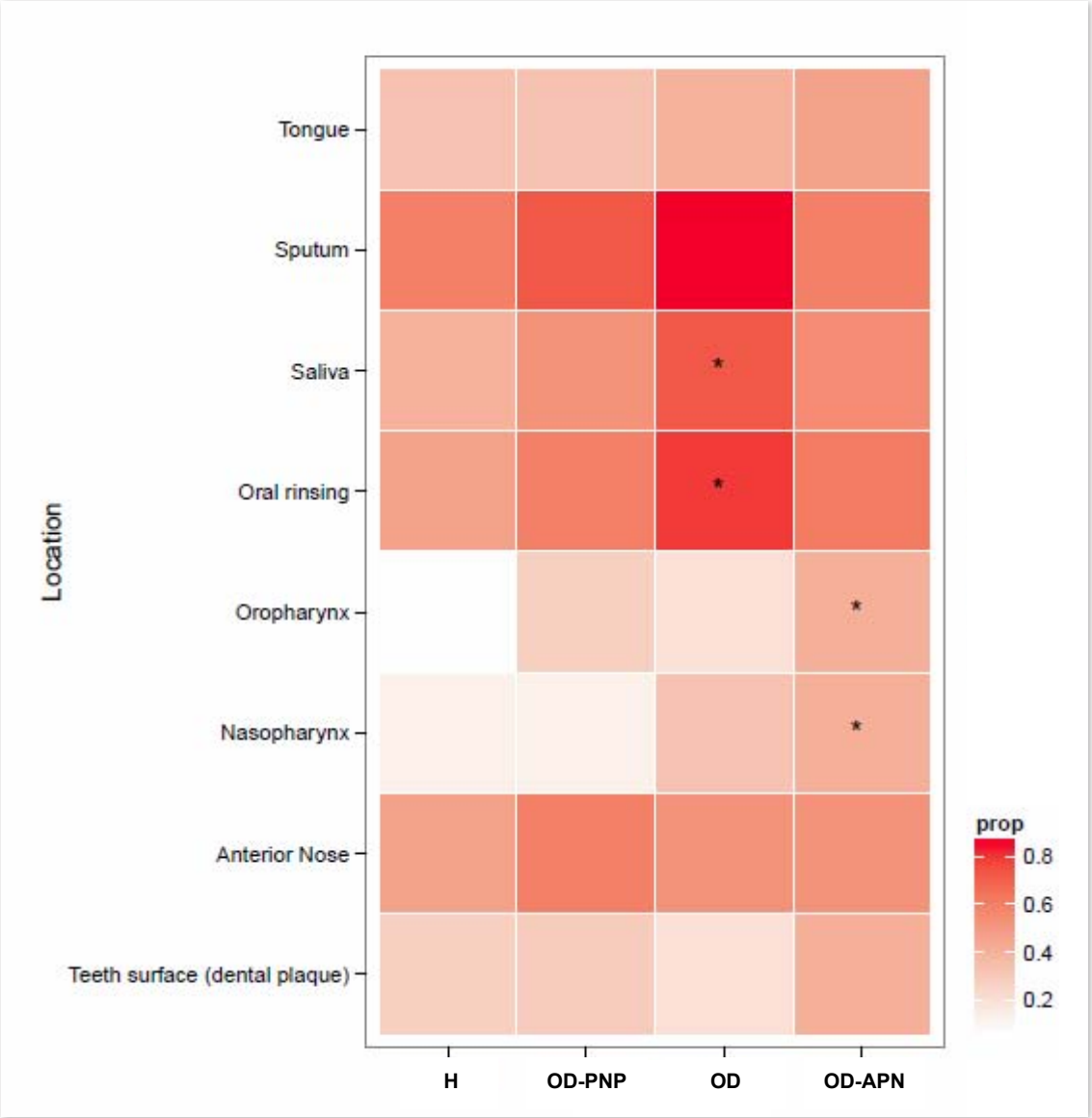


Figure 1. Proportion of patients colonized by respiratory pathogens by group and location. OD-PNP: patients with oropharyngeal dysphagia and previous pneumonia; OD: patients with oropharyngeal dysphagia; OD-APN: patients with acute pneumonia and oropharyngeal dysphagia; H: healthy elderly. * P-value <0.05 vs. group H.

2. Total bacterial load

location	p-value	method
Nose	0.289	log
Nasopharynx	0.119	np
Oropharynx	0.240	log
Oral rinse	0.897	log
Saliva	0.033	log
Sputum	0.136	np
Teeth	0.604	np
Tongue	0.667	np

Table 2. Results of comparison between groups for each location. "log" indicates testing were made on log transformed data (ANOVA on log-transformed data). "np" indicates that a non parametric test was used (Kruskal-Wallis).

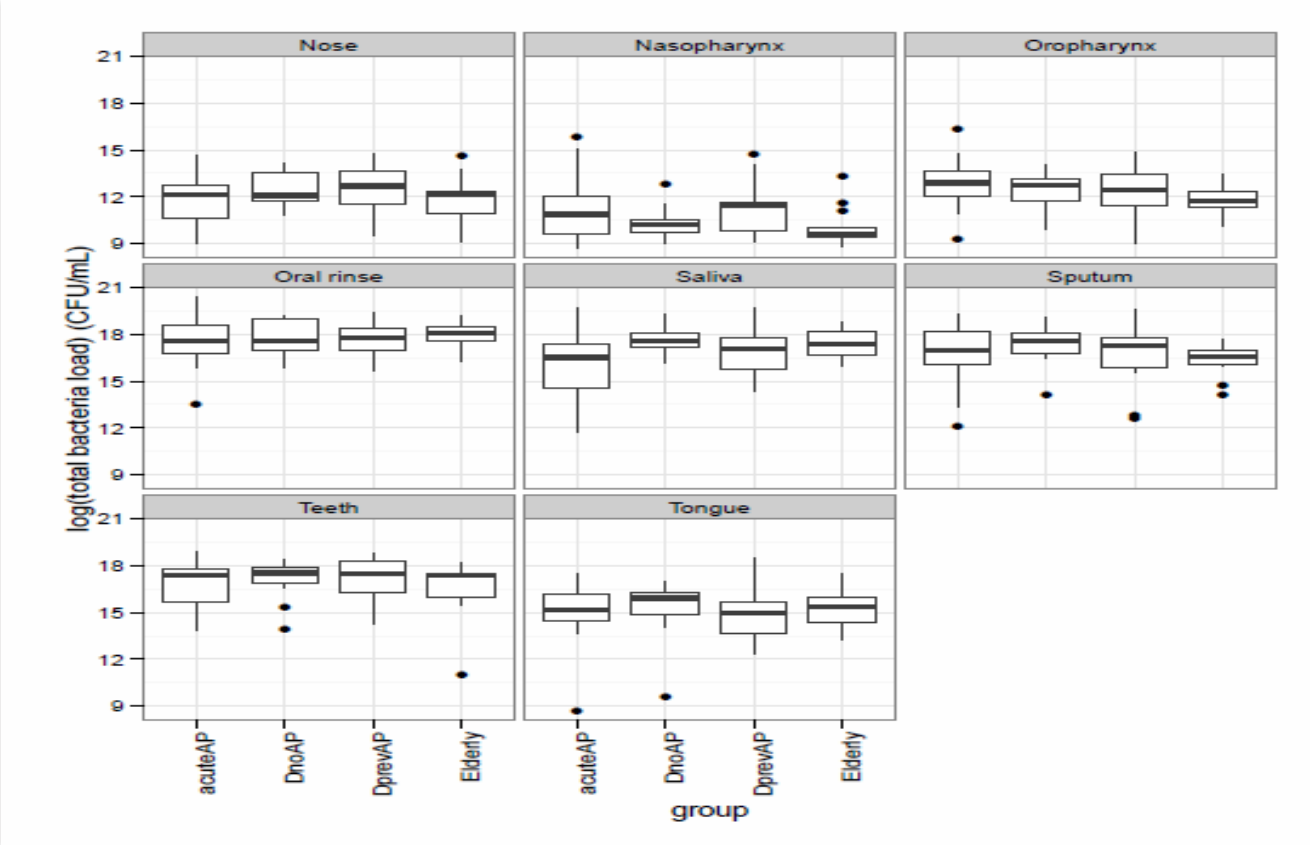


Figure 2. Distribution of total bacterial load for each group by location (on a log-scale). acuteAP: patients with acute pneumonia and oropharyngeal dysphagia; DnoAP: patients with oropharyngeal dysphagia; DprevAP: patients with oropharyngeal dysphagia and previous pneumonia; Elderly: healthy elderly.

location	n	mean	sd	min	max
acuteAP					
Nose	17	$4.25e+05$	$6.39e+05$	$8.56e+03$	$2.39e+06$
Nasopharynx	13	$9.26e+05$	$2.11e+06$	$5.86e+03$	$7.13e+06$
Oropharynx	16	$1.32e+06$	$3.05e+06$	$1.10e+04$	$1.25e+07$
Oral rinse	16	$1.14e+08$	$1.84e+08$	$7.55e+05$	$7.40e+08$
Saliva	16	$4.52e+07$	$8.99e+07$	$1.28e+05$	$3.65e+08$
Sputum	15	$5.59e+07$	$7.11e+07$	$1.88e+05$	$2.43e+08$
Teeth	17	$3.94e+07$	$3.89e+07$	$1.04e+06$	$1.52e+08$
Tongue	17	$9.48e+06$	$1.21e+07$	$6.03e+03$	$3.93e+07$
DnoAP					
Nose	15	$4.37e+05$	$4.49e+05$	$5.06e+04$	$1.46e+06$
Nasopharynx	15	$5.27e+04$	$8.80e+04$	$7.91e+03$	$3.60e+05$
Oropharynx	15	$3.89e+05$	$3.37e+05$	$2.13e+04$	$1.21e+06$
Oral rinse	15	$8.51e+07$	$7.82e+07$	$7.93e+06$	$2.08e+08$
Saliva	15	$6.36e+07$	$6.45e+07$	$9.97e+06$	$2.45e+08$
Sputum	15	$5.52e+07$	$4.93e+07$	$1.40e+06$	$1.90e+08$
Teeth	15	$4.10e+07$	$2.68e+07$	$1.08e+06$	$9.91e+07$
Tongue	15	$8.49e+06$	$6.84e+06$	$1.48e+04$	$2.38e+07$
DprevAP					
Nose	15	$6.03e+05$	$6.75e+05$	$1.33e+04$	$2.47e+06$
Nasopharynx	14	$3.29e+05$	$7.07e+05$	$8.90e+03$	$2.50e+06$
Oropharynx	15	$5.19e+05$	$6.89e+05$	$8.00e+03$	$2.72e+06$
Oral rinse	15	$8.18e+07$	$8.14e+07$	$6.62e+06$	$2.68e+08$
Saliva	15	$6.19e+07$	$9.93e+07$	$1.65e+06$	$3.47e+08$
Sputum	15	$5.33e+07$	$7.97e+07$	$2.98e+05$	$3.16e+08$
Teeth	14	$5.07e+07$	$4.46e+07$	$1.54e+06$	$1.45e+08$
Tongue	15	$1.71e+07$	$3.39e+07$	$2.23e+05$	$1.08e+08$
Elderly					
Nose	15	$3.23e+05$	$5.46e+05$	$9.34e+03$	$2.12e+06$
Nasopharynx	13	$6.91e+04$	$1.59e+05$	$6.76e+03$	$5.91e+05$
Oropharynx	15	$1.92e+05$	$1.73e+05$	$2.55e+04$	$6.87e+05$
Oral rinse	15	$7.66e+07$	$5.34e+07$	$1.14e+07$	$2.14e+08$
Saliva	15	$5.45e+07$	$4.59e+07$	$8.91e+06$	$1.40e+08$
Sputum	15	$1.85e+07$	$1.37e+07$	$1.30e+06$	$4.97e+07$
Teeth	15	$2.84e+07$	$2.22e+07$	$5.90e+04$	$8.27e+07$
Tongue	15	$8.91e+06$	$1.21e+07$	$5.66e+05$	$3.97e+07$

Table 1. Summary statistics of total bacterial load by groups and location in CFU/mL. n, mean and standard deviation are presented. acuteAP: patients with acute pneumonia and oropharyngeal dysphagia; DnoAP: patients with oropharyngeal dysphagia; DprevAP: patients with oropharyngeal dysphagia and previous pneumonia; Elderly: healthy elderly.

3. Principal component analysis of microbiota

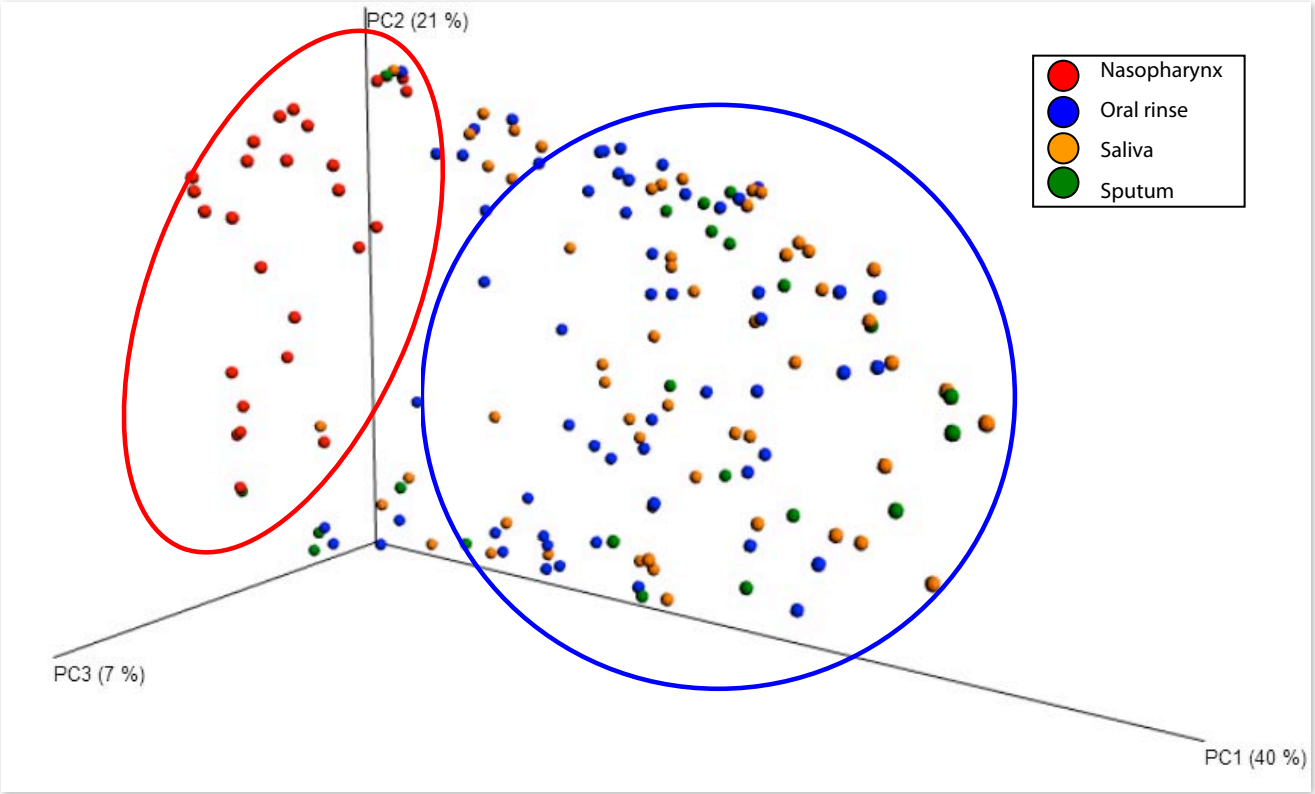


Figure 3. Principal component analysis plot of weighted unifrac distances, all groups. Note the different position of nasopharyngeal samples compared to oral samples and sputum, indicating the differences in microbiota composition between both locations.

