Translational Oral Health Research

Jukka H. Meurman
Editor

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Editor
Jukka H. Meurman
Department of Oral and
Maxillofacial Diseases
University of Helsinki
Helsinki
Finland

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Preface

The European Society of Translational Medicine defines translational research as "an interdisciplinary branch of the biomedical field supported by three main pillars: bench-side, bedside and community" [1]. In Wikipedia it is defined as follows: "As a relatively new research discipline, translational research incorporates aspects of both basic science and clinical research, requiring skills and resources that are not readily available in a basic laboratory or clinical setting." In other words, translational research brings innovation and discoveries from laboratory to the clinic.

Traditionally, dental and oral health-related research has been discipline-driven where characteristically a holistic view often has been lacking. More recently, however, the old "silothinking" has been abandoned and an interdisciplinary research has become the norm in many areas. Truly translational research, however, has been rare in dental research.

This book is the first to be devoted to translational research within the field of oral health. The aim is to expedite the transfer of knowledge gained in the laboratory to clinical practice. It is examined how basic sciences and basic research are providing new methods and materials that will enable clinicians to treat patients more effectively. Readers will gain a translational perspective on a variety of oral conditions and related systemic diseases. The importance of evidence-based research and the roles and comparative value of preclinical and clinical trials are highlighted. Knowledge of translational and clinical research is essential in understanding how new inventions and discoveries are being accomplished, and what regulations and guidelines need to be taken into account when planning studies, and not forgetting the ethical aspects of any research. *Translational Oral Health Research* is the first book to be devoted entirely to the subject, and it will be of interest to both researchers and clinical practitioners.

Finally, I herewith want to acknowledge all the authors who have contributed to this book. We were most happy to get the leading experts in their fields to share with their knowledge.

Helsinki, Finland — Jukka H. Meurman

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Contents

Translational Research in the Oral Health Sciences

1

Jocelyne S. Feine

Abstract

From basic biomedical through implementation science, the goal of health research is ultimately to improve the health of populations. In this chapter, translational health research is defined and its rationale described. A variety of cross-disciplinary, or team science, approaches to study complex health issues are also described; these include multidisciplinary, interdisciplinary, and transdisciplinary collaborations. While there is a broad and general acceptance of team science, academic institutions and funding agencies are still in the process of developing appropriate benchmarks that will enable the assessment of the contributions of individual researchers involved in team science, as well as promoting initiatives to encourage and sustain these types of research teams.

J. S. Feine, D.D.S., M.S., H.D.R., F.C.A.H.S
Oral Health and Society Division, Faculty of Dentistry, McGill University, Montreal, QC, Canada

Department of Epidemiology and Biostatistics, Faculty of Medicine, McGill University, Montreal, QC, Canada

Department of Oncology, Faculty of Medicine, McGill University, Montreal, QC, Canada
e-mail: jocelyne.feine@mcgill.ca

The primary aim of government funding for biomedical research is to improve the health of the population. While health research over the past decades has significantly increased life spans and improved population health, it is also recognized that scientific discoveries do not always translate to health [1]. Furthermore, those discoveries that do manage to reach clinical practice become obsolete relatively fast, since the length of time needed to move discoveries forward in the translation process is very long [2]. Thus, governments and funding agencies are encouraging scientists to take different approaches to research that will enhance and accelerate knowledge and product translation. The primary framework that has been proposed to accomplish this is through translational research [3, 4].

1.1 Definition of Translational Research in the Biomedical Sciences

The exact definition of biomedical translational research is still being debated [5]. However, to put it simply, *biomedical translational research* is the concept of "bench to bedside," a unidirectional view that has since been expanded to recognize the fact that translation can (and does) occur bidirectionally among the five steps of the continuum (Fig. 1.1) [6, 7]. In fact, the impetus for much of our basic research (apart from pure

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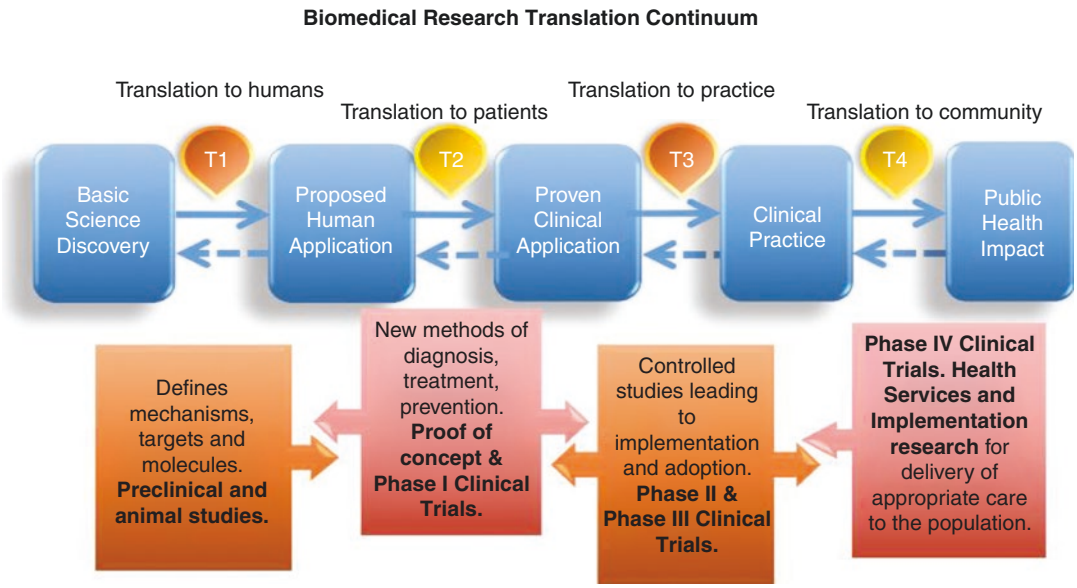

Adapted from Drolet & Lorenzi, 2011 and Fernandez-Moure, 2016

Fig. 1.1 This figure depicts the four translational phases in the biomedical research translation continuum (T1–T4) and provides examples of methodological approaches that are used within each phase

discovery research) is buttressed by clinical and epidemiologic evidence of the impact of various diseases on individuals and populations.

Most models of the biomedical translational research continuum illustrate a path for product development. The model shown in Fig. 1.1 depicts five steps (in blue) that range from "Basic Science Discovery" to "Public Health Impact." Translation occurs between each of these and can move in both directions. The boxes below the blue steps are descriptions of some of the types of research approaches that are used in each step.

Following this model, let's consider the example of a vaccine to prevent dental caries:

Basic science research uses a variety of laboratory methods to test various component(s) in the development of a vaccine product. This product is then tested in animals to determine whether it works in the intended manner, what concentration appears to be effective, and what, if any, side effects occur.

If it appears that the product is safe and effective in an animal model, then T1 (translation to humans) occurs. The product is tested in a few humans in Phase I clinical trials to determine safety, maximum tolerated dose (MTD), pharmacokinetics (what the body does to the product, e.g., absorption, distribution, metabolism, and excretion), and pharmacodynamics (what the product does to the body, e.g., biochemical and physiological effects).

Provided the Phase I testing is positive, Phase II clinical trials are undertaken to further determine safety and effectiveness. These involve very small groups of the vulnerable population(s); in the example of a caries vaccine, this population could be made up of those who are at a high risk for caries, i.e., young children or older people.

Following this, T2 can begin, involving translation to Phase III randomized clinical trials. In these studies, the vaccine is tested in a larger group of vulnerable people who are randomly assigned to either the experimental or to a control group (placebo or a standard treatment).

If the experimental treatment (vaccine) is shown to be superior to the control, then the T3 translation to clinical practice can be initiated. This is when knowledge translation becomes highly important, because changing clinical practice is an enormous undertaking. Thus, efforts in

training dental students and the development of technologies/methodologies that can assist dentists in successfully carrying out evidence-based practice are crucial to this translational process.

Once the new vaccine is accepted and used by dentists, then T4 (from clinical practice to the community) is initiated. Efforts aimed at implementation of programs to deliver the vaccine can be developed and tested; furthermore, the effects of the vaccine on the incidence and prevalence of caries in large populations can be determined, including economic analyses for the most cost-effective preventive programs.

1.2 What Is Needed to Carry Out Biomedical Translational Research?

The most viable way to facilitate research translation is through research collaborations with those in other disciplines who are interested in investigating the same topic [8]. "*Collaborative methods for scientific research are increasing in importance as scientists are tasked with researching the world's most complex problems. The complexity of these issues requires scientists to transcend their own disciplinary boundaries and create teams to assess the interconnected network of systems associated with the problem*" [9]. Known as "cross-disciplinary" or "team science," these collaborative approaches can be multidisciplinary, interdisciplinary, and transdisciplinary [10]. The terms are distinct from one another in the following ways:

Multidisciplinary—This is team science research in which a health condition is studied by experts within their own discrete disciplines, individually following separate protocols either independently or sequentially. Although these researchers meet together in order to redefine problems and develop more comprehensive assessments of the health condition, each remains fully entrenched in his/her own disciplinary approach [11].

Interdisciplinary—This is also team science research in which a health condition is examined by experts in discrete disciplines. However, these experts work together to develop and carry out protocols that incorporate the conceptual and methodological approaches inherent to each of their respective fields [11].

Transdisciplinary—Transdisciplinary research is posited to be the highest level of collaboration among scientists [9]. "*It is an integrative process in which researchers work jointly to develop and use a shared conceptual framework that synthesizes and extends discipline-specific theories, concepts, methods, or all three to create new models and language to address a common research problem*" [8].

For expert scientists to succeed in translating their research, it is important for them to understand the translational research process, in addition to having a general understanding of other research approaches along the translation continuum [12]. One can easily begin the process by asking a colleague from a different discipline how she/he thinks about a particular oral health condition. Conversations like these can inspire ideas for team research projects.

1.2.1 Graduate Student Training

A key ingredient to successful change in research practice is through graduate training. Potential graduate trainees should be screened to determine whether they have the necessary personality characteristics for translational research; this includes having the ability to communicate and collaborate with others.

Disis has described the qualities of graduate trainees who would be successful in transdisciplinary research. "Future translational researchers of all ages must be adaptable, life-long learners. They have to be highly curious about a lot of different things, collecting data and ideas from the basic literature and creatively applying these to disease solutions. This means being outside your comfort zone, reading literature that is way outside your field" [13].

What skills do we need to instill in our graduate students that will encourage and prepare them for translational research success? The first step is to give graduate trainees a solid understanding

of their own and other disciplines, along with the vocabulary used in the language of these disciplines. Since mastering one discipline is very time-intensive and is still the primary goal in our present models of graduate training, we need to develop teaching paradigms that will provide our graduate trainees with a comprehensive knowledge of other disciplines in an efficient manner. This will enable our new researchers to communicate effectively with researchers from many different disciplines in order to enable them to successfully lead or participate in team science groups.

For example, a team science course can be designed in which researchers from a variety of disciplines will describe the theoretical underpinnings, including how each of their disciplines develops hypotheses, the ethical issues specific to each, methodological approaches, analytic techniques, interpretation of findings, knowledge translation, etc.

Along with associated readings and group assignments, the student will be better equipped to expand his/her thinking about specific health issues and to better determine how to address those issues through appropriate research approaches.

Some groups have published competencies and ideas for translational biomedical graduate research training programs that provide knowledge and skills to graduate trainees [14–17]; furthermore, while there are still relatively few active training programs, some have already been assessed for success [16].

1.2.2 Publication of Translational Team Science Research

Health research journals need to be prepared to publish multiauthored translational research reports. Adequate space for these complex reports should also be made available. Editors should understand that a greater number of reviewers in the assessment of translational research reports may be needed. If a series of reports are written from one translational research project, the authors may wish to publish their work in a series. Journal editors should be open to considering a variety of publication options for these authors. Publication of translational research is a topic of interest for editors of journals in health research.

The relatively new IADR/AADR publication, the JDR Clinical and Translational Research (CTR), provides a platform in which oral health translational research reports can be published. The journal was designed specifically to allow for multiple authorships and longer reports that will serve the specific publication requirements for this type of research.

1.2.3 The Role of the Institution

Universities and funders that are interested in promoting translational research must consider and act on a variety of issues. The process of creating and sustaining translational research teams requires more than merely the desire of researchers [18]. Universities and funding agencies will need to provide reasonable, but wholehearted, financial and infrastructure support to make team sciences initiatives successful.

University promotion and tenure committees have traditionally judged the productivity of researchers through the number and quality of journal publications, order of authorship, and funding, in addition to administrative and teaching contributions. Team science research necessarily involves teamwork in which authorship order and contributions to protocol development are not clearly obvious. This means that the documentation required to support an individual team member's tenure/promotion must include descriptions of the team member's role (in relation to others in the team) for every aspect of his/her work. Universities must put effort into developing appropriate criteria and ensuring that their committee members understand how cross-disciplinary research teams function. Committee members should be trained in the best ways to judge the productivity of an investigator who carries out team research. The criteria presently used for an independent researcher who follows a traditional discipline-specific path cannot be used for team science investigators [19, 20].

Conclusion

Translational team science research has begun. While there remain many issues that must be resolved, it is imperative that we begin to think about complex oral health conditions beyond our individual disciplinary lenses.

An understanding of the biomedical translational research framework is fundamental for the effective investigation, development, and uptake of new preventive strategies and therapies to cure or mitigate disease symptoms. Translational research offers a platform for the use of team science research approaches to complex health problems in which a variety of relevant disciplinary lenses are focused on one particular condition.

> Science is a collaborative effort. The combined results of several people working together is often much more effective than could be that of an individual scientist working alone (John Bardeen [21]).

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2 How Cross-Disciplinary Research Has Increased Our Understanding of Oral Mucosal Diseases

S. J. Challacombe, H. McParland, G. Proctor, J. F. Setterfield, M. Escudier, J. Sanderson, B. Kirkham, G. Larkin, and P. J. Shirlaw

Abstract

Over the last 40 years, research in dental schools has moved from a position where clinicians were expected to make a scientific contribution to their fields on their own, to a fruitful multidisciplinary approach to answering basic questions about the disease process. At one time, clinicians hoping to make an academic contribution were expected to learn basic laboratory techniques in order to do so. Indeed, the foundation of current multidisciplinary research was that this approach was relatively successful. However, it became apparent that successful high-impact contributions required expertise that cannot any longer reside in individuals but in groups. Both the scientific questions and the means of addressing them have become increasingly complex and require a wide range of expertise. This applies not only to laboratory-based research but to clinically applied research. The former needs interactions with expert nonclinical scientists and the latter interactions with expert clinicians from different fields in order to maximise the investigation and management of complex clinical diseases. In oral medicine, there are now many examples of both types of multidisciplinary approach, which have resulted in a marked increase in our knowledge of disease mechanisms in mucosal disease which have in turn led to new approaches to treatment.

S. J. Challacombe (✉) · H. McParland
M. Escudier · P. J. Shirlaw
Department of Oral Medicine, King's College London Dental Institute, Guy's and St Thomas' NHS Foundation Trust at Guy's Hospital, London SE1 9RT, UK
e-mail: Stephen.challacombe@kcl.ac.uk

G. Proctor
Department of Mucosal and Salivary Biology, King's College London Dental Institute at Guy's Hospital, London SE1 9RT, UK

J. F. Setterfield
Department of Oral Medicine, King's College London Dental Institute, Guy's and St Thomas' NHS Foundation Trust at Guy's Hospital, London SE1 9RT, UK

Department of Dermatology, Guy's and St Thomas' NHS Foundation Trust at Guy's Hospital, London SE1 9RT, UK

J. Sanderson
Department of Gastroenterology, Guy's and St Thomas' NHS Foundation Trust at Guy's Hospital, London SE1 9RT, UK

B. Kirkham
Department of Rheumatology, Guy's and St Thomas' NHS Foundation Trust at Guy's Hospital, London SE1 9RT, UK

G. Larkin
Department of Ophthalmology, Kings College Hospital, London SE5 9RS, UK

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2.1 Introduction

In early days of dental schools, research was undertaken by relatively few members of staff, and they were invariably clinical academics. In many dental schools, the research laboratories, if present at all, were based in Departments of Oral Medicine and Pathology or in Departments of Conservative Dentistry (now known as Restorative Dentistry). These researchers were expected to master basic laboratory techniques and to apply them to their area of investigation. Usually they spent time in basic science laboratories to learn and master the techniques that they would use. In microbiological studies, for example, the clinical staff would be attached to a Microbiology Department in the main hospital and learn the techniques which could be applied to their work. Usually they were supported by skilled technical staff, but very few dental schools had any permanent nonclinical academic staff. This was a model which was prevalent in most schools until the 1980s, and there were many skilled clinical academics who mastered basic science techniques with great effect.

However, it became gradually clear that the pursuit of detailed complex research required greater expertise in a range of disciplines than could be managed and combined with clinical activities. It required experts in each of those fields who work with clinical academics to address translational questions and apply these basic techniques to the clinical questions. Thus, dental schools began to employ basic scientists to support the research strategy in the schools to be able to take that research to an international standard. Thus most dental schools will need to work closely with basic scientists in the field of immunology, molecular biology, microbiology, pathology, genomics and proteomics to be able to understand basic disease processes before they can be applied clinically.

In the field of oral medicine, the majority of oral mucosal diseases can be considered to be oral manifestations of systemic diseases. Thus in the field of dermatology, common diseases including lichen planus and recurrent aphthous stomatitis as well as less common diseases including mucous membrane pemphigoid and pemphigus vulgaris can be considered as the oral manifestations of dermatological disease. It is evident that a multidisciplinary approach of oral physicians, dermatologists and basic scientists will be able to address challenges of research into these diseases with more efficacy than oral physicians alone. Similarly in gastrointestinal diseases such as Crohn's disease, coeliac disease and ulcerative colitis which can all manifest as oral diseases, joint multidisciplinary working with gastroenterologists, oral medicine physicians, immunologists and other basic scientists can help to elucidate pathogenic mechanisms.

Perhaps the most obvious area where general progress has been made using a multidisciplinary approach, where ophthalmologists, rheumatologists and oral medicine physicians have worked closely and successfully together, is in Sjögren's syndrome. The majority of patients with Sjögren's syndrome presents with dry mouth, but the majority also have dry eyes and other ophthalmological problems and rheumatoid arthritis. Successful investigations of pathogenesis, epidemiology and identification of different phenotypes of Sjögren's syndrome require a multidisciplinary approach. Such international collaborations have been a showcase for this approach (Fig. 2.1; see below). Such multidisciplinary approaches are of obvious benefit to the clinical management of patients as well as to research leading to understanding of the pathogenic mechanisms and of more effective treatments applicable worldwide.

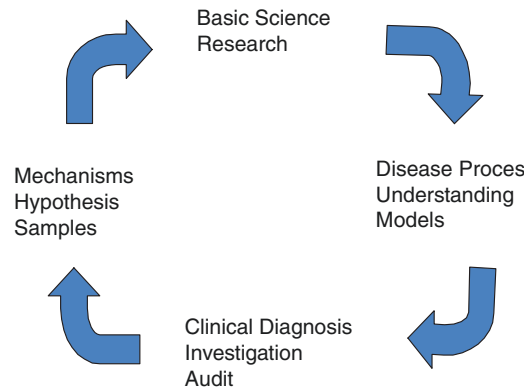


Fig. 2.1 The classical cycle of investigation, hypothesis and experimentation in clinical research

2.2 What Is Research in Medical and Dental Schools?

It can be argued that research in dental schools is actually a philosophy of investigation at a number of different levels (Table 2.1). At the clinical level, research may be the detached analysis of response to therapies, the continuous audit of outcomes of treatments, the analysis of patients and the division into clinical phenotypes, epidemiology, etc. The second area is that of applied research which is looking at the clinical trials, the disease groups, the classification of disease and the investigations using patients. This often leads on from basic research which until recently has been hypothesis-based investigation often of mechanisms of disease or cell interactions but not necessarily with an obvious clinical benefit (see Table 2.1). Thus ideally there is a continuous cycle of basic science research leading to disease process understanding and models which lead to clinical diagnosis investigation and audit which lead to hypothesis, mechanisms and samples which lead to basic research to understand these and so on (Fig. 2.1). This is a modification of the classical cycle of hypothesis to experimentation to observation to preliminary data and back to hypothesis.

Thus clinical and translational research progresses from basic research using cell molecules and genes, to clinical research using human research participants and materials, (having investigated the mechanisms, markers, drug devices and interventions) (see Fig. 2.2) and then on to the application to health and populations. This requires understanding the barriers, the feasibility, the strategy, the effectiveness and the safety as well as the quality of the translation of this research. The ideal translation is from bench to bedside, i.e. laboratory to human but then from bedside to community with the evidence allowing this to be translated into practice.

Table 2.1 What is reseach in medical and dental schools?

	A Philosophy of investigation
Clinical	Detached analysis of response to therapies, outcomes of treatments, analysis of patients, epidemiology, etc.
Applied	Disease groups, classification. Clinical trials, investigation using patients
Basic	Hypothesis-based investigation but not necessarily with obvious clinical benefit

2.3 Multidisciplinary Clinics

Multidisciplinary clinics can allow the collection of large numbers of patients with similar clinical diseases and make much more efficient the journey from applied research to changes in clinical practice. At Guy's Hospital Dental School (now King's College London Dental Institute), multidisciplinary clinics were established in the 1990s for (a) Sjögren's syndrome with the Departments of Rheumatology, Ophthalmology and Immunology; (b) orofacial granulomatosis with the Departments of Gastroenterology, Immunology and Nutrition; (c) bullous diseases with the Departments of Dermatology and with Moorfields Eye Hospital, Ophthalmology; and (d) Behcet's syndrome with the Department of Ophthalmology and Rheumatology. These multidisciplinary clinics have led to the Dental School being host to regional centres in each of these conditions and have led to changes in the management of several due to research, application of findings and assessment of clinical outcomes. It can be argued that without a robust system of disease severity measures and of measurement of clinical outcomes to management, any research leading to clinical application is unlikely to fulfil its full potential.

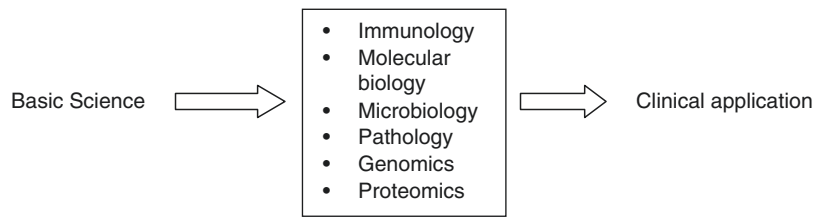


Fig. 2.2 Clinical research in oral mucosal diseases

2.4 Assessment of Disease Severity and Treatment Responses in Oral Mucosal Disease

It is a sad truism and reflection on the field that few oral medicine treatments are evidence-based, even those regarded as standard therapies. Until the last few years, there had been a lack of any method to routinely assess disease severity and thus of quantifying responses to therapies. This led to the obvious need to devise and validate oral disease severity scores for a variety of conditions seen in routine clinical practice which could also be used for assessing treatment responses.

The benefits of a scoring system for mucosal disease severity are that (a) they can indicate the severity of disease, (b) they are needed to indicate the efficacy of any treatments, (c) they may distinguish between or reveal between disease subgroups, (d) they may assist in deciding to implement or withhold treatment and (e) they are a routine clinical audit tool which can also be used for research.

Any such oral disease severity scoring systems (ODSS) must be objective and must be reproducible; they should be easy to use and they should be widely applicable. Fortunately such ODSS have been created and validated and are used for recurrent aphthous ulceration [1], lichen planus [2], pemphigus [3], mucous membrane pemphigoid [4], orofacial granulomatosis [5] and dry mouth assessment [6].

2.5 Bullous Diseases

A joint clinic with the Institute of Dermatology was established in 1994 with the Oral Medicine Department at Guy's Hospital. The dermatologists from the Institute were Dr. Martin Black and Dr. Jane Setterfield and from the Department of Oral Medicine Professor Stephen Challacombe and Dr. Pepe Shirlaw. Monthly clinics alternated between the Dental Institute at Guy's Hospital and those held in the Institute of Dermatology at St Thomas' Hospital. Later strong connections were made with Dr. John Dart in the Moorfields Eye Hospital, Ophthalmology Department, with a special interest in mucous membrane pemphigoid. This led in due course to the joint clinic becoming part of a Biomedical Research Centre leading on to clinical trials and a European collaboration. The establishment of a joint clinic led in due course to its recognition as a regional centre and to wide geographical referral to the centre resulting in the largest series of patients with mucous membrane pemphigoid (MMP) and patients with pemphigus vulgaris (PV) in the UK (Fig. 2.3a, b).

Such richness in clinical material carries with it an obligation to perform basic research, which might lead to identification of clinical phenotypes and improvement in patient management. Greater than 20 peer-reviewed research papers have emanated from this clinic along with Master students in Oral Medicine and five higher degrees (MD/PhDs).

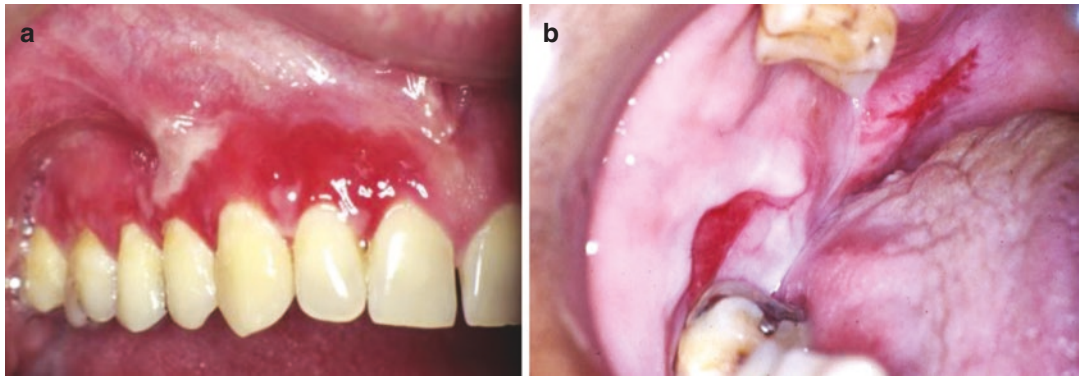


Fig. 2.3 Clinical examples of (**a**) MMP involving the upper right gingivae and (**b**) pemphigus vulgaris in the healing phase in the right buccal mucosa

Notable achievements coming from this research include the establishment and validation of an oral disease severity scoring systems for both MMP and PV (Fig. 2.4) [3], identification of different clinical phenotypes of MMP [4, 7], establishment of new assays to monitor serum antibodies to epitopes of BP180 in MMP [8, 9] and desmogleins 1 and 3 in PV [10], establishment of saliva as an alternative diagnostic fluid in both MMP and PV and the discovery of secretory IgA antibodies in saliva in MMP [8, 9].

This multidisciplinary research clinically was supported by laboratory-based investigations in immunology, immunopathology and ophthalmology. This has led to the definition of the clinical spectrum, identification of disease subgroups, the study of isotype specificity and of the role of IgA antibodies in disease severity, HLA associations and antigen specificity. The recognition that a B-cell- and antibody-mediated disease is driven by T-cell responses to antigens and epitopes has also been studied [11].

The main laboratory and clinical studies in MMP utilising a multidisciplinary approach are outlined in Table 2.2 (laboratory and clinical studies in MMP), and some key findings in MMP derived from a multidisciplinary clinic are summarised in Table 2.3.

In *pemphigus vulgaris*, laboratory-based research has allowed development of sensitive ELISAs and optimisation of substrates, while the development of an intraoral disease severity scoring system has allowed an evidence base to treatments for the first time and the translation of these findings to the clinic. For example, where the serum antibody titres have fallen and the clinical severity score has fallen, patients can be maintained on local therapy and do not need systemic immunosuppression. Key findings in pemphigus vulgaris derived from a multidisciplinary clinic are summarised in Table 2.4.

Intra-oral scoring system – MMP or PV

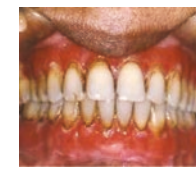
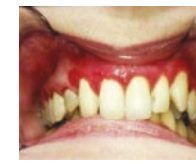
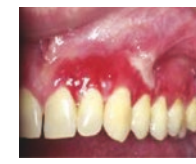
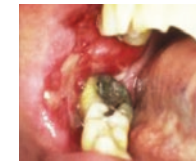
Site	Site score	Activity score (0 - 3), Double if site score = 2
Outer lips	1	 1
Inner lips	1	
L Buccal Mucosa	<50% = 1, >50% = 2	
R Buccal Mucosa	1-2	
Gingivae		 2
Lower R (distal)	1	
Lower central	1	
Lower L (distal)	1	
Upper R (distal)	1	
Upper central	1	 3
Upper L (distal)	1	
Dorsum tongue	1-2	
R ventral tongue	1	
L ventral tongue	1	
Floor of mouth	1-2	 3
Hard palate	1-2	
Soft palate	1-2	
Oropharynx	1-2	

Fig. 2.4 An oral disease severity scoring system for mucous membrane pemphigoid. Seventeen intraoral sites are each scored for the presence of disease severity between 1–3, to give a total for the patient at each visit (plus a pain score) (see [3])

Table 2.2 Cross-discipline laboratory and clinical studies in mucous membrane pemphigoid (see [4, 7–9, 11])

• Laboratory studies with immunology, immunopathology
– Target antigens in MMP in relation to clinical phenotype: BP180, a6b4, NC16a, laminin 5
– Antibody isotypes: IgG, IgA, secretory IgA
– Use of saliva as diagnostic fluid
• Clinical studies with dermatology
– Distinct clinical phenotypes of oral MMP
– Oral disease severity scoring
– Different clinical phenotypes may respond to different therapies

Table 2.3 Key findings in MMP derived from a multidisciplinary clinic (see [4, 7–9])

• Three main clinical oral phenotypes of MMP
• Two main immunofluorescent types relating to target antigens (BP180 and a6b4, laminin 5)
• Serum IgG antibody titre correlates with disease severity
• Serum IgG and IgA antibodies combined lead to a more severe disease
• Different clinical phenotypes respond to different drugs
• Salivary IgA and IgG antibodies to BP180-NC16a are diagnostic biomarkers in mucous membrane pemphigoid

Table 2.4 Key findings in PV derived from a multidisciplinary clinic (see [3, 8–13])

• The severity of cutaneous and oral pemphigus is related to desmoglein 1 and 3 antibody levels
• The transition of pemphigus vulgaris into pemphigus foliaceus related to Dsg antibodies
• Oral and genital lichenoid reactions associated with circulating autoantibodies to desmoplakins I and II
• Serum and salivary IgG and IgA antibodies to Dsg3 in mucosal pemphigus vulgaris

2.6 Orofacial Granulomatosis

A multidisciplinary clinic looking after patients with orofacial granulomatosis (OFG) and oral Crohn's was first established at Guy's Hospital in 1995. This was a joint clinic involving Oral Medicine and Pathology, Gastroenterology (Dr. Jeremy Sanderson and colleagues), Nutrition (Dr. Lomer and colleagues), Immunology (Professor Jo Spencer) and Psychology. This clinic became a referral centre for not only oral medicine and oral maxillofacial surgery consultants around the UK, but also gastroenterologists and dermatologists as a UK regional referral centre. This led in due course to the largest series of characterised OFG in the UK and possibly the world. The first 250 patients were examined in detail both in vivo and in vitro, and the majority had colonoscopies as well as oral biopsies. Clinical trials and national collaborations (Liverpool, London and Newcastle) followed, and the findings have changed clinical practice in OFG (see Fig. 2.5 and Table 2.5).

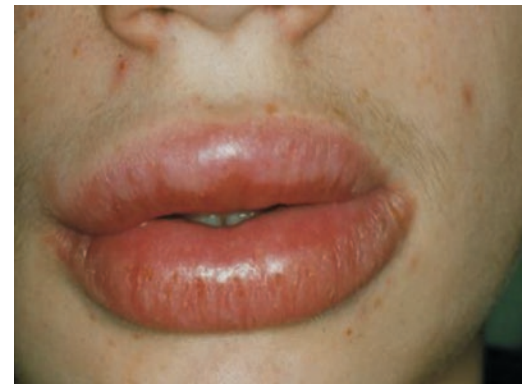


Fig. 2.5 Classical swollen granulomatous lips of orofacial granulomatosis

Table 2.5 Key recent findings from multidisciplinary OFG clinic and research

Treatment:
• Cinnamon- and benzoate-free diets as a primary treatment for OFG [14, 15]
• Oral disease severity scoring system for OFG [15, 16]
• Experience with anti-TNF-αtherapy for orofacial granulomatosis [17]
• Development of a low phenolic acid diet for the management of orofacial granulomatosis [5]
• Dietary intervention for oral allergy syndrome as a treatment in orofacial granulomatosis: a new approach [5]?
• Azathioprine is effective for oral involvement in Crohn's disease but not for orofacial granulomatosis alone [18]
Translational research:
• Subepithelial dendritic B cells in orofacial granulomatosis [19]
• Distinguishing orofacial granulomatosis from Crohn's disease: Two separate disease entities [16]?
• Clinical evidence for allergy in orofacial granulomatosis and inflammatory bowel disease [20]
• Genetic association analysis reveals differences in the contribution of NOD2 variants to the clinical phenotypes of orofacial granulomatosis [18, 21]

There was a wider source of referrals for this clinic than the other multidisciplinary clinics. Referrals have come from eight different medical and dental specialties including dermatology, gastroenterology, paediatrics, oral and maxillofacial surgery, general medical and dental practice and allergy clinics. Thorough investigations including oral examination, colonoscopies, severity scoring, haematological and immunological studies, patch testing, scratch testing and biopsies have allowed a series of novel findings which have changed clinical practice. The main findings are outlined in Table 2.5. These detailed investigations along with colonoscopies allowed a distinction to be made between those patients with oral manifestations of Crohn's disease and those with orofacial granulomatosis restricted to the oral cavity or head and neck [15, 16, 22].

The introduction of cinnamon- and benzoate-free diets by dietitians and their assessment using an oral disease severity score showed that diet alone could result in significant clinical improvement without the need necessarily of systemic immunosuppressives which had hitherto been the first line of treatment. Cinnamon and benzoate elimination diets could have significant and rapid clinical benefit in two-thirds of patients [5, 14, 15].

These investigations also allowed a fundamental difference between children and adults to be revealed. That is the question of whether OFG was a marker for the subsequent development of Crohn's disease. In adults, 10% of patients presenting with OFG develop Crohn's in the first 10 years (Fig. 2.6). Subsequent follow-up over a further 15 years showed no further development [15, 16]. In children 25% of patients presenting with OFG developed Crohn's within the first 10 years, suggesting that OFG can be a marker for these subsequent developments of Crohn's

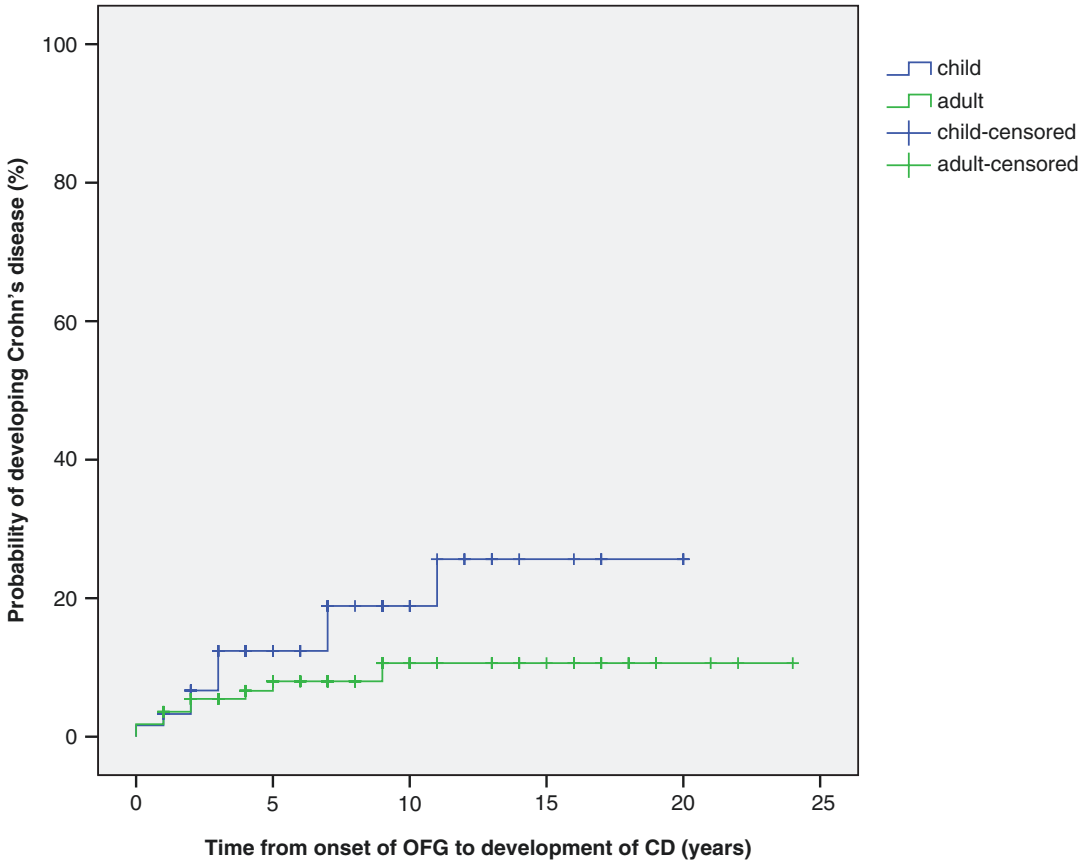


Fig. 2.6 Cumulative risk of onset of CD in childhood and adult onset of oral symptoms [15, 16]

but that the risk is much greater in children and that if 10 years have passed since the onset of OFG, the risk of subsequent development of Crohn's is very small (Fig. 2.6).

The presence of psychologists on the clinic was beneficial both for clinicians and for patients. These studies revealed a significant psychological impact of facial disfigurement and the need of psychologist assessment on a regular basis and further referral for treatment separated from the OFG clinic.

Patch and scratch testing revealed that some 40% of OFG patients were positive to one or more ingredients and that the majority of those would be responsive to exclusion diet. In scratch testing some 60% of patients with OFG were positive with silver birch being the main allergen which has led to further investigation of the aetiology [20]. Immunological investigations on this multidisciplinary clinic have led to findings with immunogenetics (an increase in NOD2) [18, 21] and a curious and as yet unexplained finding of subepithelial dendritic B cells in OFG biopsies not associated with lymphoid tissue [19]. These do appear to be associated with IgE expression, and they may have a role in immune responsiveness including IgE-mediated allergic responses. Key findings from a multidisciplinary clinic in terms of diet are summarised in Table 2.5. The additional detailed analysis of the genetics has recently been published [18] and a finding that Azathioprine is effective for oral involvement in Crohn's disease but not for orofacial granulomatosis alone [21]. This again emphasises the importance of distinguishing between OFG and oral Crohn's disease clinically.

2.7 Sjögren's Syndrome Clinic

Patients with Sjögren's syndrome are usually referred to three main specialities depending on their primary symptoms. Sjögren's is the second most common autoimmune disease and is likely to present firstly to dentists because of dry mouth. A significant proportion of patients present to ophthalmologists because of dry eyes and to rheumatologists because of associated joint pain. There is obvious value clinically to have a single referral clinic comprising oral medicine physicians, rheumatologists and ophthalmologists to coordinate both investigations and treatment plans.

The Multidisciplinary Sjögren's Syndrome Clinic was established at Guy's Hospital in 1992 initially with Rheumatology (Dr. B Kirkham, Professor G. Panayi) and later with Ophthalmology (Dr. G. Larkin). This has facilitated the detailed assessment investigation and management of a large series (approximately 600 patients) in the discovery and definition of the non-Sjögren's syndrome (SNOX) [23] and other advantages with clinical trials on saliva substitutes with GSK, basic research and understanding dry mouth [6, 24, 25] but also becoming part of national Sjögren's network (in the UK via Birmingham) and international collaboration via the NIH-funded SICCA project [26, 27]. The clinical collaboration allowed the discovery of a new syndrome (SNOX) which was shown to be non-Sjögren's but comprising dry mouth, dry eyes and the presence of primary generalised nodal osteoarthritis with non-specific sialadenitis [23]. None of these patients had rheumatoid factor or ENA autoantibodies (Ro) and (La) autoantibodies, thus distinguishing them from Sjögren's syndrome. No patient from the clinic with SNOX has developed a lymphoma which emphasises the need to distinguish SNOX from Sjögren's syndrome.

Perhaps the most important international multidisciplinary collaboration has been the NIH-funded Sjögren's International Collaborative Clinical Alliance or SICCA [26]. This ambitious alliance set up collaborations over seven countries (the USA (three sites),the UK, Denmark, Argentina, China, India and Japan) and with the aim of develop standardised diagnostic criteria for Sjögren's syndrome and establishing evidence-based criteria for the diagnosis of Sjögren's syndrome. This multidisciplinary international collaboration involved clinical leads in the SICCA research groups from oral medicine, rheumatology and ophthalmology and active collaborations with pathologists, statisticians, epidemiologists and immunologists. Standardised samples were taken from greater than 1500 cases of putative SS and analysed. This collaboration has led to the establishment of agreed international criteria for the diagnosis of SS [28, 29] but

also methodology for quantifying ocular disease [30]. Standardisation across countries allowed genetic analysis of over 1000 patients with SS [31] and longitudinal studies to be performed for the first time [31].

2.8 Clinical Oral Dryness Score (Fig. 2.7)

One of the benefits of a multidisciplinary clinic was to develop an oral dryness score which was reliable and easy to use for the routine assessment of severity of dry mouth [32]. CODS can be incorporated into the routine clinical assessment of dry mouth patients, particularly since the clinician would normally be undertaking most aspects of the clinical assessment in any case.

Investigations showed that there was good correlation between the clinical oral dryness score (CODS) and whole salivary flow rates but also interestingly with mucosal film thickness on buccal, palatal and tongue surfaces [6, 32]. The score has now been calibrated and is in widespread clinical use.

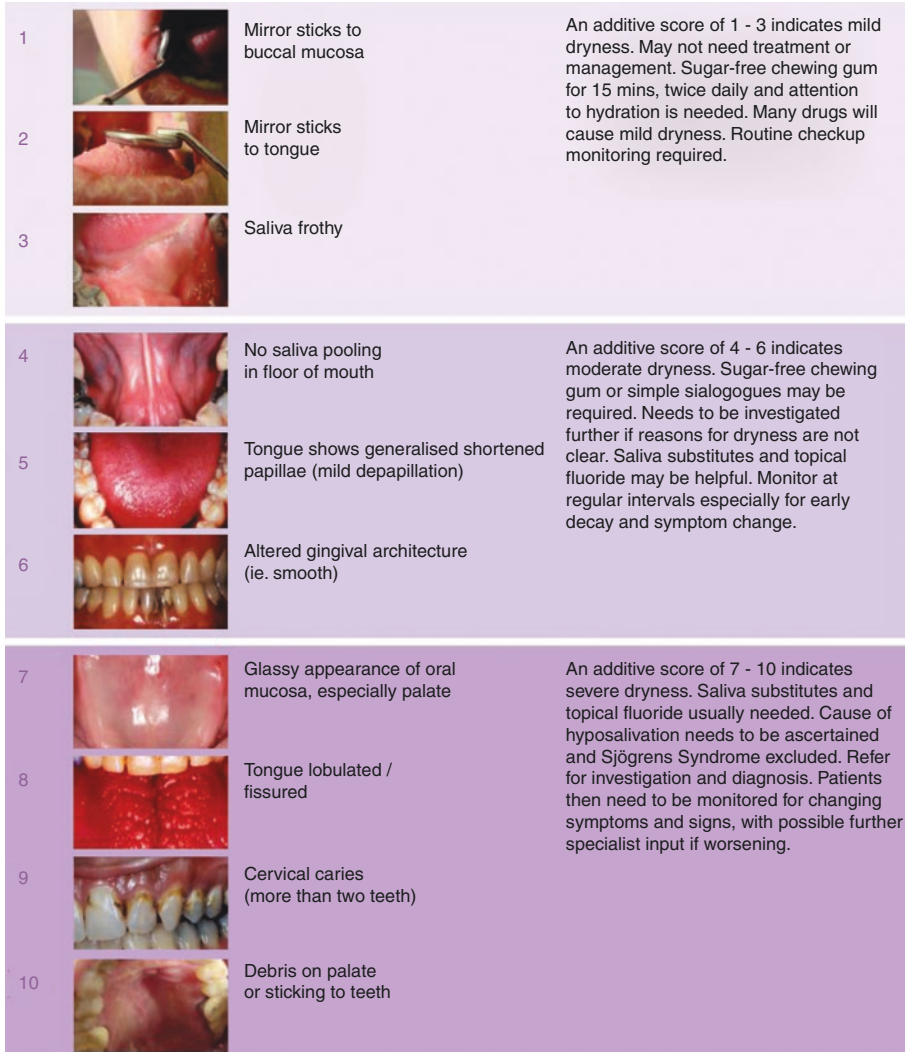


Fig. 2.7 Clinical oral dryness score (The Challacombe scale) [32]. One mark is given to each clinical feature, and scores of 1–3 indicate mild dryness, 4–6 as moderate dryness and 7–10 as severe dryness. CODS is related both to the salivary flow rate and to the index of mucosal wetness

Table 2.6 Summary of main translational research findings from the international SICCA collaboration and from a dry mouth (Sjögren's syndrome) clinic

• First prospective, protocol-directed, consistent data accession cohort to develop pSS phenotype and SS classification criteria
• First longitudinal cohort to study SS natural history (repeat examinations and biospecimen collection)
• Development of new method to assess ocular staining in SS based on multi-organ system data analysis
• First opportunity for comprehensive study of SS genome.
• New diagnostic criteria have been developed on the basis of data
• SSB-positive alone is not associated with key phenotypic features of Sjögren's syndrome [34]
• Association of CXCL13 and CCL21 expression with the progressive organisation of lymphoid-like structures in Sjögren's syndrome [35]

The advent of SICCA allowed worldwide 1500 cases of Sjögren's syndrome to be characterised [26, 33]. It was the first prospective protocol directed consistent data accession cohort to develop Sjögren's syndrome phenotypes and classification criteria. It has already developed a new method to assess ocular staining [30] and is the first opportunity for a comprehensive study of the Sjögren's syndrome genome [31] and has had a major impact on understanding the pathogenesis of Sjögren's syndrome on a global scale. Thus multidisciplinary research and clinics have led to new diagnostic criteria being developed on the basis of data. They have shown that SSB alone is not associated with Sjögren's syndrome (see Table 2.6) [28, 29, 34].

Conclusions

Cross-disciplinary research has greatly increased our understanding of oral mucosal diseases. On the clinical front, close collaborative working with consultants of different disciplines in joint clinics has resulted in a depth of investigation of oral medicine patients (e.g. colonoscopies, slit lamp eye examinations, general rheumatological examinations, skin and other mucosal site dermatological investigations) that would not have been possible with oral medicine physicians alone. Joint expertise has led to greater number of referrals and identification of clinical phenotypes. This has facilitated focussed laboratory-based research in immunology, genetics, molecular biology, etc. into the mechanisms underlying oral mucosal disease [36]. Together with the clinical research, this joint knowledge has already been transferred to the chairside and has changed and improved the management of oral mucosal diseases [36]. Thus cross-disciplinary research has not only been of direct benefit to patients but also revealed how much more needs to be done.

Acknowledgements To all those colleagues who share enthusiasm for multidisciplinary clinics including *Oral Medicine*, Jane Setterfield, Pepe Shirlaw, Michael Escudier, Anwar Tappuni, Richard Cook and Helen McParland; *Gastroenterology*, Jeremy Sanderson, Pritash Patel and Carlo Nunes; *Dieticians*, Miranda Lomer and Helen Campbell; *Oral Pathology*, Peter Morgan and Eddie Odell; *Rheumatology*, Bruce Kirkham, Francesca Barone and Gabriel Panayi; *Dermatology*, Martin Black, Jane Setterfield and Anne Marie Powell; *Immunofluorescent laboratory*, Balbir Bhogal and Matthew Gratian; *Immunology*, Charles Kelly and Jo Spencer; *Salivary Research Unit*, Gordon Proctor, Samira Osailan, Rashida Pramanik and Guy Carpenter; and *Ophthalmology*, Genevieve Larkin and John Dart.

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3 Epigenetics and Periodontitis: A Source of Connection to Systemic Diseases

Antonio Bascones-Martinez
and Jerian González-Febles

Abstract

Over the past 15 years, there has been intense research interest in periodontitis and its associations with several systemic conditions and how periodontitis can modify the expression of those diseases. The area that looks forward in those relationships is called periodontal medicine. Offenbacher described periodontal medicine as a discipline that focuses on the investigation of associations between periodontal diseases and systemic diseases and their biological plausibility in human populations and in animal models. It has been reported that periodontal disease may independently increase the risk of diabetes mellitus, cardiovascular disease, preterm or low-weight delivery, or rheumatoid arthritis. On the other hand, periodontitis is a chronic infection, which pathogenesis is orchestrated by multiple factors. Within those factors, genetics and epigenetics may have an important role in the pathogenesis. Epigenetics is a new area in research that is defined as genetic control by factors other than an individual's DNA sequence via silencing certain genes while promoting others. These processes involve regulating transcription factor and access to chromatin, as well as microRNA (miRNA) and long noncoding RNA (lncRNA) regulating the expression of mRNA. In this chapter, we are going to deal with periodontitis pathogenesis, the role of epigenetics in its process, and the new connections of periodontitis and some systemic conditions by the expression of some epigenetic factors. This basic knowledge drives to know how to understand the possible connections and some targets to cope with in the future.

A. Bascones-Martinez, D.D.S., M.D., Ph.D. (✉)
J. González-Febles, D.D.S.
Department of Oral Medicine and Surgery,
Complutense University, Madrid, Spain
e-mail: bascones@ucm.es

3.1 Introduction

Periodontitis is a chronic inflammatory disease characterized by periodontal attachment and alveolar bone loss that eventually may lead to tooth loss. It is considered the most prevalent chronic inflammatory disease that affects 46% of adults older than 30 years in the USA [1], where the prevalence of severe periodontitis is over 11% [2]. The primary etiological factor of periodontitis is the presence of specific bacteria organized in dental biofilm that leads to the triggering of several signaling routes that prompts the initiation of immune response mechanisms. The microflora that causes periodontitis is somehow complex, in which there are over 700 different bacterial species. In the recent years, some

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clinical studies have defined key pathogens that present a crucial role in the initiation and progression of periodontitis [3, 4], demonstrating that their presence is a risk factor for ongoing attachment loss. Among those bacterial species, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are the most relevant pathogens in periodontitis. Both species exhibit a wide range of virulent factors that could activate several signaling pathways, inducing a humoral immune host response [5, 6].

While periodontopathogenic bacteria are the main cause in the activation of immune response, other elements such as genetics and environmental factors may influence or modulate the host response to bacterial burden [7–9].

3.1.1 Molecular Regulation of the Immune Response

In gene expression, its central dogma is based on the DNA transcription into mRNA, which serves, in turn, as template for the protein synthesis [10]. The immune response model at the transcriptional stage starts with an external stimuli, such as bacterial burden, which gives rise to a signaling cascade within the cell cytoplasm, activating the binding of promoters and enhancers that regulates some DNA sequences. These assembles activate in turn the transcription machinery as well as the expression of specific genes. At this time, certain DNA sequence alterations, mutations, and polymorphisms may come out to alter transcription factors binding to specific areas of gene expression regulation.

Regarding the protein structure, the changes might occur within the coding regions of the gene (exons), leading to changes in the position and sequence of amino acids in the protein structure. In this sense, some novel processes in this basic model have been depicted in a recent study [11] that have suggested synergistic mechanisms between transcriptional activators bound to promoters and enhancers in different layers that are superimposed, leading to different stages in gene modification that may occur.

It has been identified specific mechanisms to be rapidly turned on and off in response to that external stimuli [11]. Those have been suggested as special pathways in creating selectivity of immune response to certain bacterial stimuli, regulating the magnitude of that response and conducting to chronic inflammation [12, 13]. Therefore, it could present a potential role in how some bacterial species may activate different signaling pathways leading to a specific host immune response.

3.1.2 Epigenetic Modifications of Gene Expression

Epigenetics is defined as changes in gene expression, which are not controlled by DNA sequence but by silencing certain genes and promoting others. These processes involve alterations of the DNA and associated proteins such as DNA methylation or modifying DNA structure (e.g., chromatin alterations), as well as microRNA (miRNA) and long noncoding RNA (lncRNA) that may regulate the expression of messenger RNA (mRNA) [14]. These epigenetic alterations may be able to modulate the host response to several bacterial stimuli and regulate the magnitude and speed of those processes [7, 9].

Genetic mutations are commonly known to control the epigenome [15], inducing several changes in DNA structure and sequence alterations that affect the protein function. Nevertheless, some epigenetic modifications such as histone modulation are often strongly correlated with patterns of inherited gene expression [16], including those that lead to gene mutations. Hence, the link or boundary between genetics and epigenetics is still not clear, driving to the concept that some researchers [15] suggest that both are two sides of the same construct, being connected.

3.1.3 Immune Response in Periodontitis and Epigenetic Modifications

The immune host response to the bacterial products starts with the innate immune system activation. This is the natural host barrier defense against

bacteria, which is mediated by subepithelial dendritic cells, neutrophils, macrophages, natural killer cells, and monocytes. Those cell activations are orchestrated by several families of proteins such as toll-like receptors (TLRs) that are involved in the bacterial recognition of their patterns.

Innate system does not require any previous exposure to a microbial product, so thus, it is so important in initiating the host response to new microbial challenges. However, some bacteria have the capacity to evade some host response mechanisms as a result from adaptation to the hostile environment [17]. Shifts within the periodontal tissues have been ascribed to the interaction between biofilms and their products and some specific receptors such as TLRs.

Afterward, when innate response is not able to control the bacterial aggression, adaptive immune response is activated. This host response is normally mediated by T- and B-cells that will provide to the host the so-called immunological memory. This adaptive response may play a crucial role in established periodontitis lesions that are characterized by high proportions of plasma cells [18], which in turn are going to generate antibodies against microbial products.

Nevertheless, the initiation and the magnitude of this interaction between biofilms, its products, and the host response may be altered by genetic traits but also by some systemic conditions and environmental factors such as smoking or stress. Within this interaction, epigenetic modifications may modify the interplay between environmental factors, genetic traits, and host immune response.

3.1.4 DNA Methylation, Histone Modulation, and Other Gene Alterations

The genome modification in host cells can often occur via DNA methylation. It normally takes place at the 5′ position of cytosine in CpG dinucleotides, called CpG islands. CpG islands are dinucleotides that involve the connection between a cytosine nucleotide and a guanine nucleotide by a phosphodiester bond. Clusters of CpG sequences appear in the promoter regions, prevent transcriptional initiation, and silence the genes [31]. There are three different isoforms of DNA methyltransferases by which DNA methylation can occur. DNA methyltransferase 3a and DNA methyltransferase 3b normally present a crucial role in “de novo” methylation of CpG residues, while DNA methyltransferase 1 (DNMT1) regulates the methylation pattern copying to new synthetized DNA strand during replication. DNA methylation is necessary for normal cell development, and it is essential in tissue-specific gene transcription [19]. Differential methylation patterns associated with lipopolysaccharide (LPS) signaling, cell adhesion, and other processes such as apoptosis or oncogenesis have been observed in untreated periodontitis tissues [20]. Recent studies have suggested that bacteria have the potential to cause alterations in the DNA methylation status, but also the environment, aging, and stress may also play a role on modifying the expression of periodontitis, involving epigenetic changes that affect disease expression or cause oral dysbiosis.

Depending on the health status of periodontal tissues, CpG islands are differentially methylated. Recent data that investigated the differences in DNA methylations between healthy gingival tissues from healthy subjects and inflamed periodontal tissues from chronic periodontitis tissues identified some CpG sites methylated in inflamed tissues different from healthy tissues [20]. Some genes such as SOCS3, VDR, MMP25, BMP4, RUNX3, interleukin-17, TNFRSF18, ZNF277, ZNF501, CADM3, and BDNF were observed hypermethylated in inflamed gingival tissues and others hypomethylated compared to healthy gingival tissues [20]. The authors suggested that these methylated CpG regions might confirm a linkage between epigenetic modifications and the immune host response. One example of DNA methylation in periodontitis is the DNA hypermethylation of prostaglandin-endoperoxidase synthase 2 promoter in chronic periodontitis lesions. This pattern is associated with a high expression of cyclooxygenase-2 in chronic periodontitis tissues, increasing the inflammation status of the tissues. Those epigenetic modifications of gingival tissues are suggested to mainly occur within the biofilm-sulcular epithelium interface. Within this interaction bacteria may

have the capacity to induce some DNA methylations (meter ref). It has been described that *Porphyromonas gingivalis* could cause the hypermethylation of a protein that regulates the chromatin remodeling, called as GATA binding protein [21]. This protein could also be hypomethylated by a *Fusobacterium nucleatum* infection, driving to the concept that one epigenetic modification may occur depending on the presence of specific bacteria.

Other epigenetic change related to oral dysbiosis is histone acetylation. A recent study conducted by Martins and co-workers [22] reported that both regulation of DNMT1 and histone acetylation were shown in oral dysbiosis. This study has proven that epigenetic changes may indeed be associated with oral dysbiosis.

In general, histone acetylation is associated with enhanced transcription of genes [31], nucleosome assembly, chromatin folding, DNA damage repair, and replication [23]. It is usually related to the chromatin structure relaxing, leading to an enhanced transcription of inflammatory genes, such as nuclear factor kappa B (NF-κB) target gene or several genes of pro-inflammatory cytokines, which are commonly upregulated in periodontitis. Normally, NF-κB is able to activate innate immune system mediated by TLRs [24], and its chronic activation may induce osteoclastogenesis that would lead to bone resorption [25]. In periodontitis lesions, it has been demonstrated that *Porphyromonas gingivalis* and *Fusobacterium nucleatum* infection may be able to induce epigenetic modifications such as histone 3 acetylation and the downregulation of DNMT1.

Moreover, histone acetylation by lipopolysaccharides (LPS) could influence p300/CBP activation. Its activation is related to the transcriptional stimulation of some pro-inflammatory cytokines such as IL-1, IL-2, IL-8, and IL-12, commonly found in periodontally affected tissues [22].

3.2 Role of MicroRNAs

MicroRNAs (miRNAs) are a group of small, noncoding RNAs that play key roles in epigenetic regulation by controlling the translation and stability of mRNAs [26]. They are crucial in developmental processes, apoptosis, and cell proliferation [27]. However, this regulation depends on the activities of other cofactors, DNA methylation and/or histone acetylation. The other cofactors include RNA-binding protein, CREB-binding protein or E1A binding protein p300, and cyclic AMP response element-binding protein (CBP). This regulation indirectly inhibits or promotes mRNA expression. Hence, these molecules may play a significant role in inflammatory processes, affecting both innate and humoral immune response to the microbial challenge [7, 26]. In addition, autoimmune diseases may be also affected by these molecules [26]. However, periodontitis is the gold standard in oral chronic inflammatory diseases, where miRNA might have a specific and detrimental role in its pathogenesis [7]. Recently, Kebschull and Papapanou have performed an extensive review about the role of miRNA in periodontal disease [7]. They have observed that miRNAs could affect different immune processes at different stages of the inflammatory response against the bacterial insult in periodontal disease.

As it has been explained, the inflammatory process starts with a bacterial challenge. The first line in host response involves the pathogen pattern recognition mediated by TLRs. The most common TLRs in periodontitis are TLR-2 and TLR-4, which have the capacity to interact with specific bacterial species and their virulent factors. *Aggregatibacter actinomycetemcomitans* and the lipopolysaccharide (LPS) from *Porphyromonas gingivalis* are the main bacterial species and products that are able to activate those receptors. After their binding, NF-κB pathway is activated. NF-κB is a family of rapid responder transcription factors that are able to induce changes in target gene expression. As a consequence of TLR activation, different cell types are triggered, such as macrophages, neutrophils, natural killer cells, or dendritic cells, developing the comprehensive innate immune response.

Several miRNAs are able to induce changes in the NF-κB signaling pathways. One of them is the miRNA-146a, which is itself regulated after NF-κB activation via TLR-2, TLR-4, TLR-5, and TLR-9 in response to the bacterial challenge, driving to a downregulation of two important cytokine receptors, receptors of IL-1 and TNFα.

This miRNA was found to be upregulated in periodontally affected gingival tissues [28, 29]. miR-146a is also able to suppress TLR-2 expression in keratinocytes [30] and macrophages [31], leading to a weak inflammatory response. However, in macrophages, the induction of miRNA-146a by *P. gingivalis* LPS does not result in a lower cytokine production [32], driving to the concept of a counteracting effect by other miRNAs [7].

One of the most important miRNAs in periodontitis is miR-155, which acts at different stages in the host response. This miRNA is able to downregulate NF-κB signaling pathway, as well as to promote cell differentiation [33]. It also mediates the response to infection by type I interferon production [34], a mechanism possibly connected to aggressive periodontitis [35], increasing inflammation and periodontal tissue destruction. Furthermore, it is able to induce the expansion and activation of natural killer cells [36] and high production of interferon-γ [37]. In macrophages, it was shown that miR-155 increases the levels of leukotriene B4, resulting in high responsiveness of TLRs [38]. However, the dysregulation of its expression and also the miR-146 expression in epithelial cells infected by *P. gingivalis* was observed to turn into an increased sensitivity of TLR signaling pathway, exponentially expanding the immune response [39, 40].

In natural killer cells, miR-30e and miR-200a are downregulated in periodontally affected tissues that lead to an increased activation of natural killer cells and a higher production of interferon-γ, driving to a higher tissue destruction [41, 42]. Other miRNAs such as miR-451, which has the ability to suppress neutrophil chemotaxis [43], or miR-486, which leads to an overexpression of NF-κB signaling [44], are upregulated in periodontitis lesions.

Dendritic cells play an essential role in the innate system, bridging the innate system and adaptive immune response. They are able to detect some bacterial species and their products, producing specific inflammatory cytokines, critical in the immune response. Recent investigations have pointed out that their functions are tightly controlled by miRNAs [45].

One example is miR-155, a miRNA that was found to control other innate system processes (see above). It was shown to have the ability to affect the function and maturation of dendritic cells, influencing the cytokine signaling and thus the inflammatory host response [46]. On the other hand, miR-451 has exhibited to reduce cytokine production by dendritic cells, which has responded to bacterial infection [47], and miR-148a was shown to damage the innate response and the antigen presentation mechanism by dendritic cells [48], both overexpressed in periodontally affected gingival tissues.

As the innate response can be regulated by several miRNAs, some of those might also influence certain adaptive response processes. B- and T-cells are the main cell strains in the adaptive immune system. This system involves their expansion and provides immunological memory. Some miRNAs, such as miR-146a, miR-650, miR-155, miR-210, or miR-455, may play a role in the control of some adaptive immune processes. Other miRNAs are upregulated in periodontitis lesions such as miR-650 that was found to influence the proliferative capacity of B-cells [49]. As seen above, miR-155, an upregulated miRNA in periodontally affected tissues, has different functions in host immune regulation. In adaptive immune, this miRNA could also have the capacity to control CD8 T-cell response [50], as well as to influence in the indirect activation of T-helper cell 17 response by dendritic cell signaling [51]. Nevertheless, others are downregulated in periodontitis lesions such as miR-210, which are related to the increase of T-cell signaling, being associated with the etiopathogenesis of periodontitis [52, 53].

In summary, miRNAs are epigenetic modifications that are crucial in immune response regulation, being bridges between different exogenous challenges and host response. While further research in miRNA functions and role in chronic inflammatory disease such as periodontal disease is needed, they are a very promising source of connection between different immune processes in different oral and systemic conditions.

3.3 Role of Long Noncoding RNAs

Long noncoding RNAs (lncRNAs) modulate cell proliferation, senescence, migration, and apoptosis. They also interact with DNA, RNA, and other

proteins and regulate gene expression and other miRNA activities. A recent publication performed by Zou et al. [54] has demonstrated the presence of some lncRNAs in chronic periodontitis lesions. As miRNAs, lncRNAs could be up- or downregulated, affecting different host immune pathways and miRNA functions. Some lncRNAs such as HOTAIR, PRDX6, IFNG, or TIRAP are associated with periodontitis lesions [54]. Periodontitis was shown to express the upregulation of HOTAIR, PI3 (lncRNAs RP3-461P17 and RP1-300I2.2), PRDX6, TIRAP, lncRNA CDKN2A, and CDKN2B. Conversely, lncRNAs NR_003716, RP11–29014.3, IFNG, lincRNA-CDON-1, and CDKN2BAS were shown to be downregulated in periodontally affected tissues. Certain lncRNAs such as lincRNA-CDON-1 appear to be involved in signaling pathways in TLR expression, crucial in the etiopathogenesis of periodontitis. Likewise, further studies to understand the role and functions of those lncRNAs in periodontitis pathogenesis are needed.

It has been found that lncRNAs possess transcribed ultraconserved regions (T-UCRs), which are a segment of DNA and considered as a novel class of noncoding RNAs. UCRs are conserved, i.e., unchanged between the species. Therefore, alteration in this area is unlikely to occur due to chance, and differential expressions have been observed in several systemic conditions such as cancers or cardiovascular diseases [55].

3.4 Periodontitis, Epigenetic Alterations, and Systemic Diseases

Nowadays, periodontitis is considered a noncommunicable chronic inflammatory infectious disease which is connected to some systemic conditions. Recent epidemiological studies have remarked important associations between periodontitis and some systemic diseases such as diabetes, cardiovascular disease, rheumatoid arthritis, or cancer [56, 57], where systemic inflammation and bacteremia are the main mechanisms. However, some epigenetic modifications commonly found in periodontitis are also related to some systemic conditions, leading to another biological mechanism of connection between periodontitis and systemic diseases.

As it has been discussed in previous items, oral dysbiosis, commonly related to periodontitis, is able to produce epigenetic modifications that, in turn, have the capacity to induce some changes in different levels of immune response, both in the innate and humoral system, leading to a chronic response that could be associated with other systemic immune responses or even changes in other sites of the human body.

3.4.1 Obesity

The association between periodontitis and obesity has been studied along years. While a systematic review published by Suvan et al. [58] has established that there are clinical studies that support this association, the magnitude of that association is still unclear, and there is a need for more prospective and intervention studies to understand the connection between both diseases. Despite the fact that recent data has demonstrated that obesity could be a risk factor of clinical attachment loss [59], the biological plausibility of that connection is still not clarified. It has been hypothesized that the high secretion of adipokines in obesity creates a pro-inflammatory state that has been positively associated with periodontitis [60]. A recent publication [61] has showed that the stimulation of macrophages with adiponectin causes the expression of miR-155, an important miRNA in the pathogenesis of periodontitis [62]. Moreover, it has been demonstrated that some miRNAs such as miR-185 were found to be strongly expressed in obese patients with periodontitis compared to non-obese periodontitis patients [62], suggesting that an obese status may aggravate periodontal tissue destruction.

3.4.2 Cardiovascular Disease

The association between periodontitis and cardiovascular disease (CVD) has been a focus of research along the last decades. Last data have established that periodontitis may be a risk factor

to control in CVD patients [57, 63–66]. Bacteremia has been hypothesized as the main biological mechanism that connects both diseases [67, 68]. Nevertheless, recent publications have pointed out a genetic susceptibility contributing to periodontitis and CVD [69]. This publication has remarked some genes that are presented in both conditions. ANRIL, CDKN2A, CDKN2B, and PLG are the most relevant genes [69–72]. In that sense, mRNA transcription of ANRIL, lncRNA, and ANRIL has been associated with atherosclerosis, periodontitis, and several types of cancers [70]. Therefore, it is important to keep investigating the influence of those lncRNAs and genes to fully understand the connection and possible target to treat consequences in both conditions.

3.4.3 Rheumatoid Arthritis

Rheumatoid arthritis(RA) is a chronic inflammatory autoimmune disease that has been extensively related to periodontitis [73, 74]. It has been hypothesized that oral bacteria, such as *Porphyromonas gingivalis*, among others [75], may play a key role in protein citrullination and ACPA formation in RA patients [76, 77], initiating and perpetuating the immune response in RA. Recent reports have shown that also *Aggregatibacter actinomycetemcomitans*, another well-established periodontal pathogen [78], is able to increase chronic exposure to citrullinated proteins and the development of autoantibodies. A number of evidence have shown that periodontitis and RA share a number of genetic and environmental risk factors [79]. The main genetic risk factor is the *HLA-DRB1* allele of the class II major histocompatibility complex (MHC-II), and the smoking habit is a common risk factor between periodontitis and RA [75].

Nonetheless, recent data have shown that periodontitis patients are exposed to citrullinated histone H3 in inflamed gingival tissues, which drives to other exposure target for the autoantibodies presented in RA [80]. It means that not only proteins citrullinated by bacteria are subjected to be a target for autoantibodies, but some epigenetic modifications of that oral dysbiosis increase targets and sources of perpetuating inflammation and citrullination.

3.4.4 Cancer

The role of oral infections in oncogenesis remains changing over time. As discussed above, chronic inflammation such as in periodontitis may have the potential to provoke epigenetic modifications [20] leading to DNA and histone methylations that contribute to oncogenesis. However, any bone modulation will involve these histone modifications [81], and periodontitis, which involves bone loss, may also cause histone modulation [14].

A recent publication by our group [14] has extensively reviewed the plausible role of oral infections such as periodontitis in oncogenesis. We have found different population-based studies, by which a plausible association between periodontitis and different types of cancer could be explained. A longitudinal study reported that serum *P. gingivalis* antibody increased the risk of orodigestive cancer mortality [82]. Likewise, some data showed antibodies to several oral pathogens to pancreatic cancer [83]. It was found that the antibodies to the commensals were associated with lower risk of pancreatic cancer suggesting that dysbiosis may be a more appropriate risk marker than the role of a few pathogens. Dysbiosis on the other hand can be a marker for abnormal immunity which predisposes to cancer development [84]. Moreover, a prospective cohort study that assessed periodontal treatment was associated with lower risk of subsequent cancers, but this study did not adjust for confounding factors such as smoking, alcohol consumption, or genetics [85]. Despite some methodological flaws of those studies, there is a plausible role of periodontal disease in the oncogenesis process.

As it has been discussed above, epigenetics might play a significant role in different host immune processes as well as other cell functions. During the process of tumor initiation and progression, the cancer epigenome is remodeled via global

hypomethylation, increased promoter methylation at CpG islands, global downregulation of miRNAs and lncRNAs, or interactions between them and alterations in the nucleosome. The imbalance between transcriptionally permissive and repressive chromatin modifications may alter gene expression and lead to cancer [14]. While DNMT1 is overexpressed in many cancers [86], oral dysbiosis drives to a reduced expression of DNMT1. Thus, the assumption that oral dysbiosis may foster oncogenesis might not be supported. On the other hand, histone acetylation has been shown to regulate tumor suppressor gene p53 or proto-oncogene c-Myb. These indicate that histone acetylation can up- or downregulate oncogenesis [87].

Notably, the role of miRNAs in oncogenesis varies depending on the mRNA they regulate and thus can be promoters or suppressors of oncogenesis [88]. Some miRNAs commonly found in periodontitis have been related to different types of cancers. MiR-31 may be found in pancreatic cancer or oral potentially malignant disorder [89, 90]. Moreover, miRNAs, miR-146a and miR-155, were related to head and neck squamous cell carcinoma [91]. Nonetheless, certain lncRNAs have been also correlated with different types of cancers. The lncRNA called HOTAIR, upregulated in periodontitis lesions, has been associated with tumor metastasis, recurrence, and prognosis in breast, colon, and liver cancers and oral squamous cell carcinoma [54, 92, 93].

However, further studies are needed to understand how they may interact between both conditions and to examine the role of oral infections in carcinogenesis via the holistic approach considering the multisystem in the whole human body (Tables 3.1, 3.2, 3.3, 3.4, and 3.5).

Table 3.1 Principal miRNAs in periodontitis lesions adapted from the publication of Kebschull et al. [7]

	Regulation periodontally affected tissues		Type of immune system affected	
miRNA	Upregulated	Downregulated	in periodontitis	Mechanism
miR-30e	–	Yes	Innate immune system	Inhibits NK cell activation. This downregulation increases NK cell activation and hence increases tissue destruction
miR-31	–	Yes	Innate immune system	Negative regulator of NF-κB and mediates osteoclastogenesis. Its downregulation produces over-activation of TLRs and decreases bone formation
miR-146a	Yes	–	Innate and adaptive immune system	Regulates NF-κB signaling pathway activation, reduces dendritic cell cytokine production, impairs dendritic cell TLRs, and controls B-cell development
miR-148a	Yes	–	Innate and adaptive immune system	Impairs antigen presentation function by dendritic cells and the whole innate response
miR-155	Yes	–	Innate and adaptive immune system	Regulates NF-κB signaling pathway, mediates type I interferon and interferon-gamma production; increases TLR sensitivity, critical in dendritic cell maturation; controls CD8 T-cell response; and indirectly influences the activation of T-helper cell 17
miR-200a	–	Yes	Innate immune system	Negative regulation of IL-12 in NK cells. Thus, its downregulation causes increased production
miR-210	–	Yes	Adaptive immune system	Its downregulation increases T-cell signaling
miR-451	Yes	–	Innate immune system	Suppression of neutrophil chemotaxis
miR-486	Yes	–	Innate immune system	Increases exponentially NF-κB signaling pathway
miR-650	Yes	–	Adaptive immune system	Regulates B-cell proliferation

Table 3.2 Main lncRNAs in periodontally affected gingival tissues

lncRNA	Regulation periodontally affected tissues		Type of immune system affected in periodontitis	Mechanism
	Upregulated	Downregulated		
HOTAIR		Yes	–	Still unknown
RP3-461P17 RP1-300I2.2	–	Yes	–	Still unknown
TIRAP	Yes	–	–	Still unknown
CDKN2A	Yes	–	–	Still unknown
CDKN2B	Yes	–	–	Still unknown
lincRNA-CDON-1	Yes	–	Innate immune system	Affects signaling pathways of TLR activation
ANRIL	–	Yes		Regulation of chromatin, ADIPOR1, VAMP3, and C11ORF10 expression. Its downregulation provokes reduced expression of those markers, increasing the risk of atherosclerosis, metabolic syndrome, periodontitis, and several forms of cancer

Table 3.3 Some examples of methylations in cancer expression

Author/year	Country	Type of cancer involved	Epigenetic modification	Results		Mechanism
				Upregulation of cancer	Downregulation of cancer	
[94]	Belgium	Melanoma	DNMT1	Yes	Yes	Transient depletion of DNMT1 can lead to long-term activation of cancer-germline genes and repression of mitosis/division-related genes at the same time
[95]	USA	Colon cancer	DNMT1	–	Yes	Interaction between a subset of lncRNAs and DNMT1 was reduced in colon cancer cells, which contributes to aberrant DNA methylation and gene expression in tumorigenesis
[96]	USA	Lung cancer	DNMT1	Yes	–	There is a cross talk between tyrosine-protein kinase KIT and DNMT1 in the development of drug resistance, which implies an upregulation of oncogenesis process by means of that interaction
[97]	China	Breast cancer	DNMT1	Yes	–	DNMT1, DNMT3A, and DNMT3B commonly or individually contributed to DNA methylation in different breast cancer cells

Table 3.4 Some examples of histone acetylations in cancer expression

Author/ year	Country	Type of cancer involved	Epigenetic modification	Results		Mechanism
				Upregulation of cancer	Downregulation of cancer	
[98]	China	Hepatocellular carcinoma	Histone acetylation	–	Yes	Histone deacetylase (HDAC) 9 increased the expression of miR-376a by upregulating the global histone H3K18 acetylation level, which is inversely correlated with hepatocellular carcinoma
[99]	Poland	Colorectal cancer	Histone acetylation	Yes	–	Histone H3 lysine 27 acetylation (H3K27Ac) is upregulated in CRC
[100]	Germany	Lymphoma, hepatoma,	Histone acetylation	Yes	–	The 5-HTT gene is epigenetically downregulated by histone deacetylation. The 5-HTT gene is usually silenced in several types of cancer

Table 3.5 Some examples of miRNAs and lncRNAs in cancer expression

Author/ year	Country	Type of cancer involved	Epigenetic modification	Results		Mechanism
				Upregulation of Cancer	Downregulation of Cancer	
[90]	USA	Pancreatic cancer	miR-31	Yes	–	Expression of enforced miR-31 significantly enhanced invasion and migration of multiple pancreatic cancer cells
[89]	Taiwan	Oral potentially malignant disorder	miR-31	Yes	–	Epithelial dysplasia and miR-31 upregulation synergistically predict the increased incidence of recurrence and/or malignant transformation in patients with OPMD. Detection of miR-31 expression is an adjuvant method for screening of high-risk OPMD
[91]	Germany	Head and neck squamous cell carcinoma	miR-146a and miR-155	Yes	–	Downregulation of miR-146a and miR-155 in blood of patients correlated with the occurrence of distant metastasis regarding tumor patients
[93]	China	Oral squamous cell carcinoma	Long noncoding RNA-HOX transcript antisense intergenic RNA (HOTAIR)	Yes	–	HOTAIR was highly expressed in OSCC tissues and facilitated the growth of OSCC cells, thus probably being an eligible molecular marker for OSCC diagnosis and prognosis determination
[93]		Oral squamous cell carcinoma	Long noncoding RNA-HOX transcript antisense intergenic RNA (HOTAIR)	Yes	–	Overexpression of HOTAIR indicated poor overall survival in OSCC patients. Knockdown of HOTAIR in OSCC cells decreased cell proliferation and colony formation, increased cell invasion and migration, and induced apoptosis in vitro

3.5 Concluding Remarks

Epigenetics is a new field that bridges genetics with environment, adapting the host response to the bacterial infection. The interaction between genetics, immunity epigenetics, and inflammation might play major roles in periodontitis, connecting it with systemic conditions. However, further studies are needed to explore these bridges, their impact and functions, not only in periodontitis and systemic conditions that have been drawn in this chapter but also with other autoimmune diseases.

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Topic: aMMP-8 Oral Fluid PoC Test

4

Solomon O. Nwhator, Anna-Maria Heikkinen, Taina Tervahartiala, Dirk-Rolf Gieselmann, Jussi Leppilahti, and Timo Sorsa

Abstract

There is rising interest about influences of periodontal diseases on systemic health, while traditional measures cannot quantify periodontal inflammation. Confusing case definitions make the need to quantify periodontal inflammation greater than ever. Modern measures of periodontal inflammation depend on metabolism-mediated changes in salivary metabolites (salivary metabolomics)—an extra mile in salivary diagnostics.

Matrix metalloproteinase-8 fits well into the latest model of chronic periodontitis polymicrobial synergy and dysbiosis (PSD). Once instituted, host-derived mediators of inflammation perpetuate periodontal inflammation through cytokines and proteolytic enzymes including MMP-8, especially in its active form (aMMP-8). aMMP-8 correlates well with periodontal disease severity and activity, is effective in disease monitoring, and is the most abundant host-derived collagenolytic matrix metalloproteinase (MMP) in the saliva, mouth rinse, gingival crevicular and periimplant sulcular fluids.

Based on these characteristics, Finnish periodontologists together with German and Finnish biotechnology companies invented a novel aMMP-8 chairside test kit using unstimulated whole mouth rinse as a substrate. The point-of-care (PoC) kit measures periodontitis quickly and accurately through a simple color change. The test detected initial periodontitis among adolescents, as well as chronic periodontitis in a variety of clinical scenarios relevant to important reproductive health parameters and had high validity and reliability. When applied to adult Nigerian and adolescent Finnish subjects, researchers stumbled upon a chance discovery—raised aMMP-8 level among close to 90% of Nigerian pregnant women.

S. O. Nwhator
Department of Preventive and Community Dentistry, Faculty of Dentistry, College of Health Sciences, Obafemi Awolowo University, Ife, Nigeria

A.-M. Heikkinen · T. Tervahartiala · J. Leppilahti
Department of Oral and Maxillofacial Diseases, Head and Neck Center, Helsinki University Hospital, University of Helsinki, Helsinki, Finland

D.-R. Gieselmann
Division of Periodontology, Dentognostics GmbH, Jena, Germany

T. Sorsa (✉)
Department of Oral and Maxillofacial Diseases, Head and Neck Center, Helsinki University Hospital, University of Helsinki, Helsinki, Finland

Division of Periodontology, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden

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4.1 Introduction

The desire to classify periodontal diseases and measure periodontal inflammation is as old as its indices, and there are close to 50 case definitions in the literature [1]. Despite this, adequate quantification of periodontal inflammation has remained elusive until recently. Links between chronic periodontitis and systemic conditions are the subject of huge research efforts. Because of the complexity of these periodontal-systemic associations and conflicting scientific reports, most of the associations remain speculative.

As long as we continue to depend on traditional classifications and indices, the periodontal-systemic associations will stay speculative. Millions of patients will continue to suffer the consequences of these associations, while academic arguments continue due to the inherent weakness of traditional indices. The better choice is for periodontologists to limit traditional indices to clinical descriptions of disease severity and embrace the use of inflammatory mediators. This requires a total paradigm shift, looking beyond periodontal pockets/attachment loss and embracing measurable continuous indices of periodontal inflammatory burden.

Of all available indices, only one; the periodontal inflamed surface area (PISA) [2] currently has such a power to precisely relate inflammatory burden to remote diseases. The message is clear; though redness, bleeding, deep pocket, attachment loss, and others are useful, they only signify past and static destruction but terribly fail to quantify and predict ongoing or future inflammation in real time. There is a need to adequately and dynamically measure periodontal inflammation using parameters/indices/mediators that take the pathogenesis of periodontitis and gingivitis/pre-periodontitis into consideration. Depending on plaque levels alone is futile since the degree of inflammation varies between low and high responders depending on genetically-determined levels of response.

We now know that individual response to inflammatory stimulus is affected by levels of proinflammatory cytokines and their balance against anti-inflammatory cytokines [3, 4]. Since it is also known that certain polymorphisms affect response levels, the most pragmatic approach should be to quantify these mediators of tissue destruction. Adequately measuring the mediators of periodontal tissue destruction is even more important because these mediators remain elevated even after antibiotic and/or manual therapy in certain individuals [4]. Targeted anti-inflammatory therapies should be selected in high responders. It has even been suggested that a proactive investigation of measuring increased inflammation susceptibility will help in ensuring more targeted, specific periodontal interventions with predictable outcomes [4]. This paradigm shift will also ensure that additional immunostimulation is instituted in low responders but avoided in high responders.

The current management protocols adopt a "one size fits all" approach which is only modified after they fail woefully in addressing periodontal inflammation. A bold attempt at ensuring this paradigm shift came with pockets of attempts at metabolomics in periodontics. Defined as a study of metabolites or small molecules generated by the process of metabolism [5], saliva gives a great opportunity for metabolomics in periodontitis. Salivary metabolomics has several benefits including early detection of pathologic conditions especially in oral cancer and periodontal diseases. Both conditions share a common fate of late presentation and accompanying poor prognosis. The simple and consistent composition of saliva and the fact that it can be collected noninvasively with a minimal chance of nonspecific interference make metabolomics a great alternative [5].

Blood/serum is useful for many biochemical assessments, but saliva gives a more objective reflection of physiological processes in the oral cavity. It is imperative to overcome the limitations of salivary metabolomics which include susceptibility to "noise" (due to its great sensitivity, complexity), and high setup costs. The need for complex informatics and multiple analytical platforms also scares intending researchers into this area [5]. Salivary metabolites can also be altered by age, sex, collection procedures, circadian rhythm, gland stimulation, diet, sample

integrity, storage conditions, sample volume, and others [5]. Despite these challenges, salivary analytes are stable and cost-effective for storage, while their collection is pain-free without the anxiety and risk of infection associated with venipuncture. Obtaining multiple follow-up samples is also easy and point-of-care technologies becoming increasingly available with easy metabolite identification and no need for preprocessing [5].

The challenges of salivary metabolomics are not enough reasons to give up on the use of salivary metabolites/markers. A point-of-care system overcomes the complexities of preprocessing, and measuring the right marker ensures adequate quantification. To be valid, the method of quantification of periodontal inflammation needs to justify its choice of marker. It also needs to compare well with traditional measures of periodontal inflammation while surpassing them in specificity, reproducibility, predictability, and accuracy. Cytokines especially interleukin-1, interleukin-6, and interleukin-10 and matrix metalloproteinases (MMPs) are of particular interest to periodontics due to their undeniable roles in the pathogenesis of chronic periodontitis [4].

Matrix metalloproteinases (MMPs) are a family of structurally related but genetically distinct zinc-dependent proteolytic enzymes. They are metalloendopeptidases which can destroy the extracellular matrix and basement membrane components. Acting in synergy, they regulate several inflammatory responses [6]. Of the growing number of MMPs, few are relevant to chronic periodontitis of which MMP-8, MMP-9, and MMP-13 are of special interest. MMP-8 is the most important collagenolytic MMP. Apart from being the most abundant MMP present in gingival and periodontal tissues, MMP-8 plays an important role in the pathogenesis of chronic periodontitis especially in its active form. Active or activated MMP-8 correlates with active phases: periodontitis and peri-implantitis [7–9]. There is also a strong correlation between the severity of chronic periodontitis and active MMP-8 (aMMP-8) which results from the cleavage of the latent inactive proenzyme/zymogene at the N-terminal peptide [7, 8]. aMMP-8 also correlates with the severity of chronic periodontitis, is effective for early diagnosis, and is relevant for monitoring periodontal health [10]. aMMP-8 levels in oral fluids (saliva, mouth rinse, gingival crevicular and peri-implant sulcular fluids) precede and predict the attachment loss [7–9].

Active MMP-8 thus meditates irreversible tissue destruction associated with periodontitis and peri-implantitis, a phenomenon described as the best-known example of site-specific "unwanted tissue destruction" [7, 8]. For periodontal health, the tissue destructive activity of aMMP-8 is balanced/kept in check by its corresponding inhibitor—the tissue inhibitor of matrix metalloproteinase (TIMP-8). The host response to the polymicrobial synergy of the dental biofilm and the eventual derangement/dysbiosis lead to the dysregulation of the balance MMPs and TIMPs. Based on overwhelming evidence, one of such secretions (neutrophil collagenase-2 or MMP-8) is implicated in the pathogenesis of chronic periodontitis. The aMMP-8 has been widely investigated as a marker of periodontal inflammation and destruction [9, 11]. Current evidence suggests, therefore, that any imbalance in favor of active neutrophil collagenase-2/aMMP-8 will cause collagenous matrix degradation which will result in the loss of periodontal supporting tissue, i.e., active periodontal degeneration, the hallmark of tissue destructive periodontitis [9, 12].

4.2 Periodontal Health Versus Periodontal Inflammation and Clinical Measuring

Our understanding of periodontal health and disease/inflammation has been radically altered. This is in the light of current evidence which suggests that the previous notion of a complete absence of inflammation in periodontal health is a mere mirage. Current evidence suggests a fragile balance of pro- and anti-inflammatory activities. This view is supported by the finding that inflammatory cells are present in apparently "periodontal healthy" patients in healthy gingival and periodontal tissues [13, 14]. This fragile

balance exists between proinflammatory mediators and their anti-inflammatory counterparts resulting in a dynamic or equilibrium [15]. As long as this "uneasy" balance is maintained, the clinical observation will be that of "clinically healthy" periodontal tissues. It becomes imperative for clinicians to bear in mind while making this diagnosis that "clinically healthy" connotes the idea of "clinically undetectable" rather than "completely absent" inflammation. The fact that inflammation could be present but be clinically undetectable underpins the limited usefulness of traditional measures, case definitions, and characterization of inflammatory, biofilm-induced periodontal diseases, namely, chronic gingivitis and periodontitis. Traditional diagnostic terms are majorly descriptive, a good example being inflammation limited to the margins of the gingiva, the so-called chronic marginal gingivitis (Fig. 4.1). Despite obvious limitations, these traditional measures are by no means useless; they are in fact useful in terms of clinical description of disease. However, their usefulness goes no further except when they can be extrapolated to reflect adequate quantification of periodontal inflammation as achievable through PISA. Beyond this, the limited usefulness of traditional measures of chronic periodontitis is becoming increasingly obvious.

To effectively manage those "difficult to explain" cases, periodontologists must embrace the shift to the measurement of periodontal inflammation through inflammatory markers and salivary metabolomics. The innovation of point-of-care kits makes this even more versatile in everyday management of inflammatory periodontal diseases. The neutrophil collagenase-2 (aMMP-8) is a promising point-of-care system in this regard.

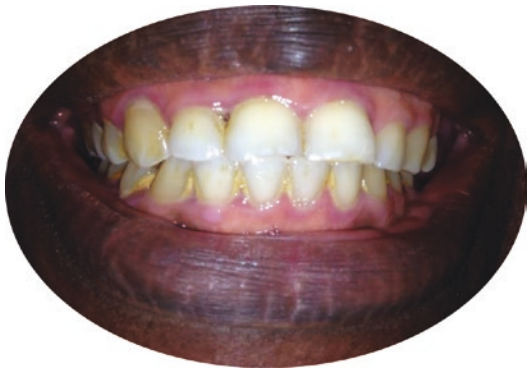

Fig. 4.1 Inflammation limited to the gingival margin in a female adult African

4.3 Validity, Sensitivity, and Specificity of Neutrophil Collagenase-2 (aMMP-8) System

The long history of dependence on clinical measures of periodontal inflammation cannot be easily discarded. The gradual paradigm shift will be expedited by validating the claims of these novel systems and ascertaining their sensitivity, specificity, and reliability. Recently there are published studies, which have investigated the validity, sensitivity, specificity, reliability, and reproducibility of a novel point of care (aMMP-8 chairside test). The studies had satisfactory results concerning initial periodontitis in Caucasian adolescents as well as chronic periodontitis among nigerians [16, 17].

The high validity of the novel system was reflected in its being 96% sensitive for poor oral hygiene, 95% sensitive for chronic periodontitis with at least two with periodontal pockets, and 82.6% sensitive for at least two sites with bleeding on probing (BOP) [16]. The validity of this novel system was replicated in Finnish adolescents with the sensitivity of 63.6% and a specificity of 100% [17]. These results among adolescents helped to confirm the repeatability (reliability) of the results (Fig. 4.2). Furthermore, according to Heikkinen et al. (2017), aMMP-8 chairside test detects initial periodontitis in adolescents with predisposing genetic background with inflammatory mediators, especially for genetic polymorphisms of matrix metalloproteinase-3 (MMP-3) and vitamin D receptor (VDR) [18, 19]. The same study group observed just lately that only TLR4 (rs498670) and TNFSF11 (rs2277438) genes could have a positive correlation with radiological findings suggestive of initial periodontitis after adjustment for smoking and visible plaque [20].

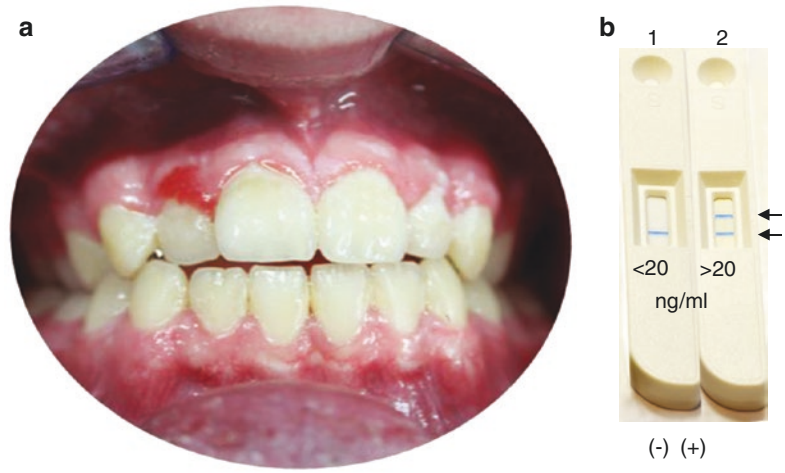

Fig. 4.2 (**a**) Inflammation in a 16-year-old boy aMMP-8 test slightly positive. (**b**) PerioSafe—test analysis of negative (1) <20 ng/ml and positive (2) >20 ng/ml adolescent patient with initial periodontitis

With the high validity and reliability of the novel aMMP-8 chairside test being established, the applicability to general health parameters was investigated, namely, reproductive health parameters [21–23].

4.4 Justification for aMMP-8 Chairside Test in Reproductive Health Parameters

The possible link between chronic periodontitis and reproductive health parameters predates the present century. The concerns have existed for over one century. This was expressed by the early focal infection theory of Charles Rosenow in 1909 [24], the focal allergy theory of Berger's in 1939 [25], and Slauk's focal toxicosis theory of the 1940s [26]. The "premature birth" of those theories ensured their "early death," but the ideas resurfaced almost half a decade later with concerns raised by the finding of Linossier and coworkers 1982 [27]. It was their isolation of sperm immobilizing factor from necrotic dental pulp which probably fueled the awakened interest within a few years. This time, the investigators were neither dentists nor periodontologists; they were in fact gynecologists =Bieniek and Riedel.

In 1986, Bieniek and Riedel [28] were curious about the finding of antibiotic-resistant bacteriospermia among patients with chronic periodontitis. This relationship between chronic periodontitis and fertility was again neglected until another decade with the landmark work of Offenbacher and colleagues linking preterm birth with chronic periodontitis in 1996 [29]. Later, Kavoussi and coworkers [30] reported links between chronic periodontitis and endometriosis in 2009 which was followed up by Kligner and coworkers who reported sperm motility in 2011 [31]. Hart in 2012 reported a link between delayed conception [32], while Oguz reported erectile dysfunction in relation to chronic periodontitis in 2013 [33].

These findings necessitated a search of the literature for associations between chronic periodontitis and specific reproductive parameters. The relationship between chronic periodontitis and reduced libido is unclear with possible mechanisms including the arginine link. A direct relationship exists between arginine—a direct nitrous oxide precursor—and libido in men and men and women [34, 35]. In men, arginine is required for achieving and maintaining penile erection making the enhancement of arginase activity by *P. gingivalis* of great importance [36, 37]. A critical biochemical pathway necessary for male sexual arousal is short-circuited by *P. gingivalis* [38]. The fact that salivary arginase activity increases in patients with chronic periodontitis while periodontal therapy reduces activity explains the link [39].

4.5 Effect of Chronic Periodontitis on Erectile Dysfunction and Sperm Count

The arginine link already explains partly the link between chronic periodontitis and erectile dysfunction which according to the American Sexual Health Association is the inability to maintain an erection suitable for intercourse [40]. Other mechanisms involved the effect of a proinflammatory state cause of host-derived mediators of chronic periodontitis. Prominent among these mediators is the proinflammatory state promoted by tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 and IL-6 [41, 42] which promotes endothelial dysfunction and injury resulting in erectile dysfunction through the vasculogenic pathway [43, 44].

Colagar and coworkers reported a link between lipid peroxidation/decreasing total antioxidant capacity and low sperm count [45]. Raised levels of IL-6, a mediator of chronic periodontitis, are linked with lipid peroxidation explaining a possible link between chronic periodontitis and reduced sperm count [46].

Kligner and others reported an association sperm sub-motility and chronic periodontitis [31].

4.6 Association Between Periodontitis and Conception

Associations between endometriosis [30], pelvic inflammatory disease [47], and increased time to conception [32] have been reported in the literature. Chronic periodontitis influences pregnancy outcomes through two possible mechanisms, first a direct access by periopathogens and second by mediators of chronic inflammation [48]. *F. nucleatum* "a potential accessory pathogen" facilitates the colonization of periodontitis-associated bacteria [31] through a "gate opener" effect. Once it translocates to extraoral sites, the "potential accessory pathogen" of *F. nucleatum* changes into an "overt pathogen" status [49].

Evidence exists for the direct hematogenous spread of *F. nucleatum* found in the subgingival biofilm of a stillborn infant whose mother had pregnancy-associated gingivitis [50]. It also crossed the endothelium to access the fetal-placental compartment in experimental models using E-cadherin-binding FadA adhesin and TLR4-dependent necroinflammatory response [51]. *P. gingivalis*, probably the most important periopathogen, induces fetal loss through its cardiolipin-specific antibody production [50–52].

4.7 Emerging Racial Differences on Chronic Periodontitis and Pregnancy Outcomes

The literature appears indifferent and at best equivocal on the effect of race on chronic inflammation especially as it relates to periodontitis. African human umbilical vein endothelial cells showed greater oxidative stress and inflammation in vitro with higher nitrous oxide expression [53]. Some workers have reported a reduced inflammatory response among Africans due to "chronic conditioning or priming of innate immunity" leading to attenuated inflammatory responsiveness [54]. Africans put up a lower inflammatory response to endotoxin [55]. This, however, might not predict acute stress response in innate immunity-mediated diseases but might apply to acute inflammation rather than the chronic endotoxemia in periodontitis [55].

Interleukin-6 (IL-6) and interleukin-10 (IL-10) relationship is important in the pathogenesis and response level in chronic periodontitis [4]. Raised interleukin levels are not enough to explain levels of without a relatively low level of the anti-inflammatory IL-10 [4]. Raised IL-6 levels have been reported, but the same study failed to demonstrate racial differences in IL-10, TNF-α, and C-reactive protein [56]. Their finding partly explains the elevated aMMP-8 among nine

of ten Nigerian pregnant women assessed with a novel aMMP-8 chairside test [21]. Since MMP activity increases in the prepartum period [57], raised MMP-8 levels are important for preterm birth. The findings of Nwhator and coworkers in 2015 [21] are therefore an important step toward understanding racial differences in preterm birth deserving further investigation.

The mechanisms mediating delayed conception and chronic periodontitis [21, 32] are unclear, and the dearth of literature on the topic is not surprising considering its novelty. However, a direct relationship exists between levels of tissue inhibitor of metalloproteinase (TIMP) and successful conception post-in vitro fertilization [58]. The likelihood of the reverse scenario, increased time to conception by raised MMP-8 levels, deserves further investigation. To further explore the mechanisms of association between reproductive health parameters and periodontitis, a novel aMMP-8 chairside test was employed amidst several clinical situations among men and women in Nigeria.

4.8 Role of aMMP-8 Chairside Test in Specific Periodontal Health Parameters

The widespread elevation of aMMP-8 among black pregnant Nigerians affecting almost 90% of women independent of demographics, educational level, and trimester was a surprising chance finding [21]. The workers further investigated the possible association between chronic periodontitis and increased time to conception among nonpregnant [22] fertility clinic attendees trying for pregnancy and 70 pregnant controls. The odds of increased time to conception were higher with suffering from periodontitis assessed with a novel qualitative aMMP-8 chairside test.

The authors had earlier investigated the effect of chronic periodontitis on seminal fluid parameters using a novel aMMP-8 chairside test and reported a significant association between subnormal sperm count and poor oral hygiene and across all age groups [23]. The sensitivity of the aMMP-8 test kit from the Nigerian study was 95% for periodontitis, 96% for poor oral hygiene, and 82.6% for bleeding on gentle probing (Table 4.1). All stated sensitivity values of the aMMP-8 test kit were for two sites with periodontal pockets or bleeding on gentle probing among adults. Values among adolescents were similar with 63.6% sensitivity and 100% specificity. It would be useful to tailor future case definitions along these lines. As stated earlier, elevated aMMP-8 levels were detected among approximately one of every ten (87%) of pregnant Nigerian women using a novel qualitative aMMP-8 chairside test kit [21]. The novel aMMP-8 chairside test kit also helped to detect chronic periodontitis in association with increased time to conception. Using the same novel aMMP-8 chairside test kit, workers were able to detect an association between reduced sperm count and poor oral hygiene [21].