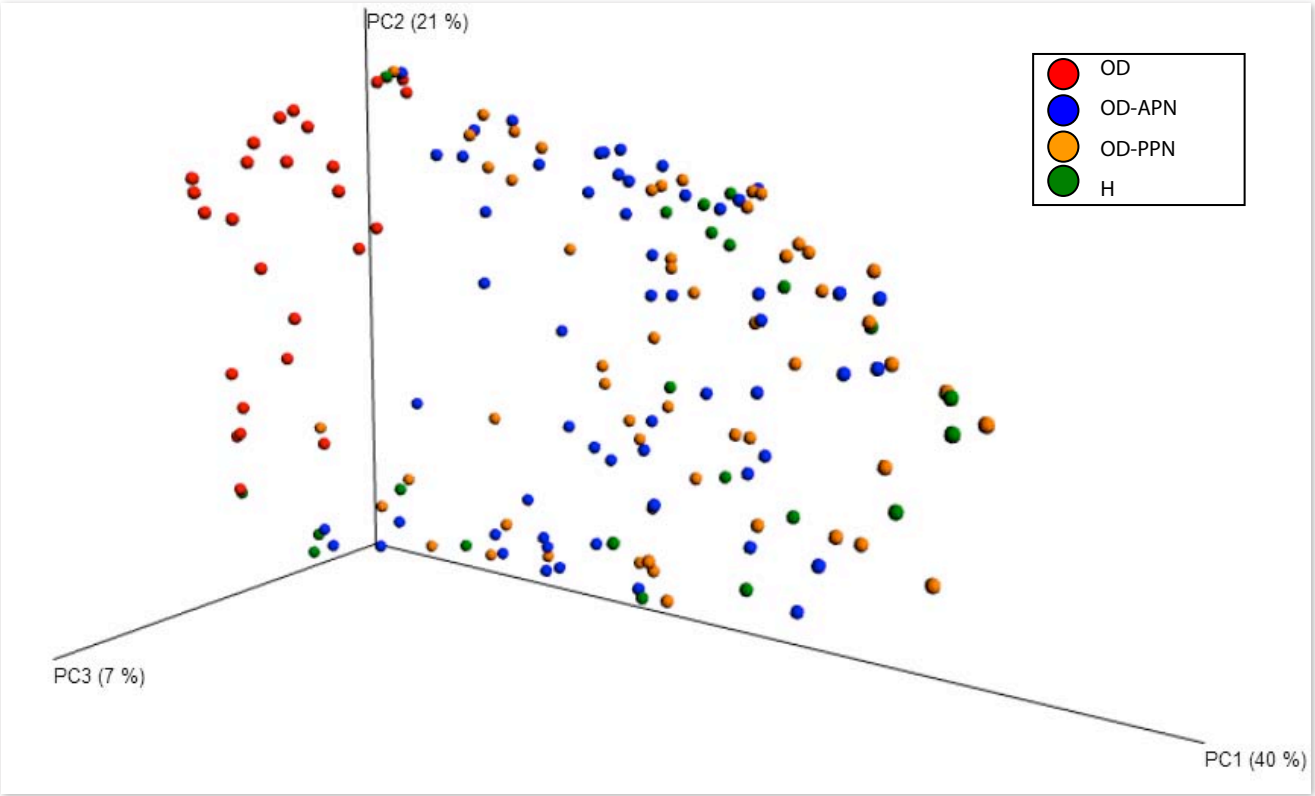


Figure 4. Principal component analysis plot of weighted unifrac distances, all niches. Note that there are no visual distribution patterns indicating a similar microbiota composition between groups. OD-PNP: patients with oropharyngeal dysphagia and previous pneumonia; OD: patients with oropharyngeal dysphagia; OD-APN: patients with acute pneumonia and oropharyngeal dysphagia; H: healthy elderly.

APPENDIX II. METHODS TO OBTAIN THE OROPHARYNGEAL SAMPLES, MOLECULAR BIOLOGY METHODOLOGY AND ADDITIONAL RESULTS.

ABSTRACT

Eight different oropharyngeal samples were analyzed by quantitative PCR and pyrosequencing in chapter II of the present doctoral thesis to describe oropharyngeal microbiota and colonization by respiratory pathogens of several groups of elderly patients with dysphagia and a control group of older patients without dysphagia. To obtain them, we used a specific methodology to ensure the location to be sampled, to avoid contamination and to properly conserve the samples for its posterior analysis at the Nestlé Research Centre in Lausanne, Switzerland. This appendix explains and clarifies the methodology used during the second study of this doctoral thesis with text and images of the procedures. In addition, in the oral microbiota study (chapter II) we analysed microbiological samples with molecular biology techniques based on DNA examination that are much more specific and sensible than classical microbiological techniques. This appendix, also describes the full methodology to obtain the samples from the oral microbiota study and how these samples were analyzed step-by-step at the Nestlé Research Center with these novel methodology and equipment. To work with molecular biology equipment first an extraction of DNA should be performed. Then DNA can be analyzed to detect and/or quantify specific pathogens, to quantify the total bacterial load or to sequence the most predominant bacterial genera among many other applications. Finally, in this appendix we present additional results from the oral microbiota study that were not published due the high amount of data generated during this study. These supplementary results will complement chapter II understanding, including results about the proportion of patients colonized, the total bacterial load and principal component analysis of niches sampled and groups of patients.

METHODS TO OBTAIN THE OROPHARYNGEAL SAMPLES

During the oral microbiota study (Chapter II) we took samples from eight different anatomical locations, all the procedures performed were done with globes, mask and sterile material to avoid contamination of samples. All samples were collected early in the morning. After collection, all samples were frozen at -80ºC as soon as possible until the extraction of DNA was performed.

1. Sampling of oral rinsing

10 mL of sterile water were given to the patients and they gargled it during 15s, after gargling, water was collected in a 50mL Falcon® tube (FALCON, Blue Max 50mL Polypropylene Conical Tube, Becton Dickenson). Following, the content of the tube was centrifuged at 3000RCF for 30 min at 5ºC. Then 9 mL liquid of water were remove using a sterile 10-mL plastic disposable pipette without disturbing the pellet (1 mL or less liquid remained in the tube). Once the liquid was removed, the pellet was re-suspended using the remaining liquid and the same pipette or the vortex.

2. Sampling of nose

Interior nose samples were obtained according to standard procedures using swabs. A sterile swab collection and transport system was used (Copan 480CE, Brescia, Italy). Each sampling set consisted of an applicator swab with flocked nylon fiber tip as well as a screw-top polypropylene vial filled with 1 mL Modified Liquid Amies Medium. The swab had a rigid shaft with a molded breakpoint which was broken off without the use of scissors. To make the procedure, the swab was introduced into the nasal openings approximately 1 cm

and rotated 4 times in each nostril. Once the specimen was collected, the swab was placed in the provided vial (Figure 1). The specimen was vortexed for 10 to 20 seconds to disperse organisms from the swab tip before freezing.

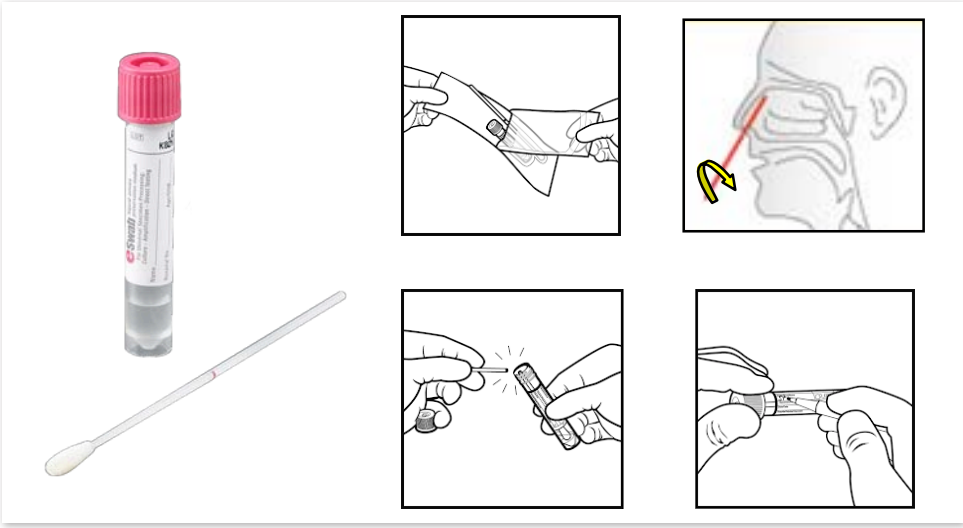


Figure 1. Procedure of nose sample collection. From Copan diagnostics.

3. Sampling of nasopharynx

Nasopharynx samples were obtained according to standard procedures using swabs. Samples were obtained with a deep nasopharyngeal sterile swab. A swab collection and transport system was used. Each sampling set consisted of a pernasal applicator swab with flocked nylon fiber tip as well as a screw-top polypropylene vial filled with 1 mL Modified Liquid Amies Medium (Copan 482C, Brescia, Italy). To obtain the specimen the patient's head was tipped slightly backward and the swab passed directly backwards, parallel to the floor of the nasopharynx. The swab should pass without resistance until it reaches the posterior pharynx which is approximately one-half to two-thirds the distance from the nostril to the ear lobe. If resistance was encountered, the swab was removed, and a new attempt was made to pass the swab through the other nostril. Once the swab was in place, it was rotated 180º or leaved in place for 5 seconds to saturate the tip before removing it slowly (Figure 2). The procedure is described in:

http://www.copanswabs.com/products/microrheologics/animation.php

Once the specimen was collected, the swab was placed in the provided vial. The specimen was vortexed for 10 to 20 seconds to disperse organisms from the swab tip before freezing.

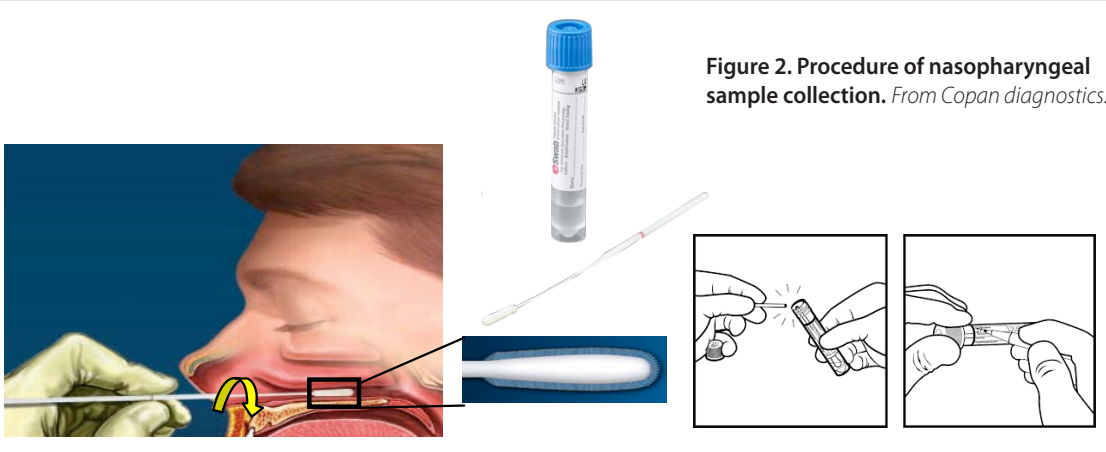


Figure 2. Procedure of nasopharyngeal sample collection. *From Copan diagnostics.*

4. Sampling of oropharynx

Oropharynx sample was obtained according to standard procedures using swabs. A swab was rubbed over the posterior pharynx and bilateral tonsillar tissue while avoiding contact with the tongue, buccal surfaces, and lips. A swab collection and transport system was used (Copan 480CE, Brescia, Italy) as described for the nasal samples.

5. Sampling of tongue

Tongue sample was obtained using the swabs by several strokes over the first two thirds of the tongue dorsum in anteriorposterior direction. A swab collection and transport system was used (Copan 480CE, Brescia, Italy) as described for the nasal samples. Freeze the swab at -20°C as soon as possible. Take 3-5 strokes in total. Sample the area surrounded by thick lines on the image (Figure 3).

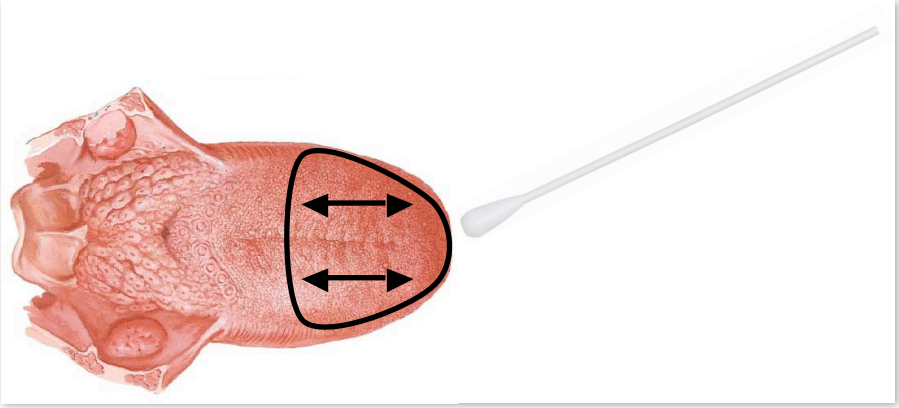


Figure 3. Procedure of tongue sample collection.

6. Sampling of dental plaque

Dental plaque samples were obtained by pooling samples obtained from the mesial buccal surface of all 2 first molars using a sterile Gracey curette 11/12 (LM-dental, Finland) (Figure 4). If these teeth were missing, samples from the nearest posterior adjacent tooth were taken. Plaque samples were taken by placing a sterile curette to the base of each pocket and, with a single stroke, pressing against the tooth removing a plaque sample into a sterile 1.5 mL Eppendorf tube with 1 mL of Liquid Amies Medium. The specimen was vortexed for 10 to 20 s.

Figure 4. Gracey curette 11/12 (LM-dental, Finland)

7. Sampling of saliva

Saliva samples were collected by spitting into a sterile plastic 50-mL tube.

8. Sampling of sputum

Sputum sample was obtained by making patients to expectorate into a 50 mL sterile container. To get a good sample, the patient was instructed to take deep breaths and then force out a deep cough expectorating the sputum into the container. If a good sample was not taken on the first try, patient was asked to continue to cough until the collection was achieved. After the collection, sputum sample was processed with the commercial kit specially designed for microbiological spu-

tum samples (SLsolution and Sputum Dipper™, Copan, Brescia, Italy):

1. A SL sterile solution tube and a Sputum Dipper™ were used to process each sample.
2. The Sputum Dipper™ was introduced into the container of sputum specimen.
3. The drill-shaped head of the Sputum Dipper™ was completely submerged and rotated in the sample for approximately 10 seconds ensuring the plastic coil of the dipper was filled up with sputum sample.
4. The Sputum Dipper™ was introduced into the SLsolution (Sputum Liquefying solution, a mucolytic agent for sputum samples) tube and the shaft of the dipper was broken-off at the molded breakpoint an the tube was closed.
5. The SLsolution tube with the sample was vortexed for 30 seconds at 2000-2500 rpm.
6. The sputum and SLsolution mixture were left at room temperature for 15 minutes and then the SLsolution tube was frozen at -80 ºC.

Finally, frozen samples (-80ºC) were sent to the Nestlé Research Center (Lausanne, Switzerland) in dry ice for analysis on 2 different batches.

Figure 5. SL solution and Sputum Dipper

REFERENCES

- *Breuninger, C., Follin, S., et al. (Eds). (2001). Handbook of nursing procedures (pp. 755 - 759). Springhouse, PA: Springhouse Corporation.*
- Sally Beattie, RN, MS, CNS, GNP : http://www.modernmedicine.com/modern-medicine/content/sputum-sample-collection.

MOLECULAR BIOLOGY METHODOLOGY

Molecular biology analysis was performed at the Nestlé Research Center (NRC) in Lausanne, Switzerland just after the recruitment of patients from study of chapter II finished. Samples were sent frozen at -80ºC to the NRC for it analysis. During the analysis period I performed a stay during more than two months at the NRC learning and doing the analysis with the microbiologist responsible of the study and the NRC technician. Below, the process extraction of DNA and analysis is explained.

1. DNA extraction

To perform the quantitative PCR analysis for the study of oral microbiota (chapter II), a DNA extraction was performed with a standardized commercial kit (QiaAamp DNa mini kit).

DNA isolation is a standardized procedure to collect DNA for subsequent molecular analysis. There are three basic and two optional steps in DNA extraction:

1. Cell lysis, to expose the DNA within (chemical and physical methods).

2. Removal of membrane lipids (detergents or surfactants).
3. Removal of proteins (protease) -> optional, almost always done.
4. Removal of RNA (RNAse) -> optional, often done.
5. Precipitation of the DNA with alcohol (DNA is insoluble in it). This step also removes alcohol-soluble salts.

DNA extraction is a critical step in molecular biology processes, so it should be done with a reliable and standardized methodology and avoiding contamination.

Extraction study protocol: for this study, DNA extraction was performed in a blinded way for all groups with the QIAGEN DNA mini extraction kit for blood and body fluid samples (spin protocol) (Qiagen, Venlo, The Netherlands). All the work was done in the labs of Food and Health Microbiology at the Nestlé Research Centre, Lausanne. Bacterial cell lysis and inactivation was performed in a biosecurity laboratory manually and the rest of the extraction was performed with the QIAGEN QIACUBE robot (Qiagen, Venlo, The Netherlands) to increase the standardization and minimize the human error.

Cell lysis protocol:

- Defrost of 12 samples (1 batch) from the -20°C freezer to 5°C (the process normally took 45min.). Samples could not be unfrozen more than 2 hours (defrost time was registered).
- Manual lysis:
  - 12 numbered (extraction number) 2mL *eppendorfs* tubes were prepared under the laminar flood hood.
  - 200µL of buffer AL were added to each *eppendorf* tube.
  - 200µL of sample were added to each *eppendorf* tube after resuspending it with the vortex during 5 seconds.
  - 20µL of protease K were added to the solution and all was mixed with the vortex during 15 seconds.
  - The tubes were removed from the laminar flood hood and incubated at 56°C for 10 min.
  - A short spin was applied to the tubes (condensation).
- Automatic extraction with QIACUBE (biosafety laboratory P1; Figure 1):
  - The machine was turned on and the protocol, the extraction material and the elution volumes (50µL+50µL) were selected. In addition, extraction solutions (Ethanol 96-100%, AW1, AW2 and AE) and pipette tips were placed.
  - Spin filters (pos. L1) and 1.5mL collection tubes with safety caps (pos. L3) were placed in the rotor adapters and then in the QIAcube centrifuge.
  - 2mL manual lysis Eppendorfs were placed in its location.
  - Subsequently, the extraction started and the machine checked that all was properly prepared showing the extraction time.
  - After the extraction process tubes were stored at -20°C in its box.

QIAcube | Protocol Sheet

General Information (January 2009)

Application	DNA
Kit	QIAamp® DNA Mini Kit (50) plus QIAamp DNA Mini Accessory Set A, cat. no. 1048145 or QIAamp DNA Mini Kit (250) plus QIAamp DNA Mini Accessory Set B, cat. no. 1048146
Sample material	Blood or body fluid
Short protocol name	Manual Lysis
Version	1
Full protocol name	Blood and body fluid spin protocol with manual lysis V1
Editable parameters	Elution volume 1: 50–100 µl in increments of 10 µl; default 100 µl Elution volume 2: 50–100 µl in increments of 10 µl; default 100 µl
Required QIAcube® software versions	**Firmware version FIW-50-001-J_FW_MB.hex and PLC program version FIW-50-002-G-PLC_MB.prs or higher; available from the QIAcube Web Portal**

Shaker

Material	420 µl manually lysed blood or body fluid (see "Comments" on the next page)
Vessel	2 ml screw-cap tube without skirted base* (use in combination with Shaker Rack Plugs)
Adapter	Shaker adapter for 2 ml screw cap tubes (marked with "S2")

* Sample Tubes CB, 2 ml (cat. no. 990382); see www.qiagen.com/MyQIAcube.

Disposable Tips

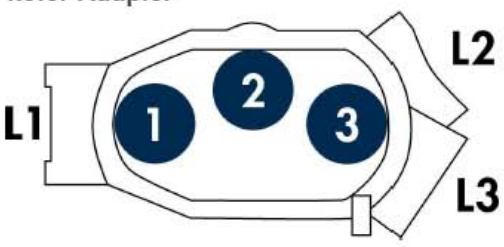
Disposable Filter-Tips, 1000 µl

Reagent Bottle Rack

Rack labeling strip	QIAamp DNA

Position	Reagent
1	–
2	–
3	96–100% ethanol
4	Buffer AW1
5	Buffer AW2
6	Buffer AE

Rotor Adapter



Position	Labware	Lid position
1	QIAamp Mini spin column	L1
2	–	–
3	1.5 ml collection tube*	L3

* Sarstedt, Micro tube 1.5 ml, Safety Cap (see www.sarstedt.com).

QIAGEN®

Sample & Assay Technologies

Figure 1. Protocol sheet of the QIAGEN DNA mini extraction kit for blood and body fluid samples (spin protocol).

1. Molecular biology techniques

After bacterial DNA was extracted, we use it to perform several molecular biology techniques: 1) quantitative real time PCR analysis was done in order to: a) quantify the total bacterial load of the samples using primers to amplify the conserved region of the 16S RNA of bacterial DNA (to know the total bacterial carriage of the samples), and b) detect and quantify five respiratory pathogens associated with AP (*Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Escherichia coli* and *Pseudomonas aeruginosa*). 2) pyrosequencing of the samples in order to determine the proportion of the predominant microbial groups (microbial genera) in each location and patient group. All analytical procedures were done in blinded fashion (lab technician was blinded towards the patient's group).

2.1 Quantitative real time PCR (qPCR)

The real time quantitative polymerase chain reaction (qPCR) is widely used in the detection of nucleic acids in the field of microbiology since it allows to accurately quantifying amplified DNA in real time. It has a high sensitivity and specificity, it is fast (generally less than one hour), cheap (volumes of the reagents used are very small) and a high quantity of samples can be processed in one single run (1).

Real-time PCR can detect specific amplificated sequences of DNA as they accumulate in "real-time" during the PCR amplification process. As this process occurs and amplification products duplicate, this technique can determine their accumulation and quantify the number of initial substrates of interest present in the sample (2;3). This method combines PCR biochemistry with a fluorescent probe for the detection of amplified products in the same reaction. To make this process possible, a segment of DNA was amplificated by using two PCR primers and TaqMan probes.

TaqMan probes (5'nuclease probes; Figure 2) are composed of a short oligonucleotide (DNA) containing a 5' fluorescent dye and 3' quenching dye. To generate a light signal, the probe must bind to a complementary strand of DNA at 60°C and secondly, Taq polymerase must cleave the 5' end of the TaqMan probe (5' nuclease activity), separating the fluorescent dye from the quenching dye. A single TaqMan probe can be used for detection of amplified target DNA well (2;3).

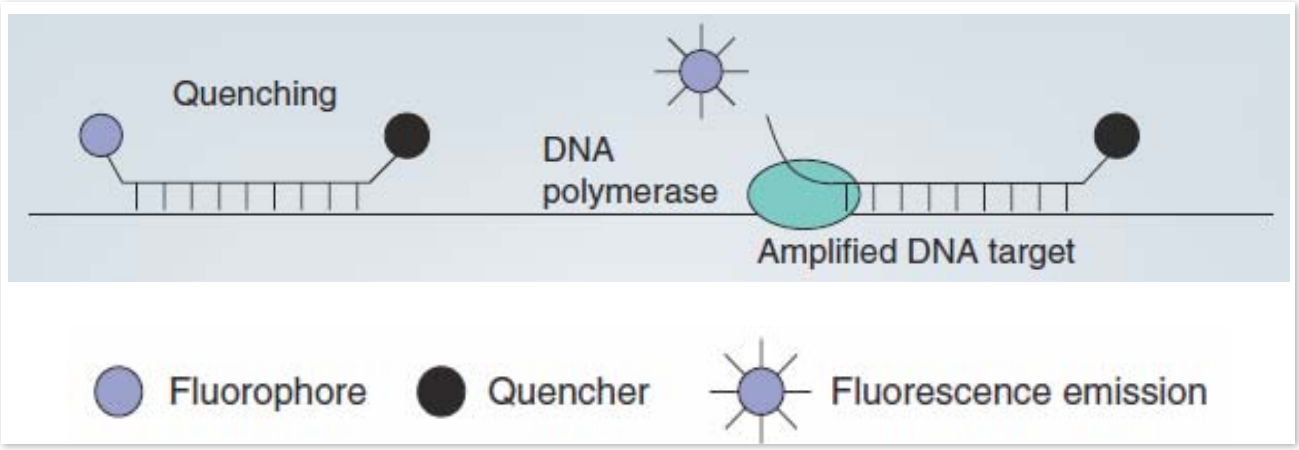


Figure 2. Real-time PCR probes. TaqMan probe (5' nuclease probe). Fluorescence occurs when the fluorophore and the quencher are separated from each other by the nuclease activity of the DNA polymerase. *Adapted from Maurin M 2012 (2).*

qPCR assays were calibrated with standard curves using commercial solutions included in the primer and probes kits to express the equivalent of Colony Forming Unit (CFU) per mL of sample. For each qPCR assay, detection limit was different and varied from $5x10^3$ CFU mL^{-1} to $3x10^4$ CFU mL^{-1} for specific pathogens, while for total bacterial load it was $5x10^4$ CFU mL^{-1}.

Design and development: both assays were performed with TaqMAN probes (FAM Dye and Black Hole Quencher (BHQ)), which are more specific than other detection methods like the SYBRgreen.

2.1.1 Total bacterial load (TBL)

TBL aims to achieve an amplification of all or almost all the bacterial DNA present in the sample (depending on the primer design and coverage) in order to know the total bacterial carriage. To perform this, a set of universal primers and probes for the amplification of the 16S rDNA of the domain *Bacteria* should be chosen.

Primers and probes

In our study 2 different sets of universal primers were evaluated; one of Sigma-Aldrich and another from the publication by Nadkarni *et al.* 2002. All the primers coverage was tested *in silico* against the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) to look for the coverage and matches of these primers. Finally the literature primer was chosen because although the amplicon was bigger the bacterial coverage was greater (Table 1) (4).

Source	Name	Amplicon	T_m	Sequence
Nadkarni et al. (4)	Probe	467	67.3	CGTATTACCGCGGCTGCTGGCAC
	F		60.2	TCCTACGGGAGGCAGCAGT
	R		60.4	GGACTACCAGGGTATCTAATCCTGTT

Table 1. Primers used for the Total Bacterial Load amplification. F: forward; R: reverse; T_m: melting temperature

Standard curve

To do the standard curves there are two main possibilities: to use a mixed solution of different bacterial strains (the problems of that option are the differences of every bacterial strain in extraction efficiency and in the amplification step); or to use one strain and comparing that with the general amplification of the entire bacterial load. Finally to do the standard curve to calculate the TBL of our samples, we chose a commercial solution of the strain *E. coli* K12 with serial dilutions from $5x10^7$ to $5x10^2$ CFU mL^{-1}.

qPCR configuration

Amplification and detection of DNA by real-time PCR was made using the ABI 7900HT Sequence Detection System (Applied Biosystems, USA) with 384-well plates and a broad-range primers–probe set to quantify the 16S rRNA gene (4). PCR was carried out in duplicate in a 25µL reaction volume containing 300nM broad-range primers and 175nM broad-range probe using the TaqMan PCR Core Reagents Kit (Applied Biosystems, USA) and using the 2x Taqman Precision MasterMix ABI gène GEX (Applied Biosystems, USA). The reaction conditions for amplification of DNA were following manufacturers' recommendations. The qPCR program was as follows: 95°C 10 min, 95°C 15sec, 60°C 1 min in a total volume per well of 25µL.

2.1.2 Respiratory pathogens

With this methodology we aimed to detect and quantify 5 different upper-respiratory tract infectious (URTI) pathogens. During the whole process, specificity of the primers is the most important aspect because lots of pathogens have many genetically related non-pathogenic strains that can interfere in the results of the analysis giving false positives. Consequently, a correct primer design must be achieved.

Primers and probes

As the pathogenic bacterial strains have many genetic related non-pathogenic strains

in common, primers must be designed to only interact with very specific parts of the pathogen related genes. So for each primer chosen, we tried for a set of target bacterial strains and a set of non-target bacterial strains to prove their specificity. The strains used to carry on that test were from the German type culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ) or were isolated pathogenic hospital strains from the Microbiology Service of the Hospital de Mataró, Spain that were sent to the NRC in Switzerland.

Two different sets of primers were tested for each respiratory pathogen; some of them acquired from a specialized company (Primerdesign, Southampton, UK) and some designed at the NRC.