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Table 4.1 Comparing the aMMP-8 test and bleeding on probing (BOP) and periodontal pockets

Periodontal parameter	Sites involved	aMMP-8 test +	aMMP-8 test −	Total	Sensitivity	*P*-value
BOP	≥2	19	4	23	82.6	
	<2	6	15	21	28.6	0.000
Periodontal pockets[a]	≥2	18	1	19	94.7	
	<2	34	23	57	59.6	0.003

The aMMP-8 test[b] was highly sensitive for at least two sites with BOP (82.6%, $p = 0.000$) and periodontal pockets (94.7%, $p = 0.000$). [b]PerioSafe[a] at least 4 mm deep periodontal pockets

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Composite Biomaterials: From Lab to Clinics

5

Pekka K. Vallittu

Abstract

Although metals, ceramics, and particulate filler resin composites have successfully been used as dental and also medical biomaterials for decades, devices made out of these materials do not meet all clinical requirements and the present understanding of the importance of minimally invasive dentistry. For instance, preparation of large amounts of tooth substance is needed, the high-strength materials lack property to be shaped in situ, and metal objects may interfere with some medical imaging systems (computer tomography, magnetic resonance imaging). There has been a lot of development in the field of composite biomaterials, which has focused to a large extent on biodegradable composites. Less focus has been paid to biostable composites although they could provide lots of benefits over the existing biomaterials. This chapter reviews the rationale of using biostable glass fiber-reinforced composites (FRCs) in several dental and surgical applications from restorative and prosthetic dentistry to cranial surgery. Biostability of dental restorations and implantable medical devices is still important to ensure success of the treatment in short and long term. Materials mechanical properties, biocompatibility, and possibility to add bioactive components to dental and surgical implants alongside with the clinical experience suggest that FRC materials are a relevant new group of biomaterials for clinical medicine and dentistry. FRCs with continuous or discontinuous glass fibers in biostable thermoset resin matrix provide high-strength and high-toughness nonmetallic biomaterial. By adding bioceramics, such as bioactive glass, to the FRC construct, the material combination supports osteogenesis and vascularization and provides antimicrobial properties, for example, to the implant. Material combination of FRC and bioactive glass is used clinically in cranioplasty and cranio-maxillofacial implants, and they have been investigated also as oral and orthopedic implants. This is a journey from lab to clinics.

5.1 Introduction

Due to several medical and dental application-related requirements and practical reasons, there is an ongoing trend toward using nonmetallic biomaterials. Biodegradable and biostable medical and dental composite materials have been

P. K. Vallittu
Department of Biomaterials Science, Turku Clinical Biomaterials Centre—TCBC, Institute of Dentistry, University of Turku, Turku, Finland

City of Turku, Welfare Division, Turku, Finland
e-mail: Pekka.vallittu@utu.fi

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developed considerably in recent decades [1, 2]. Currently, they are used in large scale in dentistry, but they can be used clinically also in some applications in reconstructive medicine. Dental reconstructions in restorative dentistry and prosthodontics utilize particulate- and fiber-reinforced resin composites (FRCs). In medical implantology, numerous different polymeric materials, such as polyethylene (PE), polymethyl methacrylate (PMMA), and polyetheretherketone (PEEK), have been and are under investigation and are also used clinically, but these materials have not proven to be perfect solution for bone reconstruction. In Europe, one million bone transplantations and worldwide 2.2 million bone graft procedures are annually performed. In particular, the need for skull reconstructions, i.e., cranioplasties, is increasing only due to an increase in decompressive craniectomies. There are large number of infections which relate to autologous bone flaps and implants of polymeric materials [3–6]. The current golden standard for cranial reconstruction is autologous bone grafting or use of titanium plates and meshes. Yet, a common complication (up to 81%) is autologous bone flap resorption due to nonviability of the flap osteocytes. Metals do have other clinical shortcomings: in long bone applications, high stiffness of metals causes stress shielding and can lead to loosening of implant. In cranial surgery, metals have limitations of imaging and radiation therapy [7].

This chapter describes fundamental properties of FRC materials which explain their suitability for dental and medical biomaterials. Tables 5.1 and 5.2 list clinically and technically relevant material properties for presently used dental and medical biomaterials. FRC materials are new group materials which have been developed and clinically tested during the last 25 years of time. Principles behind the development of FRC materials are in resolving clinical problems of bulk metals, ceramics, and polymers. Development has progressed from the level of low medical risk applications to the applications of higher risks and more demanding regulatory issues to be

Table 5.1 Clinically and technically important properties of solid biomaterials which have been used in restorative and prosthetic dentistry as bulk materials

Property	Co-Cr	Ti	Au	GC	Al_2O_3	YZP	PFR	FRC
Isotropicity	+	+	+	+	+	+	+	+/–
Anisotropicity	–	–	–	–	–	–	–	+/–
Toughness	–	+/–	+/–	–	–	–	–	+
Flexural strength	+	+	+/–	–	+/–	+	–	+[a]
Isoelasticity with dentine	–	–	–	–	–	–	+/–	+[b]
In situ[c] processability	–	–	–	–	–	–	+	+
Thermal isolation	–	–	–	+	+	+	+	+
Radiopacity	+	+	+	+/–	+	+	+/–	+/–
CT compatible	–	–	–	+	+	–	+	+
Milling processability	+	+	+/–	+	+	+	+	+
3D printable	+	+	–	–	–	–	+	–
Technical bonding[d]	+/–	+	+/–	+	–	–	–	+[e]
Thermal expansion match[f]	–	–	–	+	+	–	–	+/–[g]

Co-Cr cobalt-chromium alloys, *Ti* titanium and its alloys, *Au* gold alloys, *GC* glass ceramics, *Al_2O_3* aluminum oxide, alumina, *YZP* yttrium oxide-stabilized zirconium oxide, *PFR* particulate filler resin composite, *FRC* fiber-reinforced composite

[a]Continuous unidirectional FRC

[b]Glass FRC

[c]In situ processability = possibility to be formed and molded in direct applications

[d]Technical bonding = bonding to resin luting cements via surface conditioning

[e]FRC with interpenetrating polymer network (IPN) polymer matrix

[f]With dentine 10 [($^oC^{-1}$) × 10^6]

[g]+ along the direction of fibers, – perpendicular to fibers

Table 5.2 Clinically important properties of solid biomaterials which have been used in cranioplasty implants excluding in situ cured bone cements (modified from [94])

Property	AB	Titanium	HA	TCP	BGS53P4	PEEK	PMMA	PE	FRC-BG
Resorbability	+/−[a]	−	+	+	+	−	−	−	+/−[b]
Osteoconductivity	+/−[a]	+	+	+	+	−	−	−	+
Osteoinductivity	+/−[a]	−	+	+	+	−	−	−	+/−[c]
Neovascularization	+/−[a]	−	+	+	+	−	−	−	+/−[c]
Flexural strength >600 MPa	−	+	−	−	−	−	−	−	+
Thermal isolation	+	−	+	+	+	+	+	+	+
Bone-like radiopacity	+	−	+	+	+	−	−	−	+
MRI compatible	+	+/−	+	+	+	+	+	+	+
Antimicrobial	−	−	−	−	+	−	−	−	+
In situ moldable	−	+/−	−	−	−	−	−	−	−
Overlay structure	−	+	−	−	−	+/−	−	+/−	+

AB autologous bone, *HA* hydroxyapatite, *TCP* tricalciumphosphate, *BG* bioactive glass S53P4, *PEEK* polyetheretherketone, *PMMA* polymethylmethacrylate, *PE* polyethylene, *FRC-BG* thermoset glass fiber-reinforced composite with BG S53P4, *MRI* magnetic resonance imaging

[a]Depending upon the biointegration of the bone flap

[b]FRC, not resorbable; BG S53P4, resorbable

[c]FRC, no; BG S53P4, yes

filled. Thus, research started from removable dentures and have ended until now to clinically used bioactive FRC cranial implants. This has been a journey from lab to clinics.

5.2 Dental Applications for FRC

Development of new biomaterials toward clinical use has to follow regulations which are covering medical devices and biomaterials in Europe and worldwide. Risks, which relate to the newly developed biomaterials, can be controlled by selecting the first applications to be short term of use or the device to be removable in nature. Development of FRC has begun by introducing FRC for removable dental prostheses [8–13]. It took almost 40 years until the FRCs were started to be used in larger scale clinically. Delay in getting FRC for clinical use was due to problems in combining resin systems to reinforcing fibers, in difficulties in handling the FRC technically, and in rebuttal of accepting new type of materials by clinical dental profession and dental laboratory technicians. However, development of the FRC with new type of resin systems and understanding of designing principles behind of constructing devices, and the clinical experience, have led to the use of FRCs in a variety of disciplines and applications: in removable prosthodontics [14–18], fixed prosthodontics [19–47], restorative dentistry [46–61], periodontology [39, 62], root canal systems [63–73], orthodontics [74, 75], and in repairs of fixed prostheses [76, 77]. Critical evaluation of the available FRC materials and correct patient selection are of importance for successful use of the material.

What is rationale to use FRCs in dentistry? Although there are several proven dental materials and treatment options based on conventional dental materials, a large number of the partially edentulous patients are not treated by fixed dental prostheses to replace their missing teeth or to repair their damaged biting function. This is often due to high cost of the state-of-the-art type of treatments by fixed prostheses and due to irreversible damage by the treatment when creating space for metal and ceramic crowns by cutting enamel and dentine of abutment teeth. It is paradoxical that the dental profession is removing the most durable bonding site of teeth simply due to limitations of processing properties of conventional and some novel dental materials. Although some novel nonmetallic alternative materials, such as yttria-stabilized zirconia, have become available, the use of zirconia requires cutting equal or even larger amount of enamel and dentine of abutment tooth

than by using conventional porcelain-fused-to-metal material combinations.

To have an ideal material for dental restorations, it should be moldable in situ, it should form durable adhesion to the underlaying tooth substrate, and it should provide high strength and high toughness after being processed. From the material science perspective, FRCs fulfill these requirements. FRC is a material combination of polymer matrix and reinforcing fibers. Fibers of the composite are the reinforcing phases in the system when the load is applied to the composite. Load is transferred to be carried by the fibers, and the material becomes strong and tough (Fig. 5.1). The reinforcing fibers can be continuous unidirectional (rovings), continuous bidirectional (weaves), continuous random-oriented (mat), or discontinuous-oriented random fibers.

FRC has properties, which relate to the direction of fibers: FRC can be isotropic, orthotropic, or anisotropic. The mechanical, optical, and thermal properties of the FRC are dependent on the fiber quantity and orientation. A high-quality glass FRC material with high fiber quantity (up to ca. 65 vol%) provides high flexural strength (with E-glass ad 1250 MPa) [66]. A positive correlation exists between water sorption of polymer matrix and the reduction of flexural properties. For instance, high water sorption of polyamide (nylon) matrix causes reduction of over 50% in strength of FRC, but the FRC with less hydrophobic polymer matrix of acrylates has reduction of strength of less than 20% only. The reduction of the flexural properties is reversible, i.e., dehydration of the FRC recovers the mechanical properties. No significant reduction of flexural strength and modulus of elasticity by hydrolytic effect of water even in long-term water storage (ad 10 years) of glass FRC occurs which demonstrates the hydrolytic stability of good quality glass fibers, and their silane coupling agent mediated adhesion with the polymer matrix [78, 79].

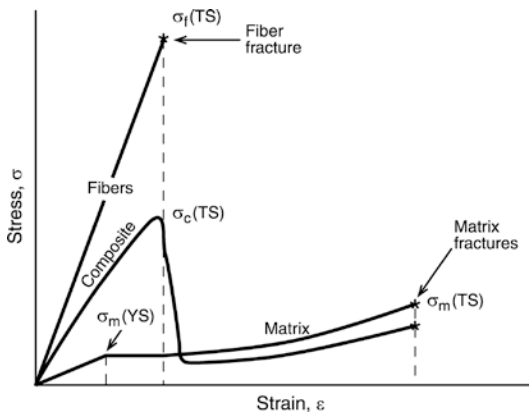

Fig. 5.1 Stress-strain curves for polymer (matrix), reinforcing fiber (fiber), and fiber-reinforced composite

The continuous unidirectional fibers provide the highest strength and modulus of elasticity for the FRC, but the property is available only in the direction of stress equal to that of direction of the fibers. Anisotropic behavior of unidirectional FRCs can also be seen in other properties, such as thermal expansion, optical properties, and polymerization shrinkage of the composite [80, 81]. Controlling the polymerization shrinkage by aligned discontinuous glass fibers is utilized in a novel filling composite resin for bilayered resin composite restorations. When the reinforcing effect of the fibers is divided into two or more directions, FRCs are called orthotropic and isotropic with regard to the thermal, optical, and physical properties.

5.3 All Started from Removable Dentures

Research on dental FRCs started in the early 1960s when first experiments on using glass fibers in denture base polymers were made. Glass fibers were selected as the most suitable fibers due to their translucency and possibility to achieve chemical bonding between the fiber and polymer matrix with silane coupling agents. Some tests were made to reinforce denture base polymers also with carbon/graphite fibers and polyethylene fibers. Only little attention was paid during that time to the reasons of limited reinforcing effects of fibers which was later showed to relate fraction of poorly impregnated fibers by powder-liquid-type denture base resins. In the 1990s, studies were published, which showed that highly viscous resin mixture of polymethyl methacrylate (PMMA) powder and monomer liquid was not able to adequately impregnate the

fibers [82–84]. Understanding the fundamental problem in resin impregnation leads to the development of preimpregnation system of the reinforcing fibers with porous PMMA. Porous PMMA between the silanized glass fibers behaves as a polymer powder in the acrylic resin mixture. It lowers the polymerization shrinkage of the resin between the fibers, and as the results, well-impregnated FRC was achieved.

The fiber reinforcements in denture bases are divided into two categories. Ladizesky and coworkers reported a method where fibers were distributed through the entire denture base. The approach by Vallittu is based on the concept that only the weakest part of the denture base (location of fracture initiation) is reinforced by precisely aligned and positioned fiber reinforcement. Two fiber-reinforcing concepts are called as total fiber reinforcement (TFR) and partial fiber reinforcement (PFR) [9]. Clinical studies have been performed with FRC-reinforced removable dentures, which suggested that PFR offers an effective and technically easy method to eliminate fractures in denture base [9].

5.4 Next Step: Fixed Dental Prostheses

Definitive fixed dental prostheses (FDPs) are expected to function for years of time in hostile oral environment. It is known that FRCs can be used to produce definitive FDPs although soon after introduction of FRC FDPs in the 1990s this was questioned. FDPs made of FRC are classified to surface-retained FDPs, inlay-/onlay-retained FDPs, full-coverage crown-retained FDPs, and hybrid FDPs (Fig. 5.2) [39]. The latter type is a combination of various retaining elements according to the specific need of the dentition. FRC FDPs can be made directly or indirectly. Direct FRC FDPs can be bonded to tooth by polymerization of restorative composite resins simultaneously with the polymerization of the FRC. Adhesive properties of FRC bonded directly to the dentine and enamel have been studied by Tezvergil et al. showing that only minor differences could be found between adhesive properties of FRC and particulate filler resin composite [85, 86]. In the FRC FDPs, the framework between the abutments is made of continuous unidirectional fibers, which offer high flexural strength. The crowns can be reinforced with woven fibers or in some fabrication concepts by making a fiber loop of unidirectional fibers to surround the abutment [25, 26, 87]. Several laboratory and clinical studies emphasize the importance of correct fiber directions of the FRC framework on the strength of the FDP construction.

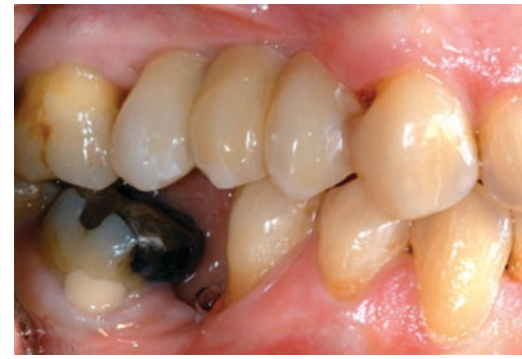

Fig. 5.2 Example of a minimally invasive glass FRC fixed dental prostheses with three pontics. Prostheses is retained by an inlay in D17 and an inlay with labial bonding wing in D13

Surface-retained FRC FDPs are typically used in anterior and premolar region. The recent laboratory investigations have suggested that optimally designed FRC FDP made on non-prepared abutments can provide even higher load-bearing capacity than conventional porcelain-fused-to-metal FDPs [43]. Inlay-/onlay-retained FDPs are made by combining the cavities of the abutments by continuous unidirectional fibers. FRC framework on the flat bottom of the cavity supports the FDP against vertical occlusal loads. The FDP can be made indirectly or directly, and this type of FDP is preferred in the premolar region and the molar region. In premolar and especially in molar region, the requirement for the FRC FDP is adequate vertical space for connectors and inlays. In the connectors, 4 mm of vertical space is needed, and in the inlays (onlays, crowns) minimum of 2 mmof occlusal space is required for the FRC and overlaying veneering resin composite.

Full-coverage crown-retained FDPs are made by layering woven FRC on prepared abutments. Abutments are connected with continuous unidirectional fibers and by having an additional piece FRC to support cusps of the pontics. Veneering is made with particulate filler resin composite. The use of full-coverage crowns as retaining elements of FDPs does not allow treatment to be according to the principles of minimal invasiveness. FRCs can also be used as reinforcements of provisional FDPs during fabrication of conventional FDPs [88].

5.5 Into the Root Canal

Root canal anchoring systems are in the same category of medical device regulatory requirements than FDPs. The first FRC root canal posts were used in Japan in 1600 century. The posts of that time were made of wood, which is a composite of cellulose fibers and lignin polymer matrix. The Father of Dentistry, Pierre Fauchard, presented silver posts for retaining crowns in the 1800s. Silver was thereafter replaced by dental gold alloys, which became standard for over hundred years of time. Metal posts are structural and due to material properties rigid in constructions, which effectively transfer occlusal loads to the fragile dentine of the root. Repeated stresses cause fatigue of dentine and can cause vertical fracture of the root. By adding so-called extraradicular metal ferrule of width of 1.5–2.0 to the crown, the root fractures can to large extent be eliminated. However, the present nonmetallic crown materials of glass ceramics and resin composites do not have metal ferrule, and thus, the root fracture elimination has to be done intraradicularly. The so-called modulus compensation is a method to lower the magnitude of local stress in root dentin. The modulus compensation is achieved by selection post material and post design, which matches to the modulus of elasticity of root. Glass FRCs fulfill the requirement of isoelasticity with dentine. The use of FRC in root canal posts to anchor cores and crowns has rapidly increased [63–73]. FRC can be used in root canal as prefabricated solid posts and individually formed posts, the latter representing to the most optimal post design [70].

The prefabricated posts are made of reinforcing fibers (carbon/graphite, glass, quartz) and finally polymerized resin matrix between the fibers which form a solid post of a predetermined diameter. Individually formed posts are made of non-polymerized fiber-resin prepregs, consisting of glass fibers and light-curing resin matrix. The rationale of the individually formed FRC post is to fill the entire space of the root canal by FRC material [70, 73]. The increased fiber quantity, especially in the coronal part of the root canal, increases load-bearing capacity of the system. Biomechanical behavior of restored tooth can also better be simulated because the fibers are located closer to the dentine walls, where the highest stresses exist. A tooth restored with individually formed root canal post system withstands cyclic loading of high magnitude for a long period of time without catastrophic failure or marginal breakdown of the crown, which can predispose to the secondary caries. For transferring the occlusal loads from crown to the individually formed FRC post, dentine, and periodontium, good bonding between the luting cements, post, and dentine is essential. Adequate bonding of resin composite luting cements to the post can clinically be achieved by using FRC post system where the polymer matrix is composed of interpenetrating polymer network (IPN) system which allows monomers of the cement to dissolve the surface of the post [63, 89–92]. Crosslinked polymer matrix of all present prefabricated FRC posts does not enable bonding of luting cements or core built-up resin composites to the post, and therefore mechanical retention of posts may be used.

5.6 To Replace Amalgam

Although amalgam has shown its many benefits as dental restorative material, its use is ending due to environmental reasons. On the population level, the replacing material should offer high cost-effect ratio for the treatment outcome. Particulate filler resin composites have fulfilled

this in terms of material cost but often failed in terms of longevity of restorations made by general practitioners. One reason for the limited longevity of restorations is low mechanical strength of the particulate filler resin composite as material and inadequately adjusted occlusion, which can cause high local stress concentrations and damage the restoration. Utilization of reinforcing fibers in filling composites to toughen the material has been tested for years [46–61]. Reasons for the poor success of previous FRC filling materials have been of selecting of too short discontinuous fibers, which were not even in theory able to increase strength and toughness of the resin composite. The current concept of using FRC in fillings is based on bilayered composite system in which FRC base is made of discontinuous fibers with length of the fibers exceeding the critical fiber length. At the same time, the fibers should be short enough to be used within the dimensions of single tooth (Figs. 5.3 and 5.4). One interesting observation of using discontinuous FRC is that fibers can be aligned during packing the composite to the cavity. It has been shown that fiber orientation can become perpendicular to the axial walls of a cavity, which reduces polymerization contraction of the composite and improves postcuring adaptation of the filling to the dentine. Fibers in the FRC increase toughness and other physical properties of the material compared to regular filling composites. Although it is known that protein and microbial adhesion of glass FRC does not considerably differ from that of particulate filler resin composites, the occlusal surface of the FRC is covered with polishable and wear-resistant particulate filler resin composite. The function of the FRC base for filling composites is to support the filling composite layer and serve as a crack propagation prevention layer.

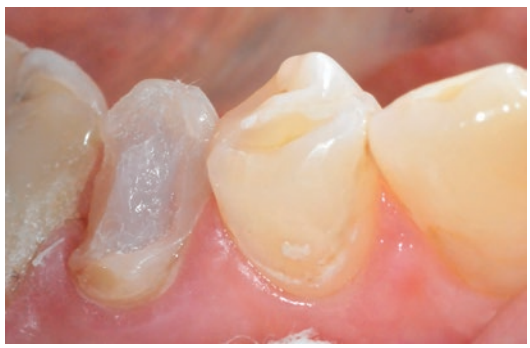

Fig. 5.3 Example of discontinuous glass FRC placed to reinforce the remaining tooth substance and the resin composite restoration. FRC base is veneered with regular particulate filler resin composite

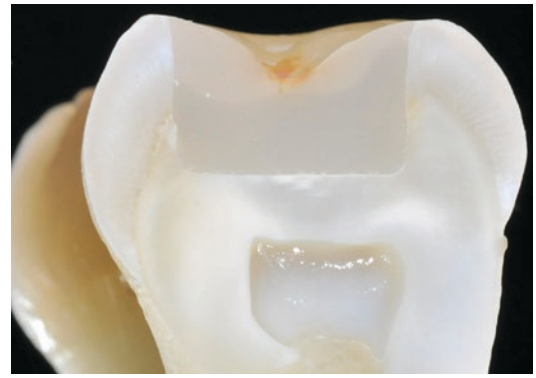

Fig. 5.4 Longitudinal section of tooth showing the bilayered resin composite filling: base is FRC and occlusal surface is regular filling composite with characterization paint

5.7 Into Bone: Surgical Applications for FRC

It can be estimated that worldwide over two million bone graft procedures of which 280,000 are because of hip fractures, 700,000 vertebral reasons, and 250,000 wrist fractures, and 700,000 various cranial bone repair reasons are annually performed [93]. The need for skull reconstructions is increasing mainly due to an increase in decompressive craniectomies, a life-saving maneuver to relieve intracranial pressure resulting from swelling of the brain due to, e.g., trauma or cerebrovascular accidents [94].

Durable and tough FRC materials have proven their suitability to cranial implantology. To improve osteoconductivity and osteoinductivity of the FRC material, particles of bioactive glass have been added to the surface or inner space of FRC implants (Fig. 5.5) [94]. Because radiopacity of glass FRC corresponds to that of the cortical

bone, there are no artifacts in the diagnostic images, but the implant can be seen in X-rays, CTs, and MRIs (Figs. 5.6 and 5.7). Radiation therapy can also be given in the presence of FRC implant. Table 5.2 lists properties of presently used cranial implant materials with respect to their clinically needed properties [94].

Presently, the most commonly used fibers in medical FRC are made of glass of specific composition, but carbon/graphite fibers have also been tested. Glass fibers referred as S-glass are basically free of leaching ions in physiologically moist environment like in living tissues with presence of extracellular liquid. Nominal composition (in wt%) of commonly used S-glass is SiO_2 62–65, Al_2O_3 20–25, MgO 10–15, B_2O_3 0–1.2, Na_2O 0–1.1, and Fe_2O_3 0.2. The use of carbon/graphite fibers has been limited due to risk of release of micro and nanometer scale carbon wear debris to the tissues. Glass fibers of diameter 15–17 μm are used in implants as continuous fibers which have been woven to textile form before impregnating and coupling with resin, and therefore release of wear debris is not a problem. In the presently used design of FRC cranial implants, both woven textile

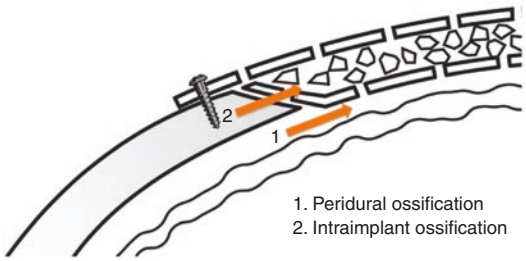

Fig. 5.5 Schematic drawing of the FRC-bioactive glass cranial implant. Biostable mesh-like FRC laminates are the outer and inner surface of the implant. Bioactive glass particles are packed between the laminates. Bone growth occurs in the implant (intraimplant ossification) and on the inner surface of the implant (peridural ossification)

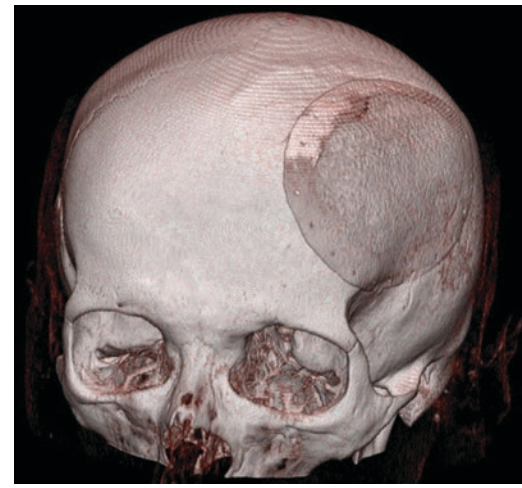

Fig. 5.6 Computer tomography (CT) image of the onlay-type FRC-bioactive glass implant in the temporal area of skull (courtesy: Professor Kalle Aitasalo, Turku University Hospital, Finland)

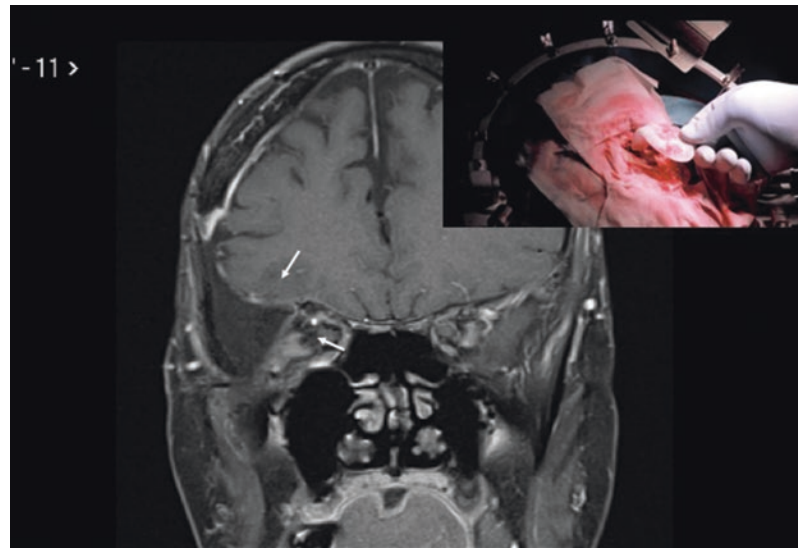

Fig. 5.7 Magnetic resonance image (MRI) of a sphenoid-orbitotemporal FRC-bioactive glass implant (white arrows) and photograph of the insertion of the implant (courtesy: Docent Ville Vuorinen, Turku University Hospital, Finland)

form of fibers and unidirectional continuous fibers are used in the implant construction. The role of continuous unidirectional fibers is to connect the outer and inner surface laminates together for providing high strength to the implant [36]. Special features of the FRC cranial implant construction are mesh-like surface laminates and the presence of free space between the outer and inner laminates, which is loaded with particles of bioactive glass (Fig. 5.5) [94].

Polymer matrix of FRC implants is biostable highly cross-linked acrylic resin. The use of biostable FRC protects brain tissues for tens of years. Before introduction of glass FRC, bulk PMMA implants were tested but fabrication of thin and tough margins of PMMA proved impossible, and the material was changed to FRC [95]. Long-term durability of the cranioplasty implant is important because according to the present best knowledge, the cranial defects need years of time to be closed by a new forming bone even in the presence of osteoinductive implant materials. This is the reason why any of the biodegradable polymers or composites cannot be used for repairs of large bone defects in the cranium. Biodegradable polymer-based materials degrade and lose the mechanical strength too fast in relation to the bone regeneration. With regard to degradable metal alloys of magnesium, there are problems in tissue healing due to release of hydrogen gas during degradation process [96].

When biostable onlay-type glass FRC implant is loaded, stress is transferred from resin matrix to be carried by the reinforcing fibers with specific orientation, and finally the load is transferred to the margins of the bone [97]. Initial load transfer from implant to the bone is achieved by fixation screws and final load transfer by osseointegration. Thermoset copolymer and the silanized glass fibers form a durable composite for fabrication of patient-specific and standard-shaped implants [98–100].

Biocompatibility of FRC implants is related to the biocompatibility of its components of polymer matrix, reinforcing glass fibers, silane coupling agent, and bioactive glass. Biocompatibility of all of these material components processed ex vivo has been proven by laboratory, preclinical, and clinical studies. By using osteoblasts on the cell culture model with FRC implants, no signs of undesired reactions of the material were found. For instance, when bone marrow-derived osteoblast-like cells were harvested and cultured on the FRC material plates and on commercially pure titanium plates and cell growth and differentiation kinetics were investigated, similar alkaline phosphatase activities on both FRC and titanium were observed [101, 102]. In addition, expression of osteoblastic markers of osteocalcin and bone sialoprotein indicated that the fastest osteogenic differentiation took place on FRC after 7 days. In contrast, a slower differentiation process was observed on titanium. It was concluded that the proliferation and maturation of osteoblast-like cells on FRC appeared to be comparable to titanium. The presence of BG on the implant surface enhanced cell maturation. Preclinical animal experiments have been carried out to show cell response to FRC in vivo followed by clinical studies (Figs. 5.8 and 5.9) [94].

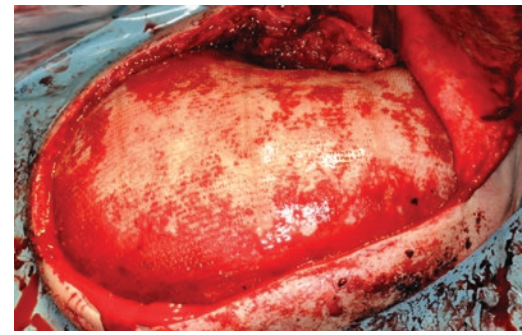

Fig. 5.8 FRC-bioactive glass cranioplasty implant during the surgery. Note the penetration of blood with stem cells and growth factors into the implant

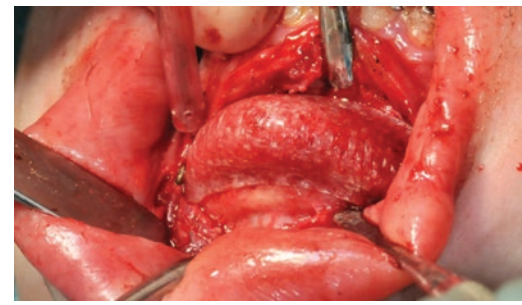

Fig. 5.9 FRC-bioactive glass genioplasty implant

5.8 Role of Bioactive Glass

In many of the FRC implant studies, there have been bioactive glass (BG) (S53P4) particles on the surface of the FRC implant [95, 97, 101–105]. BGs are synthetic dissolving biocompatible osteoconductive-osteoinductive bone substitutes. Some compositions of BGs have clinically been used because of antibacterial and angiogenesis-promoting properties [106–110]. Preclinical animal experiments with cranial implants have been made with rabbit model in which the implant design had two FRC laminates and there were particles of bioactive glass between the laminates for improving osteogenesis, angiogenesis, and antimicrobial properties [94, 111].

There are several various and clinically used compositions of bioactive silicate glasses. Out of several compositions and particle sizes of bioactive glass, clinically the most potential bioactive glass in bone augmentation indications is silicate glass S53P4 with the nominal composition (in wt%) of Na_2O 23, CaO 20, B_2O_5 4, and SiO_2 53 and average particle size on 500 μm [111]. Leaching of BG and the released ions is behind the biological function of the glass, and detailed knowledge of these reactions is a key to selecting BGs as component in FRC implants. BG S53P4 has shown to fulfill several known requirements (moderate increase in pH, bacteriostatic properties, osteoinductivity) for osteogenesis and bone remodeling.

In biological environment, ions of calcium and phosphorus are released from the BG, and they cause biomineralization on the material surface, like on the surface of glass FRC. For cells, at the early stage of osteogenesis, released ions from the BG and slightly increased pH due to ion exchange reactions are inducing differentiation of mesenchymal stem cells to cell lines for bone formation (Fig. 5.10). This, in conjunction with biomineralization, promotes bone growth. It is essential to understand the microenvironment where cell differentiation occurs. If the pH increases too much due to ion exchange by the BG, differentiation of cells does not happen and cells can eventually die. Too high increase of pH can be because of inadequate flow of interstitial liquid, too small particle size of BG, and too reactive leaching profile of BG due to its composition. Level of pH where differentiation of mesenchymal stem cells is hindered is around 8.5, whereas the effective differentiation can be seen in pH of 7.8–8.0 [111]. There is also in vitro obtained information that BG can induce vascularization, and indeed, histological analysis of the new bone around BG shows presence of blood vessels [94, 112].

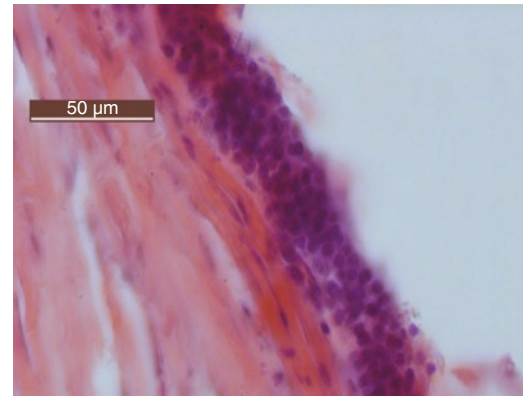

Fig. 5.10 Histological section (HE staining) of the tissue sample inside the FRC-bioactive glass implant at the time point of 2 years. Histology shows presence of active osteoblasts

With regard to osseointegration, i.e., bonding between the BG of the implant and bone tissue, a series of reactions starting at the glass surface followed by a series of biological reactions are occurring. The different reaction steps taking place at the glass surface depend mainly on the glass composition but also on the surface topography, surface area of glass, and flow of the interstitial fluid in the microenvironment close to the glass surfaces. In the subsequent steps, calcium and phosphate from the solution, and migrating from the bulk glass, form first amorphous hydroxyapatite and then crystallize at carbonate-substituted hydroxyapatite (HA) layer at the glass surface. This HA layer is compatible with the biological apatite and provides an interfacial bonding between the material and tissue [94].

Antibacterial properties of the glasses are attributed to the local rise of pH level and increased ion concentration causing increased osmotic pressure [73]. The US Food and Drug

Administration (FDA) approved BG 45S5 and BG S53P4 for certain clinical applications where antimicrobial properties are required. Increase of the alkalinity by bioactive glass 45S5 is higher than with glass S53P4, and therefore glass 45S5 is considered to be more effective in terms of antimicrobial properties. On the other hand, a balance between antimicrobial properties, i.e., increase of pH and moderate alkalinity and ion release and osteogenicity, has been found with BG S53P5. In vitro conditions in the presence of BG S53P4 showed increase of pH to the level of 7.9 [94, 113]. Antimicrobial efficiency has been shown for more than 20 microbe species, including *Staphylococcus aureus* and *Staphylococcus epidermidis*, which are the most common pathogens in periprosthetic infections [114–119].

5.9 The First Clinical Tests

To overcome discomfort and pain by cranial and facial bone reconstructions based on autologous bone transplants, and problems related to biomaterial implants, patient-specific FRC-BG cranial implants were started to be used first time in 2007 [23]. Before the time of FRC-BG implants, the first-generation implants were made of bulk polymethyl methacrylate (PMMA) which has been polymerized ex vivo and covered from the surface with exposing particles of BG S53P4 [94]. Based on the clinical experiences with the PMMA implants, further improvements in terms of allowing osteogenesis and vascularization to occur inside the implant and to have thinner and cosmetically more pleasant looking margins for the implants, studies of FRC-BG implants started (Fig. 5.5) [120–122]. Another aim to use FRC instead of PMMA was needed to have thin but tough margins for the onlay implant. FRC materials are known to have high static and dynamic strength and toughness.

The first FRC-BG onlay-type implants were loaded with BG S53P4, and the implant structure had dense outer and inner surface laminate made of glass FRC fabric, and between the layers there were porous glass FRC particles of BG. Postoperative positron emission computer tomography (PET-CT) examination with (18F)-fluoride marker has demonstrated activity of the mineralizing bone by osteoblasts especially at the margins of the implant into which the blood was penetrated by capillary forces [94]. When implant of that kind had been analyzed more in detail after being in situ for 2 years and 3 months, 3D CT reconstructions demonstrated ossification on the lower surface of implant which was considered as peridural ossification. Clinical follow-up study of this type of FRC-BG implant showed higher survival estimates than for other implant materials and autologous bone in retrospective study material [121]. FRC-BG implants are used also in CMF applications such as genioplasty implants (Fig. 5.6).

Based on the observations of the first-stage FRC-BG implants, the implant design was changed for having better interstitial liquid perfusion through the implant by pulsatile movement of dura mater, which facilitated stem cells and growth factors from the refreshed bone margins at the operation site to penetrate into the implant and become in contact to BG particles and promote osteogenesis. Release of ions and related increase of pH by the BG enhanced osteogenesis and vascularization to occur in the implant and make the implant microenvironment bacteriostatic. The present design of FRC-BG cranial implant has received good acceptance by the surgeons, and it was approved for clinical use as patient-specific implant and standard-shaped implant in Europe in 2014.

5.10 Future Aspects

Versatile properties of FRC in terms of biomechanics, possibility to add biologically active compounds to the medical device structure and into the polymer matrix of the FRC opens new horizons for the reconstructive dentistry and medicine. The limitations which are found to relate to the present and under development being biodegradale implant systems and to the to stem cell based tissue engineering approaches in cranial bone repairs can be overcome by using FRC-BG implants [123–131]. New applications

for FRC will be found from orthopedic and trauma surgery and spine surgery. These applications will utilize novel techniques to fabricate in situ moldable implantable devices. Research from lab to clinics continues.

Acknowledgments The journey from lab to clinics with FRC biomaterials has been possible by support and contribution of research network of the FRC Research Group of the BioCity Turku Biomaterials and Medical Device Research Program (www.biomaterials.utu.fi). The University of Turku and Turku University Hospital are greatly appreciated.

Disclosure The author is an inventor and scientific consultant in the dental FRC material producing Stick Tech Ltd.—member of GC Group—and has a role also as a member of the board and shareholder of the Skulle Implants Corporation.

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Translational Oral Health Research Implants and Bone Regeneration

6

M. Sanz, A. Carrillo de Albornoz, and I. Sanz-Sanchez

Abstract

Immediately after tooth extraction, physiological and eventually pathological changes affect the healing of the alveolar ridge. This three-dimensional shrinkage of the alveolar bone is determinant to the subsequent implant placement. Different surgical protocols have been proposed as a way to limit these dimensional changes and hence to optimize implant placement from an aesthetic, patient-friendly and long-term perspective. The evidence-based efficacy of these interventions, the indications and contraindications, and the advantages and disadvantages of the different methods are thoroughly discussed.

Ridge augmentation procedures are frequently performed to compensate the structural changes that occur at the residual alveolar ridge after tooth extraction, which significantly limit bone availability for implant placement. The correct selection and application of the available regenerative techniques and biomaterials are key determinants of success in modern implant therapy. The use of scaffolding biomaterials, growth factors, cell therapy and advanced surgical techniques offers promising potential in the ordinary and challenging bone defects. The evidence-based efficacy of these interventions, the indications and contraindications, and the advantages and disadvantages of the different methods are thoroughly discussed.

6.1 Introduction

Over the past decades, the placement of dental implants has become a routine procedure in the oral rehabilitation of fully and partially edentulous patients, and there is a well-established evidence supported by long-term studies (≥10 years) that different implant systems may attain high success and survival rates [1–3]. These results, however, were based on the adherence to classical surgical protocols based on the placement of dental implants in healed ridges (at least 6 months after tooth extraction) and after allowing for an extended healing time (of 3–6 months) without receiving any functional loading with the goal of attaining an optimal osseointegration.

This classical clinical protocol for the placement of dental implants has changed significantly over the past 20 years. From this initial 'osseointegration'-oriented protocol, there has been an evolution towards less stringent unloaded healing periods with the aim to 'speed the healing process' and 'improve patient's comfort and aesthetic result'. Similarly, the indications for

M. Sanz (✉) · A. C. de Albornoz · I. Sanz-Sanchez
ETEP (Etiology and Therapy of Periodontal Diseases) Research Group, Faculty of Odontology, University Complutense, Madrid, Spain
e-mail: marsan@ucm.es

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implant therapy have evolved from initially considering mostly fully edentulous patients with optimal jaw bone dimensions to the current situation, where nearly every edentulous space may be considered suitable for implant placement. The advent of implants with moderately rough surfaces has shown evidence of accelerating osseointegration, thus allowing for less healing time, even to a minimum. In fact, immediate loading protocols have been used with a high degree of predictability under specific clinical conditions [4]. Similarly, the placement of dental implants at the time of tooth extraction has significantly reduced the treatment time and patient morbidity [5, 6].

Introduction of three-dimensional imaging has led to improvements in preoperative diagnosis and implant surgery. Guided surgery has shown to improve the positioning of implants, shorten the surgical time and reduce postoperative complications [7]. However, the accuracy of these diagnostic techniques is not always ideal, and clear guidelines have to be carefully followed in order to prevent malposition of implants [8].

In situations where insufficient bone is available for implant therapy, bone augmentation interventions are routinely carried out with highly predictable results when the proper indications are selected and the appropriate surgical techniques and biomaterials are used. In particular, guided bone regeneration has become a well-accepted treatment option, either prior to or simultaneously with implant insertion [9]. In these clinical situations with deficient bone availability, other alternatives, such as the use of short or narrow implants, have also demonstrated predictable outcomes [10].

In spite of the reported excellent outcomes of implant therapy, technical, biologic and aesthetic complications may occur, and their prevention and treatment have become a key element in modern implant denstistry [11].

Dental implants may fail due to lack of osseointegration during the early healing after implant installation, and these complications are usually associated to pain, infection and occasionally neuropathy. Their aetiology may be related to local or systemic factors affecting the patient. In fact, metabolic disorders, such as uncontrolled diabetes or immune deficiencies, have been associated with early implant failures, since these conditions may interfere with bone apposition and/or remodelling at the implant–bone interface. Similarly, radiation therapy in the surgical area or medication, such as bisphosphonates and/or anticoagulants, may also affect the outcome of implant placement or increase the frequency of postoperative complications. Implant treatment in these compromised patients must adhere to strict treatment protocols [12].

After osseointegration and functional loading, peri-implant bone loss may also occur, mainly due to inflammatory changes in the peri-implant tissues usually in response to oral biofilm accumulation on the implant surfaces or prosthetic components. More rarely, peri-implant bone loss may be the consequence of fractures of the implant or the abutment components. The early inflammatory changes of the peri-implant soft tissues without causing peri-implant bone loss (mucositis) may be reversible through the establishment of therapeutic and preventive measures, mainly by the patient's adherence to strict oral hygiene regimens and professional maintenance recall programmes [13]. When the inflammatory changes coexist with peri-implant bone loss (peri-implantitis), current treatments are not highly predictable and usually require surgical access for implant surface decontamination and in specific situations peri-implant bone regeneration [14].

6.2 Surgical Placement Protocols

In the last 20 years, evidence from experimental research has clearly shown that immediately after tooth extraction physiological dimensional changes affect the healing of the alveolar ridge, and most of these changes will occur within the first 3 months of socket healing [15]. These height (apico-coronal) and width (bucco-lingual) alterations in the alveolar ridge would therefore influence subsequent implant placement. In light of this evidence, different surgical protocols have been proposed as a way to limit these dimensional changes and hence to optimize implant placement.

In the Third International Team for Implantology (ITI) Consensus Conference [16], four protocols for implant placement were defined according to the time between tooth extraction and implant installation:

- Type-1 protocol (immediate implant installation) when implants were placed in fresh extraction sockets, with the aim to engage the remaining socket walls with the implant.
- Type-2 protocol (early implant placement) when implants were placed approximately 4–8 weeks after tooth extraction. The main objective of this protocol was to ensure primary healing of the site assuring the lack of pathology when placing the implant and, at the same time, the optimization of the soft tissues available for adequate flap management mainly in situations when the socket walls have been partially destroyed and bone regeneration interventions in conjunction with implant placement were indicated.
- Type-3 protocol (early delayed implant placement) when the implants were placed once most of the dimensional changes in the alveolar ridge had occurred (12–16 weeks).
- Type-4 protocol (healed sites) when the implants were placed in a fully healed ridge (typically after more than 16 weeks from the extraction, although more likely after 6–12 months).

6.3 Immediate Implants

Immediate implant placement after tooth extraction (i.e. the type-1 implant placement protocol) has become a common surgical protocol in modern implant practice. The advantages of this protocol are in principle very attractive, since there is a reduced exposure of patients to surgery, less morbidity, reduced overall treatment period, possibility of immediate provisional prosthesis, optimal aesthetic outcomes and enhanced patient acceptance. However, the predictability and success of these outcomes depend heavily on strict selection criterion and very accurate surgical handling of the fresh extraction socket [17].

Initially, it was claimed that immediate implant placement would limit the physiological bone resorption. However, although the attaining of osseointegration was highly predictable, there was no clear clinical and histological evidence that immediate implant placement would prevent the physiological process of crestal bone modelling and remodelling during the healing of the extraction socket. In fact, experimental studies have clearly demonstrated that the resorptive changes of the residual socket walls would occur independently of the timing of implant installation. Thus, significant dimensional alterations in the buccal bone wall, both in apico-coronal and bucco-lingual directions, have been reported after implant placement in fresh extraction sockets [18]. These histological findings have been further corroborated by human clinical trials reporting significant reductions, both at the bucco-lingual and apico-coronal dimensions of the alveolar crest around immediately placed implants [19]. These changes, however, are rather variable, and different local factors have been identified to significantly influence these resorptive patterns. Among these factors, the thickness of the buccal bone wall, the implant macro-design and the location where the implant was placed (anterior/posterior) have shown a significant impact [20]. Since most of the sites in the maxilla have thin buccal ridges (approximately 87% of the buccal bone walls presented a width of $\leq$1 mm [21]), the placement of immediate implants has shown to be risky in the anterior maxilla, mainly in relation to the advent of aesthetic complications. Moreover, the buccal bone plate will suffer extensive horizontal bone resorption during healing, which may cause the loss of the convex contour of the upper maxilla, with the typical unaesthetical consequences in subjects with a high smile line. To partially compensate this horizontal resorption, the use of bone substitutes with a low resorption rate filling the gap between the implant surface and the inner bone plate has shown to significantly reduce this resorptive process. Therefore, their use should be advocated when the aesthetic demands are high [22].

Other factors, such as the use of a flapless technique, immediate provisional restorations, placement of soft tissue grafts or the use of implant-abutment connections following the platform-switching concept, have also been advocated to further counteract these resorptive changes and thus improve the aesthetic outcomes with this surgical protocol, although their specific added value is still unknown (Fig. 6.1).

From a surgical point of view, the attainment of a correct 3D implant positioning with good primary stability in sufficient apical bone availability is an essential factor for achieving successful outcomes with immediate implants. Additional stability may be obtained by anchoring the implant in the bony structures of the alveolar walls or inter-radicular septa, although an excessive buccal positioning of the implant must always be avoided as well as the choice of implants that are too congruent with the socket anatomy. An adequate gap dimension (>2 mm) between the implant and the inner surface of the buccal bone plate crestally is fundamental to allow for satisfactory bone healing [23]. In an apico-coronal dimension, it is important that the final position of the implant shoulder is placed at least 1 mm apical to the buccal ridge, in order to compensate for the expected vertical resorption [24].