To prepare the analysis, each strain was quantified. Firstly they were cultured and at the exponential logarithmic phase they were concentrated (centrifugation) and aliquoted. One part of the aliquots was frozen and with the other part, serial diluted cultures were performed to quantify the CFU mL^{-1} in the original aliquot. The final concentration prepared of each was $5x10^7$CFU/mL.

The strains tested for the primer specificity were sent to be identified by sequencing (except the ones coming from the type culture collection DSMZ) to ensure the tests. The strains tested were the following:

Escherichia coli primer test:

In the *E. coli* group 12 different *E. coli* and *Shigella* strains were tested (*E.coli* and *Shigella* are genetically related). These were from the DSMZ, the NRC or from the Hospital de Mataró. In green the target strains and in red the non-target ones; underlined the strains that were amplified.

- *E. coli K12*
- *E. coli ECOR-01*
- *E. coli ECOR-28*
- *E. coli ECOR-35*
- *E. coli ECOR-42*
- *E. coli ECOR-56*
- *E. albertii DSMZ 7582*
- *E. fergusonii DSMZ 3698*
- *E. hermannii DSMZ 4560*
- *S. flexneri DSMZ 4782*
- *S. boydii DSMZ 7532*
- *S. sonnei DSMZ 5570*

Haemophilus influenzae primer test:

In the *H. influenzae* group 10 different strains were tested; these were from the DSMZ, the NRC or from the Hospital de Mataró.

- *H. influenza DSMZ 24049*
- *H. influenza DSMZ 11970*
- *H. influenza DSMZ 9999*
- *H. influenza DSMZ 4690*
- *H. influenza DSMZ 11121*
- *H. influenzae 6266570*
- *H. influenzae 6269918*
- *H. aegyptius DSMZ 21187*
- *H. parainfluenzae DSMZ 8978*
- *H. parahaemolyticus DSM 21417*

Pseudomonas aeruginosa primer test:

In the *P. aeruginosa* group 9 different strains were tested; these were from the DSMZ, the NRC or from the Hospital de Mataró.

- *P. aeruginosa 6865906*
- *P. aeruginosa 6269012*
- *P. aeruginosa Spain-7*
- *P. aeruginosa DSMZ 50071*
- *P. aeruginosa DSMZ 19880*
- *P. aeruginosa DSMZ 24600*
- *P. aeruginosa DSMZ 24599*
- *P. otitidis DSMZ 17224*
- *P. fluorescens DSMZ 50090*

Staphylococcus aureus primer test:

In the *S. aureus* group 10 different strains were tested; these were from the DSMZ, the NRC or from the Hospital de Mataró.

- *S. aureus DSMZ 20231*
- *S. aureus COL*
- *S. aureus 6265443*
- *S. aureus Spain-6*
- *S. aureus N315*
- *S. simiae DSMZ 17636*
- *S. epidermidis DSMZ 20044*
- *S. warneri DSMZ 20316*
- *S. capitis subsp. Urealyticus DSMZ 6717*
- *S. haemolyticus DSMZ 20263*

Streptococcus pneumoniae primer test:

In the *S. pneumoniae* group 10 different strains were tested; these were from the DSMZ, the NRC or from the Hospital de Mataró.

- *S. pneumoniae R6*
- *S. pneumoniae 6269800*
- *S. pneumoniae DSMZ 20566*
- *S. pneumoniae DSMZ 11865*
- *S. pneumoniae 6272139*
- *S. pseudopneumoniae DSMZ 18670*
- *S. infantis DSMZ 12492*
- *S. mitis DSMZ 12643*
- *S. mitis 6275706*
- *S. oralis DSMZ 20627*

Moreover, a spiking test was done to asses 2 main objectives: a) to know if the sample carrying substances had any inhibitory effect on the qPCR analysis and b) to know the detection limit of the clinical samples. This test was done by doing the qPCR of known concentrations (different dilutions of 500, 5000 and 500000 CFU/mL) of 2 different bacterial target strains extractions (the ones aliquoted in the primers tests) in water (control) and in the 8 different samples of the trial. With that results, the amplification and quantification of the control group (water) was compared with the amplification and quantification of the different niches samples.

To confirm the detection limit, we took the lowest concentration of bacterial DNA in which there was a complete amplification among all the samples.

To know if the mixed pathogen populations had any effect on the qPCR, a test with a mixed pathogen known strains could be done.

Primers and probes results:

- Target strains and no target strains detection is marked above (underlined strains were amplified in the test). All *Shigella* strains showed amplification in the *E. coli* test due to the close genetic proximity between them (some authors consider them genetically identical). In *Streptococcus*, all the *S. mitis* strains were amplified.
- Detection limit: the detection limit of the assay was between 500 – 5000 CFU mL^{-1}.
- The spiking results showed slightly inhibition of the qPCR by the samples of approximately 0.5 log difference.
- The assay with best sensitivity and specificity was chosen.

Standard curves

To establish the standard curves a commercial solution included in the primer and probes kits of $2x10^5$ bacterial strain copies/µL was used. To make it, due to the very significant contamination risk, the commercial solution must be opened and handled in a separate laboratory environment, away from the other components. The standard curve contained triplicate dilutions of: $2x10^5$, $2x10^4$, $2x10^3$, 200, 20 and 2 copies/µL. To consider a valid standard curve, after the performance of the qPCR, slope values must be around -3.33 (100% efficiency) with a stabilised accept-

able margin of -3.6 to -3.1 (110 – 90%) and R^2 values (measure of replicate reproducibility) must be higher than 0.985 to be acceptable. To achieve this, the standard curve dilutions must be prepared very carefully and the triplicates have to give very similar C_T values (cycle in which there is a significant increase in reporter signal, above the threshold) between them (Figure 3). Our experiments showed that the number of bacterial DNA copies in standard curves as provided by manufacturers agreed with CFU as measured by growth on plates.

The efficiency of the amplification is calculated from the slope of the standard curve using the following formula (5):

$$E = 10^{\wedge(-1/slope)}$$

Ideally, the amount of PCR product will double during each cycle of exponential amplification; that is, there will be a 2-fold increase in the number of copies with each cycle. This translates to a reaction efficiency of 2. Using an efficiency equal to 2 in the equation above, 2 = 10–1/slope, indicates that the optimal slope of the standard curve will be –3.33. Amplification efficiency can be also expressed as a percentage (percent of template that was amplified in each cycle) with the following formula:

$$\% \text{ Efficiency} = (E - 1) \times 100\%$$

For an ideal reaction, % Efficiency = (2 – 1) x 100% = 100%. Efficiency close to this value is the best indicator of a robust, reproducible assay. Low efficiency may be caused by poor primer design or by suboptimal reaction conditions. Reaction efficiencies higher than 100% may indicate pipetting error in your serial dilutions or coamplification of nonspecific products, such as primerdimers (5).

qPCR configuration

Amplification and detection of DNA by real-time PCR was done with the ABI 7900HT Sequence Detection System (Applied Biosystems, San Francisco, USA) with 384-well plates. We used the primers specific for each of the pathogens tested with the Taqman probes included in the tube (lyophilized primers and probes) from Primerdesign (Primerdesign, Southampton, UK). PCR was done in duplicate in a 12.6µL reaction volume (7.6 µL master mix + 5µL sample (2.5 µL H_2O + 2.5µL sample) following primer and probes manufacturer recommendations. 2x Taqman Precision MasterMix ABI gene GEX (Applied Biosystems, San Francisco, USA) was used. The reaction conditions for amplification of DNA were 95°C for 10 min, followed by 50 cycles of 95°C for 18s and 60°C for 1 min.

- Plate organization: plates were designed to have 2 replicas of each sample, a standard curve with triplicates per each dilution (6 dilutions) and two negative control wells (2 NTC). 5 plates per pathogen were done to carry on the total number of samples (834) on duplicate; additionally, the fifth plate included 48 different samples from healthy volunteers and different niches (nose, saliva, tongue and nasopharynx). The master mix was added first with an electronic pipette in a volume of 7.6µL; then the samples were placed vertically in the order of extraction and pipetted with an 8 channel electronic pipette in volumes of 2.5µL (firstly 2.5µL of the sample and then 2.5µL of water were added > the samples were diluted ½); in the standard curve (5µL) and in the NTC (5µL water) the sample load was not diluted. The final volume per well was 12.6µL.
- MasterMix preparation: it was prepared at the beginning of the experiment, after cleaning the bench top with ethanol to avoid contamination and it was done in ice with the following format:

For 1 sample:

- 6.25µL 2x Taqman Precision MasterMix ABI gène GEX
- 0.625µL Pathogen primer/probe Primerdesign (specific pathogen)
- 0.625µL H_2O
- 5µL ½ Sample (2.5µL sample + 2.5µL H_2O)

It was prepared in excess (for about 10 wells more) due to have enough at the end of the pipetting.

- ABI 7900HT set up: The plates were analysed by ABI prism7900HT, with the FAM reporter with the passive reference ROX. To begin the machine was turned on before preparing the plate because it needs to be warmed up for some minutes to reach an adequate temperature. After preparing the plate, it was settled inside and a template of the plate's design was loaded with all the conditions, detectors and the qPCR cycling program. The only thing that was changed was the name of the file and the detector for each pathogen.

Analysis

The analysis of the amplification curves was done by the computer ABI 7900HT software based in the standard curves (defined before start of the analysis), but to better understand the given values and graphs, some definitions must be clear:

- The baseline is the average background. It is calculated according to the noise level in the early cycles, when there is no detectable increase in fluorescence due to PCR products. In our analysis it was automatically settled by the machine.
- The threshold is the level of fluorescence above the baseline, at which the signal can be considered not to be background. The threshold can be calculated automatically or manually. It was settled by the machine.
- The C_T value (Cp for Roche's thermo cyclers) is defined as the cycle in which there is a significant increase in reporter signal, above the threshold, i.e. the cycle in which the growth curve crosses the threshold. It is consequently related to the initial amount of DNA and shows also the sensitivity of the assay. The C_T value is consequently in inverse proportion to the expression level of the gene. If the C_T value is low, it means the fluorescence crosses the threshold early, meaning that the amount of target in the sample is high. The C_T is therefore dependent on the threshold level, and it is then important to compare the threshold value from one run to another, if no normalization method is used.

After having the results, an excel file was created (1 per pathogen) to collect all the different plate results (5 plates per pathogen). The standard curves graphics and values (slope and R^2) and the medium C_T values of all the standard curves were collected in the file as well as the sample amplification and quantification results.

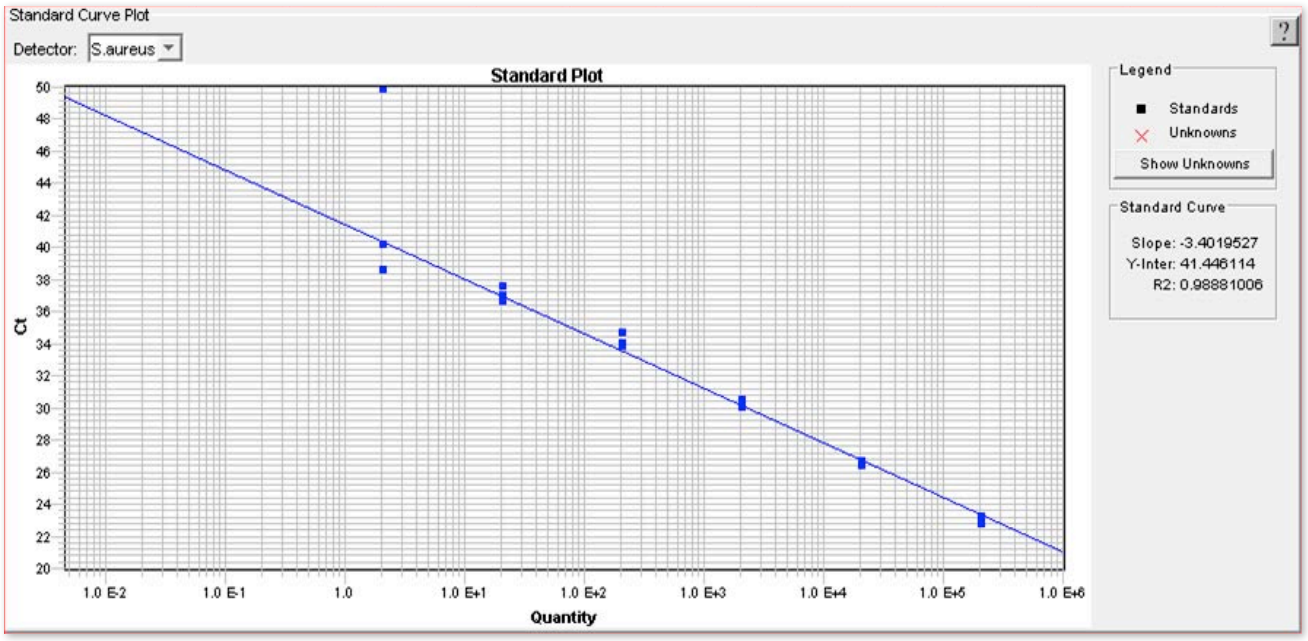


Figure 3. This example shows a standard curve of the pathogen *S. aureus* (on the top) with valid efficiency (slope) and R^2 values (-3.40 and 0.988).

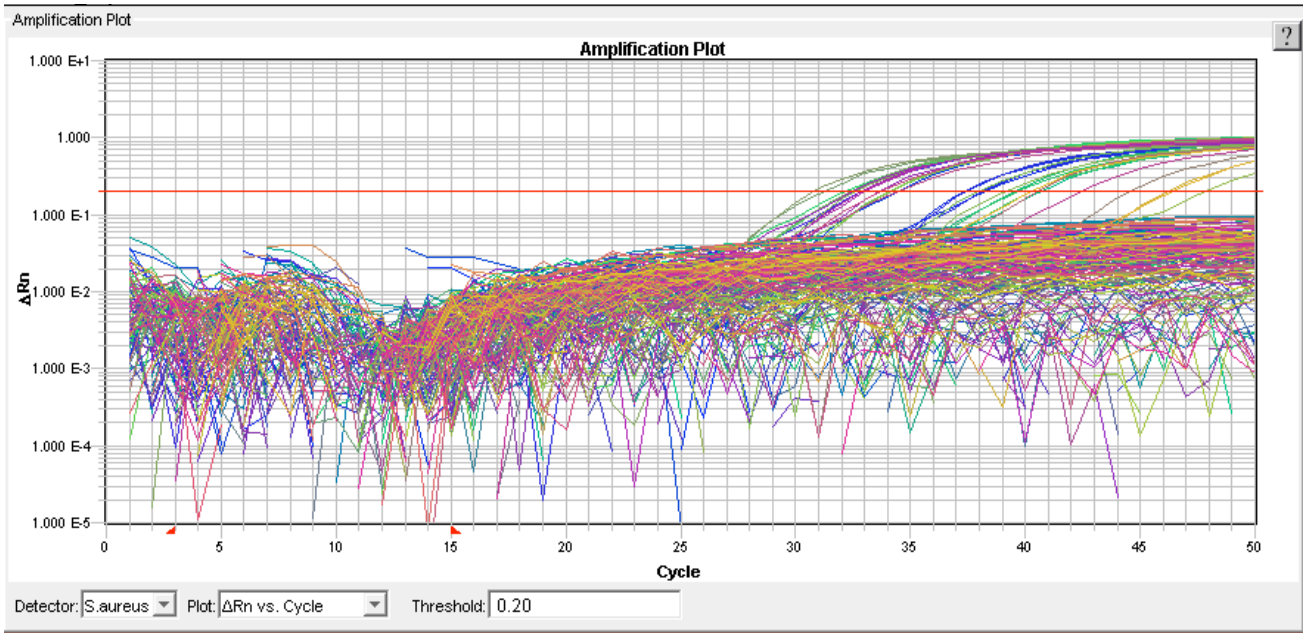


Figure 4a. This example shows the amplification curve of one *S. aureus* plate (the standard curve is in the previous page) without including the standard curves.

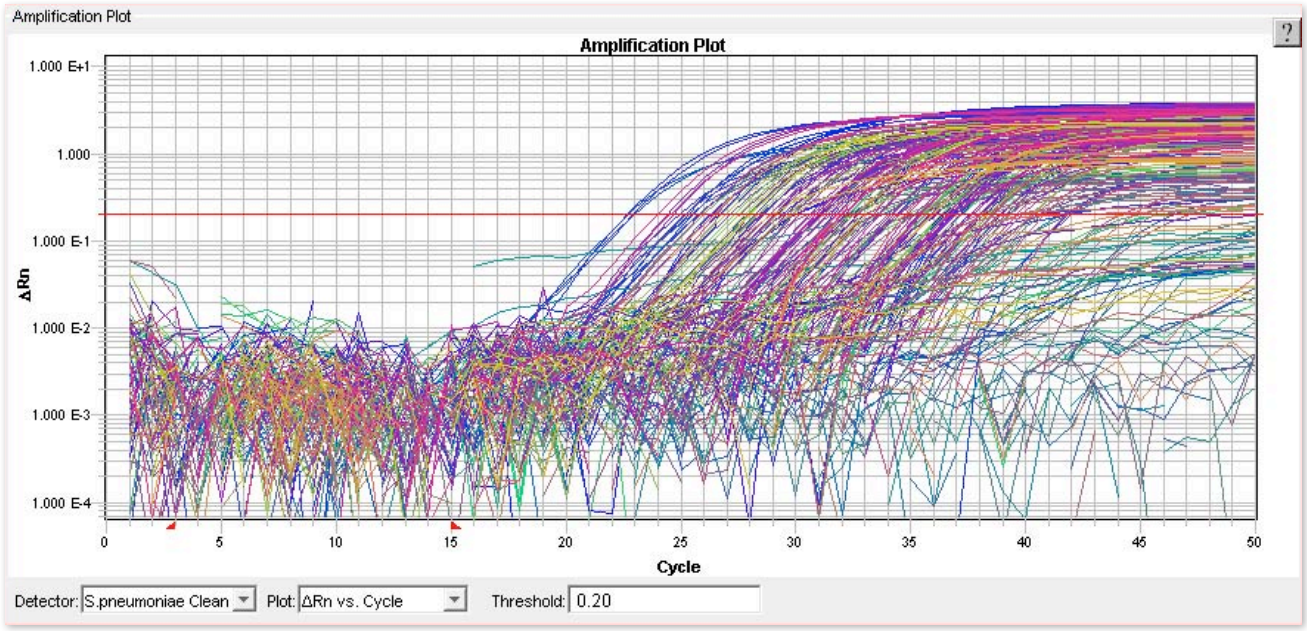


Figure 4b. This example shows the amplification curve of one *S. pneumoniae* plate without including the standard curves. Note the high amplification rates observed.

Troubleshooting during qPCR analysis

During the analysis process we found out some problems that were solved in order to improve efficiency of the analysis:

Different machines: initially, all the development tests were done in a ABI STEPONE qPCR 96 wells machine. As the sample number was so high, we tested the ABI 7900HT machine (applied biosystems) with the possibility to use 384 wells plates to gain time and reduce the costs (less volume of PCR components).

Different annealing times: we also tested different annealing times; firstly by mistake we tried 95°C during 10s (the company recommendations are for 15s), then we tried 15s and the results were better and then we tried 18s, giving us the better results (it seemed that with lower volumes, higher annealing temperatures worked better). Despite this, we've to take into account that if the annealing time is too high, the DNA polymerase efficiency could be reduced.

Streptococcus pneumoniae: that strain showed high amplification rates in all the plates done, so we decided to make some extractions of samples from healthy volunteers in different niches to see if the amplification was also high (low primer specificity). This test was done in the fifth plate of each pathogen (we tried it also in all the pathogens studied). The results showed a high amplification in all the *S. pneumonia* plates and samples. We think that maybe *Streptococcus mitis*, a common related non-pathogenic strain could be the responsible for that amplification. Meanwhile, the articles describing clinical validation of detections assays were published. Detection assay (primers and probes) were endorsed by CDC and published on their website. To solve the problem, we finally repeated *Streptococcus* analysis with those validated primers as published on CDC website.

2.2 Pyrosequencing:

Pyrosequencing analysis was performed in order to study microbiota composition of the different anatomical locations sampled. This methodology allows us to determine the predominant microbial groups present in the sample. We used the "High Throughput Sequencing" (HTS) protocol:

> Partial 16S rRNA gene sequences were amplified from the samples using the barcoded-primer approach to multiplex pyrosequencing. Primers were designed as previously proposed (6-8), primer 1061R:

*CTATGCGCCTTGCCAGCCCGCTCAGG*CC**R-RCACGAGCTGACGAC**; primer BSF517/E517F: *CGTATCGCCTCCCTCGCGCCATCAG*NNNNNNNNAG**GCCAGCAGCCGCGGTAA**, where the adapter sequences for Roche 454 FLX Titanium sequencing are *in italics*; NNNNNNNN designate the sample-specific eight-base barcodes used to tag each PCR product; underline indicates linkers, and **bold** corresponds to broadly conserved 16S ribosomal RNA gene regions.

PCR reaction were carried out in a total volume of 50 µl with 10µl of DNA extract, 1x Expand High Fidelity ^{PLUS} buffer 1 (Roche Applied Science, Basel, Switzerland), 50 µM of each dNTP (Roche Applied Science, Basel, Switzerland), 40 pmol of each primer (Microsynth, Balgach, Switzerland), 5 U Expand High Fidelity ^{PLUS} Enzyme mix (Roche Applied Science, Basel, Switzerland). The PCR parameters were 94°C for 2 min, 30 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, followed by 72°C for 7 min. After checking the amplification of the correct band, the PCR products were purified and quantified. After equimolar pooling, the amplicons were sequenced on Roche 454 GS-FLX-Titanium Sequencer.

Processing of the sequencing data

Raw sequence data were analyzed using a blend of Mothur v.1.33.0 (9) and QIIME v.1.8 (10) software packages. Pyrosequencing reads were denoised with the mothur implementation of PyroNoise (11) according to the 454 SOP described in (12). Chimeras were identified using usearch61 in QIIME (13). Then, the sequences were trimmed as described in the mothur 454 SOP in order to keep sequences overlapping the same 16S region. Subsequent analytical steps were performed in QIIME. OTUs de novo picking at 97% identity was performed using uclust (14). Taxonomy assignment of OTU representative sequences used the RDP Classifier with confidence threshold of 0.6 (15) on the Greengenes reference database v.13.8 (16). The same sequences were aligned using PyNast (17) on the Greengenes core reference alignment (18). The resulting multiple alignments was then filtered and used to build a phylogenetic tree with FastTree (19). After quality filtering (20), phylogenetic distances between all samples were computed as UniFrac distances (21). Diversity analyses based on the Unifrac distances were performed in QIIME. Permanova is a non-parametric multivariate analysis of variance and it was performed according to Anderson (2001) (22) using QIIME package.

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CAPÍTULO 3

A COMPARATIVE STUDY BETWEEN TWO SENSORY STIMULATION STRATEGIES AFTER TWO WEEKS TREATMENT ON OLDER PATIENTS WITH OROPHARYNGEAL DYSPHAGIA

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ABSTRACT

Introduction: Oropharyngeal dysphagia (OD) is a prevalent geriatric syndrome. Treatment is based on compensatory strategies to avoid complications. New treatments based on sensory stimulation to promote the recovery of the swallowing function have proved effective in acute studies but prolonged treatment has not been studied yet. Our aim was to study the effect of two sensory treatment strategies by comparing two-week treatments of each strategy on patients with OD. **Material & methods**: 28 older patients (≥70 yr) were studied with videofluoroscopy (pre/post-treatment) and randomized into two 10-day treatment groups: Group A) transient receptor potential vanilloid 1 (TRPV1) agonist (capsaicin $1{\cdot}10^{-5}$M); Group B) transcutaneous sensory electrical stimulation (TSES) (Intelect VitalStim, biphasic pulses, 300μs, 80Hz). Patients were analyzed for treatment response. **Results**: Patients were old (80.1±5 years), with many comorbidities (3±1.6 Charlson Index), and polymedication (9.25±3.59 drugs/patient), mild functional impairment (87.5±19.1 Barthel Index), and 35% were at risk of malnutrition according to the MNA-sf. Overall, patients had delayed swallow response with a laryngeal vestibule closure time of 447.1±141.1ms that improved to 372.86±142.8ms (p=0.04) after sensory stimulation. There were 64.2% responders in Group A with TRPV1 and 43% in Group B with TSES. Those responders from Group A showed an improvement in the penetration-aspiration scale (PAS, from 5.6±2.19 to 3.4±1.3; p=0.007); and the same was true for those of Group B (from 4.8±1.6 to 2.3±0.5; p=0.006). **Conclusions:** Two-week sensory treatment with capsaicin $1{\cdot}10^{-5}$M or TSES improved swallow response in older patients with OD, reducing the severity of OD in a significant subgroup.

INTRODUCTION

Oropharyngeal dysphagia (OD) is a common geriatric syndrome, with prevalence rates according to patients' phenotype: 23% for independently-living older people [1]; 47% for patients in acute geriatric units; 55% for hospitalized older patients with community-acquired pneumonia (CAP), and 56%-78% for institutionalized older residents [1-3].

Patients with OD may present impaired safety (penetrations and/or aspirations) and/or impaired efficacy (oropharyngeal residue) of swallow, which can lead to several serious complications like malnutrition, dehydration, lower respiratory tract infections, aspiration pneumonia (AP), readmissions and increase in morbi-mortality [1,3-6]. Impaired safety of swallow is characterized by delayed laryngeal vestibule closure (LVC) and impaired efficacy by impaired tongue propulsion and delayed maximal vertical hyoid motion [7].

OD in the older population is related to motor and sensory impairments. Motor impairments are mainly caused by the loss of muscular force and bolus propulsion force with aging, mainly associated with malnutrition and sarcopenia [8-10]. Sensory impairments of the pharyngeal and supraglottic areas have been related to a lack of myelinated nerve fibers of the superior laryngeal nerve [11-14]. These deficits have been related to OD and impaired safety of swallow [15,16]. In healthy persons, the swallowing center receives

high afferent input suggesting the involvement of sensory feedback during swallowing, and that input is of key importance to trigger and modulate the oropharyngeal swallow response. However, in older persons, sensory input of the pharynx and larynx is impaired and this alteration is very prevalent and associated with the presence of aspirations [11,12].

Nowadays, most older patients with OD are managed with compensatory strategies like fluid and diet adaptation with changes in bolus volume and viscosity, and swallowing maneuvers and head postures [17-19]. These strategies, although effective in compensating swallowing impairments, do not improve the oropharyngeal swallow response [7,20]. A systematic review on the effects of OD therapy concluded that "although some significant positive outcome studies have been published, there is a need for further research using randomized controlled trials" [21].

Innovative treatments are being tested to improve oropharyngeal swallow response

and avoid OD complications. One of these treatments is based on increasing the oropharyngeal sensory input through the afferent pathways by using chemical, physical or electrical stimuli [22]. The rise of sensory stimuli may increase sensory input to the swallowing center of the brain stem, leading to earlier initiation of deglutition and timely protection of the respiratory airway. Moreover, periodical sensory stimulation may reorganize the motor cortex and induce cortical neuroplasticity facilitating deglutition [22]. Transcutaneous sensory electrical stimulation (TSES) or chemical stimulation (such as transient receptor potential cation channel subfamily V member 1 (TRPV1) agonists) are two sensory stimulation strategies that can be used to that purpose. Two studies on older patients with OD showed that the supplementing the bolus with TRPV1 and transient receptor potential cation channel subfamily A member 1 (TRPA1) natural agonists significantly improved impaired safety of swallow [23,24]. However, these were acute studies and the effect of chronic treatment on older patients with OD has not been explored. Another treatment, transcutaneous sensory electrical stimulation (TSES), has led to significant improvement in the oropharyngeal swallow response (OSR) and prevalence of aspirations in several studies with post-stroke patients [25,26] but the effect on older patients is unknown.

The aim of this study was to evaluate with videofluoroscopy (VFS) and compare two different two-week interventions based on sensory stimulation of the afferent deglutition pathways (TRPV1 agonist and TSES) on older patients with OD.

PATIENTS & METHODS

Study population

Figure 1 shows the study flow chart. We studied 28 older patients (70 years or older) with OD that were prospectively recruited from the Gastrointestinal Physiology Unit of the Hospital de Mataró (Spain) between November 2012 and November 2014 (Figure 1). Inclusion criteria were to be 70 years old or older and have confirmed diagnosis of OD with VFS signs of impaired safety of swallow (Penetration Aspiration Scale >2) [27]. Exclusion criteria were active neoplasm or infectious process, epilepsy or convulsive disorders, gastroesophageal reflux disease, implanted electrodes or pacemakers, severe dementia to be currently participating in another clinical trial. All participants were informed about the study and signed the informed consent form. Study protocol was approved by the Institutional Review Board of the Hospital de Mataró (CEIC 04/12) and was conducted according to the principles and rules laid down in the Declaration of Helsinki and its subsequent amendments. ClinicalTrials.gov registration code: NCT01762228.