6.4 Early Implants

It is rather frequent that the integrity of the socket bone walls is affected by the underlying pathology that justified the tooth extraction or by the possible trauma to the bone during the extraction procedure. In these situations, where mainly the buccal bone wall is totally or partially absent, the immediate implant protocol should not be indicated, and the early implant placement protocol (type 2) has been proposed. This treatment modality reduces the risk for postsurgical complications since it allows for primary healing after tooth extraction. This proposed healing time (4–6 weeks) usually allows for soft tissue healing but at the same time permits the anchorage of the implant in the socket walls before they become fully resorbed. Since the bucal bone wall is either partially or totally absent, there is a need for regenerative interventions in conjunction with implant placement. The presence of adequate soft tissue usually allows for primary closure, thus reducing the risks of infeccion and soft tissue dehiscence. This is especially relevant in the presence of thin biotypes, where significant hard and soft tissue resorptive changes usually occur [25].

This surgical protocol is particularly suitable for bone augmentation techniques, combining bone grafts and barrier membranes (guided bone regeneration) since the tissue healing after tooth

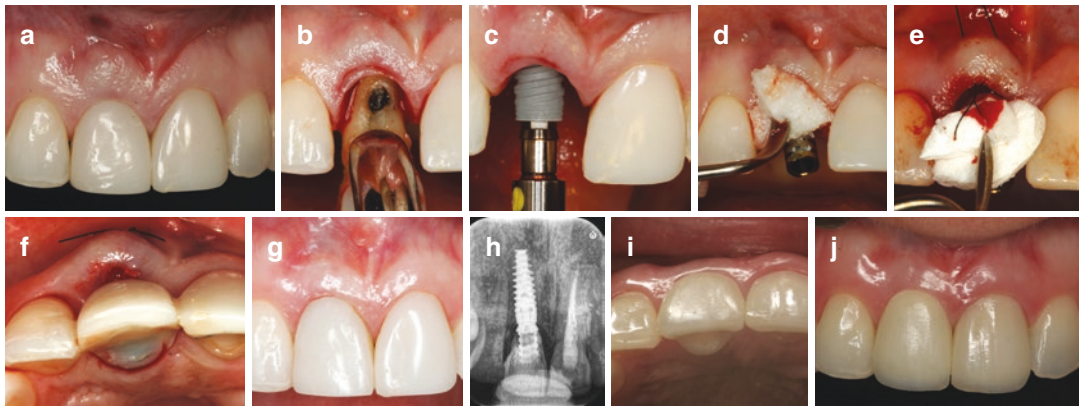

Fig. 6.1 Immediate implant placement protocol which includes (**a**) and (**b**) careful and atraumatic tooth extraction; (**c**) flapless implant placement in the ideal prosthetically driven position; (**d**) placement of a slow resorbing bone replacement graft in the buccal gap; (**e**) placement of a collagen-based soft tissue substitute to enhance the buccal soft tissue volume; (**f**) placement of an immediate provisional restoration; (**g–i**) clinical, radiographical and aesthetic outcome

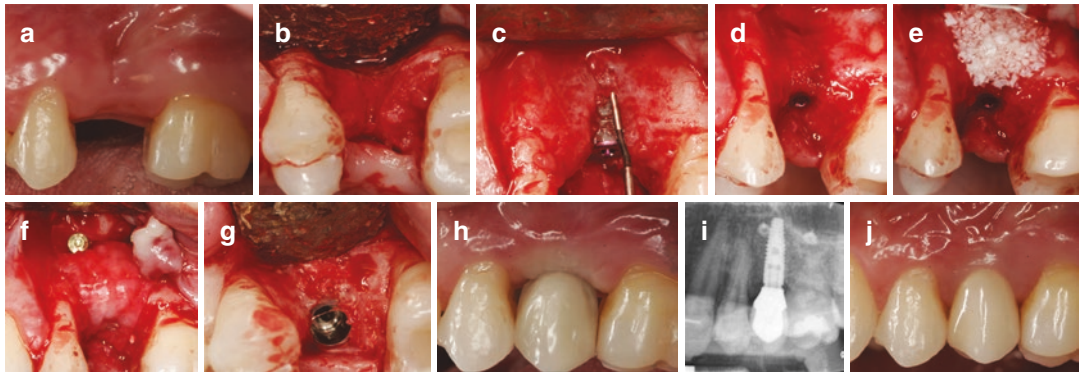

Fig. 6.2 Early implant placement protocol which includes (**a**) soft tissue healing 4 weeks after tooth extraction; (**b**) alveolar bone healing 4 weeks after tooth extraction; (**c**) implant placement with resulting dehiscence defect; (**d**) placement of an autogenous bone graft over the exposed implant threads; (**e**) over-contouring the buccal bone crest with a slow resorbing bone replacement graft; (**f**) placement of a bioabsorbable collagen membrane fixed with titanium tags; (**g**) bone regenerative outcome 4 months after; (**h**) placement of a provisional restoration; (**i**, **j**) radiographical and aesthetic outcome

extraction provides usually enough soft tissue coverage that permits primary healing of the implants and regenerative biomaterials under a submerged environment (Fig. 6.2). After 5–9 years of follow-up, a low risk of mucosal recession and the presence of an intact facial bone in 95% of the patients have been reported using this surgical protocol [25, 26]. The efficacy of early implant placement in terms of enhancing the survival of the implants placed to restore the extracted teeth has been evaluated in systematic reviews, suggesting that the early implant placement protocol may offer advantages with regard to preserving the hard and soft tissues around the implants. A meta-analysis demonstrated a pooled mean difference between groups of 13.11% reduction in defect bone height and 19.85% of reduction of defect bone width favouring the early placement group [27].

6.5 Ridge Preservation

Another surgical approach designed to counteract the resorptive changes of the residual alveolar ridge is the use of bone replacement grafts, membranes of other regenerative technologies in the fresh extraction socket immediately after tooth extraction, thus delaying the placement of the dental implant. These surgical interventions are called in general socket preservation techniques. Their efficacy has been tested in both animal and human studies. Using the dog experimental model, researchers from the University of Gothenburg evaluated different biomaterials filling the socket and evaluated histologically their impact on the healing of the socket. The use of slowly resorbing xenografts was shown to counteract significantly the ridge contraction, while other biomaterials such as autogenous bone grafts or synthetic biomaterials had minimum impact on the healing of the socket [28] (Fig. 6.3).

In humans, the application of regenerative biomaterials, such as bone autografts, allografts, guided tissue regeneration procedures, xenografts and, most recently, growth factors, has also been evaluated with varying degrees of success to maintain the anatomical dimensions of the alveolar ridge after tooth extraction. Systematic reviews have evaluated the efficacy of these interventions reporting that socket preservation techniques do not prevent the physiological resorptive bone processes after tooth extraction, although they may significantly reduce the resulting bone dimensional changes [29]. The results from the meta-analysis demonstrated statistically significant higher alveolar bone crest preservation in both height and width in the interventions for ridge preservation when compared with the healing of the untreated control socket. The

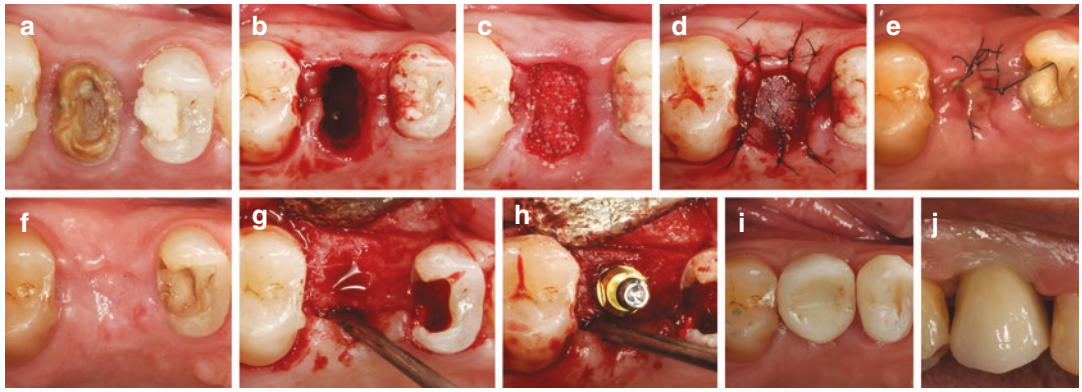

Fig. 6.3 Socket preservation protocol which includes (**a**, **b**) careful and atraumatic tooth extraction; (**c**) placement of a slow resorbing bone replacement graft inside the socket; (**d**) placement of a collagen-based soft tissue substitute to seal the socket opening; (**e**) healing at 2 weeks; (**f**) healing at 4 months; (**g**, **h**) placement of a dental implant in the ideal prosthetically driven position; (**i**) placement of a provisional restoration; (**j**) placement of the final restoration

results of the meta-regression analysis showed that the surgical procedures attaining primary closure of the socket opening (by advancing flaps of using barrier membranes or soft tissue autografts) demonstrated a significantly lesser horizontal and vertical resorption of the socket [30]. These results suggest the importance of achieving full closure and first intention healing, thus avoiding the contamination and disturbance of the healing of the biomaterial or regenerative technology.

6.6 Bone Regeneration of Residual Alveolar Ridges

Alteration of the anatomy of the residual alveolar ridge may be the consequence of the underlying pathology that caused tooth loss (trauma, chronic/acute infections or severe periodontal disease). Even in the absence of overt pathological conditions, the loss of mechanical function following tooth extraction or tooth loss will cause severe atrophic changes in the residual alveolar ridge, which may limit the availability for adequate implant placement. In fact, bone availability is the main prerequisite for safe and predictable implant placement, since an adequate amount of bone is required for attaining the functional stability of the implant needed to achieve osseointegration. Moreover, the goal of achieving adequate aesthetic outcomes requires the optimal three-dimensional position of the implant following a planned prosthetic reconstruction. The frequent presence of residual ridge deficiencies, both in horizontal and vertical dimensions, demands the indication of bone augmentation procedures, either concomitant with implant placement or as a staged intervention.

These crestal changes may be different depending on the region of the affected jaw and, similarly, the functional and aesthetic demands of the patient may vary, what advocates for an individual assessment of the possible need of bone augmentation procedures. The available bone crest, therefore, must be carefully evaluated clinically and mainly by means of a three-dimensional radiographic evaluation with the use of modern CBCT (cone beam computed tomography) technologies.

Depending on the main component of the alveolar crest defects, Seibert [31] proposed three categories:

- Class 1 defect, when the bone deficiency affected predominantly the horizontal dimension
- Class 2 defect, when the bone deficiency affected predominantly the vertical dimension
- Class 3 defect, when the bone deficiency affected both the vertical and horizontal dimensions

A careful diagnosis of the residual alveolar ridge is also fundamental for the selection of the appropriate regenerative strategy and technology, although any bone augmentation therapy must be based in a set of fundamental biological principles of wound healing, including the promotion of primary wound closure, the enhancing of cell delivery and neo-angiogenesis by attaining initial wound stability and avoiding contamination of the regenerative process.

Primary closure is, therefore, primordial to assure an undisturbed environment for healing and must be assured by appropriate flap management and suturing. This requires an adequate amount of soft tissue present before the regenerative surgery [32]. The resulting flap must cover the regenerated area, and, when sutured, it should be relatively passive and tension-free. Proper cell proliferation and differentiation are also fundamental in the wound healing process to provide the neo-angiogenesis and the differentiation of the osteogenic cells needed for regenerating the bone. The main sources of osteogenic cells include the periosteum and endosteum from the walls of the bone defects, as well as the bone marrow. These cells include osteoblasts and undifferentiated mesenchymal cells, which can be differentiated into osteoblasts in the presence of the adequate signalling molecules, nutrients and growth factors. This process requires adequate blood supply for providing not only oxygen and nutrients but also as a source of mesenchymal cells. To promote these early healing events, perforations of the cortical plate have been recommended to facilitate cell migration [33]. Another key factor that affects the wound healing is the stability of the blood clot. This is important since the clot promotes the formation of granulation tissue, which subsequently will develop into bone [34]. Moreover, the clot contains a myriad of cytokines (e.g. interleukin-1 (IL-1), IL-8, tumour necrosis factor), growth factors (e.g. platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2)) and signalling molecules that aid in recruiting cells to promote angiogenesis and bone regeneration. In most clinical situations, this clot stability can only be assured when physical space is provided by means of a scaffold and by exclusion of the soft tissue cells from the mucosal. This is usually assured by the placement of a barrier membrane between the bone replacement graft serving as scaffold and the inner surface of the flap.

The rationale behind any crestal bone augmentation procedure is not only to establish sufficient bone availability for the safe and predictable placement of a dental implant but also to provide adequate bone thickness around the installed implant. In fact, the influence of bone thickness around an implant has been evaluated during the second-stage surgery, demonstrating that thicknesses $\leq$1.5 were frequently associated with bone loss and dehiscence lesions (exposed implant surface), while as the bone thickness approached 1.8–2 mm, the occurrence of dehiscence decreased significantly [35]. Although the 'adequate' bone thickness may vary depending on the macroscopic and microscopic implant configurations, as well as in the clinical indication, it is generally agreed that at least 2 mm of the bone on the buccal side of the implant are needed to achieve long-term stability of peri-implant soft tissues and hence to attain adequate aesthetic outcomes [36].

In bone augmentation procedures of the residual alveolar crest, the treatment strategy may include the placement of an implant and the regeneration during the same surgical intervention (one-step procedure) or the delay of implant placement until enough bone volume has been augmented (staged procedure). The one-step procedure is indicated in class 1 defects, when there is enough vertical bone for placing an implant with appropriate primary stability and in the proper prosthetically driven position (lateral bone augmentation). In class 2 and 3 defects, depending on the amount of vertical augmentation needed, the staged procedure is usually indicated.

Different biomaterials have been tested as bone replacement grafts and barrier membranes in bone regenerative interventions in alveolar ridges.

As bone replacement grafts, natural and/or synthetic biomaterials have been developed, investigated and used in bone augmentation pro-

cedures. Autogenous bone grafts (autografts) have been historically the gold standard in alveolar bone regenerative therapies due to their well-documented osteoconductive, osteoinductive and osteogenic properties [37]. Their major limitation, however, is the morbidity and complications associated with their harvesting, which depends on the volume needed depending on the size of the defect. Furthermore, autografts suffer fast resorption, which requires early implant placement to provide functional loading to the regenerated bone, and even with early implant placement, significant resorption occurs. Particulate bone autografts are usually harvested from intra-oral sites and used in combination with barrier membranes following the principles of guided bone regeneration. Mono-cortical block autografts are indicated in large crestal defects, where there is a need for vertical bone augmentation, since their compressive properties allow for excellent space maintenance. These autogenous grafts may be harvested from intra- or extra-oral sites. Common intra-oral donor sites are the mandibular chin or the ascending ramus area, whereas common extra-oral donor sites are the iliac crest or the calotte. They may be used alone or in combination with barrier membranes, and they require fixation to the recipient crestal site with mini-screws to avoid micro-movements during healing. Their main disadvantage is the morbidity associated with their harvesting, and similar to particulate autografts, their resorption rate is high.

To overcome these shortcomings, natural or synthetic bone substitutes, either used alone or in combination with bone autografts, have become the standard biomaterials in bone augmentation procedures. These bone substitutes must fulfil the following requirements for their use in bone regeneration in periodontology and oral and maxillofacial surgery: biocompatibility, osteoconductivity, adequate mechanical support to provide the volume for the regenerated bone, biodegradability and being replaced by the patient's own bone, although recent studies have suggested that a slow degradation or even non-resorption may be advantageous for the maintenance of the augmented volume.

Depending on their origin, bone substitutes have been classified in three groups: allografts, from human origin; xenografts, from another animal species, usually bovine or equine; and alloplasts, which are synthetically produced. Allografts are bone grafts harvested from cadaver donors and processed by freezing or demineralizing and then sterilized and supplied by specially licenced tissue banks as bone particles or blocks. Allografts include fresh-frozen bone (FFB), freeze-dried bone (FDB) and demineralized freeze-dried bone (DFDB). Their main limitation is the potential risk of cross-infection or immunologic reactions due to their protein content, what cannot exclude the possibility of disease transmission. Nevertheless, there are no reported cases from the use of demineralized freeze-dried bone allografts (DFDBA) for dental purposes in over one million cases over 25 years [37]. DFDBA have shown osteoconductive as well as osteoinductive properties, due to the release of bone morphogenetic proteins (BMPs) during the demineralization process. Clinically, these allografts are usually combined with barrier membranes following the principles of guided bone regeneration.

Xenografts are graft biomaterials of animal origin, mainly bovine and equine. These graft materials are usually de-proteinized by means of a chemical or low-heat process. This technique preserves the original bone architecture and the inorganic mineral bone composition but removes the organic component, thus preventing any possibility of immunogenic reactions. Anorganic bovine bone grafts (ABBG) and de-proteinized bovine bone mineral (DBBM) have shown good biocompatibility and osteoconductivity in preclinical studies when used following the principles of GBR, as porous particulate granules combined with a bioabsorbable collagen barrier membrane [38]. Moreover, these xenogeneic grafts have shown a very slow resorption rate what assures their long-term stability.

Alloplastic bone substitutes are synthetic grafts that include different combinations of calcium phosphates, bioactive glasses and polymers fabricated under different manufacturing and sintering conditions, hence resulting in different

physical properties and resorption rates. Hydroxyapatite (HA) constitutes the main mineral component of the natural bone, and it is the least soluble of the naturally occurring calcium phosphate salts, what provides an osteoconductive scaffolding function, being highly resistant to physiologic resorption [39]. In contrast, tricalcium phosphate (TCP) is characterized by rapid resorption and replacement by host tissue during the early phases of healing.

Similar to bone replacement grafts, different types of biomaterials have been tested as barrier membranes for guided bone regeneration (GBR). Their specific composition falls into two broad categories: non-absorbable and bioabsorbable. Non-absorbable e-PTFE membranes have been frequently used in bone regeneration clinical applications, mainly with reinforced titanium strips, thus providing space-making capacity for increased bone augmentation. However, the need of a second surgical intervention for their removal and the frequent occurrence of postoperative complications, mainly early membrane exposure, have limited their clinical use, resulting in the much broader use of biodegradable membranes. These bioabsorbable membranes must, however, ensure that the process of membrane resorption or biodegradation does not lead to tissue reactions that may affect the outcome of bone regeneration. Bioabsorbable membranes are either natural (xenogeneic collagen type I or III), which undergo resorption by enzymatic degradation, or made of synthetic polymers, which, when inserted in an aqueous environment, undergo enzymatic degradation by hydrolysis [40]. With bioabsorbable membranes, the barrier function duration is variable, since membrane degradation depends on many factors, such as the membrane composition, pH, temperature, the polymer crystallization degree and the cross-linking in collagen membranes, and, therefore, these resorptive processes may vary and interfere with wound healing and, hence, with the bone regenerative outcome [41]. Moreover, due to the lack of stiffness and space-making properties of bioabsorbable membranes, they will collapse into the bone defect or onto the implant threads under the tension of the flap, and thus, the space available for bone regeneration will be occluded. Since crestal defects are usually non-contained defects, the use of a scaffold with either particulate or a bone block replacement graft is a prerequisite for both lateral and vertical bone augmentation procedures [42]. The achievement of primary barrier fixation when the GBR technique is utilized to correct osseous defects is also necessary mainly in large defects, as the percentage of postoperative complications increases when barrier fixation is lacking [43].

The choice of the biomaterial should be based on the clinical indication. For bone defects requiring mainly horizontal bone augmentation, the use of particulate grafts together with barrier membranes (GBR) has been indicated, especially in combination with implant placement when there is enough bone width to allow for good implant primary stability. A systematic review evaluating these interventions reported a high implant survival rate of 97.8% (78–100%) [9]. The meta-analysis performed revealed a mean defect height reduction (exposed implant surface) for all the procedures of −4.3 mm [95% CI; −4.9–(−3.7)]. The most frequently used protocol for simultaneous bone regeneration around an exposed implant surface was the combination of a particulate xenograft together with a bioabsorbable membrane, showing a mean defect height reduction of −4.4 mm [95% CI; −5.5–(−3.4)]. The primary closure of the soft tissues to protect the graft and the membrane is crucial to achieve the proper outcome. In this sense, the same systematic review showed that the exposed cases experienced a less defect height reduction of 1.01 mm [95% CI; 0.39–1.64] (Fig. 6.4).

For bone defects requiring extensive horizontal augmentation, a staged bone augmentation procedure is usually indicated. In these situations, the primary outcome is the bone width gain, which allows the implant placement in a second-stage surgery. A systematic review evaluating these interventions reported a high implant survival rate of 97.8% (78–100%) [9]. The meta-analysis performed revealed a mean bone width gain for all the procedures of 3.9 mm [95% CI; 3.5–4.3]. The most frequently used protocol for stage bone regeneration prior to implant place-

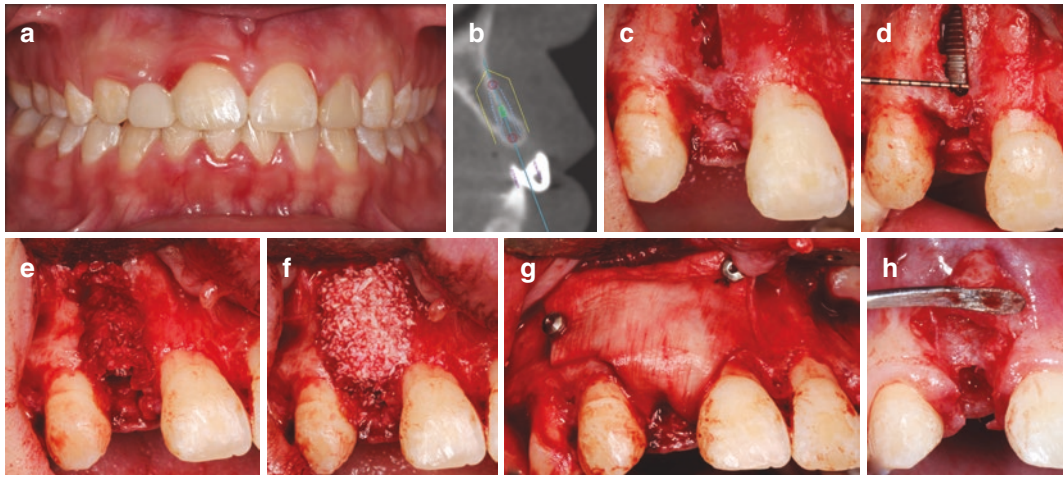

Fig. 6.4 Lateral bone augmentation protocol which includes (**a**) provisional restoration restoring the edentulous space in the upper right lateral incisor; (**b**) availability of the bone for implant placement in the right position; (**c**) implant placement with resulting fenestration defect; (**d**) placement of an autogenous bone graft over the exposed implant threads; (**e**) over-contouring the buccal bone crest with a slow resorbing bone replacement graft; (**f**) placement of a bioabsorbable collagen membrane fixed with titanium tags; (**g**) bone regenerative outcome 4 months after

ment was the use of autologous bone blocks, showing a mean bone width gain of 4.3 mm [95% CI; 3.3–4.9]. In these situations, the graft exposure was associated with a significant reduction on bone gain, with a mean of 3.1 mm less width gain [95% CI; 2.6–3.6] compared to nonexposed cases.

Ridge splitting and expansion were introduced as alternative approaches to bone grafting and GBR in narrow ridges. These techniques are based on the buccal displacement of the cortical plate through a longitudinal greenstick fracture and/or bone condensation. The major contraindication of the split crest technique regards the impossibility to modify the labio-palatal angle of implant insertion. In cases where preoperative anatomic situation involves a crest with insufficient bone volume plus extreme bone angularity, the split crest technique may jeopardize correct implant placement with respect to angulation. In these anatomic conditions, the GBR technique predictably allows implant placement in adequate prosthetically directed position [44].

Although there is clinical and histological evidence that vertical ridge augmentation may be achieved successfully, the predictability is low and the number of postoperative complications is high, mainly related to membrane exposure and the morbidity associated with the donor site when using autogenous bone grafts.

6.7 Future Technologies

Tissue engineering is making an important impact on bone regeneration therapy. The use of cell and gene therapy to enhance and direct periodontal wound healing into a more predictable regenerative path is being exploited in bioengineering efforts aimed at developing a therapeutic system to promote bone repair [45]. Various novel delivery scaffolding systems are being extensively studied and fabricated. An ideal scaffold should be made of materials that imitate the structure and properties of natural bone, include osteoprogenitor cells and provide the adequate environmental cues. However, development of optimized scaffolding matrices for the predictable regeneration of structurally and physiologically functional osseous tissues is still an elusive goal. A major obstacle is how to maximize the utility of cells/genes delivered to a passive or permissive environment where there is context for the type of cell needed but in which very few

biologic signals are given to encourage normal cell function [46]. Other obstacles, such as identifying cell sources and clinically relevant cell numbers, the integration of new cells into existing tissue matrices and the achievement of functional properties of tissue equivalents using an expanded repertoire of biomaterials, also need to be confronted in the field of tissue engineering. Practical and regulatory requirements will also need to be met before the technologies of cell and gene transfer can be applied in the clinical area.

Collectively, the cell-based, scaffold, and gene therapy methods interface and complement each other to enhance the potential to restore tissue function and structure in a predictable manner. It is expected that in the future there will be greater usage of growth factors such as bone morphogenetic proteins (BMPs) and platelet-derived growth factor (PDGF) to accelerate and enhance the healing potential of the defects, bringing about faster, easier and predictable treatment outcomes.

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7 A Critical Reason for Conducting Clinical Trials Is that Results with Humans Are Not Necessarily Predictable from Preclinical Studies

Bruce J. Baum

Abstract

In the biomedical sciences, when developing any new therapy for all types of human diseases, the typical sequence pathway followed progresses from laboratory bench research (e.g., studies in a test tube or cell culture), to experiments in an animal model, and finally to human clinical trials. The most often considered and obvious reasons for the early-stage (phase I/II) clinical trials are to ensure adequate safety and efficacy before allowing widespread administration of the therapy. However, there is another, critically important reason, not often discussed, which is that results from human clinical trials often are not entirely predictable from the preceding preclinical studies. Herein, this important reason is addressed using examples from the developing field of gene therapy targeting three quite different clinical disorders: cystic fibrosis, severe combined immunodeficiency X1, and irradiation-induced salivary hypofunction.

B. J. Baum
Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA
e-mail: bbaum@mail.nih.gov

7.1 Introduction

The last 30 years has seen an explosion in the amount of biological knowledge gained. As someone who earned a Ph.D. in biochemistry in 1974 without any training in what has become known as molecular biology, this explosion of information is part of my personal experience. While I was in graduate school, the polymerase chain reaction (PCR), reverse transcriptase, restriction endonucleases, routine and easy DNA sequencing, and the like were just simply not part of scientists' knowledge base or even in the general imagination of more than a rare few. At present these and many other molecular biological tools are mundanely used in all types of laboratories around the world. Furthermore, in many cases these tools have delivered substantial benefits related to the treatment of human disease.

I began to work in the field of gene therapy in 1991 shortly after the first paper was published demonstrating the feasibility of its use in humans [1]. It was a heady time for those working in the field, as there was unbridled optimism that the methods available generally were straightforward and that various successes in animal models would soon translate to comparable successes in the clinic. However, that beneficial translation was much slower to be achieved than expected and serious problems also occurred (e.g., [2, 3]). Even today, gene therapy can be considered a

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biological treatment that is just beginning to show return on its promises [4].

While there are now several excellent examples of gene therapy benefiting patients, for me, during the time I have worked in this field, there have been three major lessons that will be evident throughout the discussion herein: (1) it is critically necessary to appreciate the many nuances of human physiology and pathology, (2) at the current time relatively little (only a small fraction) is known about molecular biology-nucleic acid biochemistry, and (3) there are significant limitations to most of the molecular tools now available to treat patients, even those applicable to patients with "straightforward" single gene defects. Most importantly, these lessons present an overarching and critical take home lesson for all individuals trying to develop novel therapies for humans—it is essential to study humans. Results from preclinical animal studies may not predict what will be observed in early-phase clinical trials. Simply put, in order to treat human patients better, it is necessary to conduct experiments on humans. This need exists despite (1) an incomplete knowledge base about the disease and the molecular or other biological therapies being performed and (2) a lack of ideal tools with which to perform the desired treatment.

The approach employed herein will be to give specific and relevant examples from three areas of gene therapy research that have transitioned from bench toward the bedside: cystic fibrosis (CF), severe combined immunodeficiency X1 (SCID X1; "boy in the bubble" disease), and my own work on irradiation (IR)-induced salivary hypofunction. Each example will, to varying degrees, demonstrate the three lessons described above, as well as the bottom-line message of the critical importance of conducting, and learning from, human clinical trials.

7.2 Cystic Fibrosis

CF is a fairly common inherited disorder. While it affects many organs, its major and most life-threatening manifestations occur in the lung [5]. CF results from a mutation in a single gene, which encodes a chloride transport protein termed the CF transmembrane conductance regulator (CFTR). Mutations that impede CFTR's physiological role lead to altered water movement and mucociliary dysfunction in lung epithelia. What results is an accumulation of a sticky mucous, which promotes bacterial infections and pulmonary inflammation [5]. CF patients have a markedly shortened lifespan, typically <40 years, and a significantly reduced quality of life [5]. Consequently, it is no surprise that CF was one of the earliest diseases targeted for gene therapy (e.g., see [6, 7]). As reviewed recently by Sondhi et al. [5], "eleven different mouse models of CF have been developed," by knocking out the CFTR gene. However, most of these models have little to no lung disease nor do they develop the spontaneous lung inflammation characteristic of the human disease [5].

It is not difficult to transfer a gene into the lungs. For example, the human CFTR gene has been delivered directly by intratracheal instillation in many species, from mice to humans. This method provides a straightforward way to perform in vivo gene therapy to the lung, using either a viral or non-viral vector. It has been employed both for evaluating vector-directed expression in normal mice (e.g., [8]) and for use in "curing" a mouse model of CF (e.g., [9]). However, as we now know, these studies in mice had little value in predicting the success, or in this case lack of success, of this gene therapy approach in humans.

Larger animal models, e.g., pigs with knocked out or mutated CFTR genes, also have been developed [10, 11] and fortuitously exhibit lung pathology similar to that seen in humans [12]. We know from other studies (e.g., [13]) that fairly efficient transfer and expression of the human CFTR gene in porcine lung epithelial cells can be accomplished in normal pigs using an aerosolized, helper-dependent (totally defective) adenoviral vector. Furthermore, it's clear that this procedure was generally safe in pigs. However, it is not yet clear that such gene transfer maneuver will be effective in "curing" a pig exhibiting CF-like disease.

While gene delivery to the airways is reasonably efficient in normal human lungs, via

intratracheal administration (e.g., [14]), in a CF lung clogged with sputum, as well as highly inflamed and infected with bacteria, it is quite inefficient [15]. Indeed, efforts to develop strategies to remove the viscous sputum have been modestly successful in the laboratory; however, they have not yet been translated to useful clinical applicability. Sondhi et al. [5] point out that there are numerous challenges to achieving successful gene therapy for CF, via the commonly used clinical approach of intratracheal delivery (e.g., as used in bronchoscopy), including targeting the correct cell population, efficient gene expression in those cells, the normal turnover of lung epithelial cells, the vectors available for gene transfer, as well as the recollection that CF affects many other organs besides the lung. Thus, despite the existence of a huge research effort focusing on CF gene therapy for >25 years now, all that has been learned from these studies, and the initial success found with mouse models, is that the human pathology of CF and the limitations of existing gene transfer vectors have precluded any level of clinical gene therapy success for this condition [5].

7.3 SCID X1

SCID X1 is an X-linked inherited disorder in which patients have a mutation in the γ_c cytokine receptor subunit gene. Since the γ_c chain is common to many different hematopoietic cytokine receptors, e.g., interleukin-2, interleukin-4, interleukin-7, interleukin-9, and interleukin-15 receptors, its mutation results in a block in the development of T lymphocytes and natural killer (NK) cells [16]. Also, as a consequence of this mutation, SCID X1 patients have dysfunctional B lymphocytes. Since SCID X1 patients represent 30–40% of all SCID patients, it also is not surprising that this single gene disorder became an early target for developing a gene therapy [16]. In fact, the first report of any successful gene therapy in humans was for SCID X1 [17].

After years of preclinical studies, Cavazzana-Calvo et al. [17] developed the methods to perform what is called ex vivo gene therapy. With their approach they took T lymphocytes from two SCID X1 patients and inserted the correct γ_c receptor subunit gene into the DNA of those cells using a defective, and thus presumably safe, gamma retrovirus (Moloney Murine Leukemia Virus, MoMLV). Afterward, they amplified the T lymphocytes in cell culture in vitro and then infused a large number of the genetically modified cells back into the corresponding donor SCID X1 patients [17]. The original report in 2000 was greeted as essentially a miracle for the two, treated patients, each of whom had failed a bone marrow replacement therapy and, thus, were destined to spend their greatly abbreviated lives within the confines of an airtight bubble in a hospital room. After the treatment, both patients had normal levels of T, NK, and B lymphocytes, all of which also functioned normally. The two patients were able to leave the hospital and live the lives of normal children, e.g., play in a park, go to school, etc. [17].

In aggregate, this research group treated a total of nine SCID X1 patients using the MoMLV-mediated γ_c cytokine receptor subunit gene therapy strategy [18], and the therapy was successful in eight of these children. Of note, all additional patients, beyond the initial two, were apparently treated within in a few years of the 2000 publication. The reason for the limited number of patients being treated, and for the narrow time frame when the treatment occurred, was because in 2003 this group reported a very serious adverse event in four of the treated patients—the development of acute lymphoblastic leukemia [18, 19]. Three of those four patients were treated successfully for the leukemia, while the fourth died. Overall, seven of the nine originally treated SCID X1 patients, including the three surviving post-leukemia patients, remained alive and experienced a successful immune cell reconstitution [18].

Thus, despite the significant risk of developing an acute leukemia, this gene therapy represented a reasonable treatment risk for the children (and their parents) with SCID X1; there literally was no other viable alternative. However, these investigators, and many of their colleagues around the world asked what led to

the serious adverse events and what, if anything, could be done to eliminate or at least minimize the risk of their future occurrence [16]. The resulting studies were important not just for the SCID X1 patients but also for patients with many other disorders, hematopoietic and non-hematopoietic, for which an ex vivo gene therapy employed the MoMLV vector. It turned out that with the lymphocytes of the children who developed leukemia, the MoMLV vector had inserted the γ_c cytokine receptor subunit gene into a site in chromosome 11 within what is called the *LMO-2* gene locus. This locus codes for the expression of a proto-oncogene and the insertion of the foreign gene led to the aberrant expression of *LMO-2*. Aberrant expression of this gene previously was known to lead to acute lymphoblastic leukemia [18], so the reason for the serious adverse events became understood. The efforts to minimize such future occurrences led to studying retroviral integration patterns and, subsequently, the development of much better and safer retroviral vectors [16]. Indeed, at the time of this writing (May 2017), the European Union has given marketing approval for the treatment of another SCID (based on a deficiency of the enzyme adenosine deaminase; [20]), which utilizes the same general ex vivo gene therapy approach employed to treat SCID X1 patients but using a new, much safer, generation of retroviral vector.

7.4 Irradiation-Induced Salivary Hypofunction

As a group, head and neck cancers are the sixth most common malignancy worldwide, with ~500,000 cases occurring each year. Most patients are treated, at least in part, with therapeutic IR, which can damage normal tissues falling within the IR field, including the salivary glands. The end result of such salivary gland damage, for many patients, is markedly decreased saliva output and, consequently, dysphagia, a high risk for aspiration, increased oral infections (e.g., candida, caries), decreased oral mucosal wound healing, and considerable pain and discomfort. These patients, not surprisingly, experience a markedly reduced quality of life [21].

While there have been great advances in methods to deliver therapeutic IR and limit normal tissue damage, e.g., intensity-modulated radiation therapy, known as IMRT [22, 23], IR-damaged salivary glands and its sequelae still remain a significant clinical problem. There is roughly a 65% 5-year survival rate for head and neck cancer patients [24], which means many former patients are alive, thankfully, but suffering from IR-induced salivary hypofunction. Additionally, at present advanced treatment modalities, such as IMRT, are typically available at major medical centers in highly developed countries, which means many people worldwide are still treated with conventional radiation therapy and continue to be at risk for normal tissue damage.

In 1991, my research group began the long process of trying to use gene transfer technology to provide a "repair" of IR-damaged salivary glands that in turn would lead to patients with more saliva and improve their objective problems and symptomatic complaints. As indicated in the first paragraph of the Introduction, I had no background in molecular biology to support me on this endeavor. However, thanks to the help of a former postdoctoral mentor (Ronald G. Crystal, e.g., see [25]), and three outstanding postdoctoral fellows working in our group in the early days of this project (Brian C. O'Connell, Christine Delporte, Hideaki Kagami), we were able to make considerable progress in applying gene transfer to salivary glands and demonstrate proof of concept in a rat model for the "repair" of IR damage to salivary glands (e.g., see [26–28]). The general strategy that we employed used a first-generation serotype 5, adenoviral (Ad5) vector to deliver the cDNA encoding human aquaporin-1 (hAQP1), the archetypal water channel protein, via the cannulated main excretory ducts of salivary glands, a not dissimilar approach from the intratracheal administration of gene transfer vectors to the lung as described above. The vector created was termed AdhAQP1 [26]. Later studies with wonderful colleagues in my research group (Changyu Zheng, Corinne Goldsmith) and a collaboration with an excellent former postdoctoral

fellow (Songlin Wang) demonstrated the safety of AdhAQP1 and the delivery approach used (e.g., see [29]), as well as its extension to a large animal model of IR-induced salivary gland damage (e.g., see [30]).

A good thing about first-generation Ad5 vectors is that they are relatively easy to create and use, which was advantageous for a group with minimal molecular biological and virological experience such as ours in the 1990s. Another good feature of these Ad5 vectors is that they lead to high levels of functional gene transfer in the targeted tissues. However, there is an important negative feature of such Ad5 vectors: they can elicit potent immune responses (innate, cellular, and humoral) after administration. Because of the latter, first-generation Ad5 vectors, in a wide range of animal models and tissues, and in many clinical studies targeting non-salivary gland tissue, yield only transient expression of the delivered transgene, typically for no more than a week or two, with a peak response on days 2 or 3. Indeed, our studies using Ad5 vectors, including AdhAQP1, with salivary glands of mice, rats, miniature pigs, and macaques demonstrated a similar, short time course of transgene expression.

Despite the above-described shortcomings of first-generation Ad5 vectors, based on the results of our preclinical studies with AdhAQP1, and after extensive toxicology and biodistribution studies [31], we developed a protocol for a phase I/II clinical trial to test the vector in IR-damaged parotid glands of human subjects (http://www.clinicaltrials.gov/ct/show/NCT00372320?order=). The subjects enrolled exhibited grade 2 or 3 damage to their parotid glands according to the criteria of the Radiation Therapy Oncology Group [32], i.e., they had some epithelial tissue remaining in their parotid glands, but were not responsive to conventional pharmacological treatment with Salagen or Evoxac. Interestingly, because of the anticipated short expression time from AdhAQP1 as observed in our preclinical animal studies, the original purpose of the clinical trial (http://www.clinicaltrials.gov/ct/show/NCT00372320?order=) was considered essentially to be a proof of concept. We fully expected that hAQP1 gene transfer to the IR-damaged parotid glands would lead to increased fluid secretion for at most 2 weeks, with a peak response on days 2 or 3. Consequently, we thought the subjects in the trial in the event of positive results unlikely would experience long-term benefit (http://osp.od.nih.gov/sites/default/files/RAC_minutes_12-05.pdf).

Eleven subjects were treated with AdhAQP1 in this first in human clinical trial [33]. The initial findings of that trial, through day 42 post-AdhAQP1 delivery, identified 5 of 11 treated subjects as responding positively to the gene therapy. The positive response was defined as both increased salivary flow from the targeted parotid gland, as well as the improvement of two key symptomatic benefits (a subject's perception of the amount of saliva, and the level of dryness, in their mouth). Interestingly, the peak increases of parotid salivary flow rates for all responder subjects were observed at much later times than seen in animal models, from 7–42 days after AdhAQP1 delivery. The originally approved clinical protocol required subjects to be followed for 360 days. However, the protocol was amended because the first responder subject, whose initial peak response to gene transfer occurred on day 7, exhibited a second, later elevation in parotid salivary flow rate, on days 180 and 360, well above his baseline. Accordingly, the original protocol was modified to allow the evaluation of all responders to AdhAQP1 for two additional times, at least 1 and 2 years following their completion of the original 360-day protocol [34].

The AdhAQP1 clinical trial was a human study involving gene transfer to a salivary gland, and the results were unexpected based on (1) all previous clinical trials in humans with Ad5 vectors in other tissues and (2) our own studies delivering Ad5 vectors to the salivary glands of multiple animal models. Not only did the initial results show peak expression times much later than seen previously, but all five responder subjects after long-term follow-up displayed substantially elevated levels of parotid saliva flow 3–4.7 years after the AdhAQP1 administration. Furthermore, most subjects experienced relief from two key xerostomic symptoms for at least 2 years after treatment [34].

We think there were two key reasons for this unusual result. First, it is widely thought that the immune response to a first-generation Ad5 vector delivery leads to complete removal of the vector from the targeted tissue. However, we have shown that is not the case in rat salivary glands [35]. For example, after a dose of 10^9 vector particles/rat submandibular gland, 0.1% of the delivered dose was still present in gland tissue 6–12 months later [35]. Secondly, we recently showed that the human cytomegalovirus promoter used in AdhAQP1 is substantially methylated in rodent salivary glands, a modification that inhibits its ability to function as a promoter and lead to transgene expression. This does not occur in human cells [36]. The combined results of both Zheng et al. [35, 36] studies imply that the AdhAQP1 vector will be (1) present in human parotid glands long after its administration and (2) able to direct the expression of functional hAQP1 [34]. Thus, the results of the AdhAQP1 clinical trial could not have been clearly predicted from preclinical animal studies, including those with miniature pigs and nonhuman primates.

7.5 Key Differences Between Human and Animal Models

Aside from the obvious physical differences between humans and the animal models used in preclinical studies, there are some key biological differences that doubtless influence responses to viral vector-mediated gene transfer such as described above. Of particular note are immunological and genetic differences. This point can be clearly appreciated when comparing humans with mice, since the latter are the most commonly employed preclinical animal models of disease treatment. However, as noted by Davis [37], while mice have been extremely useful for developing an understanding of basic immunology, they have been less helpful in understanding human disease. Following that perspective, by studying human immunology directly and not just extrapolating from results obtained with inbred strains of mice, Su et al. have found numerous differences in T-memory cell mechanisms [38]. Similarly, Benitez et al. have shown key differences in the dynamics and mechanisms for nonmemory B cells between mice and humans [39]. Thus, it would not be surprising to find that immunological responses to a viral or non-viral gene transfer vector could be markedly different when studied in mouse models from that found in actual clinical trial subjects [40].

A comparable pattern of differences emerges from genetic studies of mice and humans. For example, inbred strains of laboratory mice share most of their protein-coding genes with humans [41]. However, as reported by Yue et al. [41], "the Mouse ENCODE Consortium mapped transcription, DNase I hypersensitivity, transcription factor binding, chromatin modifications and replication domains throughout the mouse genome in diverse cell and tissue types," and compared those results with data from humans. They found considerable "divergence of sequences involved in transcriptional regulation, chromatin state, and higher order chromatin organization" [41]. Many other studies lead to the same general conclusion, i.e., that there are quite important differences related to genetic regulation between mice and humans, such as in the patterns of gene expression seen during development [42] or RNA expression profiles for both coding and noncoding regions [43], which the authors state, "likely reflects fundamental physiological differences…".

While it certainly is not surprising that differences exist between humans and mice in key aspects of immunological and genetic regulation, similarities in these biological processes do exist between the species. What then becomes important for investigators is to understand which mechanisms are similar between humans and mice (or any other preclinical animal disease model) and which are different, so that preclinical studies aimed at developing novel therapies can be best designed for likely translation into effective clinical human treatments. For example, the work of Godec et al. [44] and Li et al. [45] have generated data sets of genetic patterns of immune reactivity in humans and mice for different conditions, e.g., sepsis and inflammation, that

show both differences and similarities. These then can be used by investigators to target specific genes or mechanistic pathways that are best conserved between the species and, thus, likely to be most valuable in novel therapeutics discovery. The same is true when looking at specific genetic components of diseases, e.g., when trying to develop the best animal models for use as models of human neurological diseases [46] and human DNA repair [47]; there are both similarities and differences between humans and different animal models.

Conclusion

As noted at the outset of this chapter, results from human clinical trials often are not as expected from the preceding preclinical animal studies. Animal experiments are still necessary in the process of developing new therapies. However, there is a critical need to study humans not only to determine if therapies developed in animals are safe and effective but also to appreciate physiological differences, manifested in genetic, immunological, and other characteristics, that will permit the development of better therapies in the future. Human clinical trials are experiments, and in order to move forward, we need to understand human biological mechanisms with as much detail as we can possibly garner from experiments in mice and other animal models.

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8 Evidence-Based Approach in Translational Dental Research

Sok-Ja Janket, Martha E. Nunn, Erdjan Salih, and Alison E. Baird

Conclusions which are merely verbal cannot bear fruit, only those do which are based on demonstrated fact. For affirmation and talk are deceptive and treacherous. Wherefore one must hold fast to facts in generalizations also, and occupy oneself with facts persistently, if one is to acquire that ready and infallible habit which we call 'the art of medicine'.

Hippocrates (c. 460–c. 370 BC)

Abstract

Many laboratory scientists are not trained in epidemiology and are lacking the ability to interpret the relationship of the observed results from the laboratory bench to the outcomes. For example, cytochrome p450 27B1 (CYP27B1) is an enzyme that converts 25-hydroxyvitamin D (Calcifediol) to 1,25-dihydroxyvitamin D (calcitriol), the bioactive hormonal form of Vitamin D in the kidney. Several studies reported that vitamin D insufficiency may facilitate development of cancers. This simplistic way of thinking asserts that lack of vitamin D will cause cancer.

However, lipopolysaccharide (LPS) or Toll-like receptor2 increased the expression of CYP27B1. We know that precedent infections, obesity, or both would increase LPS and TLR2. We also know that the cause must occur before the outcome. Therefore, what happened earlier (infection and/or obesity) would be the real cause and the low level of vitamin D may be a marker for low immune responses. Unless we compare vitamin D, infection/obesity side by side in the same statistical models, we will never identify the real cause. This example clearly suggests that to be able to establish a causal relationship correctly, the bench scientists involved in translational research need to learn the basic epidemiologic principles. Otherwise, their conclusion might be incorrect or biased. In this chapter, we introduce the basic epidemiologic concepts and techniques needed to assess and infer causal relationships in translational research.

S.-J. Janket, D.M.D., M.P.H. (✉)
Translational/Applied Oral Science, Forsyth Institute, Cambridge, MA, USA
e-mail: sjanket@forsyth.org

M. E. Nunn, D.D.S., Ph.D.
Periodontics, School of Dentistry, Creighton University, Omaha, NE, USA

E. Salih, Ph.D.
Periodontology, Goldman School of Dental Medicine, Boston University, Boston, MA, USA

A. E. Baird, M.D., M.P.H., Ph.D.
Neurological Disorders and Stroke, SUNY Downstate Medical Center, Brooklyn, NY, USA

8.1 Introduction

8.1.1 What Is Translational Research

Translational medicine (TranM), according to the European Society for Translational Medicine

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definition, is "an interdisciplinary branch of the biomedical field supported by three main pillars: bench-side, bed-side and community." The goal of TranM is to combine disciplines, resources, expertise, and techniques within these pillars to promote enhancements in prevention, diagnosis, and therapies of diseases, including oral diseases. Our own interpretation of TranM is that "TranM is a branch of science where what has been learned from the laboratory bench is tested in the clinical settings and finally is applied to the population to reduce human diseases." Thus, translational research (TranR) pertains to all the research activities preceding TranM including laboratory assays and their applications to individual patients and the populations at large. In the application steps, it is vital that at all steps of the principles of epidemiology, especially the evidence-based principles, are adhered to.

Even as early as 400 BC, Hippocrates recognized the importance of precise fact-finding methodology in medicine. To assess scientific facts precisely, one needs to utilize appropriate methodologies which often require accurate laboratory procedures which generate reliable, consistent, and reproducible data to measure biomarkers or other pertinent molecules. While both simple/classical and state-of-the-art technologies can meet the prerequisites of robust result acquisition, they are both, nevertheless, subject to the inherent need to utilize the principles of the evidence-based approach. Using elaborate laboratory experiments that employ sophisticated technology does not negate the necessity of recognizing and applying the principles of the evidence-based approach. These principles include:

1. Development of a scientifically sound rationale
2. Establishing a causal relationship
3. Controlling for other competing factors (confounding)
4. Minimizing biases both in human subjects and instruments
5. Reducing measurement errors
6. Utilizing the appropriate statistical methods

Traditionally, not all laboratory scientists are well-informed in these areas of evidence-based methodology, and those who are may frequently overlook these principles as they become weakened/obscured by various competing interests. Therefore, evidence-based principles remain critical and invaluable component of the translational research that demand within the context of this chapter elaboration. In this chapter we will detail the importance of an evidence-based approach in dental TranR and highlight some examples where these principles were applied successfully and not so successfully. Also, we hope to guide laboratory and bench researchers, who, while well versed in complex molecular pathways, are not fully aware of these principles so that this information can be carried and applied to the clinical setting.

For example, our own cross-sectional study "Salivary immunoglobulins and prevalent coronary artery disease" reported an association between salivary immunoglobulin A (sIgA) and coronary artery disease (CAD) [1]. The word "prevalent" coronary artery disease in the title suggests that it is a cross-sectional study where the predictor and outcome were assessed at the same time. Therefore, this study could not have assessed causality. Prevalence is disease occurrence at one time point without the consideration of the time when it occurred. Therefore, the disease could have occurred before the predictor assessment. Meanwhile incidence refers to an occurrence of new cases starting from the study beginning when the predictor has been assessed. Therefore incidence rate indicates a longitudinal assessment of the relationship of predictor and the outcome. However, longitudinal relationship does not automatically establish causality. We will discuss the causality establishment in detail in the Sect. 8.3.