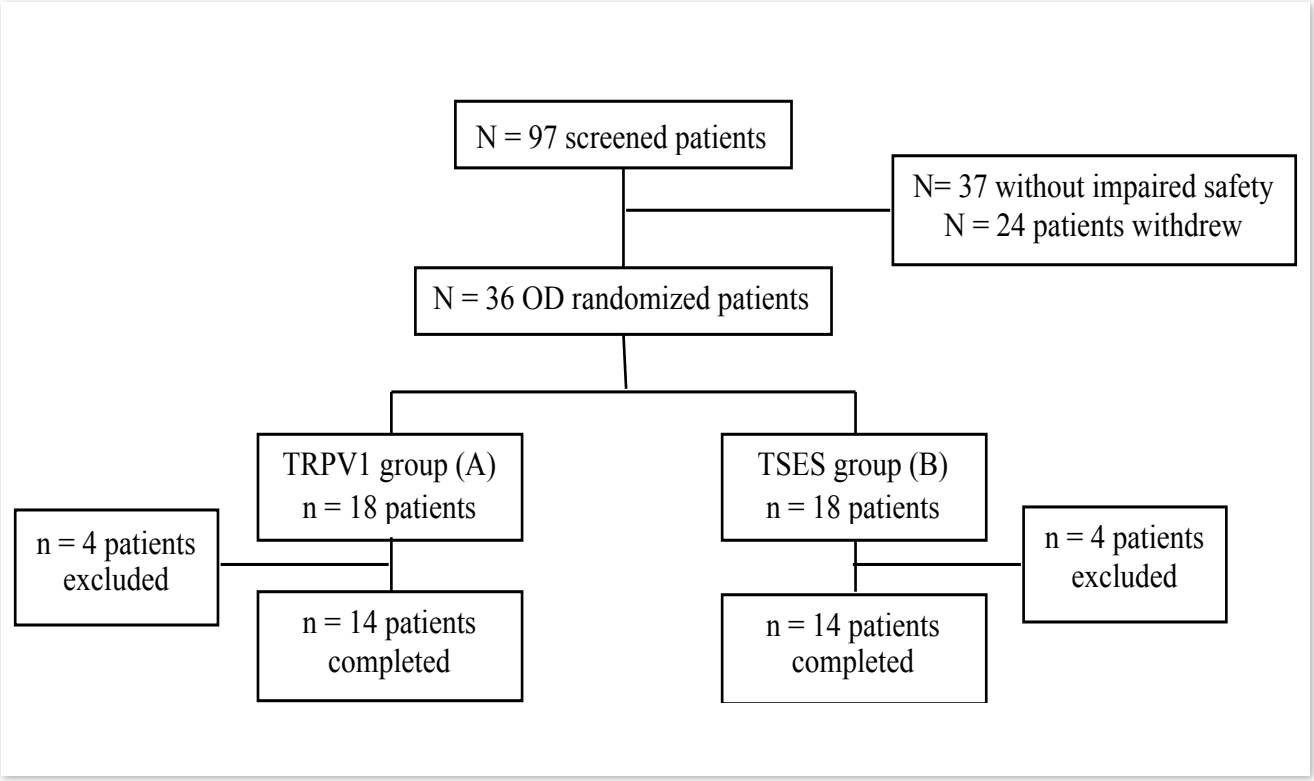

Figure 1. Study flow chart. TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation

Experimental Design

A prospective randomized clinical trial was designed to evaluate and compare the effect of 2 two-week treatments based on sensory stimulation in older patients with OD according to our previous studies [24,26] (Figure 2). During the pre-treatment visit, we collected the following data: sociodemographic characteristics of the population, functional capacity according to the Barthel Index [28], frailty status according to the L. Fried criteria [29], comorbidities with the Charlson Comorbidity Index [30] and nutritional status according to the short form of the Mini Nutritional Assessment (MNA-sf) [31]. Clinical symptoms of OD were measured by using the Spanish version of the Eating Assessment Tool (EAT-10) [32-33]. In addition, the Volume-Viscosity Swallowing Test (V-VST) was used as a clinical tool to diagnose clinical signs of OD [20]. If the clinical evaluation revealed a sign of impaired safety of swallow, then a VFS was performed. Patients with a score higher than 2 on the penetration–aspiration scale (PAS) [27] were included in the study and randomized using a software program (GraphPad QuickCalcs 2012) into one of the two treatment arms:

a) TRPV1 agonist: 10 treatment days of chemical sensory stimulation with a natural TRPV1 agonist solution (natural capsaicinoids 1x10-5M). Capsaicin solution was obtained from an alimentary capsaicinoid sauce (McIlhenny Co, Avery Island, Louisiana, USA) containing 185.5µg/g of capsaicinoid. This concentration was previously determined using liquid chromatography (AOAC 995.03 method [24]. Final concentration (1x10-5M) was obtained by dissolution of the capsaicinoid sauce in 10mL tomato juice to obtain a nectar-like solution. This product had a moderate-strong spicy taste and patients were advised of this characteristic. Treatment (10mL nectar-like solution) was taken by the patient three times per day before each meal and five days per week (Monday to Friday) for two weeks. Study product was prepared once a week, collected by patients every Monday and preserved refrigerated (4ºC).

b) TSES: 10 treatment days of electrical stimulation applying an intensity of 75% of the motor threshold using the thyrohyoid position [26]. Treatment consisted of the application, at rest, of 80 Hz of transcutaneous electrical stimulus (biphasic, 700 µs) using the Intelect VitalStim device (Chattanooga Group, Hixson, TN, USA). Patients with e-stim came to the hospital to perform the therapy 1 hour per day, 5 days a week (Monday to Friday) for two weeks [26].

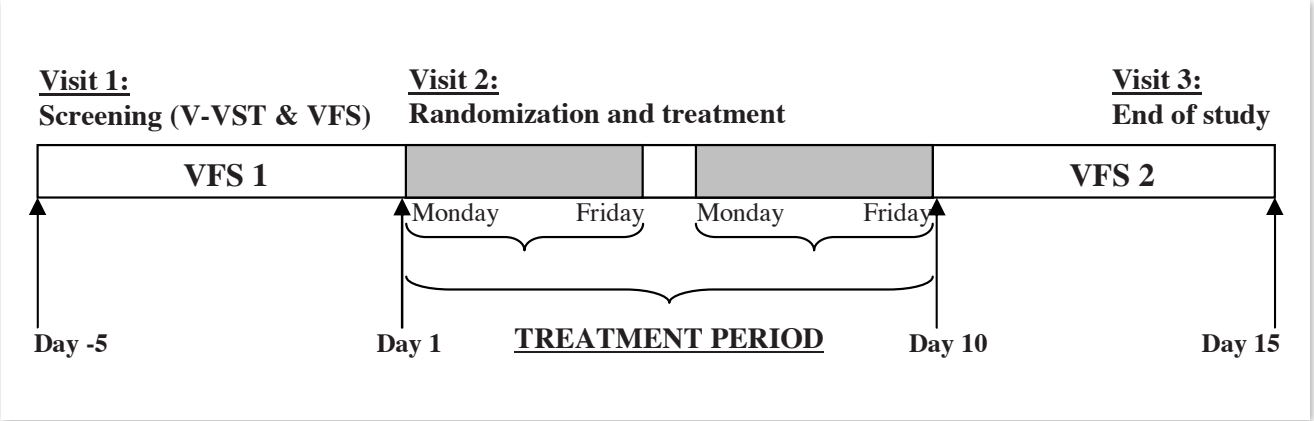

Figure 2. Study design. Treatment with TRPV1 agonist was taken three times per day for 10 days. Treatment with TSES was applied 1 hour per day for 10 days.

V-VST: Volume-Viscosity Swallowing Test; VFS: Videofluoroscopy.

Efficacy measurements of the treatment

Clinical symptoms of OD

A swallowing symptom questionnaire (EAT-10) was administered to the patients before and after the two-week treatment. The EAT-10 is a self-administered symptom-specific outcome survey consisting of 10 questions measuring the severity of clinical dysphagia with answers graded 0-4 (0: no problem, 4: severe problem) on the symptoms and clinical and social impact of OD [32,33].

Clinical signs of OD

The V-VST was used to clinically assess signs of impaired efficacy and safety of swallow. It is a validated and accurate clinical assessment tool that uses three volumes and three viscosities with pulsioxymetry to assess clinical signs of OD. The characteristics of this tool have been described elsewhere [7,20]. It was administered before and after the two-week treatment.

Videofluoroscopic study

VFS was performed before and 5 days after the treatment to measure its efficacy. Patients were studied seated, in a lateral projection which included the oral cavity, pharynx, larynx, and cervical esophagus. Videofluoroscopic recordings were obtained with a Super XT-20 Toshiba Intensifier (Toshiba Medical Systems Europe, Zoetermeer, The Netherlands) and recorded at 25 frames/s using a Panasonic AG DVX-100B video camera (Matsushita Electric Industrial Co, Osaka, Japan). Patients were studied during the deglutition of series of 5, 10 and 20mL nectar (274.42±13.14 mPa.s), liquid (20.40±0.23 mPa.s) and pudding boluses (3931.23±166.15 mPa.s). Nectar and pudding viscosity were obtained by adding 3.5g and 8.5g respectively of thickener Resource ThickenUp (Nestlé Nutrition, Barcelona, Spain) to 100 mL of liquid composed by 1:1 mineral water and the X-ray contrast Gastrografin (Bayer Hispania SL, Sant Joan Despí, Spain). Boluses were carefully offered to patients with a syringe. Videofluoroscopic signs of safety and efficacy of deglutition were identified accordingly to previously accepted definitions. Safety signs were laryngeal vestibule penetrations and tracheobronchial aspirations, classified according

to the PAS, assessed in each deglutition. Unsafe swallow was considered when the PAS score was higher than 2 [24,27].

Safety of the treatment

During the study any adverse events (AE) or serious adverse events (SAE) were recorded and notified to the Institutional Review Board of the Hospital de Mataró.

Data analysis

Prevalence of clinical signs and symptoms of OD

Prevalence of clinical or VFS signs of impaired efficacy and safety of swallow was measured as the ratio between the number of patients presenting signs divided by the total number of patients per group. Prevalence of patients presenting a VFS sign of impaired efficacy or safety at each specific bolus volume and viscosity was divided by the number of patients that performed that specific swallow (Figure 3).

Oropharyngeal swallow response

OSR was measured with the 5 mL nectar bolus because all patients performed this swallow. OSR measurements have been described previously by our group [7]. To describe the biomechanics of OSR we used the time to LVC, to upper esophageal sphincter opening (UESO), final kinetic energy (KE) of the bolus, bolus propulsion force and mean bolus velocity.

Response to the treatment

After the two-week treatment, patients were divided in responders (R) and non-responders (NR) in order to know which factors make patients respond better to each treatment. Responders were defined as those patients who after treatment, achieved safe swallow at a lower level of viscosity or, at the same viscosity level, improved at least one point in the Penetration-Aspiration Scale (PAS) [57].

Statistical analysis

Qualitative data are presented as relative and absolute frequencies and analyzed by the Fisher's exact test or the Chi-square test. Continuous data are presented as mean ± standard deviation (SD) and compared with the parametric T-test or Paired T-test; for non-parametric data we used the Mann-Whitney U-test or the Wilcoxon paired test. To assess normality we used the D'Agostino and Pearson omnibus normality test. Statistical significance was accepted if P values were less than 0.05. Hyoid data was compared by two-way ANOVA of repeated measures analysis. Statistical analysis was performed using GRAPHPAD PRISM 6 (San Diego, CA, USA).

RESULTS

Patient demographics

Both groups of patients with OD showed similar demographic, phenotypic and clinical characteristics: they were patients around 80 years old, presented high numbers of comorbidities, mildly impaired functional status, polymedication and the majority of them belonged to the pre-frail phenotype according to the L. Fried Frailty Classification [29]. Nearly 2/3 (64%) were well-nourished according to the MNA-sf and the main cause of OD in the whole population was the aging process (Table 1).

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
Age	80.07±4.9	80.85±5.25	79.36±4.75	0.434
Sex (♀)	60.71% (17)	57.14% (8)	64.29% (9)	1
Charlson	3±1.6	3±1.75	3±1.52	1
0	7.1 (2)	11.1 (1)	14.3 (2)	0.936
1-2	25 (7)	33.3 (3)	21.43 (3)	
3-4	53.6 (15)	44.4 (4)	50 (7)	
≥5	14.3 (4)	11.1 (1)	14.3 (2)	
Barthel	87.5±19.07	89.64±12	85.36±24.53	0.963
Optimum (100) (%)	50 (14)	50 (7)	50 (7)	1
Sub-optimum (<100) (%)	50 (14)	50 (7)	50 (7)	1
Fried	1.39±1.33	1.28±1.14	1.5±1.16	0.626
Robust (%)	28.57 (8)	28.57 (4)	28.6 (4)	0.535
Pre-frail (%)	57.14 (16)	64.29 (9)	50 (7)	
Frail (%)	14.28 (4)	7.14 (1)	21.4 (3)	
MNA-sf	11.96±2.06	12±2.15	11.93±2.06	0.889
Well-nourished (%)	64 (16)	64.28 (9)	64.29 (9)	1
At risk (%)	28 (7)	28.57 (4)	28.57 (4)	
Malnourished (%)	8 (2)	7.14 (1)	7.14 (1)	
BMI (Kg/m^2)	26.97±3.46	28.06±3.82	25.88±2.78	0.095
Dysphagia cause				
Elderly (%)	53.57 (15)	57.14 (8)	50 (7)	0.785
Stroke (%)	21.43 (6)	21.43 (3)	14.29 (3)	
NDD (%)	21.43 (6)	21.43 (3)	28.57 (3)	
RX-CH therapy (%)	3.57 (1)	0	7.14 (1)	
Medication	9.25±3.59	10±4	8.5±3.08	0.276

Table 1. Health Status. Demographical, clinical and nutritional data of the population p-value corresponds to the comparison between GA and GB.

MNA-sf: mini nutritional assessment-short form; BMI: body mass index; NDD: neurodegenerative disease; RX-CH therapy: radio or chemotherapy; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

Baseline clinical assessment of OD

a) Clinical symptoms and signs of OD

Both groups of older patients presented similar results on the EAT-10 score, showing a population at high risk of OD. The V-VST further confirmed these results showing that both groups presented similar high prevalence of clinical signs of impaired efficacy and safety of swallow (Table 2).

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
Impaired Efficacy (OR+PR) (%)	92.86 (26)	100 (14)	85.71 (12)	0.481
Oral Residue (%)	89.3 (25)	92.9(13)	85.71 (12)	1
Pharyngeal Residue (%)	67.9 (19)	78.6 (11)	57.1 (8)	0.419
Impaired Safety (%)	100 (28)	100 (14)	100 (14)	1
Penetrations (%)	75 (21)	78.57 (11)	71.43 (10)	1
Aspirations (%)	28.57 (8)	28.57 (4)	28.57 (4)	1
Silent Aspirations (PAS = 8) (%)	21.43 (6)	21.43 (3)	21.43 (3)	1
Higher PAS score	5.14±1.76	5±1.88	5.29±1.68	0.675

Table 2. Baseline clinical questionnaires and clinical signs of OD (V-VST). *p*-value corresponds to the comparison between GA and GB.

EAT-10: eating assessment tool; V-VST: volume-viscosity swallowing test; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

b) VFS signs of OD

VFS results showed that both groups of patients presented high and similar prevalence of signs of impaired efficacy of swallow. In addition, both groups presented similar maximum PAS scores and a high prevalence of patients with silent aspirations (21.43%), (Table 3 and Figure 3).

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
Clinical Questionnaires				
EAT-10	12.89±8.9	11.57±8.64	14.21±9.27	0.442
V-VST				
Impaired Efficacy (%)	82.14 (23)	71.43 (10)	92.85 (13)	0.325
Impaired Safety (%)	71.43 (20)	64.29 (9)	78.57 (11)	0.677

Table 3. Baseline VFS signs of OD. Prevalence of patients with VFS signs of impaired efficacy and safety of swallow and mean higher PAS score. p-value corresponds to the comparison between GA & GB.

OR: oral residue; PR: pharyngeal residue; PAS: Penetration-aspiration scale; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

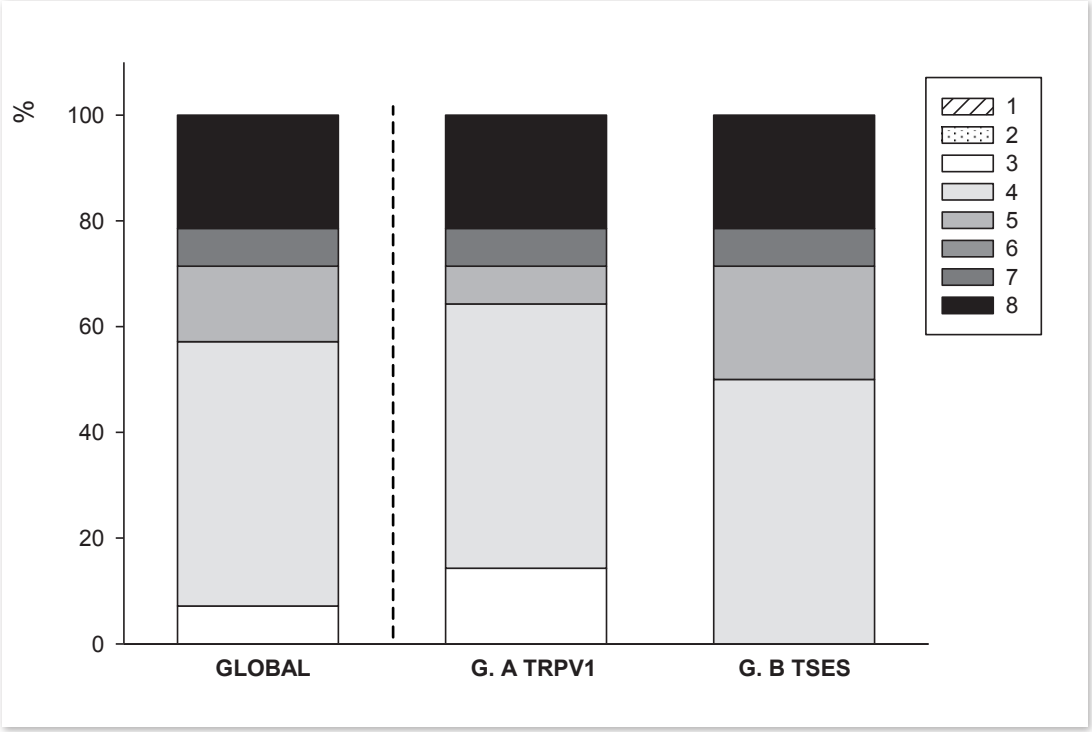

Figure 3. Baseline Penetration-Aspiration Scale Score. Distribution of patients at each level of the PAS during the first VFS. 1-2 safe swallow; 3-5 penetrations; 6-8 aspirations (8: silent aspirations).

TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

During baseline VFS, increasing bolus viscosity improved patients' safety of swallow. Pudding viscosity significantly achieved the highest safety among the three tested viscosities. On the other hand, efficacy was impaired by increasing bolus volume and viscosity as residue increased (Figure 4).

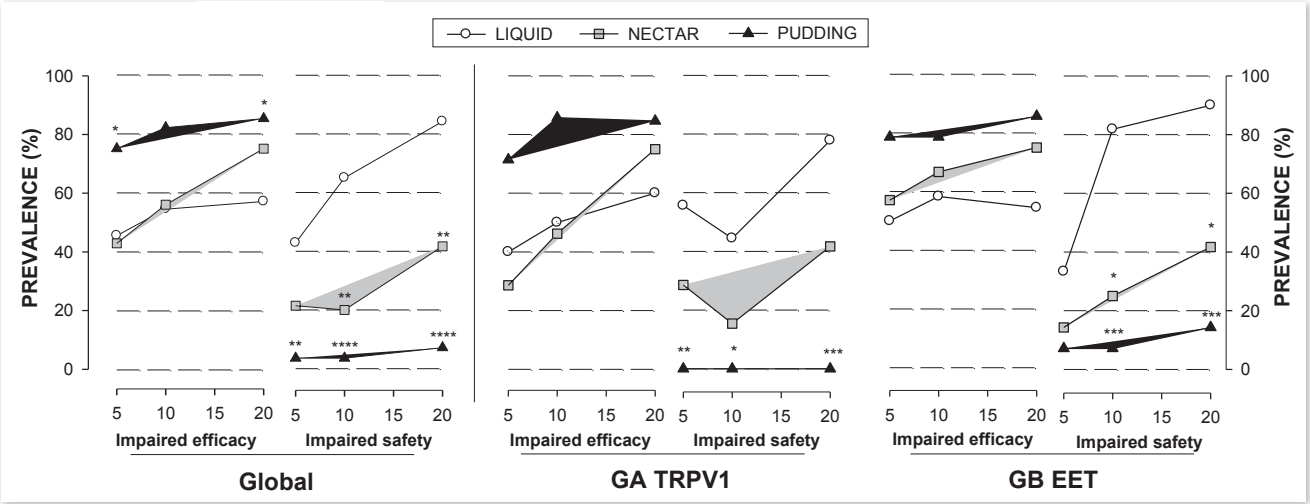

Figure 4. Baseline VFS signs of OD. Impaired efficacy was determined by the presence of oral and/or pharyngeal residue. Impaired safety of swallow was expressed as percentage of patients who presented penetration and/or aspirations during VFS. * p <0.05, ** p <0.01, ***p<0.001, **** p <0.0001 vs. thin liquid. TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

c) OSR

Table 4 shows the results of the physiology of the swallow response of the study population at baseline. Both groups presented similar delays in LVC and UESO, and similar impairments in bolus KE, force and bolus velocity.

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
OSR				
LVC (ms)	447.14±141.13	428.57±139.83	465.71±145.16	0.496
UESO (ms)	325.71±122.06	314.29±136.65	337.14±109.5	0.629
KE (mJ)	0.57±0.50	0.59±0.57	0.56±0.43	0.872
Force (mJ)	8.78±6.93	8.70±7.82	8.86±6.21	0.872
Mean Bolus velocity (m/s)	0.21±0.11	0.21±0.13	0.20±0.08	0.818

Table 4. Baseline OSR. *p*-value corresponds to the comparison between GA and GB.

OSR: oropharyngeal swallow response; LVC: laryngeal vestibule closure; UESO: upper esophageal sphincter opening; KE: kinetic energy; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

d) Hyoid movement

We have found that both groups of patients presented impaired baseline hyoid movement. However, patients from Group B (TSES) had shorter baseline maximal vertical extension (P=0.032) and maximal anterior extension (P=0.062) compared to patients from Group A (TRPV1 agonist) (Table 5).

BASELINE	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
Maximal vertical extension (mm)	23.83±7.57	26.82±5.58	20.3±8.31	**0.032**
Maximal anterior extension (mm)	39.76±6.15	41.9±6.13	37.24±5.37	0.062
Maximal vertical extension time (s)	0.49±0.23	0.53±0.27	0.45±0.17	0.45
Maximal anterior extension time (s)	0.52±0.25	0.5±0.27	0.54±0.23	0.677

Table 5. Baseline maximal vertical and anterior hyoid extension and time. P-value corresponds to the comparison between GA and GB.

TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

Post-treatment dysphagia status

a) Clinical symptoms and signs of OD

When we assessed the effect of the treatment on the clinical symptoms of OD we found a significant improvement in Group A (EAT-10: 11.57±8.64 vs 7.79±7.77, p=0.01) and Group B (EAT-10: 14.21±9.27 vs 10.29±10.74, p=0.021) indicating a self-perceived improvement by patients. Regarding V-VST clinical signs of OD, there were no differences between baseline and post-treatment assessments. After the treatment, there were no differences between groups on clinical symptoms and signs of OD measured by swallowing questionnaires and the V-VST. (Table 6).

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
Clinical Questionnaires				
EAT-10	9.04±9.26	7.79±7.7	10.29±10.74	0.565
N	27	13	14	
V-VST				
Impaired Efficacy (%)	74.07 (20)	76.92 (10)	71.43 (10)	1
Impaired Safety (%)	70.37 (19)	69.23 (9)	71.43 (10)	1

Table 6. Post-treatment clinical questionnaires and clinical signs of OD (V-VST).

EAT-10: eating assessment tool; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

b) VFS signs of OD

After the treatment, no differences were found between baseline and post-treatment evaluations although severity of OD measured with the PAS score improved from 5.14±1.76 to 4.32±2.09 (p=0.058) when both treatment groups were analyzed together.

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
Impaired Efficacy (OR+PR) (%)	96.43 (27)	92.86 (13)	100 (14)	1
Oral Residue (%)	86 (24)	86 (12)	86 (12)	1
Pharyngeal Residue (%)	68 (19)	86 (12)	50 (7)	0.103
Impaired Safety (%)	75 (21)	78.57 (11)	71.43 (10)	1
Penetrations (%)	83 (15)	64.28 (9)	42.85 (6)	0.272
Aspirations (%)	21 (6)	14.28 (2)	28.57 (4)	0.648
Silent Aspirations (PAS = 8) (%)	14 (4)	7.14 (1)	21.43 (3)	0.595
Higher PAS	4.32±2.09	4.21±1.8	4.43±2.41	0.792

Table 7. Post-treatment VFS signs of OD. Prevalence of patients with VFS signs of impaired efficacy and safety of swallow. P-value corresponds to the comparison between GA and GB.

OR: oral residue; PR: pharyngeal residue; PAS: Penetration-aspiration scale; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

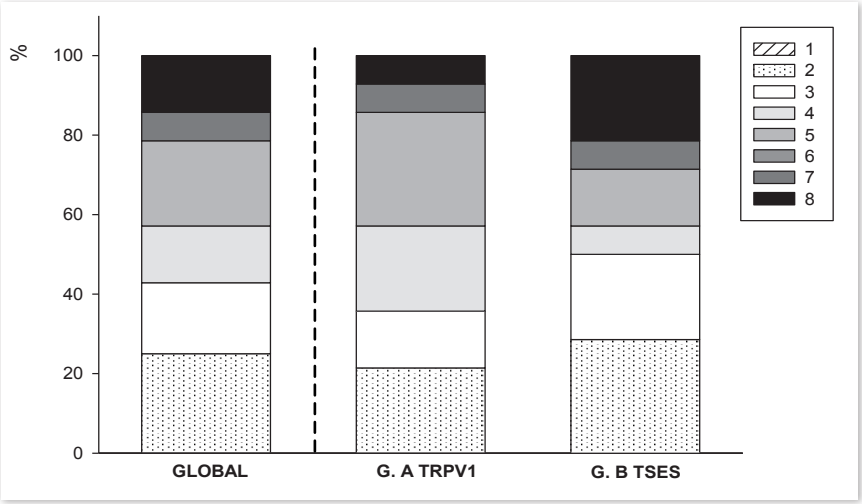

Figure 5. Post-treatment Penetration-Aspiration Scale Score. Distribution of patients at each level of PAS during the second VFS. 1-2 safe swallow; 3-5 penetrations; 6-8 aspirations (8: silent aspirations). TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

c) OSR

We found no differences between baseline and post-treatment between Group A and B. Taking all patients together (N=28), LVC significantly improved from 447.1±141.1 ms in baseline assessment to 372.86±142.8 ms post-treatment; (p=0.04). When we assessed post-treatment OSR, we found no significant differences between Group A and B in parameters such as UESO, KE, force and mean bolus velocity.

	GLOBAL	G. A TRPV1	G. B TSES	*p*-value
N	28	14	14	
OSR				
LVC (ms)	372.86±142.8	377.14±100.11	368.57±179.69	0.877
UESO (ms)	319.29±144.89	304.29±129.9	334.29±162	0.659
KE (mJ)	0.72±0.73	0.84±0.95	0.59±0.40	0.613
Force (mJ)	11.38±10.84	12.78±14.07	9.98±6.47	0.872
Mean Bolus velocity (m/s)	0.22±0.10	0.23±0.12	0.21±0.07	0.597

Table 8. Post-treatment oropharyngeal swallow response. *p*-value corresponds to the comparison between GA and GB.

OSR: oropharyngeal swallow response; LVC: laryngeal vestibule closure; UESO: upper esophageal sphincter opening; KE: kinetic energy; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

d) Hyoid movement

Post-treatment hyoid maximal vertical and anterior extension and time was very similar to baseline and not affected by the treatment (Table 9). In addition, hyoid motion curves showed no significant differences between baseline and post-treatment evaluations.

POST-TREATMENT	GLOBAL	G. A TRPV1	G. B EET	*p*-value
N	28	14	14	
Maximal vertical extension (mm)	21.99±9.47	22.80±10.71	18.97±9.72	0.154
Maximal anterior extension (mm)	39.93±5.79	38.92±12.51	37.59±5.06	0.067
Maximal vertical extension time (s)	0.48±0.22	0.57±0.26	0.41±0.17	0.163
Maximal anterior extension time (s)	0.61±0.26	0.69±0.28	0.51±0.21	0.087

Table 9. Post-treatment maximal vertical and anterior hyoid extension an time. *p*-value corresponds to the comparison between GA and GB.

TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation.