Although our IgA study adjusted for all pertinent confounding, the cross-sectional study design prevents the inference of causality [1]. Nevertheless, this study is quite important in other ways. If infections are considered a causal driver of inflammation involved in atherosclerosis, the assessment of the infection should be done on the mucosa where

pathogens first contact the host. Thus, IgA, a marker of mucosal immunity, is an appropriate marker for infection. The importance of the appropriate immunoglobulin usage was ignored by many renowned investigators [2–4], but only few recognized [1, 5]. For this reason, Chlamydia pneumoniae IgA was significantly associated with coronary heart disease [5] while Chlamydia pneumoniae IgG was not [4]. Our study has proven that oral mucosal immunity measured by salivary IgA was positively while systemic immunity measured by salivary IgG was inversely associated with CAD [1].

In non-causal association, the risk is usually reported by the odds ratio (OR), while the causality study usually reports incidence rate (IR). However, incidence rate does not always establish causality. For example, a newly published study reported that canakinumab, an interleukin-1β inhibitor, decreased the incidence of lung cancers [6]. Although canakinumab lowered the number of new cases of lung cancer, this does not mean inflammation inhibited by interleukin-1β is a causal factor for lung cancer. Because lung cancer has a long (30–50 years) latency [7], this suggests that the true cause had initiated pathogenic processes 30 plus years ago. Thus, what was observed in 2 years cannot be a causal factor, although it is possible that canakinumab could have modified the disease progression after the initiation of pathogenesis. Because the cancer microenvironment creates a low immune milieu to avoid the immune system's detection and destruction of cancer cells [8]. Cancer might have been caused by other factors, but inhibiting the inflammatory process might have decelerated lung cancer manifestation. In summary, dental and medical researchers need to be critically aware of differentiating between association and causality.

8.2 Development of Sound Rationale

This criterion is of foremost importance. No matter how well other criteria are fulfilled, without a sound rationale, the end results can be misleading or may impose potentially serious undesirable diversion of the health resources. This oversight can be quite subtle, and even very experienced scientists may fail to notice the problems in the ill-conceived rationale at the study initiation stage. If the study involved is a randomized clinical trial (RCT), the repercussions are even greater, because RCTs are considered the gold standard, and it is assumed that all the confounding factors will be balanced across the groups under comparison. However, the bias resulting from an ill-conceived rationale cannot be corrected by the study design or statistical analyses.

One prime example is the Women's Health Initiative (WHI) group's estrogen replacement therapy (ERT) and cardiovascular diseases trial [9]. Everything in this trial was done following the principles of RCT epidemiology. However, the investigators failed to recognize irreversible changes that occur with age and included women 70 years or older in the study. Older women not only have subclinical vascular changes that precede cardiovascular events but also thrombotic tendency dramatically increases with age [10].

Thus, with additional estrogen which increased the thrombotic activities and resulted in the increased risk of cardiovascular disease (CVD) seen in that study. Consequently, it is not clear whether estrogen is the culprit or old age is the cause for the increased risk for CVD. The uproar following the publication of the results of this trial forced the WHI investigators to reanalyze the data and published the results of post hoc subgroup analyses including only those women who were given estrogen immediately after menopause. The results of post hoc analyses showed no detrimental effects of ERT given at the right time to appropriate cohorts and may even have had some beneficial effects on various inflammatory diseases [11–14]. The key point is that age is an effect modifier in ERT, and specific time and appropriate cohort should be considered.

Another important example is the clinical trials as well as their meta-analysis using bisphosphonates (BISPs) alone or with adjuvant as chemo-/hormonal therapy in cancer patients

which revealed inconsistencies in results regarding whether BISPs have anticancer effects or not [15–21]. In a more recent report of clinical trials, it was found that at doses used for osteoporosis, neither alendronate nor zoledronic acid reduced the risk of breast cancer [22], contrary to reports of a protective effect seen in several observational studies [18, 19]. Furthermore, data analysis from adjuvant bisphosphonate trials showed no effect on local recurrence or contralateral breast cancer incidence [23]. Hence, over the several years, many clinical trials were conducted, yet the inconsistencies remain to date. Multiple deficiencies in studies were observed:

(a) Ill-designed rationale.

(b) Lack of clear understanding of pharmacokinetics of the drug.

(c) Impact of performing clinical trials with mixed patient background such as postmenopausal (ages ~50–70) and premenopausal (ages 35–50), as well as pooling those who are undergoing hormonal therapy or without hormonal therapy.

(d) Predetermined biased attitudes of the investigators based on the findings from animal experiments, which showed beneficial effects were at least ~100-fold higher than that of the maximum possible dose for human use.

(e) Different drug dose usage in different clinical trials and variations in length of time for observations.

(f) Different analytical methods used for evaluation of the outcome of drug action.

8.2.1 Preconditions for Mendelian Randomization

A currently popular longitudinal study equivalent is using genetics to determine the subsequent risk of disease occurrence called Mendelian randomization. This method was hailed as an alternative to longitudinal study to circumvent a long follow-up, confounding, and biases without conducting a traditional randomized trial. Because genes are present at birth, genes will always precede any disease that can occur later in life. However, many studies ignored the fact that the disease of interest must be under strong genetic influence. One recent study reported that the gene loci associated with obesity such as *FTO*, *MC4R*, and *TMEM18* did not predict periodontitis [24]. In our opinion, periodontitis is influenced by epigenetic and lifestyle factors such as aging, smoking, diabetes, and general immune dysfunction. This study showed that genetics play a minor role in the relationship between periodontitis and obesity.

Only 20% of BMI can be explained by genetics [25]. The underlying causes for obesity include complex interactions between genetic traits, low physical activity levels, excess caloric intake, and type of diet that encourages certain microbial growth in the gut, as well as environmental factors such as access to affordable, healthy food, and socioeconomic status [26]. Some twin studies report that 60–70% of BMI can be explained by genetics [27, 28]. However, it should be noted that a cohort of twins cannot be considered as an independent population, and this result should not be applied to heterogeneous populations. Moreover, sophisticated gene sequencing cannot overcome a misguided study rationale. Conversely, it could be said that epigenetics and lifestyle factors such as smoking, physical activity, diet quality, and caloric intake may have stronger influence in developing high BMI or periodontitis than genetics. These epigenetic factors are modifiable risk factors, and further understanding of epigenetic mechanisms may help prevent burgeoning BMI and/or periodontitis.

In another study, even the leptin receptor gene predicted only small portion of body weight in a genetically homogeneous population [29]. If lifestyle factors have stronger impacts on disease phenotype, then the genes associated with BMI such as FTO (rs1121980), MC4R (rs17782313), and TMEM18 (rs6548238) will not show any association with periodontitis. Consequently, null results will not clarify whether "BMI is truly not related to periodontitis" or "BMI to periodontitis relationship is not strongly affected by genes" and merely pointing an inappropriate study rationale. One example in an inappropriate study rationale can be found in "studying sexually transmitted disease in nuns" for obvious reasons.

Another early example of misguided use of Mendelian randomization was the first report of this kind regarding the causal role of C reactive protein (CRP) in CVD. Two studies reported null results discrediting CRP in causal relation to CVD [30, 31]. Our argument is not based on the fact that whether CRP is a cause for CVD or not. Rather, we question the validity of examining the genes to determine CRP's role in CVD. CRP levels change largely due to epigenetic and metabolic influence such as increased BMI [32–35] which is a modifiable CVD risk owing to an imbalance in caloric intake and expenditure. As was reported only 20% of BMI can be explained by genetics [25] and CRP, which is a BMI-driven inflammatory marker, and the gene associated with CRP may not show any relationship to CVD. In other words, these studies missed 80% of CRP's role in CVD pathogenesis.

8.3 Criteria for Causality

To reduce human disease, it is necessary to identify the factors that cause the disease and clarify how to minimize the exposure to these causative risk factors. In 1965, Sir Austin Bradford Hill, an English epidemiologist, suggested a set of criteria that may suggest a potential causal relationship between the factors. These six criteria are:

(a) Temporality
(b) Strength
(c) Consistency
(d) Specificity
(e) Biological gradient
(f) Biologic plausibility

8.3.1 Temporal Relationship

This criterion is the most important of all criteria for causality establishment and must always be satisfied. In other words the cause must precede the outcome at all time. What happens after the disease manifestation cannot be the cause for the disease. This seems obvious, but often the disease has long latency, and subclinical pathology is going on for a long time, and it can be difficult to determine whether the predictor is the cause or the result of the disease. For example, self-reported periodontitis recently found to be associated with non-Hodgkin's lymphoma in a prospective follow-up study [36]. Non-Hodgkin's lymphoma (NHL) in this study included several slow-growing lymphatic malignancies such as chronic lymphocytic leukemia, small lymphocytic lymphomas, diffuse large B-cell lymphomas, and follicular lymphomas. Certainly, the temporality requirement has been satisfied, i.e., predictor periodontitis was assessed before the diagnosis of NHL. However, causality is not as clear in this case because non-Hodgkin's lymphoma has a long asymptomatic latency which accompanies low immunity [37]. Thus, immune dysfunction prior to the cancer diagnosis is quite possible due to many immature lymphocytes which cannot generate strong immunity crowding the circulatory system. As such, periodontitis may be one manifestation of low immunity originating from yet to be diagnosed NHL in this case. In fact, anemia and leukemia manifest in the periodontium as periodontitis and gingivitis [38]. Therefore, reverse causation is quite possible in the relationship of periodontitis and non-Hodgkin's lymphoma [39].

Due to the temporality requirement, cross-sectional studies which assess the predictor and the outcome at the same time cannot prove a causal relationship. Unfortunately, in dental research, this causality consideration is often neglected. The caveat is that a significant predictor-outcome relationship even in a longitudinal study does not certify causality [40]. All other confounding variables must be controlled, and the rationale has to be sound and biologically plausible.

8.3.2 Strength (Effect Size)

Although small effect size does not preclude causality, a large effect size is more likely to suggest a causal relationship. For example, if smokers are eight times (800%) more likely to have periodontitis than nonsmokers, then smoking may be a

causal risk factor for periodontitis [41]. On the contrary, if the association has only a 20% increase in risk as in the case of having periodontitis and the risk of future CVD [42], it contains a high likelihood of having a non-causal relationship such as due to residual confounding, measurement errors, or even chance occurrence.

8.3.3 Consistency (Reproducibility)

If different scientists at different time periods report similar findings, this suggests the likelihood of a causal relationship. However, this assumption of reproducibility as a marker for causality must be interpreted with caution. It is possible that if several groups used the similar flawed methodology, the consistency does not support causality. Rather, it supports the theory that flawed methods consistently generate similar erroneous conclusions. One example refers to a study where a questionnaire was used to assess periodontitis and tested whether having periodontitis increased the risk of CVD. They observed no relationship (null result) [43]. A second study used exactly the same questionnaire and found similar null results [44]. A subsequent meta-analysis has proven that using an imprecise questionnaire in predictor assessment caused underestimation of the relative risk due to non-specific misclassification [42]. Non-specific misclassifications will move the results toward the null: in other words, the contrast between the compared groups will diminish due to the mix-up in the categorization of the exposure.

8.3.4 Specificity

Causation is more likely if the association occurs in a specific population and specific tissues or organs with no other overlapping factors. One negative example is C-reactive protein (CRP). Minor CRP increases (2 mg/L) are observed in about 50% of the US population [45] and associated with over 100 biological conditions including aging and strenuous physical activites [46, 47]. Minor increases in CRP are presumed to indicate cell stresses that may or may not be pathologic [47]. Thus, holding CRP responsible for one disease may be a difficult task because it is necessary to control for over 100 other comorbidities or pathologies. Similarly, IL-6 is a pleiotropic signaling molecule involved in many biologic actions. It plays an important role in the immune response, hematopoiesis, inflammation, oncogenesis, and other transcription factor expressions. Thus, IL-6 is not specific enough to prove its role in one disease or in one pathway.

8.3.5 Biological Gradient

This is also called dose-response. Lower level exposures would generate less serious outcomes, while greater exposures will bring about more severe outcomes. Dose-response does not always mean causality. In some disease, there may be a distinct threshold rather than a dose-response, and yet, the predictor may be a causal risk factor. For example, some causal risk factors show significant risks in the top quartile but no increased risks in the lower levels.

8.3.6 Biological Plausibility

Many bench scientists can conjure up biological plausibility. However, we must consider other parallel possibilities. For example, recent theorem that trimethylamine N-oxide (TMAO), a metabolite of the gut microbiotas of choline increased the CVD risk, generated considerable interest [48]. Several reasons prevent us from getting overly excited about the role of TMA or TMAO in human diseases. First, there are 100 trillion microbiotas in a human body with complex interactions involving the huge quantities and diverse range of microbes. Thus, identifying one or several microbes in a disease relationship is nearly impossible. Second, the gut microbiome is not readily accessible without special procedures. Many researchers use the fecal microbiome to estimate the alteration in gut microbiome. This is a gross violation of the temporality requirement of causality. The fecal microbiome is at the terminal end

of the alimentary track and does not precede gut microbiome. Thus, the fecal microbiome cannot be the cause for the biologic activities in the gut. Third, many foods generate TMAO, and the results were too non-specific. Consequently, the biologic plausibility appears to be weak. One recent study actually reported that TMAO analyses may be biased: in stroke patients, TMAO levels were lower than asymptomatic persons and presented dysbiosis showing more opportunistic pathogens, such as *Enterobacter*, *Megasphaera*, *Oscillibacter*, and *Desulfovibrio*, but fewer commensal or beneficial genera including *Bacteroides*, *Prevotella*, and *Faecalibacterium* [49].

All these criteria may not be present, but causality is still possible or vice versa. In other words, satisfying all six criteria does not assure the relationship is causal nor does satisfying some of the criteria preclude causality. However, the foremost minimal criterion is that temporality must be satisfied in a causal relationship. This means the cause must occur before the outcome in all causal relationships. But we must keep in mind that satisfying the temporal relationship does not ensure causality [40]. Rather, the temporality criterion is the minimum requirement, but causality has to be evaluated in each case by carefully adjusting competing factors.

Another case in point deserves consideration: a popular topic in research at present is fecal microbiome analyses to determine the causative microbiota for inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis. Are alterations in the fecal microbiome the cause for inflammatory bowel disease or the consequence of it? Anatomically, feces come after the gut and cannot be the cause for the pathology in the gut. However, many prominent scientists analyze fecal microbiome to evaluate the cause for IBD.

Fecal analyses suggested that fecal bacteria that produce butyric acid are associated with health, and human colonic butyrate producers are predominantly Gram-positive *Firmicutes* but are phylogenetically diverse. The most abundant groups that generate butyrate are *Eubacterium rectale*, *Eubacterium ramulus*, and *Roseburia cecicola.* These bacteria were enriched in healthy individuals [50]. However, other studies reported that *Firmicutes* were increased in obesity [51–53]. Does this mean obesity is a sign of health? The main question is "are these bacteria bringing health?" or "are they the results of health?" Certainly, examining fecal microbiome could not answer this causality question. Microbiome diversity changes according to the diet [54]. Thus, the eventual causal factor may be the diet. And yet, millions of health research dollars go to fecal microbiome sequencing studies.

Here we list the inconsistencies in fecal microbiome sequencing studies: Backhed et al. reported that germ-free mice were protected from developing obesity [55]. The mechanism includes (1) decreased absorption of glucose, (2) generation of short-chain fatty acids from the gut lumen, (3) the associated reduction in hepatic lipogenesis, (4) increase in fatty acid oxidation, and (5) decrease in deposition of triglycerides in adipocytes. The same group reported after gastric bypass surgery, the patients' fecal microbiome had changed independent of BMI. When these patients' feces were transplanted to germ-free mice, the mice microbiome promoted less fat deposition [56]. This indicates certain microbiotas may be associated with obesity, and weight loss may be due to forced dietary changes post gastric bypass surgery, and microbiome may be the consequence of these dietary changes. Again, the temporality of diet, gut microbiome change, and obesity has to be determined to identify the true cause for obesity. Others, however, reported that obesity caused spontaneous endotoxemia, i.e., elevated serum lipopolysaccharides (LPS) level and subsequent microbiome alteration [54, 57]. Thus, diets that induced obesity appear to initiate this cascade. These sequences of events and jumbled cause-effect relationship in the role of diet, obesity, microbiome, and metabolic inflammation need to be elucidated in the future.

One other baffling example of biologic plausibility in causal context is dysbiosis. Dysbiosis can be defined "An alteration of microbial community composition from a normal healthy state." It has been suggested that dysbiosis may cause periodontitis [58, 59]. However we must prove dysbiosis precedes periodontitis to be a causal

risk factor. So far, we have not seen a longitudinal assessment of oral dysbiosis causing periodontitis. Let us be reminded of Hippocratic comment that "Conclusions which are merely verbal cannot bear fruit, only those do which are based on demonstrated fact."

8.4 Controlling for Confounding

"Confounding" can be defined "other competing factors" that are related to both the predictor and the outcome. A prime example is smoking in the relation of periodontitis to CVD.

Smoking promotes periodontitis development via low immunity due to reduced interferon, antigen presenting cells, and immunoglobulin production [60] and is also a strong risk factor for CVD by itself. Therefore, we must control for the smoking effects in the relationship of periodontitis to CVD. By the same token, obesity and diabetes also increase the risk of periodontitis, and they themselves are directly increasing the risk of cardiometabolic diseases. Thus, the confounding must be controlled in the relationship of periodontitis to CVD, as is illustrated in Fig. 8.1 (described by a red dotted x).

In a complex biological system such as human physiology, the permutations of confounding factors can determine health versus disease state and usually are enumerated with large individual variations. Therefore, while difficult to achieve absoluteness, there are means to reduce or eliminate some of their impacts as illustrated in Fig. 8.1 as well as use of cross-correlation approaches to optimize the final results.

Although we previously assumed that innate immune system is activated by invading pathogens only, as our knowledge expands, we now know that obesity and diabetes endogenously activate innate immunity and generate low-grade inflammation [61, 62]. Pischon et al. reported that periodontal treatment resulted in decreased e-selectin levels. Unfortunately, this study did not provide pretreatment characteristics of the cohort. We have no way of knowing whether metabolic inflammation could have biased the results. Although it was a "self as control" study design, metabolic inflammation would have altered the serum inflammatory markers. Thus, it is important to adjust some measure of metabolic inflammation [63].

In recent years, the gut microbiome was publicized as "a new organ" causing obesity [53].

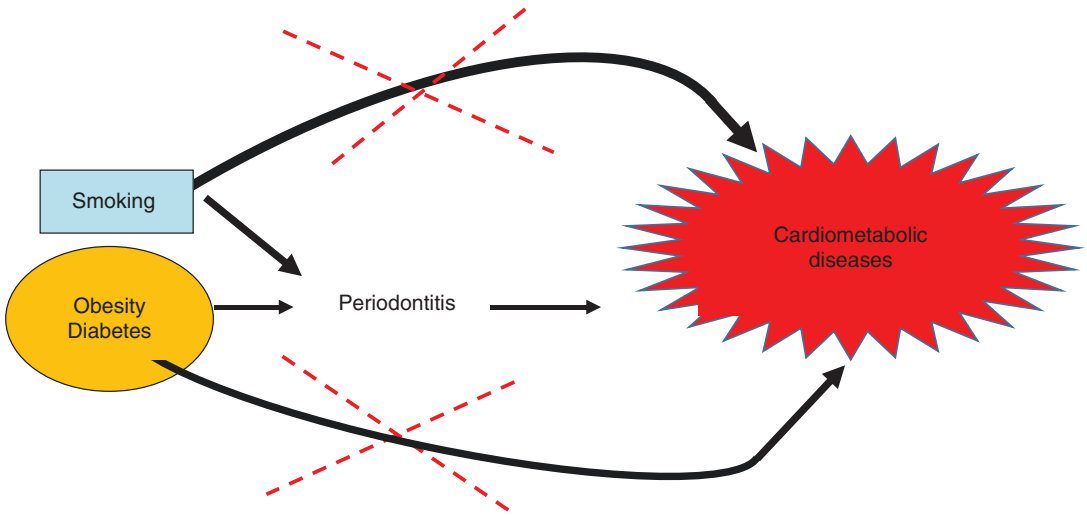

Fig. 8.1 It has been reported that periodontitis increases the risk of cardiometabolic diseases. It also has been proven that smoking or obesity increases periodontitis and that they independently contribute to cardiometabolic diseases. Thus, smoking or obesity becomes a confounding factor for periodontitis. Therefore, if we wish to establish the unbiased relationship between periodontitis and cardiometabolic diseases, the effects of smoking or obesity that coincides with periodontitis must be controlled

Diet will provide substrate for gut microbiome and will alter the intestinal microbial composition. Indeed, African children who eat a high fiber diet showed a significant enrichment in *Bacteroidetes* and depletion in *Firmicutes* ($P < 0.001$), with an abundance of bacteria from the genus *Prevotella* and *Xylanibacter*. These bacteria are known to have genes that hydrolyze cellulose and xylan. Meanwhile, these findings were not observed in European children [64].

It appears two opposing theories are conflated suggesting a third factor may be involved in "diet drives microbiome change" or "microbiome alters dietary absorption" leading to obesity. A recent study explained that "microbes are highly varied between individuals and fluctuate within an individual." [65] Furthermore, another study reported "no simple taxonomic signature of obesity in the microbiota of the human gut" [66]. In a meta-analysis, Sze and Schloss concluded that most of these sequencing studies are underpowered and used inappropriate statistical methods, and more importantly, they may show associations but not causality [67].

Often many dental researchers who are not knowledgeable in the concept of confounding combine the groups together like those who have diabetes and periodontitis or those who smoke and have periodontitis and claim that periodontal treatment improved CVD or glycemic control. In these cases, the confounding by diabetes or smoking must be controlled meticulously, or the results will be biased.

Another point that should be considered in data management is when smoking is dichotomized; it should never be smoking = "yes/no." Even though CVD risk declines with increasing time from smoking cessation, past smokers are at an increased risk of having CVD, and this dichotomy wrongly places them in the "no" category. If one must dichotomize smoking, it should be "ever smoke = yes/no." In this scenario, the past smokers and current smokers are grouped together which is more appropriate. Alternatively, a continuous measure of smoking exposure, such as pack-years, can be employed to distinguish those with little smoking exposure to those with heavy smoking exposure.

A recent classic example of a hidden confounder that has misled the biomedical research and clinical trials is the fact that at high-dose regimens of bisphosphonates (BISPs) for cancer patients and repeat doses over 3–5 years, the cumulative dose on the bone reaches to high enough level that it impacts bone osteocytes and bone lining cells. This approach in preclinical and clinical trials led the investigators evaluating the cancer bone metastasis and cancer bone burden to observe ~20–30% reduction. This was interpreted in terms of BISPs having direct anticancer effect. Remarkably, the impact of BISPs on the bone which reflects degeneration of the local bone cells and bone vitality becomes a confounding factor since the dead bone cannot support cancer colonization, and hence reduction in cancer bone burden was not directly attributable to the effect on cancer cells.

8.5 Minimizing Biases

Recently elaborate 16r RNA sequencing was done in the subgingival crevicular fluid of patients who have systemic lupus erythematosus (SLE) with and without periodontitis. They observed dysbiosis in the group with periodontitis [68]. The question still remains: "Is dysbiosis due to periodontitis?" or "Are both dysbiosis and periodontitis the phenotypes clustering immune-suppressive treatments of SLE?" In translational research or in any research activities, the ultimate goal is reducing diseases. To achieve this goal, we must decrease the exposure to causal risk factors. Therefore, it is of utmost importance to find causal factors if we wish to lower human diseases occurrence.

8.5.1 Simpson's Paradox

Simpson's paradox is defined as "the results indicate the reverse of the true relationship because a confounding factor is not considered." A source of bias in some translational research originates from the lack of epidemiologic understanding among bench scientists. Some researchers

reported, "Obesity alters gut microbial ecology" [51]. The same group also reported "gut microbiome contributes to energy harvest from the diet and energy storage in the host (i.e., caused obesity)" [69]. These two theorems have opposing cause-effect directions. Backhed et al. also reported that "introduction of a gut microbiota into adult germ-free mice caused a 57% increase in body fat" [69].

Alternatively, many researchers reported that diet-induced obesity alters gut microbiome [26, 57, 70–73] and this process involves toll-like receptor activation followed by cytokine production that is manifested in metabolic inflammation [74–77]. Utilizing antibiotics and changing the gut microbiome in leptin-deficient ob^{-}/ob^{-} mice suppressed metabolic endotoxemia, inflammation, and associated disorders [54]. The absence of CD14 in the same mouse group brought similar effects to antibiotics suggesting that innate immune sensing is involved in obesity and that CD14 acts as a co-receptor (along with the Toll-like receptor TLR 4 and MD-2) for the detection of bacterial lipopolysaccharide (LPS). We must remember that the innate immune system can be activated by both microbial and metabolic stimuli [61, 78–80]. Indeed, Fleissner et al. reported that absence of intestinal microbiota does not protect mice from diet-induced obesity [81], and another study refuted the highly cited claim by Turnbaugh et al. [53] that energy harvest from short-chain fatty acids by microbiota in the gut caused obesity [82]. Murphy and colleagues observed a progressive increase in *Firmicutes* which was confirmed in both HF-fed and ob/ob mice (we interpret this as the diet and obesity altered gut microbiome). But the changes in the microbiota were not associated with the marker for energy harvest [82]. At this time, it is not clear whether an obesogenic diet causes gut microbial changes or gut microbiome alteration caused obesity.

A potential Simpson's paradox is possible in the case of childhood infections or antibiotic use causing preadolescent obesity [83, 84]. All these studies ignored the fact that infectious inflammation can be confounded by metabolic inflammation [61, 85]. In a recent longitudinal study, infection and antibiotic use in infancy were reported to be causally associated with obesity in the adolescent [86]. However, this study ignored the main culprits of obesity, namely, obesogenic diet, insufficient physical activities, and the resultant energy imbalance [26, 61].

The mainstream thesis on the cause for obesity is still the excess energy due to high caloric intake and lack of physical activity [26]. One study compared high and low Toll-like receptor 5 (TLR5) gene expression and obesity. The group with high TLR5 gene expression was obese because of the flagellin-producing microbiota detected by TLR5 [87]. However, a Simpson's paradox may have occurred because those who have high expression of TLR5 gene were fatter at baseline (BMI = 30.6 vs 20.7, $p = 0.04$), much more insulin and leptin resistant. This finding is directly opposite to that of a highly touted animal study in molecular science [88]. In the latter study, Vijay-Kumar et al. reported that TLR knockout mice developed spontaneous obesity and metabolic syndrome [88] and the transfer of the fecal microbiome from TLR5-deficient mice to wild-type germ-free mice caused obesity and metabolic syndrome. We are not certain whether mice and humans will have the same innate immune response triggered by TLR5 activation or if there are some built-in biases in these studies. Clearly, more incisive review of the studies using TLRs in animals and humans are needed. Our opinion is that TLR activation is the result of obesity which also caused metabolic inflammation as we and others have reported [61, 89]. Numerous studies support this thesis that TLR activation is the result of obesity: TLRs were activated in nonalcoholic fatty liver disease [90] and other obesity-related cardiometabolic diseases [91, 92].

In another study, the third trimester (T3) stool of pregnant women showed the strongest signs of inflammation and energy loss. When their fecal microbiotas were transferred to germ-free mice, T3 microbiota induced greater adiposity and insulin insensitivity compared to that of first trimester [93]. This study ignored the fact that pregnancy is an immune-tolerant state, and as the fetus grows, inflammation increases due to more relaxed immunity not to reject semi-allogeneic

fetus. Thus, it is plausible that T3 stool would display more prominent metabolic dysfunction and inflammation.

8.5.2 Conundrum in Microbiome Research

Lately, popular topics in research include gut microbiome and gene sequencing. These clinical and translational research studies hold significant potential impact for leading to improve understanding and ultimate application to dental disease and cardiovascular disease. But since this is a new area of research, it is fraught with many deficiencies arising from inappropriate methodology, misconceived study rationale, and misinterpretation of results. Consequently there are many conflicting reports. Beyond potential reverse causation due to using feces to estimate gut microbiome function, additional reasons for this disparity exist: the high functional redundancy in host-microbiome interactions, normal individual variation in microbiome composition, differences in study design, diet composition, the host system between studies, and inherent limitations to the resolution of rRNA-based microbial profiling [94].

Initial evidence for obesity-altered microflora came from an animal study that leptin-deficient *ob/ob* mice displayed a decrease in *Bacteroidet*es and a proportional increase in *Firmicutes* compared with lean siblings (ob/+) given the same diet [51]. Confounders in the relationship of gut microbiome and obesity are diet and the genetic lineage of the animals [71].

Some microbiome studies used germ-free mice to prove gut microbiota cause obesity. We question the validity of using germ-free mice and extrapolating the results to humans. Although infection can change metabolism, the germ-free state in humans is unnatural, and its clinical interpretability is limited. At birth, the gut of a human newborns is sterile but by passing through the birth canal, subsequent breast feeding, and introduction of solid foods, the infant's gut is colonized with a microbial community [95]. This colonization has multiple benefits because microbiome educates the developing immune system [96] and trains it how to distinguish harmful pathogens from harmless commensals, or part of self, and to react accordingly [97, 98]. If this introduction of microbiota is disturbed, some autoimmune disease, such as Type 1 diabetes, may occur [99]. Another benefit of having well-colonized microbiome is breaking down indigestible food components, degrading potentially toxic food compounds like oxalate, and synthesizing certain vitamins and amino acids [100]. Additionally, a more powerful driver of obesity and metabolic syndrome is diet and physical activity balance [26]. Diet changes gut microbiome and intestinal permeability which allows some microbiota to translocate into the blood stream [57].

Additionally, the gut microbiome is usually assessed in the feces and is likely to be the results of obesity rather than the cause. For this reason, the fecal transplant from lean persons improved insulin sensitivity, and it is plausible because lean donors have the normal microbial community, which is not affected by obesity [101]. However, it is not clear whether these changes are permanent or if insulin resistance will return as soon as the recipients resume their obesogenic diets. Moreover, microbiome change is transient [102], and perhaps, it is prudent to make dietary change and increase energy expenditure by exercising more. As we have stated in the previous section, that fecal microbiome assessment is not appropriate for establishing the causal role of microbiota in the gut immunity. In addition, modulating gut microbiome by antibiotics appeared to improve insulin signaling and glucose tolerance by reducing circulating LPS levels and inflammatory signaling in mice [103]. However, this phenomenon was not duplicated in humans in a recent randomized trial [104]. Moreover, using antibiotic treatment to mimic germ-free state in an attempt to prevent obesity via changing gut microbiome in humans has some obvious problems such as the development of resistance to antibiotics. Additional problems using antibiotics are gut microbiome is necessary to protect the host from invading pathogens, energy extraction, and developing immune system [100]. Moreover,

reduced exposure to important gut bacteria may result in higher incidence of human allergies and autoimmune diseases [105].

Current knowledge of the mucosa-associated bacterial communities in the intestine and colon is limited because the knowledge was largely based on fecal microbiome analyses. It was reported that the lumenal and fecal bacterial communities were significantly different [106, 107]. It was demonstrated that the cecum contained 100 times more bacteria than the terminal ileum [108]. Admittedly, collecting colonic samples is difficult because of their viscosity and the difficulties in ensuring adequate anaerobic conditions. This proves potential sources of discrepancy in aerobic fecal and anaerobic intestinal microbiome. Only intubation and pyxigraphy can be performed in healthy subjects, and both should be repeated to study the stability of the flora or the influence of various parameters on its composition [106]. Some microorganisms, such as the methanogens, represent <0.003–0.03% in the right colon, and the same bacteria are present at 5–12% or more of all bacteria in the feces. Strict anaerobes analyzed using probes specific for the *Bacteroides* (*Bacteroides*, *Porphyromonas*, *and Prevotella* spp.) and *Clostridium* groups (*Clostridium*, *Eubacterium*, *and Ruminococcus* spp.) revealed that these bacteria represented 44% of fecal bacterial rRNA and only 13% of cecal bacterial rRNA. These differences suggest that studying the right-sided colonic flora would be more appropriate than studying feces for the diseases involving the right part of the colon, such as ileocecal Crohn's disease [106].

Many studies report the dietary intake shaping gut microbiome [109] as well as causing obesity [54, 110, 111]. However, equally numerous studies report gut microbiome causing obesity independent of dietary effects. Some studies, once scrutinized carefully, erroneously reported gut microbiome causes obesity although dietary factors precede alteration in gut microbiome [53, 94, 112]. Clearly, the lack of understanding of the causality principle, namely, the temporal relationship, made them to refer an imprecise association as causal relationship [53, 112]. Furthermore, the gut microbiome consisting of approximate 1000 species and their composition can change due to antibiotics, illness, stress, aging, bad dietary habits, and other lifestyle factors [113]. Gut microbiome evolves with the human development from germ-free state of newborn infants to approximating adults' microbiome by the age of 1–3 years [114]. Incidental environmental exposures play a major role in determining the distinctive characteristics of the microbial community [114].

A recent randomized trial utilizing *Lactobacillus rhamnosus* GG has been shown to decrease neuropsychiatric disorders later in the childhood by stabilizing gut permeability and restoring epithelial barrier function by tight junction control, mucin production, and antigen-specific immunoglobulin A production [115]. The underlying pathology in many autoimmune or allergic disorders is the increased intestinal permeability that brings dysregulation of immune responses as well as dysbiosis in response to ubiquitous environmental antigens. It should be noted that obesity causes increased intestinal permeability [116]. Thus, finding the very first initial trigger may prove to be a causal factor.

8.5.3 Toll-Like Receptors in Infection and Metabolism

Germ-free mice [55] were protected from developing obesity even with a high-fat diet while Toll-like receptor 5 (TLR5) knockout mice became obese and hyperphagic [88]. These results prompted the conjecture that infection or the gut microbiotas may be at the root of obesity [88]. However, in an in vitro study, subcutaneous adipocytes cultured and exposed to saturated fatty acids expressed increased TLR4 and MyD88 and upregulated NF-kB activity with significantly increased secretion of IL-6 and TNF-α [117]. This suggests fatty acids caused TLR4 expression, and not TLR4 caused fat-related inflammation observed in obesity. This further proves that TLR4 can be activated by metabolic factors [61].

As we wrote in the previous section, a high-fat diet (HFD)-fed mice expressed increased LPS in

the serum (metabolic endotoxemia) [57] and activated TLR4. TLR4, in turn, induced enteric neuronal apoptosis in a p-JNK1 dependent pathway [118]. The authors also observed that the HFD-fed mice had a statistically significant reduction in *Bacteroidetes* ($P < 0.001$) and a significant increase in *Firmicutes*, *Bifidobacteria*, and *E. coli* ($P < 0.001$) relative to mice fed a regular diet. When they supplemented the mice's diet with oligofructose (prebiotic), the level of endotoxemia decreased in HFD-fed mice. The researchers interpreted prebiotics restored dysbiosis, but we consider that prebiotics prevented high-fat diet induced intercellular permeability which resulted in a lesser degree of dysbiosis. Again, it was proven that the high-fat diets initiated increased intercellular permeability, metabolic endotoxemia, and gut dysbiosis and also activated TLR4, which in turn induced intestinal neuronal apoptosis resulting in gut motility reduction [118]. It is important to recognize which factor initiated the sequence of the events and that factor should be considered as the cause.

A recent human trial largely refuted all the animal studies reporting that gut microbial colonization may cause obesity. Reijnders and colleagues manipulated gut microbiota by antibiotics (7-day administration of amoxicillin, vancomycin, or placebo) and observed host metabolism in 57 obese, prediabetic men. Vancomycin, but not amoxicillin, decreased bacterial diversity but did not affect tissue-specific insulin sensitivity, energy/substrate metabolism, postprandial hormones and metabolites, systemic inflammation, gut permeability, and adipocyte size. More importantly, energy harvest, adipocyte size, and whole-body insulin sensitivity were not altered at 8 weeks of follow-up, despite considerable alteration in microbial composition [104]. We interpret this as antibiotics, or the lack of innate immune sensor such as TLR5 may alter the gut microbiome but may not affect metabolism or obesity. This was also the view of an expert who first reported diet-induced endotoxemia, increased serum levels of LPS due to increased intestinal permeability [119].

Germ-free mice colonized with *Bacteroides thetaiotaomicron* had improved host nutrient absorption and thus potentially increases the possibility of developing obesity [120]. However, the multicomponent ileal/cecal flora produced no significant change in levels of either mRNA compared with germ-free controls [120]. We interpret these results as "germ-free mice being colonized with one or two microbes may introduce bias because it is acting as an infection while multimicrobial inoculation may have balancing effects among the microbes" and produced less detrimental impacts.

8.6 Reducing Measurement Errors

Traditionally, translational studies tend to have a lesser degree of measurement error than large epidemiologic studies. However, it is still possible that measurement errors may lead to paradoxical results. For example, the gut microbiome includes over 1000 microbial species [121], and identifying a few microbes that are causally associated with the disease of interest is truly a daunting task. This also applies to all the large-scale proteomics studies by mass spectrometry and/or protein arrays where definition of a biomarker for causal or diagnosis is evaluated.

Traditional culture-dependent methods have numerous drawbacks such as the time and money required, difficulties in identifying the different colonies grown in agar, the lack of sensitivity, predilection for the most common culture conditions favoring fast-growing and easy-growing species, and ignoring those in low concentration or requiring unusual culture conditions, such as anaerobic conditions. Conversely, the cutting-edge analytical method of 16S rRNA also has several limitations: Firstly, the accuracy of identification is directly dependent on the completeness of the reference database. Secondly, the identification power is lower at the species than higher taxonomic levels. Thirdly, many studies use a fragment of the gene, which restricts its discriminatory power even more. Fourthly, many bacterial species have more than one copy of 16S rRNA, and inter-copy sequence variations may be present [121]. In addition, the presence of

microbe in the diseased tissue does not prove that microbe caused the disease. Some microbes have the unusual capability of slipping through intercellular spaces and are ubiquitously present in many diseased and non-diseased tissues. Some examples are *Fusobacterium nucleatum* (*F. nucleatum*) and *Porphyromonas gingivalis* (*P. gingivalis*). Whether they are innocent bystanders or truly causal microbiotas has yet to be proven. The reason for that is the majority of the studies have some methodological flaws. For example, oral gavage with *P. gingivalis* resulted in intestinal dysbiosis in mice [122]. This study provided a novel concept that orally ingested microbial species can cause gut dysbiosis linking the oral cavity to the gut microbiome. However, to prove that *P. gingivalis* is unique in causing gut dysbiosis, the control group should have been other microbiotas, such as *Salmonella*, *Escherichia coli*, or *Staphylococcus*. Using saline as control, they had proved that ingestion of "bacteria," not specifically *P. gingivalis,* caused dysbiosis which is not unlike food poisoning. Additionally, ingesting *P. gingivalis* is not the same as *P. gingivalis* present in human periodontitis. To be a cause for an infection, microbiota must overcome several obstacles [123]: First, they must outcompete the huge number of commensal bacteria [124]; second, they must disrupt epithelial barrier function [125]. In the manipulation of epithelial barrier function, several mechanisms have been recognized. One is via over expression of IL-6 [126] or manipulation of the actin cytoskeleton [127]. Here we need to be reminded that obesity and metabolic inflammation overexpress IL-6 [128, 129] and also increase intercellular permeability.

Another example of the use of inappropriate control group is highly touted "Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER)" trial where in a cohort who were overweight, many of them smoked, and were hypertensive but had not yet developed heart disease, these subjects were given rosuvastatin and the results were compared to the control group who took placebo. Certainly this cohort needed to lower their body weight and decrease smoking and hypertension by lifestyle changes. Thus the appropriate comparison group should have been lifestyle changes comparable to pharmaceutical intervention. Moreover, the outcome of cardiac events included "hospitalization for unstable angina" in the arithmetic sum of all cardiac events. This means "hospitalization for unstable angina" had equal weight as did myocardial infarctions or cardiac deaths. This is clinically inappropriate. When we look at the major cardiac events only, the cardiac event rate in the rosuvastatin group was 83/8901 and numerically 0.009 which means less than 1% had a cardiac event. The placebo group event rate was 157/8901 which can be translated as 0.018 which is less than 2%. And yet, the relative risk decreased about 50% (0.009 vs 0.018) with rosuvastatin administration. Despite the low actual number of events involved in this cohort, now statin treatment for the asymptomatic population is accepted as a standard of care. Some minute improvement in outcome can be manipulated to be highly significant by increasing the sample size because the power of a study (i.e., the probability of obtaining a statistically significant result) is dependent on the sample size. *P-value* is calculated by a *Z*-score which indicates how many standard deviations the observed value is away from the mean.

Let us review how *P*-value is derived. In the standard normal curve, when a value is located about ±1.96 standard deviations away from the mean, that value is deemed to be significant because only 2.5% on each end (5% combined) of standard normal curve will assume this or similar values. (This is why we set the α-level at 0.05.) The *Z*-score is calculated shown in the equation below.

$$Z = \frac{\bar{X} - \mu}{\sigma / \sqrt{n}}$$

X: sample mean; μ: population mean or true mean; σ: standard deviation (SD); *n*: sample size.

As we know *X*, sample mean, and σ, standard deviation, come from the experiment results which *should not be changed*. However, the sample size can be manipulated by recruiting a large number of participants. If the sample size (*n*) increases, the denominator becomes smaller

because denominator is SD divided by the square root of the sample size. If the denominator is small, even minute changes in the enumerator (the outcome) can generate a large *Z*-score, and the *P-value* becomes significant. Thus, it is important to realize that although the *P-value* is significant, sometimes the results may not be clinically meaningful. Inevitably, with a large sample size, often the measurement will be done by using questionnaires or proxies that are less precise. Consequently, their results are often imprecise but highly significant. Nevertheless, journals and funding agencies tend to believe results from studies with large sample sizes. Hence, it is always important to ask whether statistical significance is actually clinically relevant. An extension of the above concept can be also found in the misleading conclusions of the experimental data being different by only 10–20% between the comparators with *P*-value <0.05 and hence touted as "statistically significant". In most cases a difference of 10–20% between the compared groups, although it may be statistically significant, such data or changes are frequently not biologically significant or relevant.

8.7 Utilizing the Appropriate Statistical Methods

Translational research often involves small sample size because the elaborate laboratory methodology requires time and money to conduct. Also, the results are affected by the techniques used (mass spectrometry or polymerase chain reaction, etc.), researchers' skills to perform the experiment, and the animal models or species used. In some research, using the appropriate animal model is important. For example, in short-chain fatty acids assessment, murine models may be of limited value, while pigs or dogs are much better to estimate the human relationship with short-chain fatty acids and gut microbiotas.

The pervasive problems in translational studies are sample sizes are too small and using inappropriate statistical methods. When the sample size is five or six in each group, we cannot expect that these data will have a normal distribution. However, many researchers use the *t*-test which assumes that the underlying data has a normal distribution. Also, multigroup comparisons often use ANOVA, but the ANOVA requires that *each compared group* must have a normal distribution and each group must have the same sample size. Even a total sample size of 32 (each group consists of $N = 8$ in four groups), the each group ($n = 8$) must be normally distributed. In addition, for ANOVA, homogeneity of variance assumption is crucial to obtaining valid statistical results. Particularly in laboratory studies involving count data, variance may increase exponentially with group means, which can be problematic. Especially, in some 16s rRNA sequencing, the usual sample size is less than ten due to the constraints of cost, time, and computing ability. So, the sample size issue has been raised with regard to the gut microbiota research in a recent meta-analysis, and the median classification accuracy for predicting obesity by the gut microbiome composition was very modest, being between 33.01% and 64.77% [67].

One of our own students conducted a four-group comparison where how various reagents affect microbial growth. The total sample size was over 200, but the underlying assumption of homogeneity of variance was violated, and several different experimental variations were involved such as the timing of adding reagents, different numbers of microbes added at the beginning of the experiment, and differing composition of microbes. Due to these limitations in study design and data distribution, we could not use parametric regression methods. Thus, we created each subgroup reflecting on the variation in the methodology and compared to appropriate reference via nonparametric methods. In addition to concerns about sample size and variance, problems arise when the underlying distributions under comparison are highly skewed. In most biological data where the groups being compared are highly skewed, it is generally more appropriate to utilize nonparametric testing.

Some randomized trials select egregiously poor reference groups to amplify the efficacy of

their interventions. For example, if the intervention is giving milk to school children and assessing obesity outcome, the correct reference group should be drinking water [130] or diet soda [131]. If the chosen reference group is sugared soda which has been established as obesogenic [132], the results may not substantiate much health benefit from drinking milk.

Another example of using an inappropriate reference group can be found in a heart failure medicine trial. In a pharmaceutical company sponsored PARADIGM-HF trial [133], a new added ingredient LCZ696 to a previously marketed angiotensin receptor blocker valsartan was tested. Since the new product has a new added ingredient to valsartan, the appropriate reference group should be valsartan without LCZ696 and substantiate that added ingredient is safe and equally efficacious or better. However, they compared its efficacy to enalapril, an early angiotensin-converting enzyme inhibitor with well-known side effects of cough in many patients. To our opinion, since this drug will be given to advanced heart disease patients, the safety issue should be tested carefully. We do not comprehend why this point is not recognized by the leaders of the American Cardiologists group.

In conclusion, even in translational research, all of the epidemiologic principles such as developing scientifically sound rationale, establishing causal relationship, controlling for confounding, minimizing biases and measurement errors, and using appropriate statistical methods are of paramount importance.

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9 Tissue Engineering in Oral and Maxillofacial Surgery: From Lab to Clinics

Riitta Seppänen-Kaijansinkko
and Bettina Mannerström

Abstract

Tissue engineering has been considered as a third treatment modality complementing medicine and surgery. It was first defined in 1993 by Langer and Vacanti (Science 260:920–926, 1993). Today it is a rapidly growing field of research expanding to all disciplines in medicine. Currently, it is possible to engineer both hard and soft tissues in oral and maxillofacial surgery.

Tissue engineering consists usually of three components: scaffolds, cells, and regulating factors. Scaffolds are preferably biodegradable, i.e., they resorb when they are no longer needed, while regenerated tissue fills the space of resorbed material. Different types of cells can be used depending on the type of required tissue. Both stem cells and differentiated cells have been used, while regulating factors are most often growth factors. The growth of vascularization, to enable adequate delivery of nutrients and oxygen, must also be taken into account when constructs are of large size and diffusion is not enough to keep the construct vital. Regulating factors can be proteins, such as growth factors, culture media ingredients, and structural or physical elements. The required tissue can be manufactured either in the body, on the site of the defect, or ectopically, for example, in the muscular environment from where it is transplanted later, when mature enough, to the defect site. In this chapter, the past and the present of tissue engineering in oral and maxillofacial surgery will be elaborated, not forgetting the future perspectives.

9.1 Background

Regenerative medicine aims at the functional restoration of tissue malfunction, damage, or loss and can be divided into three main approaches: firstly, the cell-based therapies, where cells are administered to reestablish a tissue either directly or through paracrine functions; secondly, the often referred to as classical tissue engineering, consisting of the combined use of cells and a biodegradable scaffold to form tissue; and thirdly, the material-based approaches, which have made significant advances which rely on biodegradable materials, often functionalized with cellular functions [1].

In 1993, Langer and Vacanti [2], determined tissue engineering as an "interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve

R. Seppänen-Kaijansinkko (✉) · B. Mannerström
Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

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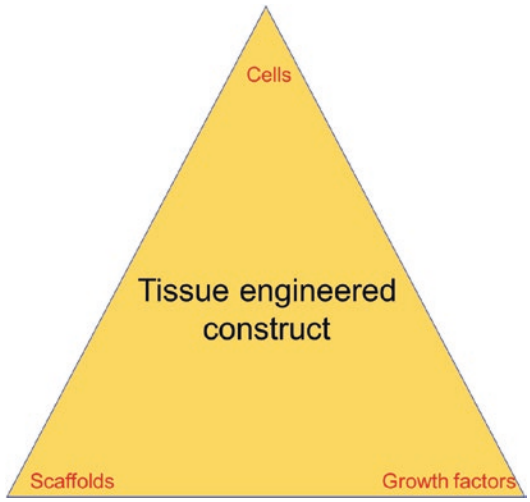

Fig. 9.1 Classical triad of tissue engineering: cells, supporting scaffold and growth factors

tissue function." They published this definition in science in 1993.

Tissue engineering has been classically thought to consist of three elements: supporting scaffold, cells, and regulating factors such as growth factors (Fig. 9.1). Depending on the tissue to be regenerated, all three vary. Currently, it is known that many other factors may have an effect on the outcome of the regenerate. These include factors enabling angiogenesis, physical stimulation, culture media, gene delivery, and methods to deliver patient-specific implants (PSI) (Fig. 9.2).

During the past two decades, major obstacles have been tackled, and tissue engineering is currently being used clinically in some applications, while in others it is just taking its first baby steps.

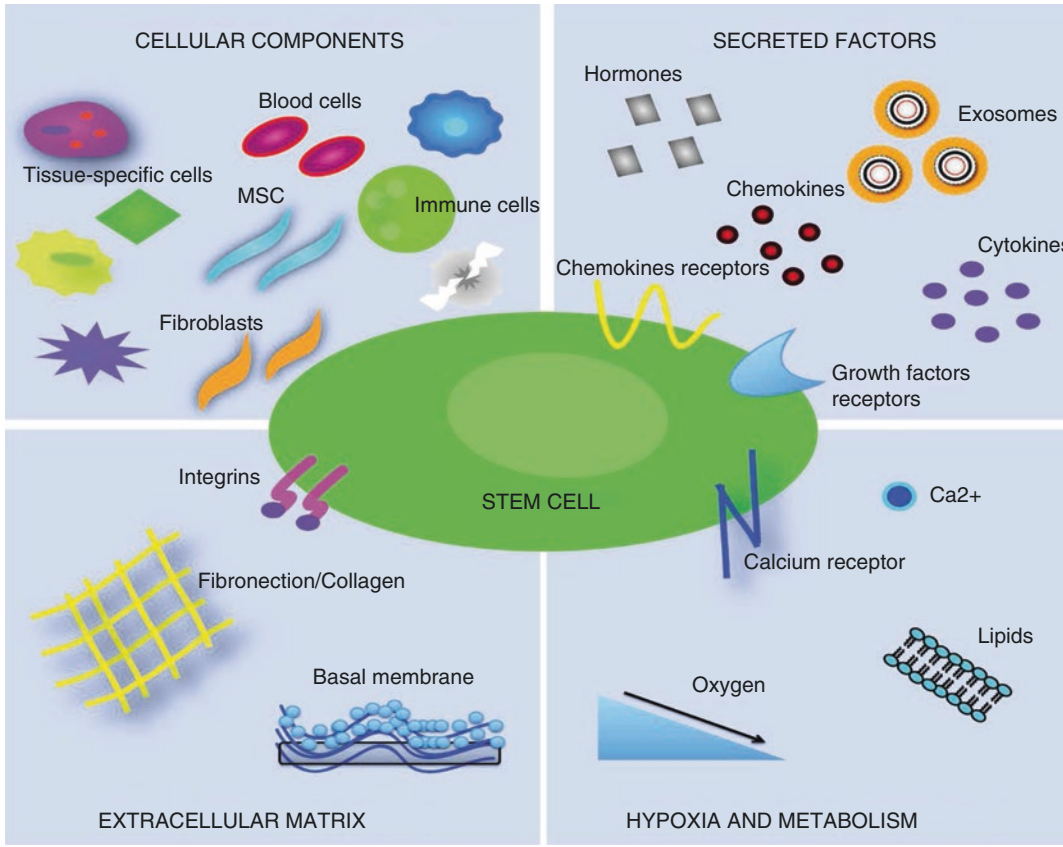

Fig. 9.2 Current concept of tissue engineering. The cells communicate with its environment with structural, physical, chemical, and cellular components which brings complexity to tissue engineering. Exosomes are new area of interest, with potential applicability in stem cell therapy and tissue engineering. Image from https://www.escience-central.org/ebooks/ebookchapter/resident-stem-cells-stimulation-new-promise-for-tissue-regeneration--165/3

9.2 Materials

Materials in tissue engineering can be manufactured with different techniques. The most common ones are molding and 3D printing. Scaffolds can also be porous granules inserted into a hollow container or biodegradable mesh, which provides the shape and size of the construct. The scaffold acts as extracellular matrix, which later is resorbed and replaced by proper extracellular matrix synthetized by cells.

9.2.1 Used Materials

A large variety of materials have been introduced to aid tissue engineering both in soft and hard tissues. They can be used as scaffolds to keep the cells and growth factors in the desired area or only to give size and shape to the construct (a mesh filled with the scaffold material).

In soft tissue engineering, collagen and freeze-dried cadaveric human dermis are currently most used material as scaffolds in oral and maxillofacial surgery [3]. They will resorb in due time leaving the cells to replace the missing tissue. In some applications, it is important to use a laminated scaffold to enable the growth of a 3D structure with different tissue types.

In bone tissue engineering, the most commonly used materials are biodegradable granules of beta-tricalcium phosphate (ß-TCP), hydroxyapatite (HA), and bioactive glass (BAG) [4–8]. The mesh is usually made out of titanium or bioresorbable materials such as polylactic acid (PLA) or its composites. The scaffold materials as well as the bioresorbable mesh will degrade, and the space is filled with newly formed bone. The speed of degradation depends on the used materials.

The mesh is often shaped manually or with indirect printing, where a template of the defect (and required reconstruction) is manufactured and the mesh shaped to surround it. It can also be manufactured with the aid of direct 3D printing.

9.2.2 3D Printing

With 3D printing, it is possible to produce custom-made scaffolds and mesh for tissue engineering. By using of computer-aided design and manufacturing (CAD-CAM), the scaffolds will be very precise and accurate. As facial structures are quite complex, this has brought huge advantages to managing different materials. Most used techniques are inkjet printing, laser-assisted printing, extrusion printing, and stereolithography. All these require top-class imaging and manipulation the data as well as fine algorithms. CAD-CAM in medicine consists of four consecutive phases: (1) CT 3D imaging data; (2) data conversion, CAD; (3) planning of surgery/manufacturing of implant; and (4) actual surgery accordingly [9]. After the scaffold has been manufactured, it is possible to seed the scaffold by cells and add growth factors, if needed.

9.3 Cells

In tissue engineering, both differentiated primary cells and stem cells can be used as a source.

Primary cells are cells taken from living tissue. They have undergone only a few divisions and, hence, represent the cells in the original tissue. The main obstacle when using these cells is the limited number that can be obtained.

9.3.1 Stem Cells

Stem cells are cells which can make perfect copies of themselves (self-renewal) or differentiate to specialized cell types. Several cell types are gathered under the same umbrella called “stem cells”: (1) human embryonic stem cells (hESCs); (2) human-induced pluripotent stem cells (hiPSCs), which are in fact reprogrammed somatic cells; and (3) adult stem cells, which cover several types of cells of hematopoietic and mesenchymal origin. Mesenchymal stem cells (MSCs) and tissue-specific progenitors reside in the

human body in most tissues throughout an individual's life and generally have a limited expansion and differentiation.

Pluripotent stem cells can differentiate to all specialized cell types. The main source is the inner cell mass of human embryonic stem cell (hESC), but lately hiPSCs have gained interest in clinical cell therapy and regenerative medicine (Fig. 9.3). When making iPSC lines, somatic cells are genetically reprogrammed using transcription factors [10, 11]. When using both hESCs and hiPSCs, there is a risk of mutations already in the laboratory, due to the lengthy in vitro culturing time and extensive cell manipulation [12, 13]. In vivo reports of tumorigenicity have raised concern for safety in using these cells in clinical work [14].

Some of the most promising research in regenerative medicine is focusing on the use and applicability of stem cells. The main stem cells used in tissue engineering are tissue derived, so called adult stem cells, which can be extracted from most adult tissues. They can be transplanted back to the same individual (autologous transplantation) avoiding risks of disease transfer or immunological reactions. They can also be transplanted to another individual (allogenic transplantation) [15].

Mesenchymal stem cells (MSCs) are of great interest to both clinicians and researchers,

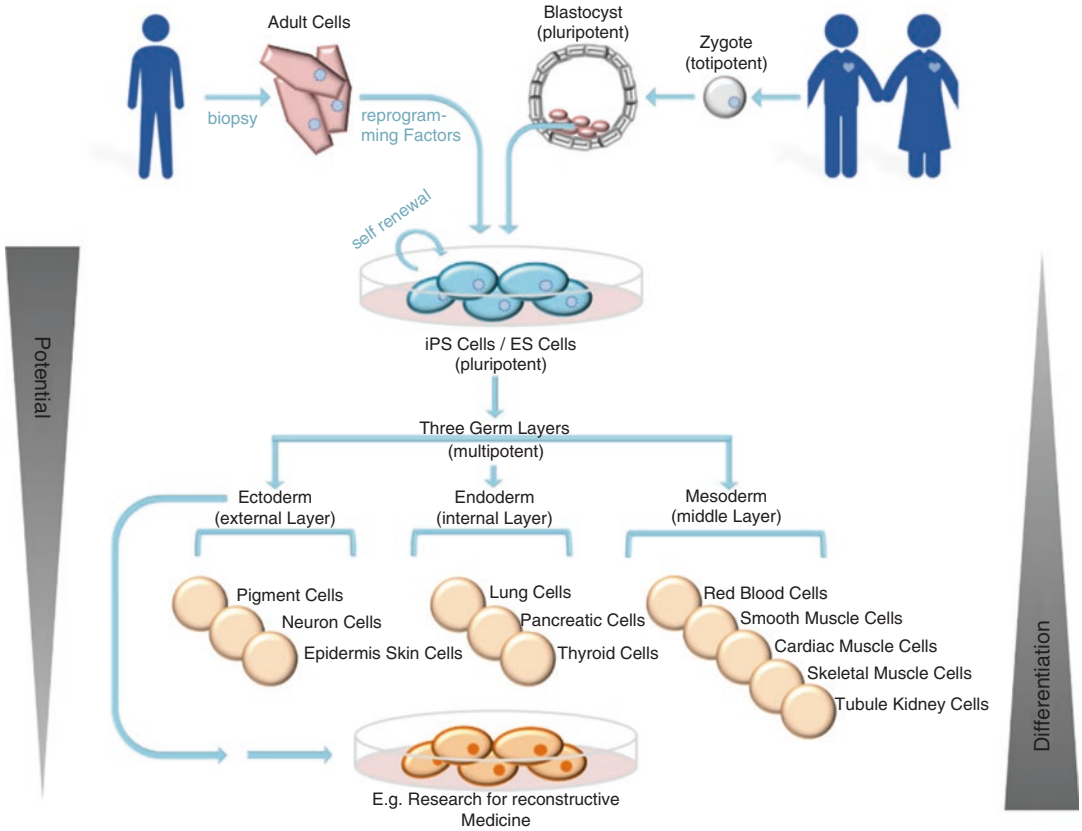

Fig. 9.3 Stem cell hierarchy. Zygote and early cell division stages to the morula stage are defined as totipotent. At the blastocyst stage, only the cells of the inner cell mass (ICM) and the embryonic stem cells retain the capacity to build up all three primary germ layers, as well as the primordial germ cells, and are pluripotent. Stem cells in the fetal tissues exist during fetal development, and some stem cells are retained until adult age. In adult tissues, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. Figure downloaded from http://synentec.com/imagetypes/content_image_full/stem_cell_development_function_3.png

due to their potential in tissue engineering. Their ease of isolation, manipulation and potential for differentiation are qualities that have gathered interest. Despite their frequent use in research, detailed standardized criteria are called for as to the identification of these cells from their various locations, as they are not all the same. The International Society for Cellular Therapy has published a set of guidelines attempting to standardize the expansion of these cells, but more standardization is still needed [16–18].

The most commonly studied MSCs are derived from bone marrow (bone marrow stem/stromal cells; BM-MSC) and adipose tissue (adipose tissue stem/stromal cells; AT-MSCs). While both BM-MSCs and AT-MSCs have an approximately equal potential to differentiate into cells and tissues of mesodermal origins (i.e., adipocytes, cartilage), AT-MSCs have a distinct advantage: they are more readily accessible than BM-MSCs. While comparative analysis of the two subtypes of MSCs has shown that there is no difference in regard to morphology, immune phenotype, isolation success, and colony frequency, differences do arise in regard to osteogenic and chondrogenic differentiation, with AT-MSCs exhibiting smaller potential for osteogenesis and chondrogenesis than BM-MSCs [15] (Figs. 9.4 and 9.5).

MSCs are currently the focus of clinical and scientific research due to their exceptional abilities for their immunomodulatory properties. However, MSCs cannot be considered truly hypoimmunogenic; rejection of allogeneic MSCs, immunosuppressive potential and immunogenicity are influenced by levels of systemic or local inflammatory cytokines. High immunosuppressive potential simply allows MSCs to suppress inflammation and delay allogeneic rejection through suppression more efficiently than other allogeneic cell types. Many believe that the

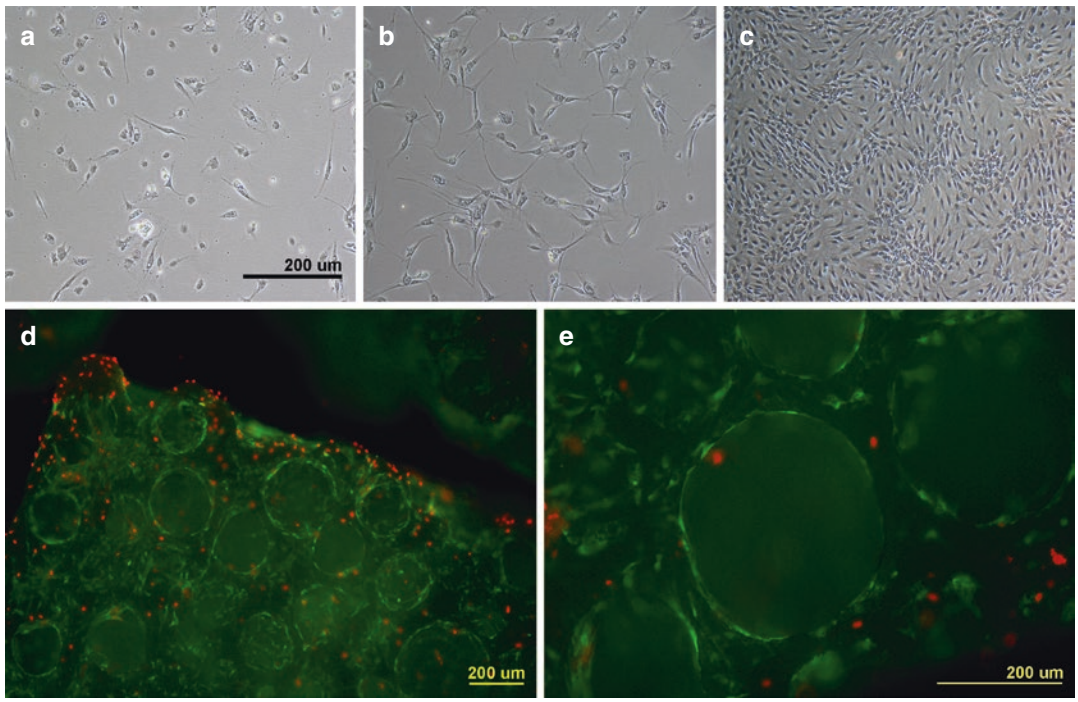

Fig. 9.4 Light microscopic images showing adipose stem cells (AT-MSCs) proliferation at day 1 (**a**), 2 (**b**), and 4 (**c**) post isolation. Fluorescence microscope image of live/dead staining of ASCs and biomaterial, with live cells (green) and dead cells (red) depicting cell adhesion to the biomaterial and cell viability (**d**) and (**e**). (Reprint from Mesimäki et al. [6] granted by Elsevier)

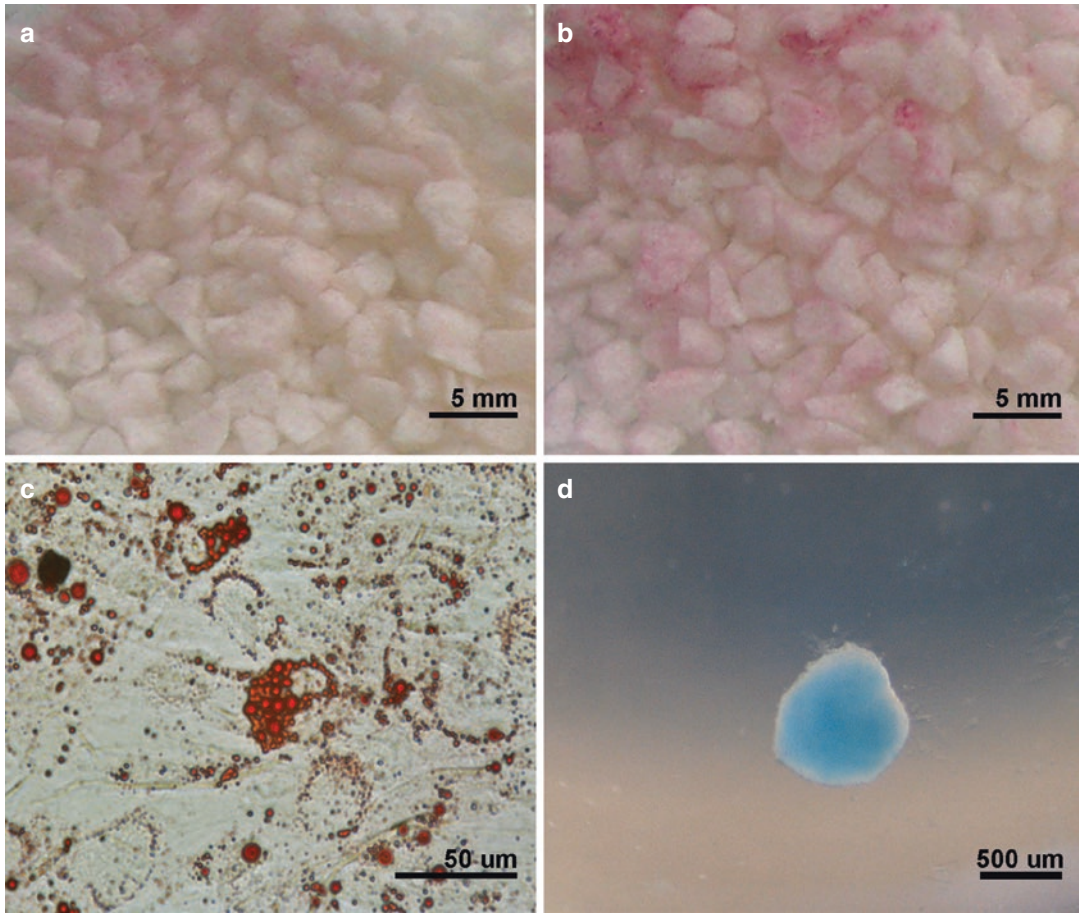

Fig. 9.5 Confirming the multipotent nature of AT-MSCs. Alkaline phosphatase staining on osteogenic differentiation cultures of AT-MSCs and biomaterial, control culture (**a**), and differentiation culture (**b**). Oil red O staining confirming adipose differentiation of AT-MSCs cultured in monolayer (**c**). Alcian blue staining confirming chondrogenic differentiation of AT-MSCs cultured in micro mass culture format (**d**). (Reprint from Mesimäki et al. [6] granted by Elsevier)

therapeutic effect of MSCs is due to a "hit-and-run" mechanism mediated by the production of extracellular vesicles (EVs) or exosomes or secretion of trophic and immunomodulatory factors. Nevertheless, a comprehensive understanding of MSC mechanism of action in cell therapy is still under investigation, and many questions remain to be answered [19].

Adult stem cell-based therapies are already clinically available; additionally, more than 3000 trials associated with stem cells are currently registered in the World Health Organization International Clinical Trials Registry Platform (http://apps.who.int/trialsearch/). The majority of these are adult stem cell-based therapies, but the registry also includes the first pluripotent-based (hESC/hiPSC) clinical trials, associated with eye diseases such as macular dystrophy or degeneration. Albeit that the technology is in place to produce a wider range of therapies, especially safety issues are not completely understood, subsequently there is a cautious transition from bench to bedside and application of technologies [19].

9.3.2 Bioprinting

The cells can also be printed together with certain biomaterials combined with growth factors, if needed. The first bioprinting technique was inkjet printing, which is fast and cost-effective. The resolution is high but the quality of vertical structure is poor. Cell density is also low. It has been used to engineer blood vessels, bone, cartilage, and neurons [20].

When using laser-assisted printing, the cost is high, while cell viability is excellent (>95%). The resolution is high and the vertical structure quality is fair. This technique has been used in blood vessel, bone, skin, and fat regeneration. The high cost has prevented this technique becoming very common. The printers are mostly complex and cumbersome compared to other types of printers. All in all, this technique is still immature for clinical work [21, 22].

Stereolithography is also relatively cheap and fast. The resolution is high, and the quality of vertical structure is good with good cell density and cell viability (>85%). It has been used in blood vessel and cartilage engineering [22, 23].

Bioprinting by extrusion, which is modified inkjet printing, can be used when biomaterial is very viscous. It is of moderate cost but slow. Cell viability is also moderate (40–80%) and resolution moderate, but vertical structure is of good quality with high cell density. Currently, most commonly used bioprinters are based on extrusion technology [22].

9.4 Growth Factors

Bone morphogenetic factors (BMPs) play a central role in bone and cartilage development, as well as in adult homeostasis of bone metabolism, but are also known to play crucial roles in all organ systems. Therefore, it has even been suggested that BMPs should be named *body* morphogenetic proteins [24]. Currently, the BMP family is comprised of several members from BMP-2 to BMP-18.

Though BMPs were initially discovered to induce bone formation, BMP-3 for instance has been shown to be a negative regulator of bone density. Some BMPs may have redundant roles in bone formation, as conditional deletion of BMP-7 from limb has no noticeable effect [25]. Further, the osteogenic potential of BMPs has reported contradictory results in in vitro studies. In two- and three-dimensional cultures of AT-MSCs, supplementation of BMP-6, BMP-7, and vascular endothelial growth factor (VEGF) and their combinations showed no significant enhancement of the osteogenic differentiation [26], while others reported a synergistic effect of BMP-6 and VEGF on the osteogenic differentiation of the same cells [27, 28]. In a study using periodontal ligament cells, supplementation with BMP-2 or BMP-6 showed no enhanced osteogenesis [29].

BMP-2 is best studied in the context of osteogenesis and has been indicated to have potential in bone formation; however, the reports have been contradictory both in vitro and in vivo. Nevertheless, studies suggest that source of serum supplementation of the in vitro cultures may play a role, with human serum supplemented media showing accelerated biochemical responsiveness to BMP-2 compared with fetal bovine serum (FBS) in AT-MSC cultures [30]. Further, the species origin in which the growth factor is produced is also important for its response, with BMP-2 of mammalian origin showing enhanced response in vitro compared with BMP-2 of bacterial origin [30]. These results may give one explanation to the existing contradiction in the reported BMP-2 studies on AT-MSCs in vitro.

Due to their approval for clinical use, BMP-2 and BMP-7 are probably the most studied growth factors for bone tissue engineering. However, the translation of BMP-2 and BMP-7 to clinical use has been hindered by various problems, including high administration dosage, safety issues, short half-life, and high cost relative to efficacy [31, 32]. The safe administration dosage may be one the reason to the complications and safety issues concerning

BMP-2 in off-label use in the cervical spine to increase bone growth and bony fusion. The FDA published a safety communication on recombinant human BMP-2 use in 2015, especially for pediatric patients, with recommendation to cautiousness in the use of BMPs until further safety evidence is available.

9.5 Enhancing Osteogenesis Using Physical Stimulation

The bone is a tissue capable of modifying its structure and mass according to the mechanical loading it is exposed to. This process occurs through the intrinsic balance in the mechanosensitive activation of the osteoblasts and the osteoclasts [33].

Several studies have provided robust evidence that reduced mechanical loading results in decreased bone mass and that increased mechanical loading can be utilized to enhance bone formation in vivo as well as in clinical cases. Further, animal and human studies have led to the examination of mechanical loading in cell models. Studies have shown that bone cells and their precursors and also MSCs exhibit mechanosensitivity in vitro in response to various mechanical stimuli; however, there is no consensus on the most effective combination of vibration loading parameters for osteoinduction [34].

Further, osteogenesis can be enhanced in vitro using another potential strategy, by application of conductive polymers as a functional surface coating on biomaterial scaffolds. One of the conductive polymers (CPs), polypyrrole (PPy), has shown great promise in tissue engineering, not only because it can mediate electrical currents, but also because it may enhance the bioactivity of biomaterials in bone applications. Reports show that AT-MSCs cultured on polymer scaffolds coated with PPy induced with electric stimulation supported the viability and proliferation of the AT-MSCs as well as supported osteogenesis [35, 36].

9.6 Experimental Studies

In preclinical studies, regeneration of many tissues in the oral and maxillofacial has been studied. Of these, teeth, salivary glands, and nerves have not yet been explored in clinical applications.

9.6.1 Teeth

Considering the teeth, many different types of cells are needed, including dental pulp regeneration and dentino- and amelogenesis [37–39]. In the future, it might be possible to regenerate the root of a tooth, but at the moment it seems impossible to guide the shape and color of the crown of a tooth. Hence, dental regeneration at least at the moments, cannot replace dental implants.

9.6.2 Periodontal Ligament

Infection leading to loss of periodontal ligament is very common in adults, and the treatment methods need improving [38]. In long-term cultures, surprisingly, estrogen seems to have properties to retain the stemness of periodontal ligament stem cells [40]]. New materials such as zein (protein derived from corn) can be electrospun with gelatin and seems to support the growth of periodontal ligaments cells. This, however, is not yet in clinical use [41].

9.6.3 Salivary Glands

Salivary gland regeneration has been studied quite extensively; however, no clinical studies have been reported yet. Salivary hypofunction can be caused by systemic diseases such as Sjögren's syndrome, but it may also be due to radiotherapy in the head and neck region. Secretion of saliva is extremely important for the well-being of oral mucosa and teeth; however, no adequate saliva substitutes to replace the

hypofunction of the glands have been developed. For tissue-engineered salivary gland, it is necessary also to be able to connect the secretion of saliva to the complex ductal system to deliver the saliva to the oral cavity. However, function of salivary glands including secretion of bioactive molecules in case of radiotherapy might be feasible when using AT-MSCs administrated by systemic routes, i.e., blood stream, by paracrine mechanisms which will provide growth factors helping neovascularization and promoting epithelial proliferation as well as angiogenesis [42]. Dental follicle-derived stem cells have been shown to be able to differentiate to salivary gland and duct cells, which might be a promising future treatment modality [43].

9.6.4 Cartilage

In cartilage tissue engineering, committed chondrocytes, ESCs, and MSCs have been used. Based on the results and availability of cells, MSCs seem to be a viable choice for this application. The regenerated cartilage in the joint will have to bear large contact area strains and stresses. It must also allow growth of functional tissue by providing appropriate cell-scaffold interactions [44].

To enable long-term survival of cells inside the scaffold, the scaffold must be either porous or woven. These properties will challenge the appropriate strength of the scaffold. In oral and maxillofacial surgery, the need for cartilage is usually in the temporomandibular joint (TMJ).

Mäenpää et al. [45] studied the regeneration of TMJ discs in rabbits. The bilayer scaffold disc comprised of a non-woven mat of resorbable PLA and a PLA membrane plate. AT-MSCs were seeded in the discs and cultured in parallel in control and chondrogenic medium for 6 weeks. Relative expression of the genes, aggrecan, type I collagen, and type II collagen, normally present in the TMJ disc extracellular matrix, increased in the discs in the chondrogenic medium. They concluded that the PLA discs seeded with AT-MSCs have potential in the development of a tissue-engineered TMJ disc. The same group later used these discs in ten rabbit TMJs. The original TMJ disc was bilaterally removed, and the AT-MSC-seeded PLA disc was used to replace the removed original disc on one side. On the other side, the cell-seeded PLA disc was pretreated in chondrogenic differentiation media. Unfortunately, the cone beam computed tomography and histology showed that most of the discs had dislocated and caused sclerotic changes and condylar hypertrophy in the joints. The pretreated discs seemed to function slightly better than the non-pretreated discs. No signs of foreign body reaction, infection, or inflammation could be seen. The authors conclude that better disc design and fixation technique might lead to better results [46].

To be able to regenerate mandibular condyle, it must be realized that both the bone and the cartilage must be produced and bound together. Chondrocytes and osteoblasts can be harvested or differentiated from abovementioned many sources: the properties of the scaffold needed are different for the bone and for the cartilage. The growth factors used need to differ as well. However, if this could be safely and predictably performed, this approach would give great relief to patients suffering from major TMJ disorders and diseases [47].

Nasal cartilage has also been a target for tissue engineering. Chang et al. [48] used autologous chondrocytes injected in fibrin glue to rabbits' dorsal nasal bones. The histological result was identical to that of normal auricular cartilage. The concentration of fibrinogen and thrombin as well as chondrocytes plays a crucial role in the formation of the cartilage. If cartilage cells are not available, bone marrow- and umbilical cord-derived stem cells have been studied. The umbilical cord-derived cells seemed to produce more type I collagen and aggrecan compared to bone marrow-derived cells, a finding which warrants further studies also in a sandwich-type construct for osteochondral reconstruction [49, 50].

9.7 Clinical Work

There are two main objectives in maxillofacial reconstruction: surgery should provide form and function of oromaxillofacial area. As facial skeleton has a very complex structure, reconstruction should restore volume, shape, bone continuity, and symmetry of bone skeleton. On the other hand, soft and hard tissues in this area enable several functions like articulation, mimics, mastication, swallowing and breathing. When the reconstruction is carried out, esthetic and reconstructive aims need to be met.

Clinically, the applications have been mainly in bone regeneration as well as in epithelial defect repair. Currently, the aim is also to avoid all animal-derived materials and replace them with synthetic or human-derived materials, such as recombinant human BMP (rhBMP) and human serum.

9.7.1 Soft Tissue Regeneration

Currently, surgeons often use collagen sponges and freeze-dried cadaveric human dermis to replace missing soft tissue [3]. However, tissue-engineered approaches have also been introduced, and they will be elucidated below.

9.7.1.1 Oral Mucosa

Oral mucosal grafts have also been used in clinical work. EVPOME®, a tissue-engineered ex vivo-produced full-thickness mucosal graft, has shown the ability to create keratinized mucosal surface epithelium when grafted on an intact periosteal bed. EVPOME® is manufactured in the laboratory from the patient's own keratinocytes (from biopsies of the palate) seeded and cultured on an acellular dermal matrix. It has proven to be safe and has potential to augment keratinized mucosa around the teeth [51].

9.7.1.2 Lips

On top of esthetics, lips provide several other functions. These include eating (closure of the oral cavity), breathing (by nose), articulation, and sensation (hot, cold, etc.). They also play an important role in facial expressions.

Regeneration of lips is very difficult especially when there is a large defect which cannot be repaired with local flaps. To regenerate an avulsed lip, both the surface structures (underlying muscles and surface structures such as skin and mucosa) have to be considered [52].

For tissue-engineered construct to be successful, it needs adequate blood supply to enable survival of cells. A sufficient fixation of different tissues plays also a crucial role in the healing and regeneration process.

9.7.1.3 Skin

Already approximately one million patients, mainly burn patients and patients with diabetic ulcers, have received tissue-engineered human skin. Dermagraft® (PGA + neonatal fibroblasts) was one of the first commercially available products for skin defects [53]. Currently, allogenic skin grafts are available as off-the-shelf products. However, they are somewhat immunogenic and may transfer diseases, although the risk is minimal after extensive testing required for these products. Skin grafts can be manufactured to replace only epidermis or dermis of both. Unfortunately, hair follicles or sweat glands are not yet included in these grafts.

9.7.2 Hard Tissue Regeneration

9.7.2.1 Cartilage

Our own research group has used tissue engineering to produce cartilage to the nasal septum. The two operations, in which a resorbable Chronos® sheet was seeded with patients' own ASCs, were successful. However, one of the patients continued her nose picking with artificial nails, and after the initial healing period, the graft was lost [54].

9.7.2.2 Bone

Bone transplants are the second most used tissues in clinical work after blood transfusions [55]. However, if autologous bone is used, usually another surgical site is required which causes

more morbidity to the patient as well as extends the length of the operation. Bone banks provide solution for this is some cases as allogeneic bone can quite safely be used even though there is a small risk of immunologic reactions and disease transfer. Bone grafts usually resorb partly; hence, in oral and maxillofacial area, it might in some cases be difficult to predict how much bone needs to be transplanted.

Sinus Lift

Sinus lift is one of the most common procedures to enable placement of dental implants in the edentulous maxilla. Traditionally it is carried out by using autologous bone harvested in the craniomaxillofacial skeleton or iliac crest. However, it was one of the first applications where bone regeneration was attempted by tissue engineering. The used carries for cells and/or growth factors are resorbable fleeces, HA, bovine bone, and, naturally, autologous and allogenic bone.

Schimming and Schmelzeisen [56] used periosteal cells on a resorbable (polyglactin 910 combined with polydioxanone) fleece in 27 patients for augmentation of edentulous posterior maxilla. They used good manufacturing practice (GMP)-class expanded periosteal cells from mandibular angle and the fleece was soaked with cell suspension. Bovine thrombin in FBS was used to seal the cells in the fleece. Cells were cultured for nearly 2 months after which they were transplanted in the sinus floor. One patient had to be dropped out due to an infection. In 18 patients the result was excellent; however, an unsuccessful result was seen in eight patients (30%) needing further supplementary autologous bone transplantation.

Meijer et al. [57] augmented sinus floors or walls prior to dental implant insertion in six patients. BM-MSCs were harvested from the iliac crest and cultured for a week on porous HA in an osteogenic culture medium, containing also xenogeneic materials such as FBS. The cells were then transplanted and the augmentation effect studied 4 months after augmentation. Of the 11 biopsies taken, bone formation was observed only in three patients (50%). It can be speculated that inadequate vascular supply might have been the reason for failures.

Other Small Local Defects

In oral and maxillofacial surgery, large bone defects, caused by cysts, are often filled with autologous bone, bovine bone, or synthetic materials such as hydroxyapatite (HA), ß-TCP, or BAG. In a study published by Stoor et al. [7] 21 bony cavities in 20 patients were filled with BAG S53P4, some even in the presence of infection (65%). The authors state that the use of this material provides and infection-free and reliable bone regeneration. When cells have been used, autogenous osteoblasts seeded in biomaterials have shown to be an excellent choice to fill these defects compared to iliac crest bone grafts [58]. Unfortunately, the need for a GMP-class facility to produce the tissue-engineered filling materials is very labor-intensive and not very cost-effective hindering their use to become more widespread. According to current legislation, iliac crest bone graft can be obtained simultaneously during the same operation when the cyst is removed, hence lowering the costs markedly.

Continuity Defects

Continuity defects are caused mainly due to tumor ablation of trauma. This can include only the bone, sometimes teeth and some soft tissue. If the soft tissue coverage and blood supply to it is adequate, it is possible to tissue-engineer the transplant directly in the defect site. However, if there is a major loss of soft tissue, the construct needs to be transplanted first to an ectopic site and after maturation transplanted again to the defect site either as a microvascular flap or a pedicled flap.

9.7.2.3 On-Site Regeneration

Zétola and his coworkers [59] published a case report in 2010 of a repair of a mandibular defect repair after resection of an ameloblastoma using recombinant human morphogenetic protein-2 associated with collagen sponge, autogenous bone chips, and synthetic HA and β-TCP blocks. No cells were transplanted. Titanium reconstruction plate and titanium scaffold filled the

abovementioned combination was implanted into defect area. Collagen with rhBMP-2 was superposed above open titanium mesh to allow muscle cells and periosteum to migrate to defect area. After 7 months, the patient had a stable occlusion. Control CT showed good bone formation directed to the center of the defect. The authors concluded that the reported reconstruction technique gave a satisfactory result with less invasive surgery and with minimum morbidity. However, studies with larger number of patients are required to indicate the treatment modality as a routine in cases of bone continuity defects.

In 2015, Park et al. [60] reported a case study where a large continuity defect after resection of ameloblastoma in the angle of the mandible was reconstructed with iliac bone and autologous BM-MSCs. The iliac bone served as a scaffold, fixed with titanium plates and screws, with cancellous bone removed. The gap was then filled with cultured BM-MSCs and fibrin glue covered with collagen membranes. Later three dental implants were placed in the graft resulting in uneventful healing.

Stoor et al. [8] used direct CAD–CAM technique and tissue engineering to repair mandibular defects in 14 patients immediately at the time of ablation surgery. Most of the patients had squamous cell carcinoma or ameloblastoma. The surgery was simulated and patient-specific implant (PSI) designed on virtual model. The PSI was a combination of scaffold and reconstruction plate with screw holes. The scaffold was filled with β-TCP and autologous bone. In four patients with ameloblastoma or drug-induced osteonecrosis cases, BMP-2 soaked in a sponge was placed to cover the cage to improve the bone formation. Finally, PSI was covered with collagen membrane or sponge (ten patients) and either radial for arm or anterolateral thigh (ALT) microvascular flap (12 patients). The follow-up was between 9 and 24 months. The overall recovery of the patients was favorable considering how demanding the patients were. Nine patients had an uneventful recovery. The main reasons for failure were infection and dehiscence of the mucosa or the microvascular flap. In these cases, extreme caution should be exercised to avoid soft tissue injury or dehiscence during the surgery and follow-up.

It is noteworthy that all these reports can be estimated having been successful due to sufficient coverage of the regenerate with vascular soft tissue enabling oxygen and nutrient supply to the healing area.

9.7.2.4 Ectopic Prefabrication

One of the first clinical papers was published by Warnke et al. [61]. They reconstructed a mandibular continuity defect using vascularized custom-made bone flap with indirect technique in which patient's CT data was uploaded to CAD software, and the defect reconstructed in the mandible was virtually simulated. A virtual implant to repair the defect was designed and converted into solid 3D Teflon replica, which was used as a model when manually shaping titanium mesh around it. The shaped mesh was filled with bovine bone mineral blocks combined with growth factor rhBMP-7, bovine collagen type-1, and autologous iliac crest bone marrow. The filled mesh was implanted into patient's back muscle (latissimus dorsi). A microvascular flap was raised 7 weeks later, and 4 weeks after the implantation, the patient was able to use her mandible and was satisfied with the aesthetic outcome. The authors concluded that ectopic bone formation is possible and causes less burden to the patient compared to conventional bone grafts.

Mesimäki et al. [6] reconstructed a major hemimaxillary bone and soft tissue defect caused by removal of recurrent keratocyst in a middle-aged male patient. The patient was very unhappy with his removable obturator prosthesis. The construct consisted of ß-TCP as scaffold material seeded with patient's autologous adipose-derived stem cells expanded in a GMP-class laboratory and commercially available growth factor BMP-2. The material was inserted into a titanium mesh preformed to fit the size and shape of the defect. The construct was first implanted into the patient's rectus abdominis muscle, where it was let to mature for 8 months. After maturation, the construct together with the surrounding muscle was transplanted using microvascular technique (TRAM-flap) to the

site of the defect and connected with titanium plates and screws to the adjacent bones. The anastomosis of flap recipient vessels was performed to the neck vessels, and flap was fixated with titanium plates. After uneventful healing, four dental implants were inserted into the regenerated bone, and a fixed bridge was used to reconstruct the masticatory function. The histology obtained at the time of fixture operation confirmed normal bone tissue in heterotopic bone area. The follow-up has been uneventful for a decade; only some small pieces of titanium mesh have had to be removed as they have protruded through the thin oral mucosa (unpublished results) (Figs. 9.6, 9.7, and 9.8).

The same group performed similar reconstruction to a male patient after total maxillary defect caused by ablation of a large squamous cell carcinoma (SCC). The combination of AT-MSCs, β-TCP granules, and rhBMP-2 in

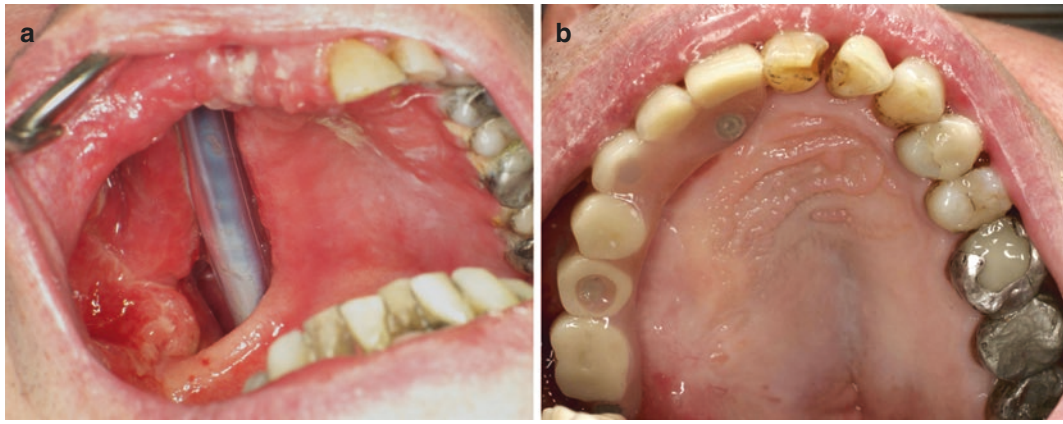

Fig. 9.6 (**a**) Preoperative clinical status 28 months after removal of keratocyst by hemimaxillectomy (**a**). Final result 1 year after bone and soft tissue reconstruction, with temporary dental implant rehabilitation (**b**). (Reprint from Mesimäki et al. [6] granted by Elsevier)

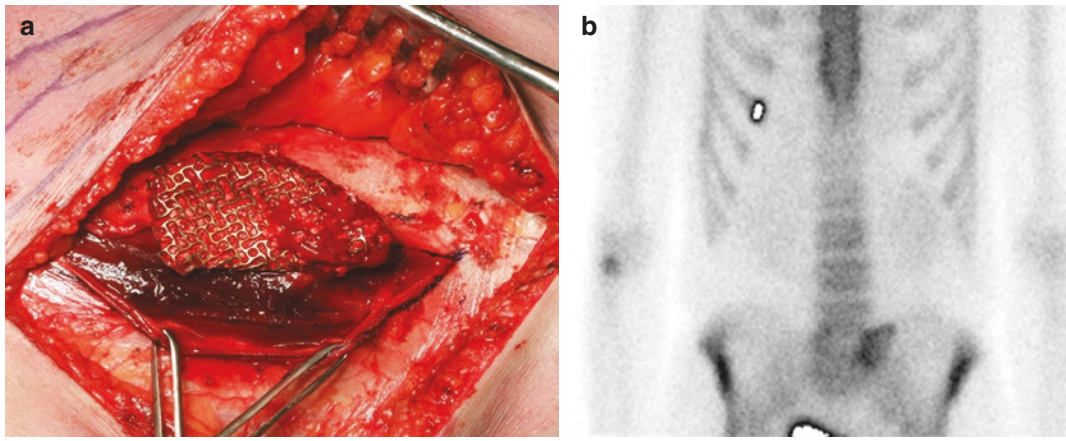

Fig. 9.7 Second and third operation. (**a**) The titanium cage filled with beta-tricalcium phosphate and adipose stem cells, before insertion in the prepared rectus abdominis muscle pouch. (**b**) The bony regenerates in the rectus abdominis muscle. Skeletal scintigraphy was performed to confirm bone activity. (**c**) The rectus abdominis free-flap raised, muscle pouch, and titanium cage opened. Note bleeding from the bone. The tissue-engineered bone was clinically confirmed to be rigid. (**d**) Histological section of the biopsy from the tissue-engineered bone showing normal mature bone structures. (Reprint from Mesimäki et al. [6] granted by Elsevier)

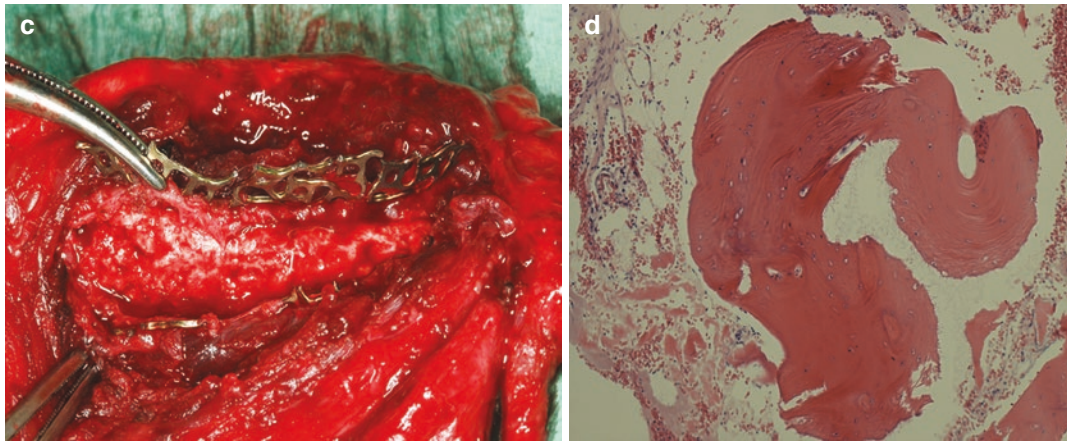

Fig. 9.7 (continued)

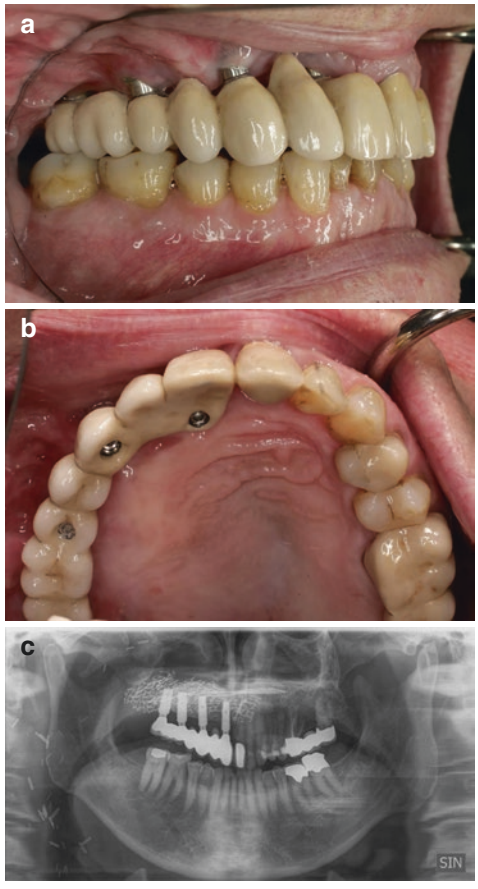

Fig. 9.8 Final reconstruction. The flap was left to epithelialize and prosthetic rehabilitation was carried out with a fixed bridge. Part of the titanium cage was removed to enable placement of implants. (Courtesy of Dr. Jari Mauno)

polylactide scaffold was implanted into the ALT-flap, and shaping of a resorbable polylactic scaffold was done with computer-aided design, with indirect technique. Patient-specific (PS) titanium reconstruction plates were used to fix the future "neomaxilla." After maturation of 7 months, the microvascular ALT flap with heterotopic bone was raised and placed into area of resected maxilla. Dental implants were inserted after uneventful healing of 5 months. The prosthetic reconstruction was established with removable prosthesis (unpublished results).

The largest number of patients with using autologous stem cells in 13 consecutive cases of cranio-maxillofacial hard-tissue reconstruction has been published by Sándor et al. [54]. The reconstruction was carried out with expanded autologous hAT-MSCs, β-TCP, and, in most cases, BMP-2. The group reported on reconstruction of defects at four anatomically different sites: cranial bone (5), frontal sinus (3), mandible (3), and nasal septum (2). In the mandible, continuity defect repair was carried out using computer aided surgical planning and AT-MSCs. If scaffolds were needed, titanium mesh or β-TCP sheet was used. The patients were followed between up to 51 months. Two patients with mandibular reconstruction received a total number of seven dental implants later, which are being loaded in masticatory function. The authors concluded that although

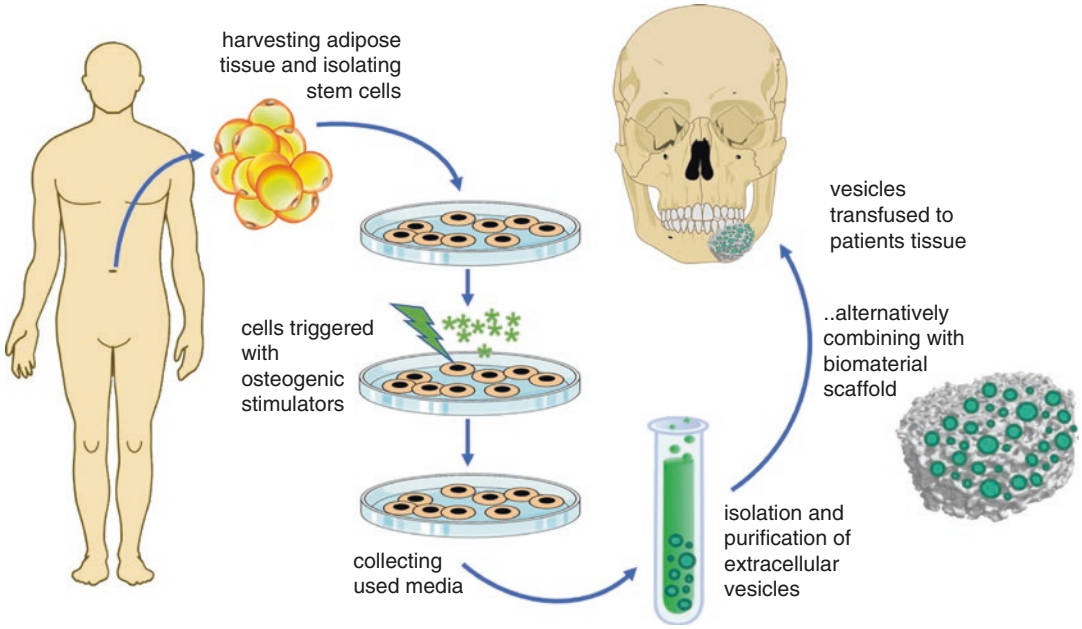

Fig. 9.9 In the future, extracellular vesicles (EVs) secreted by stem cells could be used in tissue engineering avoiding some adverse effects in the use of stem cells. EVs could be harvested from, e.g., fat tissue or blood either autologous or allogeneic, and triggered to enhanced bone formation is a defect site in a similar fashion as MSCs would, thus avoiding potential adverse immune reactions

results are promising, further research is needed with animal studies and long-term human series. This view is supported by other research groups, too (Fig. 9.9).

Matsuo et al. [62] used indirect technique in mandible defect repair. After surgical simulation, a PLA PS mesh tray was manually prepared and filled with HA. Intraoperatively, particulate cancellous bone and marrow was harvested and with platelet-rich plasma (PRP) placed into the tray. Two patients with mandible defect were included to the study. The follow-up was 28 and 33 months. One of the patients received dental implants after 10 months of the initial surgery. The heterotopic bone was macroscopically well formed. The CT evaluation showed good bone quality, and the screws used to attach the resorbable mesh tray did not hinder placement of dental implants.