Therapeutic effect of the treatment

a) Response to the treatment

After the two-week sensory treatment and second VFS evaluation patients were divided into responders and non-responders according to our previous definition. We found rates of responders of 64.2% and 43% from the capsaicin (1·10-5M) group and the TSES group respectively (ns).

b) Responder phenotype

We analyzed several demographical and clinical parameters for differences between responders and non-responders from both groups (sex, age, comorbidities, functional status, frailty, nutritional status, OD etiology, medication and OD status). We did not find any particular characteristic differentiating the groups of patients.

c) Clinical symptoms and signs of OD

Clinical symptoms measured by the EAT-10 significantly improved after 10-day treatment in responder patients from Group A (13.44±8.55 vs. 9.22±9.23, p= 0.021) but not in non-responders from the same group (8.2±8.64 vs. 5.2±2.71, p Regarding clinical assessment (V-VST), there were no changes in the prevalence of clinical signs of impaired efficacy or safety in responder patients from both groups between baseline and post-treatment assessments.

d) VFS signs of OD

When we analyzed VFS signs of impaired efficacy of swallow, we found no differences in the prevalence of oropharyngeal residue between the first and second VFS when we compared responders and non-responders. However, when signs of impaired safety of swallow were analyzed, we observed trends of improvement in responder patients from Group B (TSES) (100% vs. 33%; p=0.06) and in the prevalence of aspirations in responder patients from Group A (TRPV1) (44.4% vs. 0%; p=0.08). For the most severe PAS score during VFS, we determined a reduction of 38% and 51.8% in Group A (TRPV1) and B (TSES) respectively (P<0.01) (Figure 6). When responders from both sensory stimulation groups were analyzed together, we also found a significant reduction of OD severity measured by PAS (Figure 6).

e) Oropharyngeal swallow response

When patients were grouped in responders and non-responders and OSR was analyzed, we did not find significant differences in UESO time, KE, propulsion force or mean bolus velocity. However, we obtained significant improvement in responder patients for the time to LVC with reductions of 15.1% in Group A (TRPV1) (p=0.07) and 45% in Group B (TSES) p<0.05) (Figure 4). When responders from both groups were analyzed together, we also found significant reduction in the time to LVC (Figure 6).

f) Hyoid movement

Hyoid movement was not affected by either treatment. We compared the maximal vertical and anterior extension of the hyoid bone as well as the time when patients achieved that maximal points and we did not find changes between baseline and post-treatment in responder patients. We only found a trend of improvement of the vertical hyoid movement between VFS1 and VFS2 (Table 10) when responder patients from both groups were analyzed together. Regarding vertical and anterior hyoid movement curves, our results did not show any difference between baseline and post-treatment VFS in both groups of responder patients (Figure 7).

Safety of the treatment

During the study we had 5 serious adverse events (SAEs) (2 in the capsaicin group and 3 in TSES group) for hospitalization for various reasons, in-

cluding: respiratory infection, pneumonia and stroke. However, all patients recovered and none of the SAE's were related to the study treatments. We concluded that our treatments were safe for our older patients with OD.

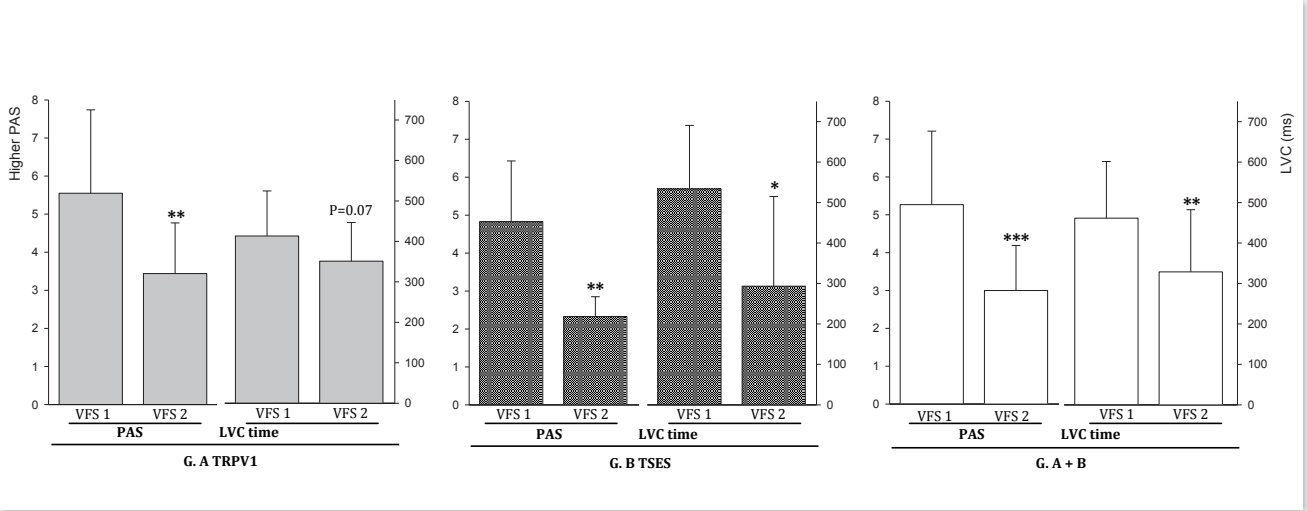

Figure 6. Effect of the treatment in responder patients. PAS: penetration aspiration scale; LVC: laryngeal vestibule closure; VFS: videofluoroscopy; TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation. *p<0.05, ** p<0.01, *** p<0.001 (VFS1 vs. VFS2).

Group A Resp. (N=9)	VFS1	VFS2	*p*-value
Maximal vertical extension (mm)	25.58±6.62	22.75±9.38	0.349
Maximal anterior extension (mm)	41.09±5.38	40.83±5.07	0.866
Maximal vertical extension time (s)	0.53±0.3	0.50±0.26	0.742
Maximal anterior extension time (s)	0.48±0.3	0.59±0.29	0.415
Group B Resp. (N=6)	VFS1	VFS2	P-value
Maximal vertical extension (mm)	20.64±6.67	18.20±7.31	0.437
Maximal anterior extension (mm)	35.93±5.91	35.62±4.37	0.812
Maximal vertical extension time (s)	0.57±0.12	0.47±0.14	0.104
Maximal anterior extension time (s)	0.73±0.12	0.62±0.21	0.187
Group AB Resp. (N=15)	VFS1	VFS2	P-value
Maximal vertical extension (mm)	24.11±7.02	21±8.63	0.096
Maximal anterior extension (mm)	39.1±5.94	38.82±5.32	0.772
Maximal vertical extension time (s)	0.55±0.26	0.49±0.21	0.207
Maximal anterior extension time (s)	0.58±0.27	0.6±0.25	0.776

Table 10. Post-treatment maximal vertical and anterior hyoid extension and time in responder patients from Groups A, B and both groups together. *p*-value corresponds to the comparison between VFS1 and VFS2.
VFS: videofluoroscopy

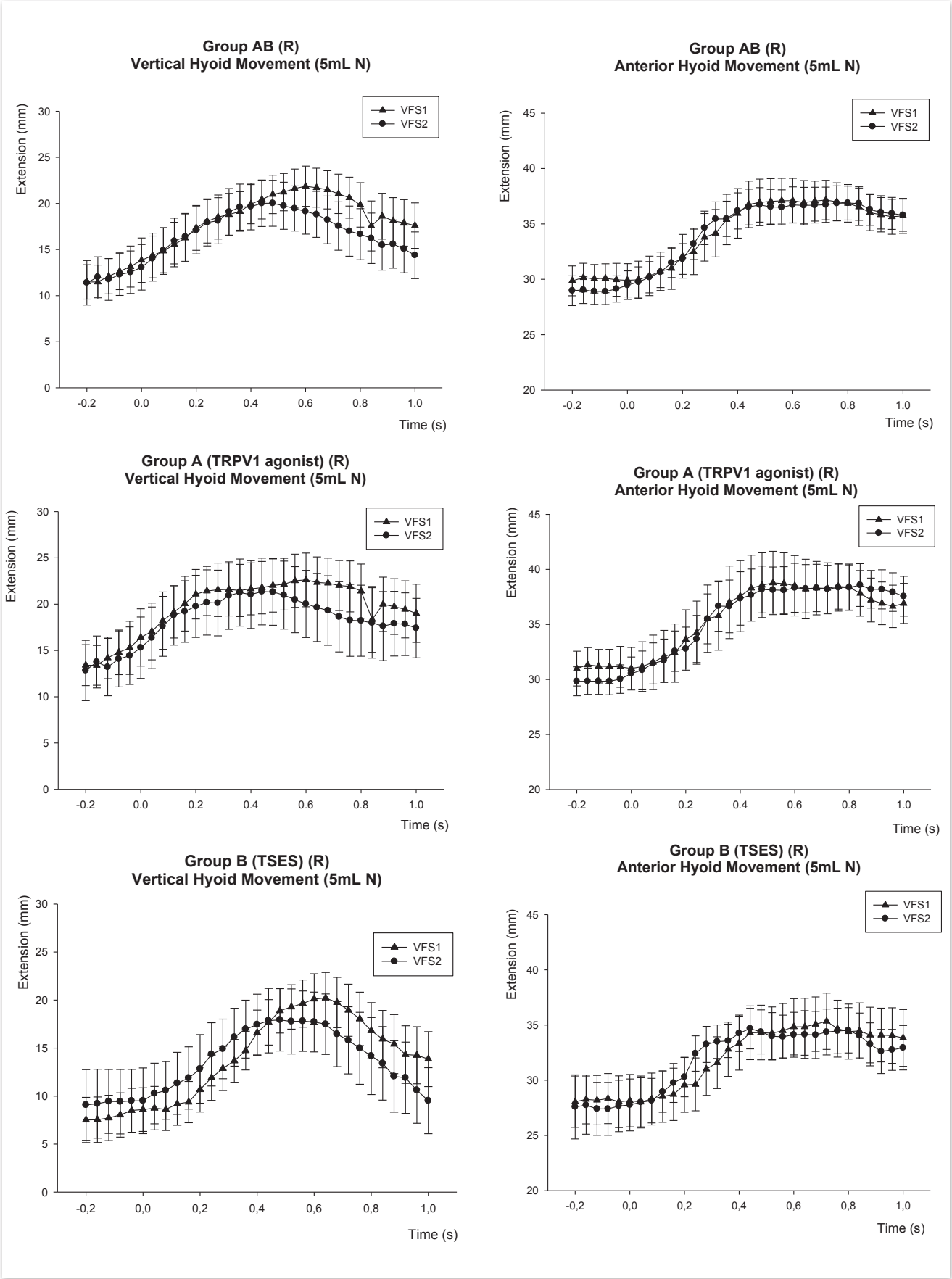

Figure 7. Vertical and anterior hyoid movement. TRPV1: transient receptor potential vanilloid 1; TSES: transcutaneous sensory electrical stimulation; VFS: videofluoroscopy; R: responders.

DISCUSSION

In this proof of concept study we found that ten days treatment with sensory stimulation strategies improved swallowing physiology and time to LVC of older patients with OD. Capsaicin $1{\cdot}10^{-5}$M and TSES were effective in 64.2% and 43% of patients respectively, significantly improving impaired safety of swallow according to PAS. In addition, both treatments were found to be safe according to our results as no AE/SAE presented by patients was related to the study therapies. Our results suggest that chronic sensory stimulation might promote the recovery of swallow function in a significant subgroup of older patients with OD, even though we were not able to identify any phenotypic characteristic linked to the response to treatment. As far as we know this is the first study to compare the effect of two chronic sensory treatments to improve swallowing physiology in older patients with OD. Conventional treatment, based on compensation, is not able to improve patient's swallow physiology and so patients are condemned to use thickeners, postures and/or manoeuvers [21]. This compensatory effect was significantly confirmed in our study, as it has in previous studies [19,20,34,35], as our population, who presented impaired safety when swallowing thin liquid, avoided 100% and 85.71% of unsafe swallows in groups TRPV1 and TSES respectively when using pudding viscosity.

Our study population was old, had a high number of comorbidities, mildly impaired functionality, took a large number of drugs and most had an appropriate nutritional status. In addition, nearly 2/3 of them were pre-frail according to L. Fried criteria. Swallowing function was strongly impaired in both groups of patients as all of them had impaired safety of swallow, with 21.43% presenting silent aspirations and 92.86%, impaired efficacy of swallow with oropharyngeal residue. OSR was also severely impaired in both groups of patients with a delayed LVC and weak bolus propulsion forces leading to a slow mean bolus velocity and delayed hyoid elevation and movement. This is a very similar result to a previous study with frail older patient with OD that we performed, indicating a population at high risk of nutritional and respiratory complications [7]. Baseline maximal vertical and anterior hyoid extension was low in our population as in that one [7], indicating low elevation of the larynx and therefore reduced airway protection. In addition, maximal vertical extension time was delayed. The delay in hyoid elevation and movement is a strong predictor of aspiration as it contributes to delayed LVC [35]. Hence, these patients are at high risk of suffering from respiratory infections and aspiration pneumonia.

After the treatment and second VFS, patients were divided according to their response to therapy based on physiological (PAS improvement) and/or clinical improvements (viscosity improvement). One of the main reasons to split both groups according to response to the treatment was to discover which clinical characteristics made a difference to whether a patient responded or not. This is very important in order to be able to select the best therapy for each phenotype of older patient with OD.

Our treatments aimed to stimulate the main afferent/sensory deglutition pathways (maxillary branch of the trigeminal nerve, the glossopharyngeal nerve and the superior laryngeal nerve) that project to the supra-medullar structures and to the *nucleus tractus solitarius* in the brainstem [36] to promote cortical plasticity and improve OSR. Impaired safety improvement after the treatment is mediated by an earlier LVC time. It has been reported that 10 days treatment with motor transcutaneous electrical stimulation on post stroke patients with OD improved the efficacy and safety of swallow and OSR while transcutaneous electrical stimulation at sensory level (using the same parameters as our study), improved only the safety of swallow and OSR [26]. In that earlier study, patients responded better to sensory stimulation than in our present study. This might be due to the brains of post-stroke

patients having greater neuroplasticity and being able to reorganize themselves to move functions to undamaged areas, and thus respond better to the different treatments than the brains of older patients.

An earlier study also evaluated the effect of chronic treatment with TRPV1 agonists (10^{-6}M) on older patients and reported a significant improvement in the latency of swallow clinically evaluated, after one month supplementation with oral capsaicin before every meal [37]. However, the improvements were not verified with instrumental assessment such as videofluorscopy or fiberoptic endoscopy.

Over the whole sample, even when patients were divided according to response to treatment, no improvement in efficacy of swallowing was found in any group of patients. That finding is logical as impaired efficacy is mainly related to low bolus propulsion force and impaired pharyngeal clearance caused by muscular weakness of the tongue and pharyngeal muscles, due mainly to age-related sarcopenia [6,9]. However, our therapeutic approach was not directed to improving muscle function like motor rehabilitation, but primarily focuses on promoting cortical plasticity to improve swallow safety.

Unfortunately, in our study we have not found conclusive differences between phenotypes of responder and non-responder patients. This might be due to the fact that the response to the treatment is related to different parameters than the ones that we analyzed (such as differences in cortical capacity to respond to a stimulus) or to the small sample size. Further studies including a larger sample size and analyzing neurophysiological parameters are needed in order to determine and define the responder phenotype for each of the tested treatments.

The effect of the treatment was shown to last for at least five days after the last treatment session, as the second VFS was performed in that period of time. However, further studies are also needed to measure the duration of the treatment over longer periods of time and to adjust treatment session frequency if needed.

The main limitation of the study, although it was a proof of concept, was the small sample size and the absence of a control group to discard placebo effect. Large controlled randomized clinical trials should be performed to assess the effect of these two therapeutic approaches on older patients with OD and to assess the duration of the effect of the treatment. Furthermore, it would be interesting to use neurophysiologic techniques in order to know the mechanism of action of these treatments on our target population and the potential role of cortical plasticity.

In conclusion, both sensory stimulation therapies have proved to induce objective improvements in swallow response of older patients with OD. In addition they are easy to learn and to use, with no safety concerns. These novel therapeutic strategies open a new treatment paradigm for geriatric patients affected with OD, as they have the potential to improve swallowing physiology rather than compensating it.

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