The neovascularization with prompt recovery of nutrition is considered to be a key issue of bone regeneration. Kokemueller et al. [63] reported a clinical case of hemimandibular defect that was reconstructed with the combination of autologous iliac crest bone marrow, β-TCP, and rhBMP-2 in titanium scaffold. The reason for the defect was chronic osteomyelitis. The patient suffered from several other comorbidities as well. They designed and produced β-TCP cylinder that had central passage with the diameter of 7 mm. The blood-soaked cylinders were implanted into the latissimus dorsi muscle. Perforator vessels were placed into central passage to enhance capillary growth. After 6 months, the flap with heterotopic bone was raised and transferred to oral and maxillofacial (OMF) defect and covered with titanium mesh. The mesh was fixed with titanium screws. There was no complication during the follow-up of 1 year. The authors conclude that the use of autologous bone marrow and β-TCP block with central vessels to repair OMF bone defects is reliable and well tolerated. Furthermore, most of the donor site morbidity can be avoided with this technique. The research group performed also experimental studies with the same protocol, and the results confirmed the clinical achievements.

9.8 Regulatory Aspects

Tissue engineering and regenerative medicine are regulated by FDA in the USA and by EMA in Europe. The manipulated cells are considered as advanced therapy medicinal products (ATMP), and the regulation is very strict. The engineered tissues must be manufactured according to current good manufacturing practices (cGMP) standards, which includes a flawless quality control system including testing of raw materials, establishing a detailed standard operating procedures (SOPs), detecting any deviations of these procedures, detecting possible contaminations, and maintaining reliable testing laboratories and follow-up systems.

9.8.1 Products Available in EU

In Europe, only four products have received manufacturing license, namely, ChondroCelect®, Zalmoxis®, MACI®, and the only one using stem cells Holoclar®. Of these, only two are available in the market at the moment.

ChondroCelect® is a product where patient's own cartilage samples are surgically removed from "less important" parts of the cartilage, and the extracted chondrocytes are expanded in a GMP-class lab. The expanded cells are the inserted surgically to the defect site. For unknown reason, the marketing authorization for ChondroCelect has been withdrawn at the request of the marketing authorization holder in 2017.

MACI® is quite similar to ChondroCelect®, i.e., using patient's own chondrocytes expanded and seeded in a collagen matrix, which is transplanted to the defect site. At the moment, it is not available as the manufacturing site has not received an approval from EMA. If a new manufacturing site will be approved, MACI® could be available to the patients.

Zalmoxis® is used for patients as an add-on treatment in adults who have received a hematopoietic stem cell transplant due to leukemia or lymphoma from a partially matched donor. Before receiving the transplant, the patient's own cells from the bone marrow have been removed, hence predisposing the patient to infections. Zalmoxis® contains genetically altered T-cells which will help the patient in restoring his/her immune system. Zalmoxis® is an orphan medicine and has been granted manufacturing license in 2016.

Holoclar® is the first and only stem cell-based ATMP product to enter the European market and received the manufacturing license at the end of 2014. It is aimed to help patients with a moderate or severe limbal cell deficiency in the eye, which can eventually cause blindness. Holoclar® is based on patients own limbal stem cells.

9.8.2 Products Manufactured in the Operating Room

On the other hand, surgeons may combine cells, growth factors, and materials in the operating room without any ATMP approvals as long as everything occurs during the same surgical procedure and the cells are used for homologous (i.e., same tissue type) reconstruction. The main obstacle causing high prices is the labor-intensive work carried out in GMP-class laboratories as well as the extensive preclinical and clinical trials required for manufacturing license [64].

9.9 Future Perspectives

In order for tissue engineering and regenerative medicine to be feasible treatment methods, more detailed experimental studies as well as controlled clinical studies are needed. The manufacturing of these products is very expensive, and often the end products are patient specific, preventing large scale manufacturing which could lead to cost-effectiveness.

It is still unclear, whether cells or growth factors are needed if local neighboring cells can initiate healing or growth factors are abundant in the area. This might be the case with bone defect and locally preserved periosteum.

Growth factors and stem cells have been speculated to be a risk for malignant transformation.

With mesenchymal stem cells, this has not been proven. When using growth factors, there still is a suspicion of hypertrophic growth and malignant transformation [65, 66]. Hence, they should not be used in patients with malignancies.

Currently there is a shift occurring in the field of regenerative medicine, with less of the focus on cell-based treatment options, and more weight is put on strategies that allow functional restoration of damaged tissues by cell-free approaches. Recent insights suggest that the structural contribution of stem cells to regenerated tissues is limited and that it is rather the stimulation of local healing processes plays an important role; research has increasingly focused on the paracrine hypothesis, exploring the factors released by the cells, including growth factors, cytokines, and EVs (for a review, see [1]).

EVs are lipid membrane vesicles, containing a variety of RNA species, proteins, and potentially even DNA. EVs function in many processes, including cell-to-cell communication, immune modulation, angiogenesis, and cellular proliferation and differentiation. Cells release several types of vesicles with different physiological properties and function [1]. Generally, the term EV is used when discussing exosomes or microvesicles, or a combination of these vesicle populations albeit conflicting definitions still exist [67]. Extracellular vesicles are able to affect several cell processes in a paracrine manner. These paracrine effects of EVs have a potential benefit in regenerative medicine, as EVs can be incorporated in treatments, for example, by injection, mixing with hydrogels, or coating scaffolds with EV using fibrin gels or specific linkers. Subsequently, EV shows great potential for a role in regenerative medicine (Fig. 9.9). Given the supportive role of EV in tissue regeneration, further studies may lead to the discovery of novel regenerative therapeutics, as well as methods to improve current techniques.

Despite of all regulatory and other hurdles, every year more clinical trials end up successful and eventually will lead to clinical applications. Multidisciplinary collaboration plays a key role in this process—we need clinicians, scientists, engineers, regulatory experts, and many more specialists to provide patients the best possible care.

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Translational Research in Sjögren's Syndrome

10

Arjan Vissink, Fred K. L. Spijkervet, F. G. M. Kroese, and H. Bootsma

Abstract

Sjögren's syndrome (SS) is an autoimmune inflammatory disorder of the exocrine glands, particularly affecting lacrimal and salivary glands. Hallmark symptoms are dry mouth and dry eye, often in conjunction with general symptoms, such as malaise and fatigue. Lymphomas could develop in 5–10% of the patients. As SS is a rather complex syndrome with many features, the one patient being diagnosed with SS may suffer from a different complex of complaints than another SS patient and may thus be in need of a different treatment approach. To better classify SS patients and to personalize their treatment, many clinicians and researchers are currently working on efforts (1) to refine classification of SS patients, (2) to ease the diagnostic work-up of SS, and (3) to better understand the etiopathogenesis of SS. Latter knowledge is essential to understand the course of the disease. This way clinicians will be able to identify patients who are at risk of developing SS or lymphomas; can intervene at an early stage of the disease to prevent damage to, e.g., the glands; as well as can personalize treatment with, e.g., biologicals. In this chapter, current major achievements are discussed, and promising new directions are indicated.

10.1 Introduction

Sjögren's syndrome (SS) is an autoimmune inflammatory disorder of the exocrine glands, particularly affecting lacrimal and salivary glands. Hallmark symptoms are dry mouth and dry eyes, often in conjunction with general symptoms, such as malaise and fatigue. Lymphomas could develop in 5–10% of the SS patients.

Recent studies indicate that the prevalence of SS in the general population is about 7 per 100,000 person-years [1], which shares SS among the most common systemic autoimmune diseases. SS is nine times more frequent in women than in men. SS is mostly recognized after the age of 40 but can already present in childhood. SS can be a primary condition (dry eyes, dry mouth, recurrent swellings of salivary glands: primary SS (pSS)) or co-occur with another autoimmune disease, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and scleroderma (pSS combined with

A. Vissink (✉) · F. K. L. Spijkervet
Department of Oral and Maxillofacial Surgery, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
e-mail: a.vissink@umcg.nl

F. G. M. Kroese · H. Bootsma
Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

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another autoimmune disease: secondary SS (sSS)).

With regard to SS, there are currently a lot of efforts with a translational character, e.g., (1) to better classify SS patients so that results from studies can be better compared with each other and the outcome can be better generalized, (2) to ease the diagnostic work-up of SS by either applying biomarker assays that are specific for SS or by applying (more) simple diagnostic clinical tools, and (3) to understand the etiopathogenesis of SS. Latter knowledge is essential to understand the course of the disease. This way clinicians will be able to identify patients who are at risk of developing SS or lymphomas; can intervene at an early stage of the disease to prevent damage to, e.g., the glands; as well as can personalize treatment with, e.g., biologicals. For example, Lendrem et al. [2] identified four distinct pSS clinical phenotypes. These phenotypes were defined on the basis of hierarchical cluster analysis of patient-reported pain, fatigue, dryness, anxiety, and depression. Importantly, these four phenotypes exhibited marked differences in a variety of biologic parameters. Presumably, SS patients within these clusters will differently response to a particular treatment, both in terms of subjective and objective parameters. In this chapter, current major achievements are discussed, and promising new directions are indicated.

10.2 Characterizing SS Patients

Over the years, many classification criteria for SS have been developed of which the 2002 revised American-European Consensus Group (AECG) classification criteria are currently the most frequently used. The AECG criteria combine subjective symptoms of dry eyes and dry mouth with the objective signs of keratoconjunctivitis sicca and xerostomia [3]. In 2012, endorsed by the American College of Rheumatology (ACR), a new preliminary classification criteria set was introduced that purely focused on objective measures [4]. As the ACR criteria were not well received by the international SS community, a joint working group consisting of members from ACR and European League Against Rheumatism (EULAR) developed a new classification criteria set (Table 10.1; [5]). This criteria set was endorsed by both ACR and EULAR and was well received by the international SS community. The ACR-EULAR classification criteria for pSS are a step in the right direction. Further refinement is needed, however, to increase their utility [6].

To increase the utility and to make the criteria even more commonly applicable for clinicians, lots of effort are currently applied to test whether salivary gland ultrasonography (sGUS) is more specific and more sensitive than measuring salivary secretion or other objective tests applied or could replace more invasive tests such as salivary gland biopsies. This to show that sGUS is a good diagnostic tool to add to the ACR-AECG classification criteria and even might have the potential to replace (some of the) current objective tests in future [7, 8]. The focus is on assessing the diagnostic potential of sGUS as sGUS is a well-tolerated, noninvasive, inexpensive, non-irradiating technique that is widely available in rheumatologic outpatient clinics. Recently, it was shown that a combination of positive sGUS and presence of anti-SSA/Ro antibodies highly predicts classification according to the ACR-EULAR classification criteria [8]. When applying the ACR-EULAR criteria for monitoring disease activity and treatment evaluation, it is recommended that the same ultrasonographer follows the same patient as a function of time as the interobserver variation of ultrasonographers is rather large (Fig. 10.1; Delli et al. [10]). Furthermore, it has to be assessed whether sGUS images of SS patients are grossly dissimilar from those of patients with diseases involving the salivary glands that mimic SS, such as sarcoidosis, hepatitis C, and HIV.

Table 10.1 A comparison of the AECG [3], ACR [4], and ACR/EULAR [5] classification criteria sets

Criterion	AECG[a]	ACR[a]	ACR/EULAR[a]
Subjective			
Sicca eye	+	–	Entry[b]
Sicca mouth	+	–	Entry[b]
Objective			
Ocular signs:			
Schirmer's test	**+**	–	**+**
van Bijsterveld score (VBS)[c]	**+**	–	**+**
Ocular staining score (OSS)	–	+ (≥3)	+ (≥5)
Histopathology:			
Labial gland focus score ≥1	**+**	**+**	**+**
Salivary gland involvement:			
Unstimulated whole saliva (≤0.1 ml/min)	**+**	–	**+**
Parotid sialography	+	–	–
Salivary gland scintigraphy	+	–	–
Autoantibodies/serology:			
Anti-SSA positive	**+**	**+**	**+**
Anti-SSB positive	+	+	–
ANA	–	+	–
Rheumatoid factor	–	+	–

Criteria that are present in the ACR/EULAR and in original criteria sets are depicted in bold

[a]For patients without any potentially associated disease, pSS may be defined as follows: (1) The presence of any four of the six items is indicative of pSS, as long as either histopathology or serology is positive. (2) The presence of any three of the four objective criteria items. For patients with a potentially associated disease (for instance, another well-defined connective tissue disease), the presence of a subjective item plus any two from the four objective items may be considered as indicative of sSS. Exclusion criteria AECG: history of head and neck radiation treatment, active hepatitis C infection, acquired immunodeficiency disease, pre-existing lymphoma, sarcoidosis, graft versus host disease, and use of anticholinergic drugs (since a time shorter than fourfold the half-life of the drug). Exclusion criteria ACR: similar to AECG with exception of pre-existing lymphoma and addition of amyloidosis and IgG4-related disease. Exclusion criteria ACR/EULAR: similar to AECG with exception of pre-existing lymphoma and addition of IgG4-related disease

[b]The ACR/EULAR criteria are applicable to any patients with at least one symptom of ocular or oral dryness, defined as a positive response on the validated sicca questions as defined for the AECG criteria

[c]VBS ≥4 equals OSS ≥5

10.3 Biomarkers and SS

Although a variety of diagnostic and therapeutic biomarkers has been proposed to classify pSS and its subsets over the years [11, 12], there is still a crucial need for novel specific biomarkers to ease diagnostics, to diagnose SS at an early stage, and to predict which patient might be helped with a tailored, targeted treatment or is at risk of developing specific comorbidity [13, 14].

10.3.1 Serology

A great variety of biomarkers is known to be present in serum of SS patients. The most frequently detected and most widely used are antibodies directed against nuclear components (ANA), antibodies directed against intracellular antigens (Ro52/SSA, Ro60/SSA, La/SSB), and rheumatoid factor (RF). These autoantibodies can already be detected many years before SS has become clinically apparent, which imply their

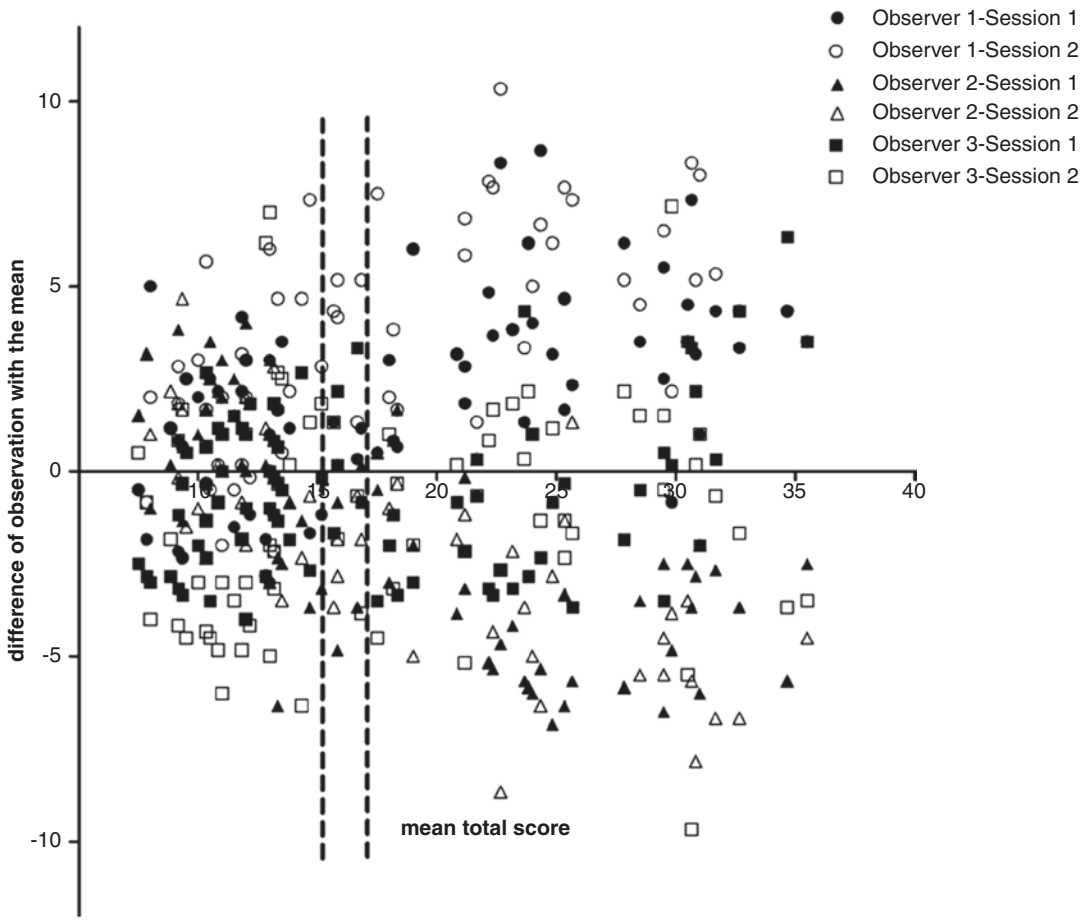

Fig. 10.1 Systematic differences in sGUS total score in pSS patients using the Hocevar scoring system [9]. For each patient, the mean of the six observations (three observers, two sessions) and the difference of these six observations with the mean were calculated and plotted against each other. The intermittent vertical lines indicate the different cutoff points applied. While the interobserver variability is rather low, the intra-observer variability is considerable. Therefore, it is recommended that the same ultrasonographer follows a patient when performing follow-up or treatment evaluation studies [10]

potential use to predict onset of pSS (Fig. 10.2; [15, 16]). It has to be mentioned, however, that it is not yet set which subject that is positive for anti-Ro/SSA and/or anti-LA/SSB will indeed develop SS in future.

Anti-Ro/SSA is found in 70–90% of SS patients and may predict the course of the disease. E.g., positivity for anti-Ro/SSA is linked with a younger age at diagnosis, a longer disease duration, a higher incidence of recurrent parotid gland swelling, a higher focus score, and a higher prevalence of extraglandular manifestations [17, 18]. Sole positivity for anti-La/SSB is rare.

Of the other antibodies present in SS patients, RF and cryoglobulins are most common, respectively, in 35–70% and 5–10% of the patients [19, 20]. Both antibodies have been linked to the development of lymphomas [21, 22]. When these antibodies are present in SS patients with parotid gland enlargement, palpable purpura, and low C4 levels, these patients are at a rather high risk of developing a lymphoma or have already developed a lymphoma. Therefore, there is currently much research ongoing to detect which biomarkers are the best markers to predict which SS patient has a high lymphoma risk. This in addition to the presumed predictive

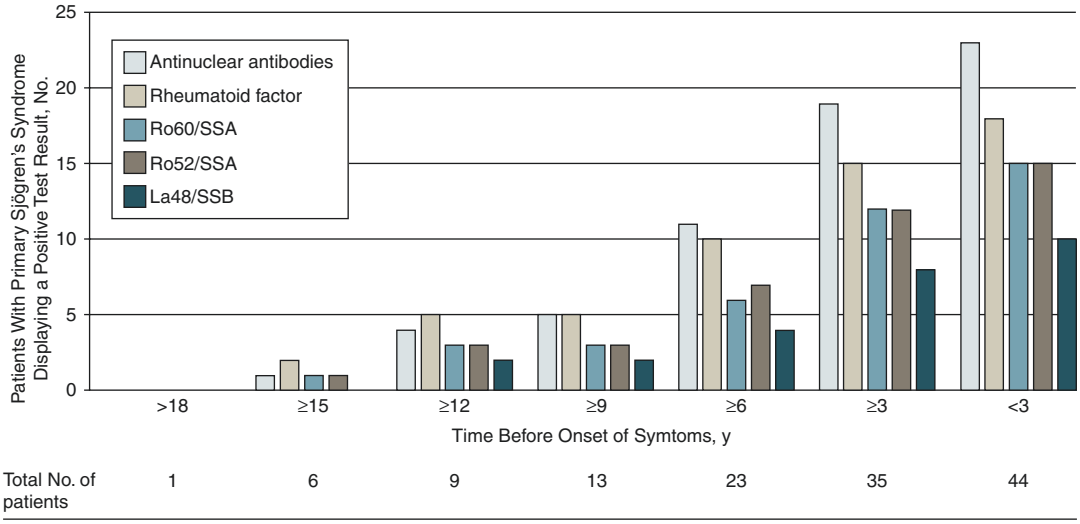

Fig. 10.2 Cumulative number of patients with pSS with autoantibodies before clinical onset (y: years). Anti-Ro/SSA and anti-La/SSB can be present many years before pSS is clinically diagnosed [15]

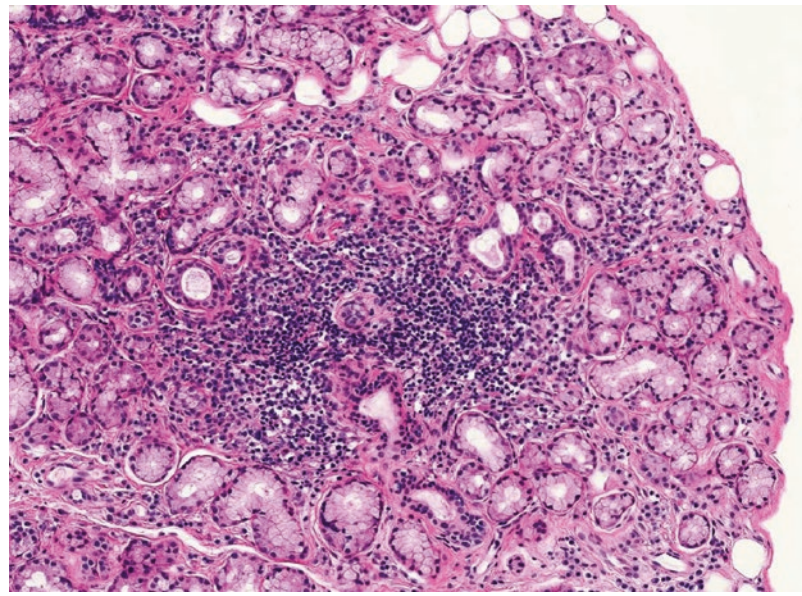

Fig. 10.3 Biopsy of a labial salivary gland (H&E stain) showing focal lymphocytic sialadenitis. Centrally in the image a focus is present, i.e., an accumulation of more than 50 lymphocytes around a duct

value of salivary gland biopsies ([23]; see section on histopathology), which is questioned by other authors [24]. It also has to be set which SS patient with a lymphoma needs treatment or just has to be closely monitored [25].

10.3.2 Histopathology

An issue that is currently heavily discussed is whether a biopsy of the labial salivary gland has to be taken to histopathologically confirm the SS diagnosis. Patients can be classified as suffering from SS syndrome when this biopsy shows focal lymphocytic sialadenitis with a focus score ≥1, i.e., the presence of at least one accumulation of 50 or more lymphocytes per 4 mm^2 (Fig. 10.3; [26]). Parotid biopsies can serve as a proper alternative to labial biopsies in the SS diagnostic work-up [27]. A major advantage of parotid biopsies is that in parotid biopsies lymphomas, mostly mucosa-associated lymphoid tissue

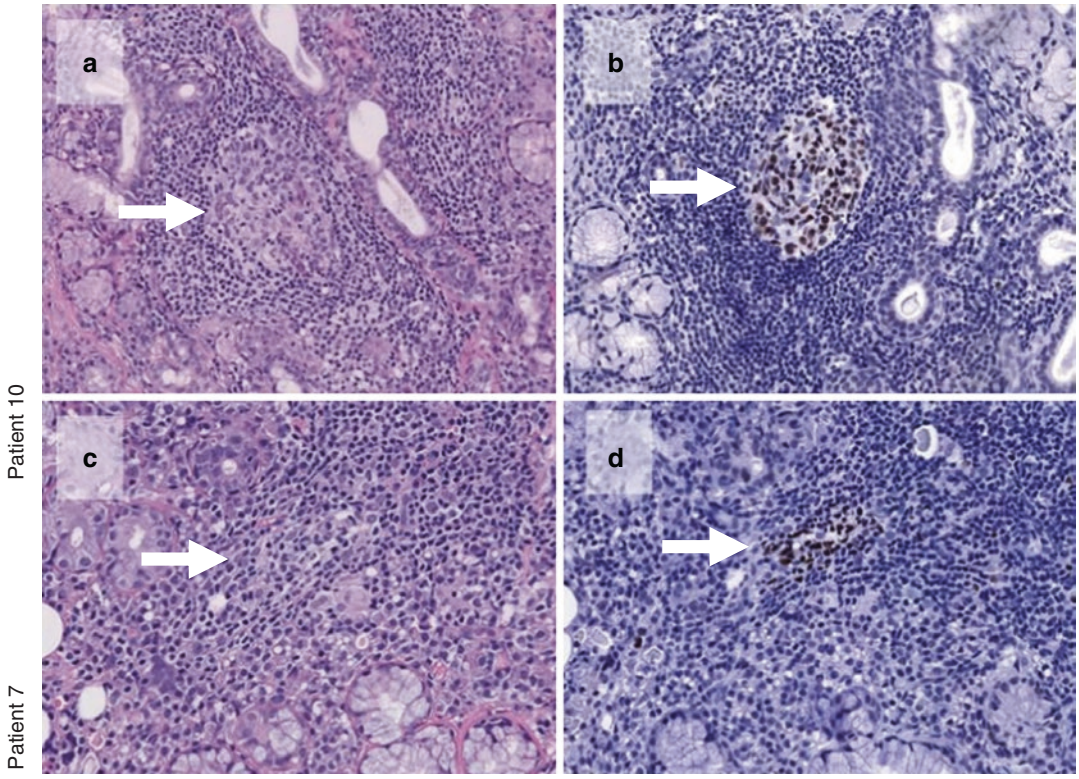

Fig. 10.4 GCs present in diagnostic labial salivary gland biopsies of pSS patients who developed a parotid MALT lymphoma later on. Arrows point to GCs. (**a**) Clearly visible GC in a periductal focus of a labial gland (H&E stain). (**b**) Bcl6 staining of the same GC as in (**a**) in a serial section. (**c**) Suspicious GC in a periductal focus of the labial gland (H&E stain). (**d**) Bcl6 staining in a serial section of (**c**) shows a small GC [24]

(MALT) lymphomas, are easier to detect as parotid glands are more commonly affected (70–80% of the lymphomas in pSS patients are MALT lymphomas in the parotid glands; Fig. 10.4). Moreover, the same parotid gland can be biopsied more often which is an important asset for treatment evaluation studies (Fig. 10.5; [28]). Theander et al. [23] and Reksten et al. [29] posed that the presences of germinal center (GC)-like structures by light microscopy in SS diagnostic salivary biopsies are also highly predictive for non-Hodgkin lymphoma development, but Haacke et al. [24] recently showed in a more extensive study that the GCs in labial gland biopsies does not differ between patients with pSS that develop parotid MALT lymphoma and patients with pSS who do not develop lymphoma (Fig. 10.6). Thus, the presence of GCs in labial gland biopsies is probably not a predictive factor for SS syndrome-associated parotid MALT lymphomas. From which cells these lymphoma's originate needs further study (see also section on lymphomas).

Recently, Delli et al. [28, 31, 32] showed that the histopathologic characteristics of parotid gland biopsies may predict which pSS patients are probably responsive to treatment with anti-CD20 therapy (rituximab) and which patient is not (Fig. 10.7). This observation brings targeted treatment within reach. In fact, the possibility of

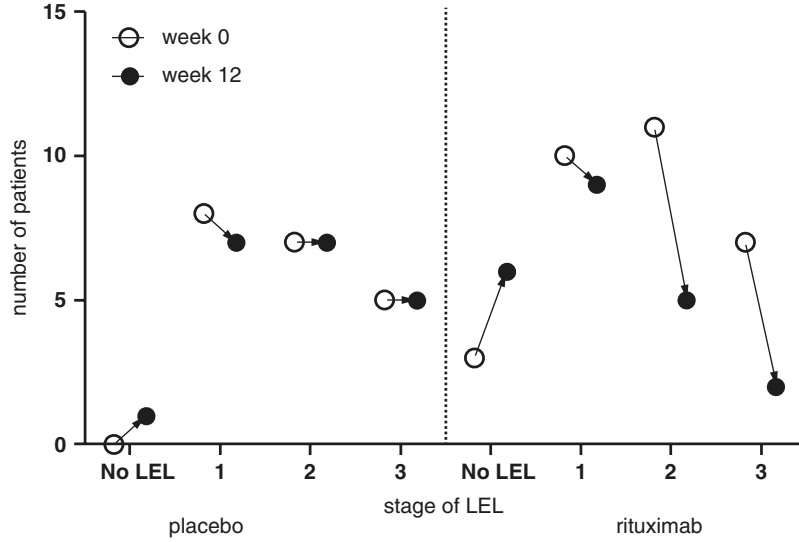

Fig. 10.5 Parotid glands allow for repeated biopsies from the same glands in pSS patients which ease comparison, e.g., for treatment evaluation studies. This figure shows the presence of three stages of LELs/mm² in placebo and anti-CD20 therapy (rituximab) treated patients at weeks 0 and 12: *Y*-axis indicates the total number of patients, while *X*-axis indicates the presence of different stages of LEL (a higher number reflects a more severe stage). Twelve weeks after anti-CD20 therapy, the number and severity of the LELs have decreased [28]

considering the composition of the inflammatory infiltrate as a tool in clinical trials to recruit homogeneous subsets of patients and to get information on the efficacy and mechanisms of action of novel drugs is challenging and very promising but still in childhood and in need of thorough studying and standardization [33]. For example, Tavoni et al. [34] showed a discrepancy among different readers in the interpretation of minor salivary gland biopsies in clinical practice. Currently, efforts are taken by a working group of EULAR to standardize histopathologic evaluation of salivary gland biopsies, including how to detect GCs [35].

10.3.3 Progress in Biomarker Research

Among the many biomarkers that are currently studied, a promising novel biomarker is the interferon (IFN) type I signature [36]. Dysregulated genes of IFN pathways, both in salivary gland tissue and peripheral blood, are considered to be an asset in diagnosing pSS and monitoring its disease activity [37–39]. E.g., presence of myxovirus resistance protein A (MxA) in cell may reflect presence of IFN type I and is correlated with EULAR SS disease activity (ESSDAI) score and levels of immunoglobulins and autoantibodies [40]. Also type II IFN seems to be involved in the pathogenesis of pSS as the focus score is higher in type II IFN pSS patients [41]. Furthermore, a higher IFNg/IFNa mRNA ratio in minor salivary gland tissue seems to be a predictor for lymphoma development [42]. However, the IFN type I signature is not specific for SS. It also could be just a biomarker for, e.g., disease activity. So to prove whether the IFN type I signature indeed is of additional value, the identification of different patient categories awaits long-term analysis of a larger cohort of patients [43].

Another key pathogenetic cytokine is B-cell-activating factor (BAFF). BAFF is present in

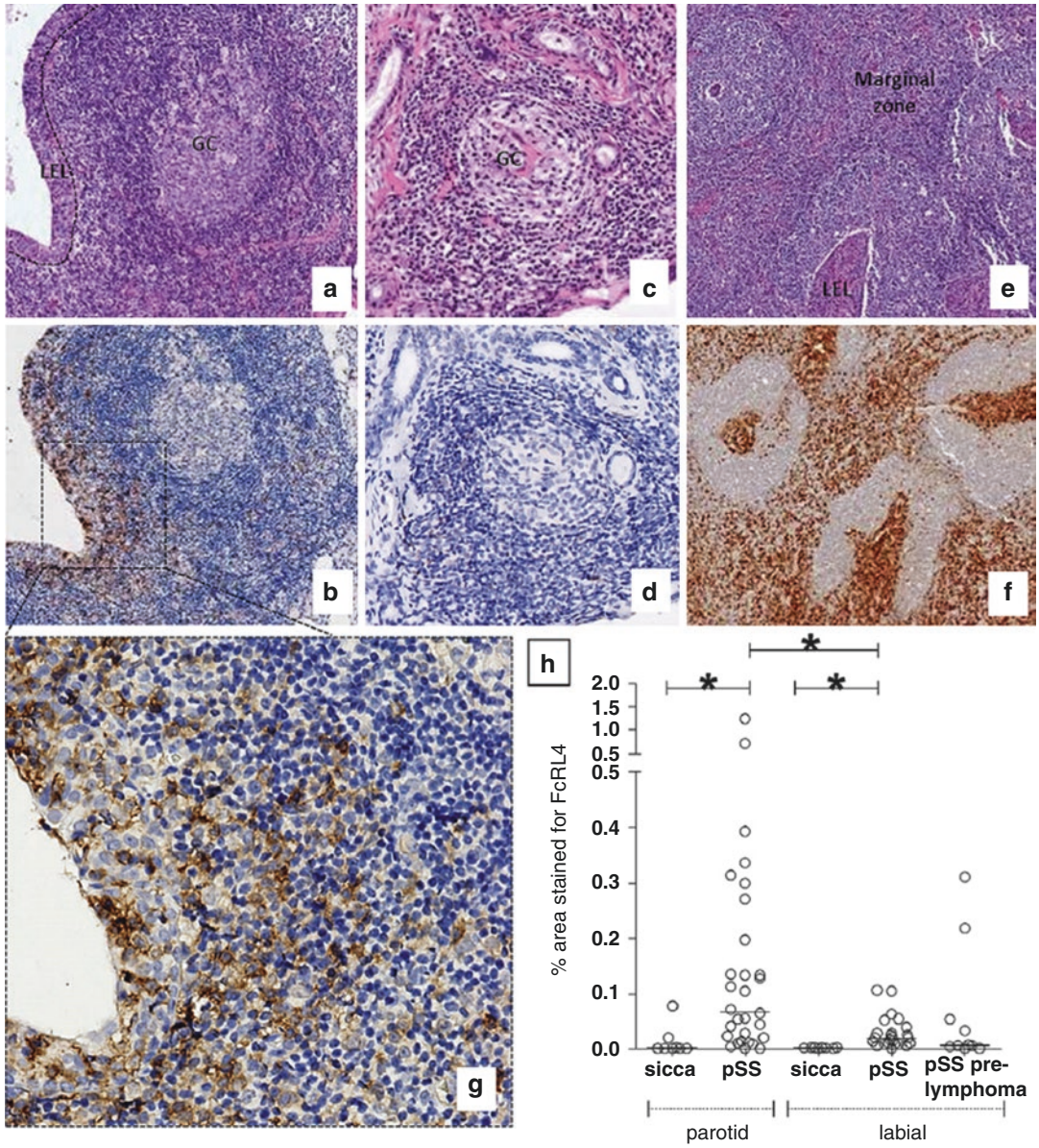

Fig. 10.6 FcRL4 B cells in parotid and labial salivary glands as well as in parotid MALT lymphomas. (**a**) Parotid gland biopsy of a pSS patient with a LEL and GC (H&E stain). (**b**) FcRL4 B cells in the same parotid gland. FcRL4 B cells are in close association with the ductal epithelium. Less FcRL4 B cells are found in the infiltrate with lower intensity of the FcRL4 stain. (**c**) Labial gland biopsy of a pSS patient (H&E stain). (**d**) FcRL4 B cells in the same labial gland. (**e**) MALT lymphoma in the parotid gland of a pSS patient (H&E stain). (**f**) FcRL4 stain same MALT lymphoma. The FcRL4 B cells cluster in and around LELs and in the marginal zone. Few FcRL4 B cells with low intensity are found despite the presence of a periductal infiltrate. (**g**) High magnification of FcRL4 B cells in the parotid gland. (**h**) Quantification of FcRL4 B cells, by measuring relative surface of FcRL4 staining. Amount of FcRL4 staining is higher in pSS patients compared to non-pSS sicca patients. In the parotid glands of pSS patients, significantly more FcRL4 positivity is present compared to labial glands of pSS patients. In diagnostic labial gland biopsies from pSS patients who developed a parotid MALT lymphoma and pSS patients who did not, FcRL4 staining was similar. *Mann-Whitney U, $p < 0.05$ [30]

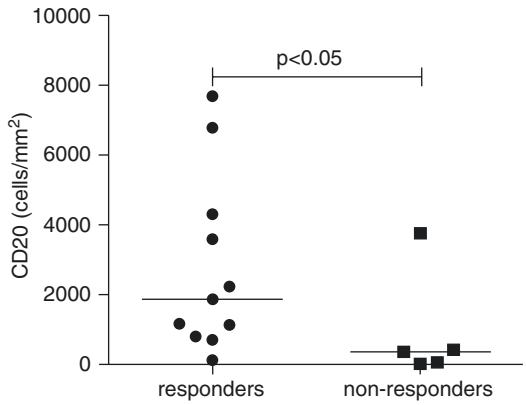

Fig. 10.7 Number of baseline $CD20^+$ B-cell/mm^2 is higher in clinical responders to anti-CD20 therapy than in non-responders [28]

peripheral blood monocytes and salivary gland tissue of SS patients. BAFF controls B-cell maturation, tolerance, and malignancy. It has been shown that BAFF levels are higher in pSS patients with higher systemic disease activity [44, 45]. It also has been shown that BAFF-driven B-cell activation may negatively affect the clinical response of pSS patients to treatment with anti-CD20 therapy [46]. Adding a BAFF blocker to anti-CD20 therapy might increase its efficacy [47].

-Omics aims at the collective characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of an organism or organisms. Currently, much effort is put on how to apply genomics, proteomics, and metabolomics of serum, saliva, tears, and salivary gland tissue for diagnosing and better understanding SS as well as for patient stratification [12]. Particularly, saliva, and to a lesser extent also tears, is a very attractive biofluid for searching candidate biomarkers for pSS. Saliva is probably a more direct agent than serum as it is produced by glandular tissues that are directly affected by the disease process. Moreover, when compared to blood, saliva (as well as tears) can be collected repeatedly and noninvasively. When using glandular-specific saliva, the biomarkers detected even can be directly linked to the underlying autoimmune inflammatory deregulation and thus to mechanisms in the pathogenesis of SS.

Mass spectrometric analysis of saliva has revealed a variety of biomarkers that are preferably involved in the pathogenesis of pSS (Fig. 10.8; [48–50]). An increased expression of inflammatory and immune response-related salivary proteins is observed as well as that the secretion of other proteins is reduced, probably related to the destruction of acinar and ductal structures. Further study is needed, but it is becoming within reach that the diagnosis SS can be made on analysis of a drop of saliva. In this respect the results of a trial focusing on the application of salivary biomarkers for SS detection (NCT01807689) are eagerly awaited.

Micro-RNAs (miRNAs) are well-preserved, small non-coding RNAs of 19–25 nucleotides involved in posttranscriptional regulation of gene expression. Alevizos et al. [51] suggested that miRNAs may serve as a set of biomarkers for pSS. Research in this field is ongoing and is presumed to have a high potential. For example, it has been shown that the risk for developing pSS is related to miR-146a expression [52]. As such, miR-146a expression is a potential biomarker to be used in the diagnostic work-up of patients with a SS suspect.

Another promising approach is laser microdissection coupled with RNA-seq analysis. With this technique acini, ducts, and inflammatory foci of pSS subjects can be isolated for RNA-seq analysis. Tandon et al. [53] showed that marked differences in gene expression occur in the ductal and infiltrating cells compared to acinar cells. In particular, two chemokines involved in immune cell trafficking to secondary lymphoid tissue, viz., CCR7 and CCL21, had a markedly increased expression. The authors suggested that these chemokines may contribute to the recruitment of diverse immune cells to the salivary glands, causing inflammation and loss of secretory function that is commonly observed in SS patients.

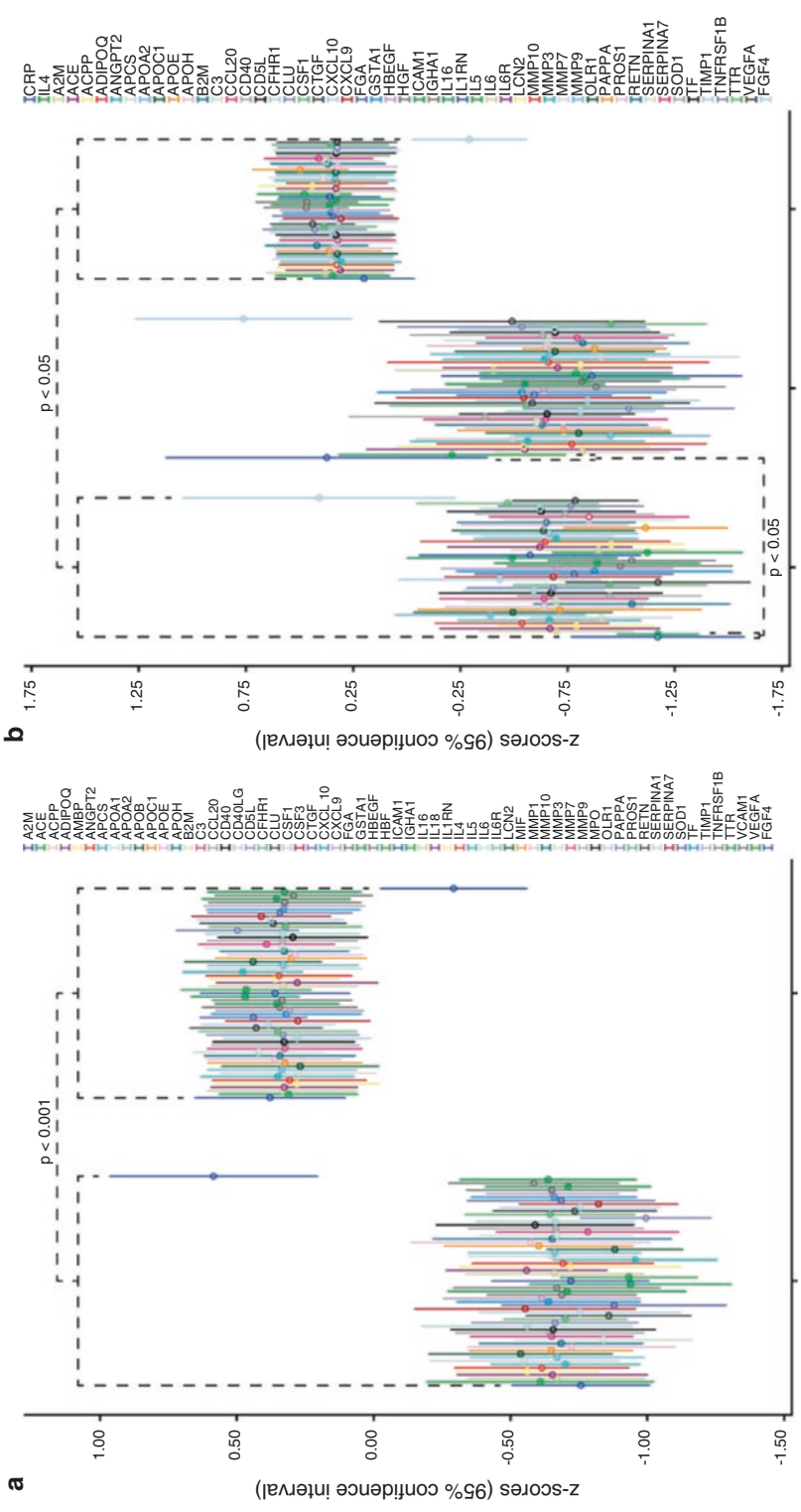

Fig. 10.8 Significant alterations in the quantity of specific proteins in saliva identified by multi-analyte profiles (MAPs). (**a**) Proteins yielding p< 0.001 by Student's two-tailed *t*-test when comparing SS patients ($n = 48$) to non-SS subjects ($n = 24$). (**b**) Proteins yielding p< 0.05 by Dunnett's two-tailed posttest (subsequent to one-way analysis of variance [two-tailed p< 0.001 were considered significant]) when comparing rheumatoid arthritis patients ($n = 12$) and SS patients ($n = 48$) to asymptomatic controls ($n = 12$). All values are represented as standardized values (Z scores); error bars indicate the 95% confidence interval for each protein. MAP proteins are annotated with their HGNC-compliant symbols [48]

10.4 Personalized Treatment: Which Biological Might Be Effective?

Presence of anti-Ro52/SSA and/or anti-La/SSB, elevated plasma levels of gamma globulins and RF, higher expression levels of Bruton's tyrosine kinase in B cells, and an increased risk of developing lymphomas, particularly mucosa-associated lymphoid tissue (MALT) lymphomas, all point toward a major role for B cells in the pathogenesis of pSS [54]. This B-cell hyperactivity seems to largely be T-cell dependent, and in particular Tfh cells play a role in SS [55].

Because of the role of B cells, anti-CD20 therapy has been considered as a potentially potent biologic disease-modifying antirheumatic drug (DMARD) to reduce disease activity. Anti-CD20 therapy results in significant depletion of $CD20^+$ B cells via several mechanisms. Open label and smaller randomized placebo-controlled trials (for a summary, see Van Nimwegen et al. [56] as well as the larger TEARS trial [57]) revealed that anti-CD20 therapy (rituximab) shows beneficial effects for pSS patients, while in a larger multicenter placebo-controlled trial, the TRACTISS trial [58], anti-CD20 therapy apparently was not effective at all. Thus, the question raises whether anti-CD20 therapy is indeed a failing therapy or whether anti-CD20 therapy is only effective in a selected category of pSS patients [59]. Probably, the same limitations apply to other biologicals that have been tested, are tested, or will be tested in SS. In other words, pSS patients that are treated with anti-CD20 therapy or other biologicals should be better selected to enhance success of a promising treatment modality. E.g., notwithstanding the negative TRACTISS trial [58], anti-CD20 therapy apparently has beneficial effects as has been reported at a clinical, biological, histological, and ultrasonographical level [56, 57, 60]. Post hoc analyses even have identified possible predictors of response, which might serve as a guide to select patients that likely will respond to a treatment with a particular biological (Fig. 10.5; [28, 31, 32]). Targeted patient inclusion will probably make, particularly when studying a disease with many manifestations as SS is, a biological to a failing or successful trial.

The central position of B cells as target for therapy is further illustrated by other recent positive trials with biologicals that are not based on the direct depletion of B cells but that do target, either directly or indirectly, these cells [56]. These biologicals comprise belimumab that binds to BAFF [47], resulting in less survival and less activation of the B cells, and abatacept [61] that blocks the co-stimulation of T cells and as a consequence the T-cell-dependent activation of the B cells [55]. Although several cytokines and chemokines decrease after anti-CD20 therapy, BAFF levels increase, likely as the result of the relative unavailability of B cells, expressing BAFF-binding receptors (Pollard et al. [62, 63]). Since high BAFF levels have been associated with humoral autoimmunity, the effect of adding belimumab to rituximab on the efficacy of rituximab is currently assessed (NCT02631538). First results are promising [47].

10.5 How to Design and Select Patients for Trials

As mentioned by Vissink and Bootsma [6], the new ACR-EULAR classification criteria for pSS do not guarantee that the proper pSS patients are selected for studies. Either these criteria need refinement or specific inclusion and exclusion criteria have to be added to the ACR-EULAR criteria for a particular study. So, when designing a trial to show efficacy of anti-CD20 therapy (rituximab) or other biologicals, the first step should be to define what specific baseline characteristics a pSS subject should have to be included in a particular trial. The information derived from previous studies with anti-CD20 therapy or other biologicals is worthwhile to identify subpopulations of SS patients that likely will respond to a particular biological.

Applying very strict criteria to include SS patients in a particular trial to make it successful also has the hazard that recruiting eligible SS patients will slowly progress. For example, Oni et al. [64] showed that when applying specific

measures of outcome, such as an EULAR SS patient-related index (ESSPRI) score ≥5 and an ESSDAI score ≥5, with requirements for unstimulated whole saliva flow >0 and anti-Ro/SSA positivity, the pool of eligible participants will greatly reduce. On the contrary, when making the inclusion to general, the result will be a failing trial unless the biological tested has such a general beneficial action that it is effective in most subcategories of SS patients.

Another critical step in trial design is to identify centers which have the tools to properly select SS patients with the required specific characteristics as well as have the experience to reliably apply the outcome parameters. For many outcome parameters, specifically trained rheumatologists (experienced in scoring ESSDAI), pathologists (targeted histologic evaluation), ophthalmologists (trained in ocular staining score), and oral and maxillofacial surgeons/specialists in oral medicine (experienced in assessing salivary gland function and taking the required type of salivary gland biopsy) are needed. It is recommended to perform trials in those expert SS centers that are able to include reasonable numbers of patients and have the needed expertise in house. This is because, in particular in multicenter trials, the inherent inter-individual variety in applying inclusion criteria and assessment tools cannot be tackled by training and calibration of clinicians when participating centers include only a few subjects. Such issues probably underlie the negative outcome of the larger randomized clinical trials performed in SS: patient selection was not sufficiently strict, and too many participating centers recruited too few patients and/or lacked the needed in-house expertise to cover all needed tests.

10.6 Lymphomas: Why Are They a Commonplace in SS Patients

Lymphomas develop in approximately 5–10% of SS patients. SS patients have an 18.8 (CI 9.5–37.3) times increased risk of developing lymphomas over the life span [65]. In most cases, these lymphomas are marginal zone B-cell lymphomas occurring in the salivary glands, in particular the parotid gland, the so-called MALT lymphomas. Lymphomas in SS patients are generally localized and follow an indolent, rather benign, clinical course, and if treatment is needed, they are very responsive to therapy [25]. In a minority of SS patients, aggressive non-Hodgkin lymphoma (NHL) is present. Even Hodgkin's disease has been described.

As mentioned before, risk factors for the development of lymphoma in SS patients include the presence of systemic activity, cytopenia, cryoglobulins, low complement C4 levels, and palpable purpura [66–68]. Whether the presence of GCs in salivary gland biopsies are predictive for the development of lymphoma is a continuing debate, but, as mentioned before, the larger study of Haacke et al. [24] could not confirm the presumption of Theander et al. [23] that GCs are indeed linked to the development of lymphomas in SS patients (see also section on histopathology).

Haacke et al. [30] tried to shed light whether MALT lymphomas preferably develop in parotid salivary glands. They showed that B cells expressing Fc receptor-like protein 4 (FcRL4), a protein that normally is expressed on a small subset of mucosa-associated B cells as well as on MALT lymphoma B cells, were present in salivary gland tissue of pSS patients where they were closely associated with ductal epithelial cells forming lymphoepithelial lesions (LELs). Remarkably, FcRL4+ B cells were far more frequent in parotid gland than in labial gland tissue (Fig. 10.6). As expected, the FcRL4 mRNA expression level in parotid MALT lymphoma was increased compared to parotid gland tissue of pSS patients without lymphoma. On the contrary, numbers of FcRL4+ B cells in labial gland biopsies taken at the time of pSS diagnosis were not predictive for later development of MALT lymphoma. The enrichment of FcRL4+ B cells in parotid gland tissue may explain why MALT lymphomas preferentially develop at this specific location pSS patients.

10.7 Etiopathogenesis

The most prominent histopathological finding in salivary gland tissue is the presence of focal mononuclear infiltrates of T and B cells and other cells, including plasma cells, macrophages, myeloid and plasmacytoid dendritic cells, and follicular dendritic cells. These infiltrates develop progressively in association with the striated ducts within glandular tissue. The result is impaired function of the glands and ultimately irreversible destruction of glandular tissue. It is also mentioned before that B cells play a central role in the immunopathogenesis and exhibit signs of hyperactivity. Hyperactivity of B cells is the consequence of the coordinated and integrated action of stimulation of the B-cell receptor, CD40, and toll-like receptors (TLR) in the presence of appropriate cytokines. The elevated levels of the B-cell receptor signaling molecule Btk, in B cells of pSS patients, illustrate the hyperactive status of B cells [69]. Overexpression of type I IFN and BAFF on one hand and IL-6 and IL-21 on the other hand is critically involved in the enhanced plasma cell formation in pSS patients. Hyperactivity of B cells results in secretion of autoantibodies and production of various cytokines [55].

As also mentioned previously, in many pSS patients, type I IFN and type I IFN-induced genes and proteins are overexpressed, resulting in the so-called type I IFN signature of pSS [70–72]. This observation also suggests involvement of viruses in the pathogenesis of SS. A variety of viruses, e.g., Epstein-Barr virus (EBV), coxsackievirus, and cytomegalovirus, are thought to play a role in onset or triggering of pSS [73]. Especially reactivation of latent EBV in genetically and hormonally susceptible individuals is presumed to play a role in the initiation and perpetuation of the chronic inflammatory autoimmune response in exocrine glands. Inoue et al. [74] postulated that binding of the exogenous ligand dioxin to the aryl hydrocarbon receptor causes lytic reactivation of EBV in B cells and salivary gland epithelial cells, resulting in immune responses in the salivary glands and possibly pSS.

10.7.1 B-Cell Hyperactivity and Role of Chemokines

Histopathologically, pSS is characterized by the presence of progressively developing focal lymphoid infiltrates around salivary gland striated ducts (lymphocytic sialadenitis; Fig. 10.3) as well as the development of LELs, in particular in parotid glands (Figs. 10.4 and 10.6). These lesions are formed by hyperplasia of the epithelium in association with lymphocytes. The histopathological features reflect the autoimmune process and manifestations of local B-cell hyperactivity. The occurrence of GCs, which are predominantly seen in the major salivary glands, is a clear sign of local activation of B cells. Another characteristic histopathological feature of pSS and witness of aberrant B-cell activity is the marked increase in the number of IgG (but not IgA)-secreting plasma cells in the exocrine glands [75]. These IgG plasma cells are predominantly present within the infiltrate, whereas IgA plasma cells dominate in the parenchyma. Chemokines are the driving force for the recruitment of lymphoid cells to sites of inflammation. As such these molecules underlie the immunopathological process in exocrine glands and contribute to B-cell hyperactivity, characteristic for pSS.

10.7.2 Germinal Centers

In approximately 25–30% of pSS patients, structures resembling GCs of secondary lymphoid organs are found within the (organized) ectopic lymphoid tissue of minor (labial) and major (parotid) salivary glands (Figs. 10.4 and 10.6) [76–80]. GCs arise after T-cell-dependent antigenic stimulation, and the presence of these structures obviously reflect local activation of B cells. In salivary glands of pSS patients, GCs are more likely to occur with increased focal infiltration and are associated with more severe disease [81].

10.7.3 B-Cell Hyperactivity and Clonal Expansion

Clonal expansions of B cells and plasma cells are increased in the salivary glands of pSS patients. These expansions are composed of IgA and/or IgG expressing cells [81]. Almost all obtained IgG and IgA sequences are somatically hypermutated, suggesting a post-GC origin of the cells. The occurrence of these clonally related cells as well as the intraclonal switching implies local activation and proliferation of B cells within the glandular tissue. Neoplastic transformations of clonally expanded cells may ultimately lead to the development of lymphoma in 5–10% of the pSS patients [82].

10.7.4 Pathogenetic Function of B Cells in pSS

The pathogenic role of autoantibodies in pSS is still largely obscure. As mentioned, the best known are anti-Ro/SSA and anti-La/SSB, both directed against ribonucleoproteins. The glandular epithelium is an important source for these autoantigens [83–85].

Besides their classical role as antibody-producing cells, activated B cells also have the ability to produce and secrete cytokines which are able to modulate immune responses [86, 87]. Herewith, B cells also play an antibody-independent role in tolerance and autoimmunity. TLR signaling appears to be critically involved in the signal required for human B cells to produce cytokine-producing cells [87]. Two subsets of cytokine secreting B cells can be identified, regulatory B cells and effector B cells. Regulatory B cells produce mainly IL-10 and TGFβ, and effector B cells produce cytokines such as IL-2, IL-4, IL-6, IL-12, IFNγ, and TNFα [86]. IL-10 producing regulatory B cells is thought to play an important role in dampening immune responses. Furuzawa-Carballeda et al. [88] showed that patients with pSS have an increased frequency of IL-10 producing circulating regulatory B cells, defined as $CD19^{+}CD38^{hi}CD24^{hi}IL\text{-}10^{+}$ cells, compared to controls. Importantly, the proportion of regulatory B cells was higher in clinically inactive pSS patients, compared to clinically active pSS patients suggesting that these cells may downregulate autoimmune inflammation to induce homeostasis.

10.8 Epilogue

The understanding of the pathogenetic mechanisms of pSS in general, and the role of B cells and plasma cells, in particular, is rapidly expanding. As discussed, -omic approaches will be another asset to elucidate further the complexity of the pathogenesis of pSS and to establish known and novel biomarkers for early diagnosis, measurement of disease activity, and definition of subgroups of pSS patients that might be susceptible to a particular treatment.

Many biological DMARDs are currently available and even more are in development to target various molecules involved in the cascade of hyperactive B cells and plasma cells including biologicals that can interfere with a large number of relevant cytokines and chemokines. In addition, non-biological drugs that inhibit B-cell receptor signaling molecules and cytokine receptors have become available. Because B-cell receptor signaling plays an important role in the autoimmune process, targeting important molecules of this pathway, such as Syk and Btk [89], is presumed to be a promising new approach for treatment of pSS too. A major potential disadvantage of all these therapies is that not only harmful autoimmune responses are affected but also beneficial humoral responses.

Besides a better understanding of the pathogenetic process and the availability of biological and synthetic DMARDs, assessment of disease activity in pSS is an essential step to rate efficacy of the treatment. With the development and validation of ESSDAI and ESSPRI, important tools have become available for rating the disease activity and patients' complaints in pSS. Both indices are complementary and should be used together in addition to objective measurements of dryness and biological markers of disease activity [90]. A change of ESSDAI of at least three

points or a change of ESSPRI with at least one point or 15% seems reasonable to show a clinically relevant effect (Seror et al. [91]). The ClinESSDAI, a modification of ESSDAI to score disease activity independent of B-cell biomarkers, should be used (1) in biological/clinical studies to avoid data collinearity, (2) in clinical trials, as secondary endpoint, to detect change independent of biological effect of the drug, and (3) in clinical practice to assess disease activity for visits where immunological tests have not been done [92]. In a real-life cohort, it was shown that ClinESSDAI is indeed a valid tool to assess clinical disease activity in pSS and may be a useful secondary endpoint in clinical trials [93].

The increased knowledge on the way how to assess patients for a particular therapy, along with the emergence of new targeted therapies, will stimulate the conduction of trials and the development of effective treatment option in SS.

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11 The Secrets of Jönköping: Why Do Most 80-Year-Olds Have More Than 20 Remaining Teeth, and Why Are There Very Few Edentulous?

Ola Norderyd and Åsa Wahlin

Abstract

Repeated epidemiological studies have been performed in the city of Jönköping, Sweden, every 10 years since 1973. The studies were initiated in order to describe the changes in oral health in the population. Basic preventive dental care and supplementary programs were extensively performed in the population, especially among children and adolescents. In this population, the percentage of individuals with sound teeth (no caries or restorations) continuously increased each decade. The main finding regarding periodontitis is the significant increase in individuals having no or minimal periodontitis experience. In 2013, 20–60-year-olds had nearly complete dentitions (28 teeth), and the individuals in age groups 70 and 80 years had a mean number of teeth of 23 and 21, respectively. Edentulous individuals having complete dentures in the age groups 40–70 years decreased from approximately every sixth individual in 1973 to none in 2013. The continuous improvement in oral health and the reduced need for restorative treatment will have an impact on dental health-care and dental delivery systems in the near future.

11.1 Introduction

Poor oral health has a major impact on public health and well-being worldwide. Oral diseases cause suffering and, in the worst cases, can be lethal. In addition, they are among the most prevalent chronic diseases [1]. Taken together this led to high costs for both the individual and society. In the early 1970s, the oral health situation in Sweden was poor with extensive caries, periodontitis, and tooth loss, and edentulousness was widespread [2]. Most available resources were used for restorative treatment, not for prevention of caries and periodontitis.

In 1974, a new dental act was introduced, which made Swedish counties responsible for providing full dental services free of charge for all children and adolescents up to the age of 20 years. In addition, the 1974 National Dental Health Insurance Act instituted an insurance system for the adult population. Swedish dental care

O. Norderyd (✉)
The Institute for Postgraduate Dental Education, Jönköping, Sweden

Faculty of Odontology, Malmö University, Malmö, Sweden

Department of Biomedicine and Natural Sciences, Centre for Oral Health, School of Health Sciences, Jönköping University, Jönköping, Sweden
e-mail: ola.norderyd@rjl.se

Å. Wahlin
The Institute for Postgraduate Dental Education, Jönköping, Sweden

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is provided by general dental practitioners, who are either employed by the public dental service or work in private practices. Publicly employed dentists treat children and adults, while most private practitioners mainly provide dental care for adults.

Jönköping is one of the ten largest cities in Sweden. The city is expanding and has increased from 110,000 inhabitants in 1973 to 136,000 inhabitants in 2017. Jönköping is situated in southern Sweden and characterized as the administrative center of the region as well as a center for industry, commerce, transportation, and education. The population is somewhat younger (mean age 40.3) than the Swedish population as a whole. About 13% of the inhabitants in Jönköping are born abroad, the majority in Iraq, Syria, in former Yugoslavia, and in other Nordic countries. Repeated epidemiological studies with a random design have been performed in Jönköping since 1973 [3]. The studies were initiated in order to describe the changes in oral health in the population. In addition to clinical and radiographic examinations, questionnaires on diets and dental care habits and attitudes have been used.

11.2 Oral Health Development

11.2.1 Caries and Restorations

A significant oral health improvement can be seen over the 40-year period, reflected in a decreasing number of carious lesions and restorations [4]. This dramatic reduction in caries may be exemplified by the number of decayed (sum of initial and manifest caries) and filled surfaces (DFS) in 15- and 20-year-olds, who in 1973 had 28 and 35 DFS and in 2013 had 3 and 6 DFS, respectively. This is a very clear improvement. The number of DFS decreased decade by decade in all age groups but for the 70- and 80-year-olds. The latter can be explained by the increasing number of existing teeth. The most obvious change was the decrease in number of filled surfaces (FS). Among 15-year-olds, 18 FS were registered in 1973 compared to 1 in 2013. In the 40-year-olds, the corresponding figures were 51 and 13, respectively. The percentage of individuals with sound teeth (no caries or restorations) also increased through the years and was 43% for the 15-year-olds in 2013 (Fig. 11.1). Regarding

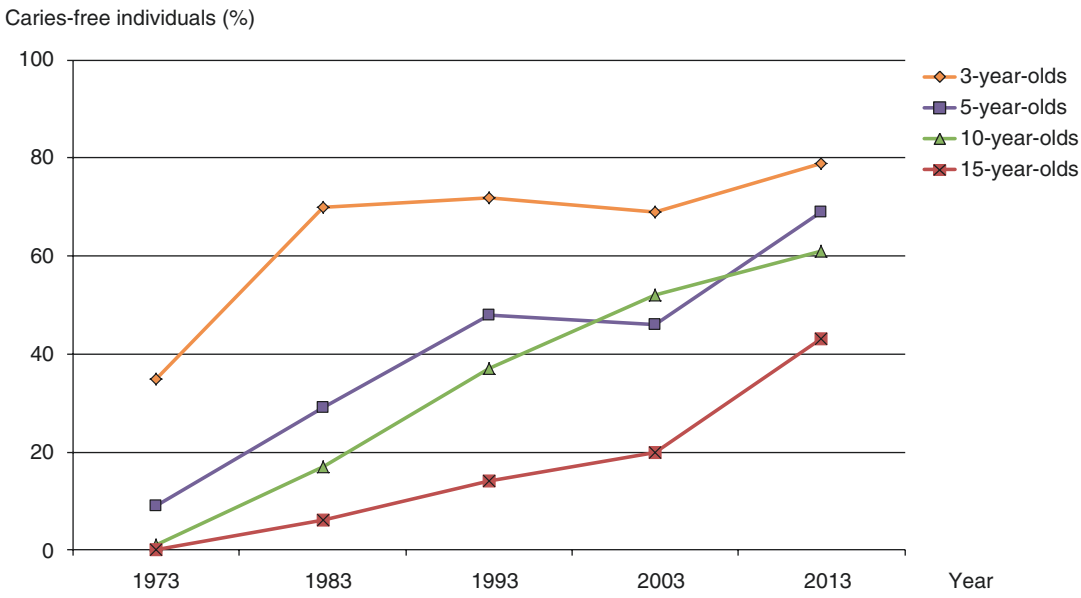

Fig. 11.1 Frequency (%) of children and adolescents without caries and restorations in 1973, 1983, 1993, 2003, and 2013

crowned teeth, the most significant change is the decrease in the number of crowned teeth in the age groups 40–60 years. The percentage of crowned teeth for the 50-year-olds was 25 in 1973 and 4 in 2013.

11.2.2 Periodontitis and Gingivitis

Between 1983 and 2013, there was a significant increase in the frequency of individuals 20–80-year-olds with no or minimal periodontitis experience—from 43% in 1983 to 60% in 2013 [5]. The number of individuals having pocket depth ≥6 mm also decreased in the total population between 1983 and 2013. There was a continuous decrease in the percentage of individuals classified with severe periodontitis from 16% to 11% between 1983 and 2013. In 1973, the prevalence of individuals in age groups 20–70 years with severe periodontitis experience was only 3%. This low number of individuals with severe periodontitis experience was due to a high number of edentulous subjects. In addition, 80-year-olds were not part of the study in 1973. An interesting finding was that among all periodontal disease groups examined, the severe group is the only group showing an increase in the number of teeth between 2003 and 2013. The frequency of tooth sites with gingivitis was generally lower in the last 20 years compared with the years 1973–1993 (Fig. 11.2). In the 50-year-olds, the percentage of tooth surfaces with gingivitis was 39% in 1973 and 15% in 2013.

11.2.3 Teeth and Edentulousness

During the 40-year period since the first Jönköping study, the mean number of teeth increased at each examination in the age groups 30–80 years (Fig. 11.3). In 2013, 20–60-year-olds had complete dentitions, and the individuals in age groups 70 and 80 years had a mean number of teeth of 23 and 21, respectively. The increase in mean number of teeth among the age groups 60–80 years was mainly explained by an increase in premolars and molars.

Oral health in the examined population has improved extensively by the decreased prevalence of edentulousness and increased number of existing teeth. Edentulous individuals with

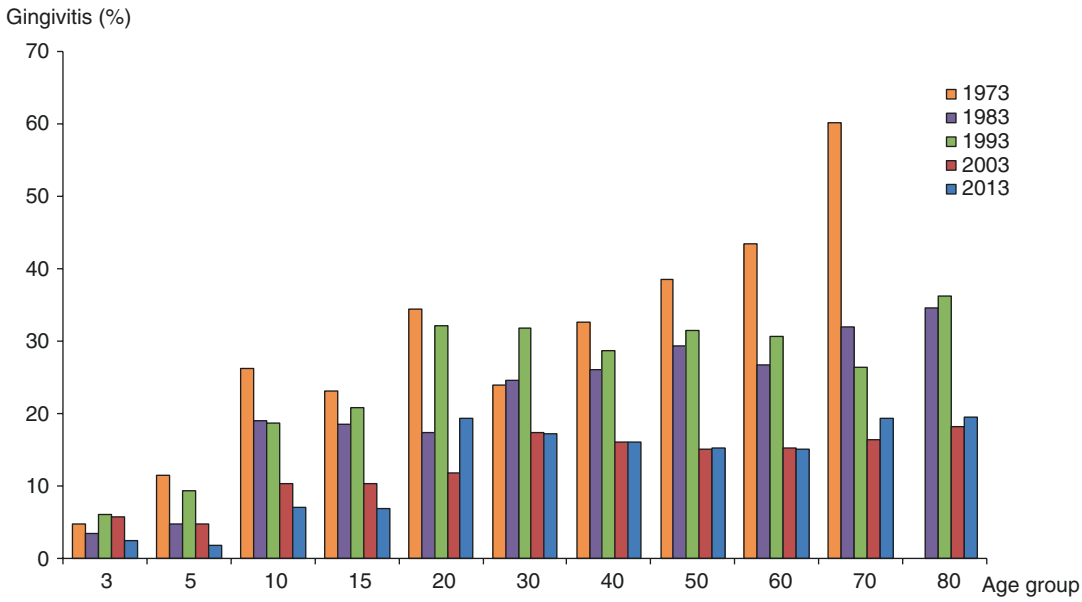

Fig. 11.2 Frequency (%) of total number of sites with gingivitis as a percentage of total number of existing sites. Means in the different age groups in 1973, 1983, 1993, 2003, and 2013

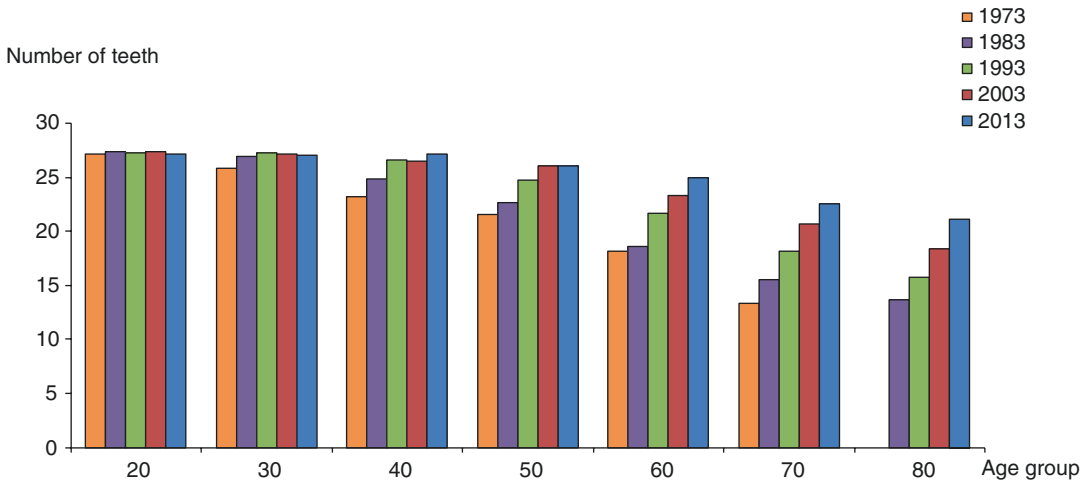

Fig. 11.3 Number of existing teeth (third molars and edentulous individuals excluded). Means in the different age groups in 1973, 1983, 1993, 2003, and 2013

complete dentures in the age groups 40–70 years decreased from approximately every sixth individual in 1973 to none in 2013. The few edentulous subjects among the age groups had been treated with implant-supported prostheses in one or both jaws. During the last decade, the number of existing teeth increased in the older age groups, 60–80 years, while only minor changes were seen among the younger age groups, where all individuals had almost complete dentitions. In the oldest age group, 80 years, the mean existing number of teeth reached one of the WHO goals, stipulating that more than half of all 80-year-olds should retain a minimum of 20 functional teeth [6].

11.3 Reasons for Improvement in Oral Health

From an analysis of the findings in the primary investigation, carried out in 1973, it was concluded that the efforts previously made had not managed to keep the two major oral diseases, caries, and periodontitis under control. It was decided that future dental health care should be based on a combination of cause-directed measures in connection with restorative treatment of high quality. To meet this aim, systematic continuous training of all dental care personnel in the county of Jönköping, within the public dental service as well as in private dental practices, was instituted in 1973 [7]. The current state of education and demand for continuous training was analyzed by means of questionnaires and interviews. From 1975 to 1979, a 5-year educational training program covering 1975–1979 was created. For dentists, three to seven courses and for dental assistants, five to seven courses were offered yearly.

From 1973 to 1979, integrated preventive dental care for children and adolescents in the county of Jönköping was developed. Aims, methods, target groups, organization, financing, and evolution of a preventive dental care organization gradually evolved for the age groups 0–16 years (4000 individuals in each age group). This was done through collaboration between dental health care and all primary schools. Basic preventive dental care programs and supplementary programs intended for individuals exhibiting a high prevalence of caries and gingivitis were presented. When implemented, these dental healthcare programs brought about a remarkable improvement in oral health among children and adolescents in the county. Over a 5-year period, the number of caries-free preschool children increased considerably and the number of caries

lesions decreased. Oral hygiene improved, and the occurrence of gingivitis decreased. It was concluded that the introduction of the continuous training programs and the dental health programs each contributed to the improvement in oral health in the population. It was also stated that the results were promising for the future, not only because of the demonstrated effects in children and adolescents but also because the adult population could benefit from these programs as well. These programs have been continuously modified over the years according to new knowledge and changes in oral health in the population. When analyzing changes in oral health since 1973, it has to be understood that nearly all of the individuals up to 60 years of age have been exposed to dental preventive programs during at least the first 20 years of their lives.

The key areas for the caries preventive programs have included fluoride (toothpaste, rinsing, and varnish), diet counselling, oral hygiene improvement, mechanical and chemotherapeutic plaque-reducing methods for risk groups, and fissure sealants for molars. For example, in the age group of 20-year-olds, the mean number of DF occlusal surfaces was reduced from 12 to 2 between 1973 and 2013. This remarkable reduction in caries and restorations in a caries-prone tooth surface is most likely a result of the general fissure-sealing programs of all permanent molars in close relation to eruption [8]. Regarding diet, the frequency of snacking between meals among children and adolescents was lower in 2013 compared to 2003. The number of 3–20-year-old individuals regularly consuming soft drinks was also reduced in 2013 compared to 2003 [3]. This could be a reflection of the multidisciplinary work within dental and general health care concerning healthy dietary habits. It is a positive change in health-related behavior, since a high soft drink consumption pattern is regarded as an additional risk for the development of both dental caries and dental erosions. It is reasonable to believe that a constant decrease in caries lesions in the long run should have an effect on the number of crowned teeth and endodontically treated teeth. In the present study, the percentage of crowned teeth was reduced from 25% in 1973 to 4% in 2013 in the 50-year-old group. The corresponding figure for endodontically treated teeth in the same age group was 17% and 3%, respectively. The reduced caries prevalence clearly indicates that the need for advanced dental procedures will decrease in the future.

Over the period of 40 years since the first Jönköping study, an increase in the number of individuals with no marginal bone loss and a decrease in the number of individuals with moderate alveolar bone loss can be seen [5]. The frequency of sites with gingivitis in all age groups was also generally lower in the last 20 years compared to 1973–1993. This is an interesting finding since more teeth are remaining even in the older age groups. Better periodontal health in subjects in Jönköping in the last 40 years can be explained by three main phenomena: increased dental awareness in the population, increased number of dental hygienists performing professional periodontal treatment, and decreased number of smokers as well as number of smoked cigarettes. The enhanced dental awareness in the Jönköping population is expressed in more regular self-performed oral hygiene of better quality (Fig. 11.4). It should be noted that visible plaque was recorded without using a disclosing agent or a periodontal probe which may underestimate the real plaque score. However, even taking a possible underestimation of the mean plaque values into account, the important finding is the positive trend of better oral hygiene over the 40-year period. The majority of the population in Jönköping attends dental health care regularly, and there are an increasing number of dental hygienists performing professional periodontal treatment. According to the statistics from the Swedish National Board of Health and Welfare, there was a threefold increase of dental hygienists in the county of Jönköping from 52 in 1995 to 168 in 2014. Those numbers cover all dental hygienists within the public dental service as well as those in private dental practices. In Jönköping and in Sweden as a whole, a decrease in the number of smokers and number of smoked cigarettes can be seen. Smoking is a well-known risk factor for periodontitis as shown in

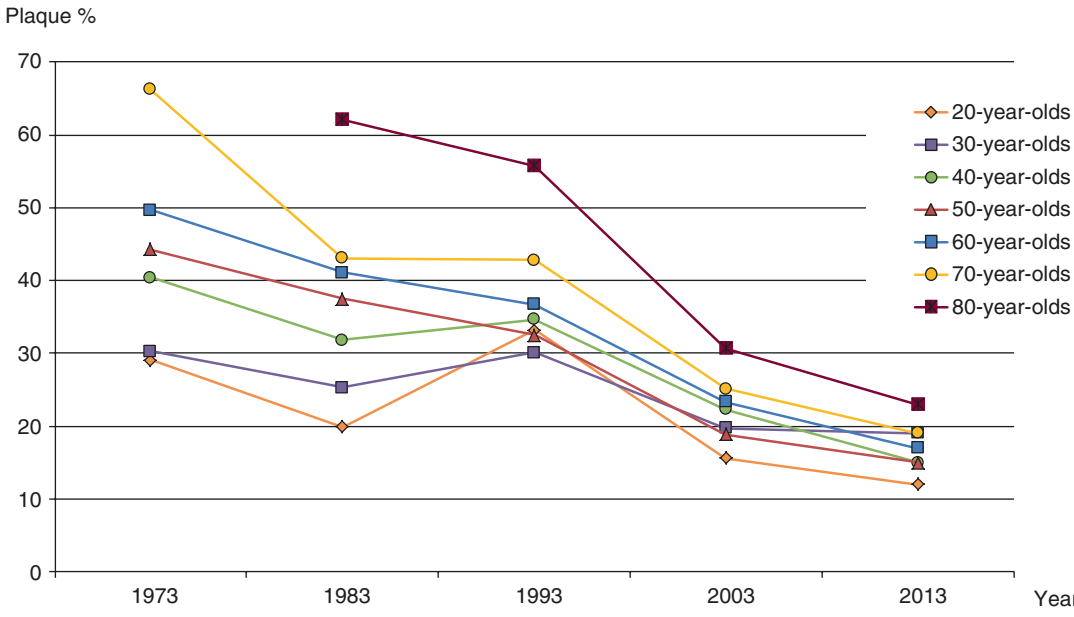

Fig. 11.4 Total number of tooth surfaces with plaque (%). Means in the different age groups in 1973, 1983, 1993, 2003, and 2013

numerous publications [9]. The Jönköping studies show that there was a reduction in smoking individuals from 16% to 8% over the last 10 years, 2003–2013. In individuals classified as having severe periodontitis experience, the number of smoking individuals was 36% and 20% in 2003 and 2013, respectively.

11.4 Conclusions and Future Challenges

The last 40 years have presented a great and continuous improvement of oral health and a decrease of dental treatment need in individuals in the city of Jönköping, Sweden. This has had an impact on most parts of the dental delivery system, such as the number of dental personnel, the dental insurance system, undergraduate and postgraduate dental education, and further research activities. It will be difficult for new dentists to get enough experience of advanced dental care in great enough volume. It is a demanding and serious challenge for the dental profession. Over the next decades, the increasing amount of treatment needed in the age groups 70–80 years and older due to more remaining teeth and the larger number of restorations may constitute a problem. However, in all the younger age groups, a decreasing need for restorative treatment in the coming years can be predicted. This is already reflected now in a dramatic decrease in the number of crowned and endodontically treated teeth. This means that although we still have a number of patients with extensive treatment needs, resources will gradually be released. Another challenge is early detection and treatment of the remaining part of the population with severe periodontitis and multiple risk factors, i.e., genetics, smoking, general health, and psychosocial circumstances.

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Developing Remedies for Oral Mucosal Diseases

12

W. Peter Holbrook, Venu G. Patlolla, and Thórdís Kristmundsdóttir

Abstract

Collaboration for several years between the faculties of pharmaceutical sciences and odontology at the University of Iceland has led to the development of several drug formulations that have been tested in clinical trials for treating diseases in the oral cavity that are relatively common. While numerous worthwhile treatment advances have been made, there is still a need for commercial collaboration to bring several successful drug developments onto the market, so that patients may gain access to these new products.

12.1 Introduction

Changes in appearance and perceived sensitivity may occur in the oral mucosa. These could be local problems or manifestations of systemic disorders. Observing, and also diagnosing, these conditions is frequently a task for the dentist who usually has considerably more experience of the oral cavity than other healthcare professionals. While local treatment of such conditions may fit in well with the knowledge and experience of dentists, there is a rather unmet need for numerous topical treatments. Consequently, there is a perceived need for the pharmaceutical industry to develop preparations suitable for localized treatment of oral mucosal conditions, preferably rather focused treatments that could be used by various healthcare personnel while reducing the risk of inappropriate treatments or side effects. This prompted a collaboration between the schools of pharmacy and dentistry in the University of Iceland to develop products that could help treat a number of common oral problems. Several therapeutic preparations have been developed and tested clinically, but currently, none of these preparations is commercially available.

12.2 Background to Local Drug Delivery

Drugs may be delivered locally to the oral mucosa in order to treat oral conditions, but there has also been an increasing awareness of the value of delivering drugs for systemic treatments through the oral mucosa in order to treat systemic disorders [1]. This takes advantage of the permeability of the oral mucosa, and it enables a bypass of hepatic metabolism and avoids gastrointestinal degradation of the drug [1]. Systemic side effects

W. P. Holbrook (✉)
Faculty of Odontology, University of Iceland, Reykjavík, Iceland
e-mail: phol@hi.is

V. G. Patlolla · T. Kristmundsdóttir
Faculty of Pharmaceutical Sciences, University of Iceland, Reykjavík, Iceland

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of drugs administered through the oral mucosa are also reduced compared to other routes of administration. However, the limited amount of mucosal surface area in the oral cavity may be too little for enabling systemic delivery of some therapeutic products. The moist environment usually found in the mouth often helps solubilization and absorption of drugs, and mucosal glycoproteins can enhance drug adherence to the oral mucosa. Self-administration of drugs by the patient is usually possible and is particularly helpful when treating oral conditions. The main disadvantages of drug delivery to the oral mucosa are that saliva can wash away the locally applied drug, and chewing, drinking and even talking can interfere with drug delivery. As the oral mucosa is a sensitive tissue of the body, it is necessary for drug delivery formulations or devices to be acceptable to the patient. The environment is highly enzymatic, and this could damage some preparations that are intended for local delivery.

Drugs are delivered to, and through, the oral mucosa in several different formulations. Mouthwashes are aqueous formulations that are easy for patients to use and are usually without side effects. Lozenges are dissolved in the mouth, and this can increase effectively the time of local delivery and local treatment. Transmucosal delivery can sometimes be made using tablets that are dissolved in the mouth and give an effective systemic delivery. Mucoadhesive patches are being developed further by Patlolla et al. (unpublished data) as they can provide efficient and constant delivery to the oral mucosa. Different types of buccal films with varying residence times and drug release time have been developed including immediate release films, sustained release films and non-dissolving films. The main aim was to optimize the drug release from the films and mucoadhesion capacity and, most importantly, to stabilize doxycycline in the buccal films at room temperature. Doxycycline was complexed in a solid state with suitable excipients by utilizing a freeze-drying technique. The complexed doxycycline was then suspended in polymeric blends, and microparticles were developed. Microparticles were then loaded onto the films. The addition of microparticles to buccal films affected mucoadhesion capacity, in vitro drug release and tensile strengths and improved the stability of doxycycline. Almost 75–80% of the drug was protected by the end of 7.5 months at 25 °C, whereas in films containing only doxycycline or non-complexed doxycycline polymeric microparticles, there were no traces of doxycycline or its degradation compounds. This stability was achieved in chitosan-based films, which is extremely unfavourable due to its high acidity. A suitable technique to stabilize doxycycline was invented, and many types of buccal films were developed. By incorporating the above microparticles in films other than chitosan, the stability might even be greatly improved. The developed drug complex and microparticles were further studied for complexation efficiency between drug-stabilizing excipients-polymers using techniques such as differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR). The DSC is a thermal analyser which measures temperature and heat flow relative to the material transitions as a function of time and temperature of broad spectrum of materials ranging from −150 °C to 600 °C. It can also measure the quantitative and qualitative data on endothermic and exothermic heat flows of materials due to physical transitions in materials brought about by phase changes, melting, oxidation, glass transition, crystallization or other heat-related changes. The data obtained from DSC can be further supported by FTIR. FTIR is a preferred method of infrared spectroscopy (IR). In IR the sample is exposed to infrared radiation, and the sample absorbs some of these wavelengths, and the rest is transmitted. By analysing the resulting spectra of molecular absorption and transmission, a molecular fingerprint of the sample can be generated. The size of the microparticles was assessed by microscopy, and the presence of intact microparticles in the buccal films was studied by scanning electron microscopy. The developed films were further optimized based on the disease type, location and pathology [2, 3]. Several drugs are delivered to the oral mucosa in gel formulations that adhere to the oral mucosa and can thus increase the delivery time. Such applications are also easy for the patient to carry out and can be helpful in treating lesions in the oral mucosa. Chewing gum is a relatively effective stimulant

of salivary flow and can increase the time of drug delivery.

The aim of the collaborative research between the faculties of pharmaceutical sciences and odontology in the University of Iceland has been to develop pharmaceutical preparations that could be delivered to the oral mucosa for treatments of inflammatory and ulcerative oral conditions. Two main pharmaceutical products have been developed in this research and have been tested in several clinical trials. Initially, however, two other products were developed by this research group and were placed on the local market in Iceland. The first product was a chlorhexidine mouthrinse that had a lower concentration of chlorhexidine than similar preparations on the market in Iceland at that time. This was intended for use by children who were known to have high levels of dental caries. The aim was to reduce the high counts of *Streptococcus mutans* and lactobacilli frequently found in oral samples taken from these children [4]. The mouthwash had a non-sucrose sweet taste and was a popular and effective mouthrinse. Another mouthwash was developed to treat aphthous ulceration and was registered and marketed in Iceland. This was an aqueous hydrocortisone mouthwash that used cyclodextrin to dissolve the steroid. It proved to be an effective treatment of recurrent aphthous ulcers [5], and side effects of this treatment were very uncommon.

12.3 New Developments of Treatments for Disorders of the Oral Mucosa

12.3.1 Topical Treatment with Doxycycline

Numerous disorders that appear in the oral mucosa are related to inflammation and ulceration, often linked to infection and sometimes also linked to the activity of the local immune system. Tetracycline mouthrinses have been recognized as helping with the treatment of aphthous ulceration for many years, although the mechanism of the drug action was not clear. While the aetiology of oral ulceration and inflammatory conditions in the oral mucosa may be varied, there is a clear link to the increased activity of matrix metalloproteinases (MMPs) [6]. Such increased activity of MMPs has been found in periodontitis and aphthous ulceration. Reduction of this enzyme activity was found to occur with treatments containing tetracyclines (Golub et al. [7]) at concentration levels often below the required to have significant antimicrobial activity. As a result of these findings, the research team in the University of Iceland developed a bioadhesive gel, suitable for topical applications, that contained low concentrations of doxycycline. This preparation was shown to inhibit matrix metalloproteinase activity in vitro and was then used in a double-blind placebo-controlled clinical trial for treating aphthous ulceration (Skulason et al. [8]). In the initial clinical trial, the gel was applied four times per day to the ulcer for 2–3 days, and the presence of ulcer and the degree of discomfort and altered sensation in the mucosa were recorded. The results clearly showed good healing of the oral mucosa and a significantly shorter ulceration time for patients using the active topical drug preparation containing doxycycline (Figs. 12.1 and 12.2) [8]. The stability of doxycycline in the trial preparations was not sufficient, and this led to further research resulting in considerably improved stability of this clinically helpful preparation. Doxycycline degrades due to multiple factors: oxidation, epimerization, photodegradation and body or even room temperatures as well as being mixed in aqueous formulations. Continuing research is under way in order to stabilize doxycycline in hydrogels and to provide more constant drug release to the oral mucosa, for example, from buccal films. A mouthrinse incorporating minocycline has been found to be effective for treating aphthous ulceration in a clinical trial conducted by Yarom et al. [9]. Finally, after many attempts, the team succeeded in developing an in situ forming and temperature-sensitive mucoadhesive hydrogel containing stabilized doxycycline hyclate in sub-antimicrobial concentrations just enough to inhibit the MMPs. The doxycycline was found to be stable beyond 4 years in some of the developed hydrogels, which is the highest stability (Fig. 12.3) achieved for a doxycycline aqueous formulation to date whereas the stability

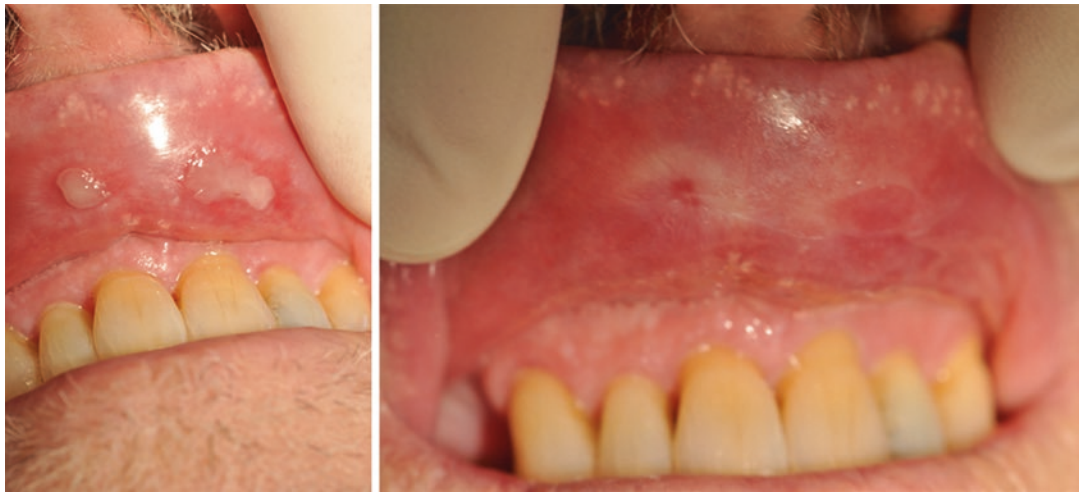

Fig. 12.1 Healing of aphthous ulcers on the upper labial mucosa following 2 days of treatment with locally applied doxycycline gel (see Ref. 8). The gel was applied to the ulcer four times per day for 2–3 days. Patients recorded the presence of ulcers, taste, discomfort and altered sensation daily in a diary

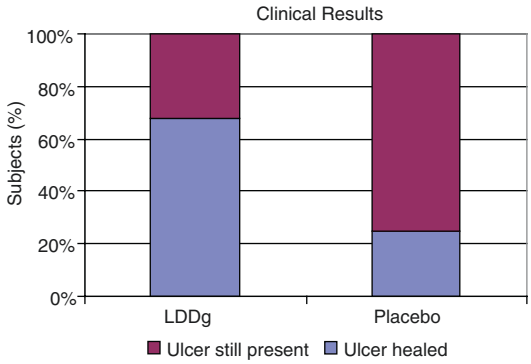

Fig. 12.2 Results of the clinical trial for the doxycycline gel treatment of aphthous ulcers (see Ref. 8). 70% of ulcers had healed by the third treatment day in the test group but only in 25% of subjects receiving the placebo ($p < 0.005$). The hydrogel was well accepted and reduced discomfort, even in the placebo group

of doxycycline when added to water is only for a few days. As the degradation of doxycycline is by multiple pathways, it was very difficult to stabilize in aqueous formulation, for example, it is sensitive to higher temperatures, but storing in refrigerator did not help in preventing the degradation. It was sensitive to exposure to light, but storing in amber-coloured bottles or covering the containers with aluminium foil did not prevent the degradation and, most importantly, adding antioxidant did not halt the oxidation as not all antioxidants were beneficial [10]. All the above-mentioned factors could be solved, but prevention or halting the epimerization was difficult as no known excipients could halt epimerization. In tetracyclines epimerization reaction was known to be reversible [11]. The chemical conversion of one epimer to another (its chiral counterpart) is called epimerization. In stereochemistry an epimer is one pair stereoisomers. Isomers are different compounds but have the same molecular formula. A particular type of isomers that differs from one another only because of the special orientation of atoms is called stereoisomers. The pharmacological activity of epimers differs. After considering many important contributions from previous authors, like incorporation of *non-ionic* surfactant [12], co-solvent [13], antioxidants [10, 14], chelating agents [10], complexing agents [15], cyclodextrins [15–17], dehydrating agents [18], etc., and also from our team's intuitive experience with the doxycycline aqueous stability, a stable formulation at 4 °C with long-term shelf life was developed [19]. The developed hydrogel was optimized for mucoadhesion capacity, which can effectively improve the residence time of formulation. The drug release time and mechanism of drug release from the polymer matrices were studied, as this helps in the optimization of drug release at the site of

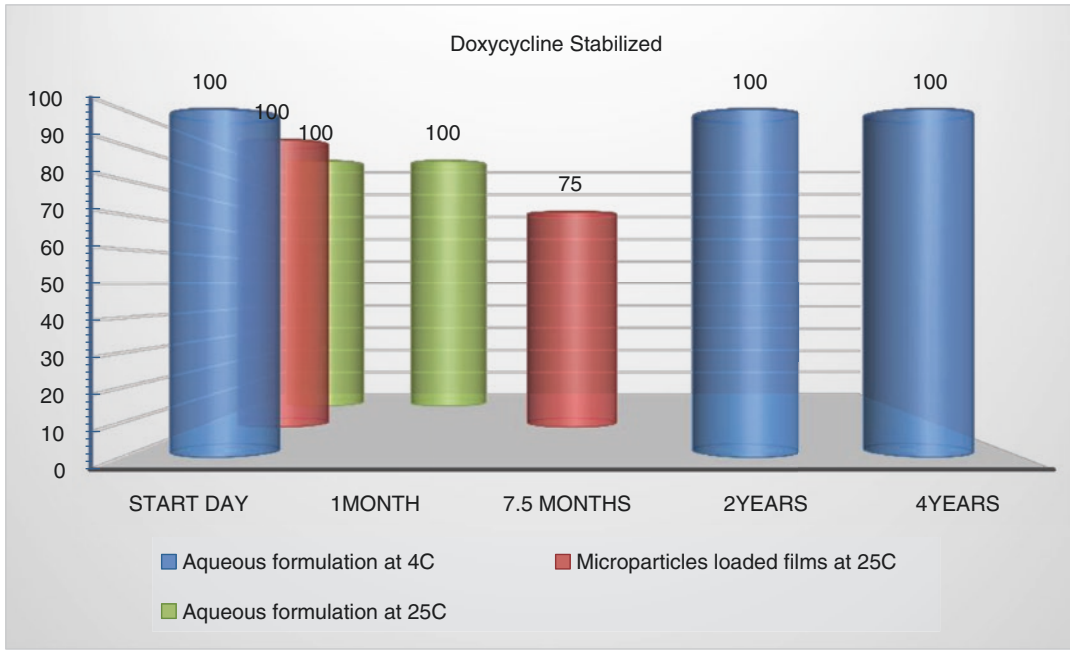

Fig. 12.3 The stabilities achieved in developed doxycycline formulations (Patlolla et al. unpublished data). The highest stability is achieved in an aqueous based formulation at 4 °C that was 100% stable even after >4 years, whereas the stability of doxycycline is only for few days when in contact with water molecules. At 25 °C the aqueous formulation was 100% stable for 1 month, whereas stability is only for a few days when doxycycline solution is left at room temperature. Even at 25 °C the precomplexed doxycycline containing microparticle-loaded buccal films was found to be 75–80% stable after 7.5 months whereas no trace of doxycycline was seen in only doxycycline containing films. The formation of degradation product 4-epidoxycycline was completely prevented in precomplexed microencapsulated doxycycline microparticles at 4, 24 ad 40 °C after 7.5 months

application. Texture profile analysis of the hydrogels was also studied, and the developed hydrogels were optimized for in vitro performance, ease of applicability, its ability to withstand deformative forces within the oral cavity and retention time at application site. Furthermore, the team is working to develop doxycycline formulations that are stable even at room temperature (25 °C) for ease of storage and commercial transport which does not require refrigeration. Thus studying the solid state stability of doxycycline has been further carried out (Patlolla et al. unpublished data).

12.3.2 Topical Preparations Containing Monocaprin

Another area of interest to this research collaboration was the development of topical preparations of monocaprin, an antimicrobial monoglyceride that was known to be effective against enveloped viruses, particularly herpes simplex virus, and also to hinder growth of *Candida albicans* (Thorgeirsdóttir et al. [20–24]). Monocaprin (Fig. 12.4) is a natural breakdown product of triglycerides that are commonly found in plant oils, particularly coconut oil. It is classified as "Generally Regarded As Safe (GRAS)" by the US Food and Drug Administration. This product is quite different from commonly used antimicrobial compounds and, therefore, was unlikely to promote drug resistance to such microorganisms if it became widely used for treating clinical conditions. The activity of monocaprin against herpes simplex virus was tested in a topical preparation that also included doxycycline. Consequently, the research team were combining the antiviral activity together with the inhibition of matrix metalloproteinase by doxycycline in a

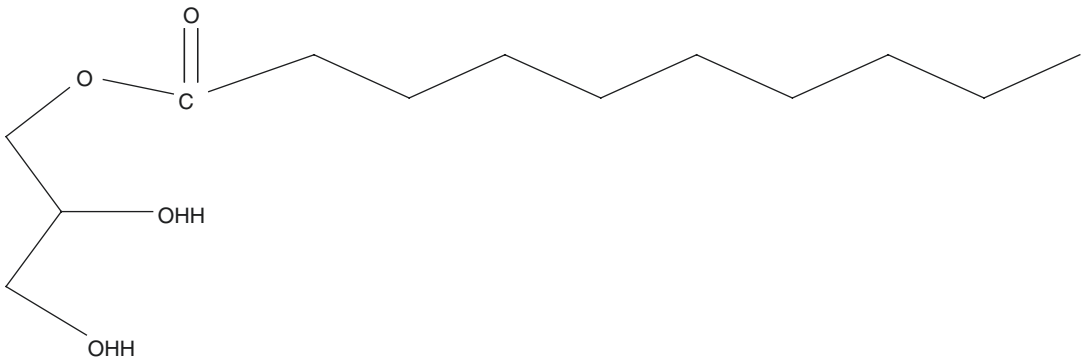

Fig. 12.4 Monocaprin: a monoglyceride that has been found to kill enveloped viruses and various bacteria, both Gram-negative and Gram-positive, and the yeast *Candida* spp. (see Refs. 20, 23)

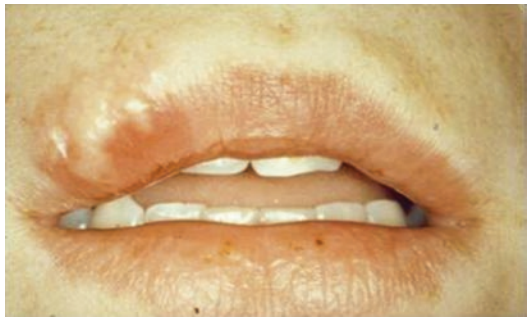

Fig. 12.5 Clinical appearance of a cold sore ("herpes labialis"). Treatment with topical application of a gel containing monocaprin and doxycycline shortened the time to complete the healing of cold sores both at the prodromal stage of the cold sore or after vesicles have first appeared. The treatment time was reduced by 2 days for both stages of the cold sore ($p < 0.05$) (see Ref. 25)

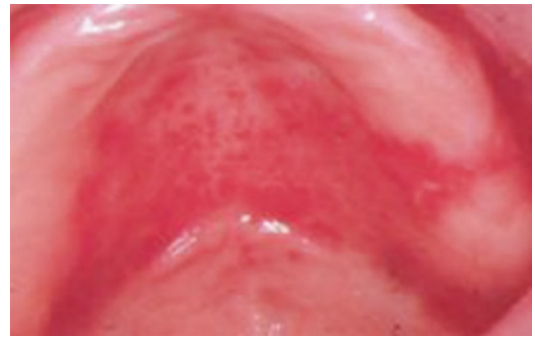

Fig. 12.6 *Candida*-associated stomatitis in an elderly denture-wearing patient where regular topical antifungal therapy is often not very effective (see Ref. 22)

treatment for cold sores (secondary herpes infections; Fig. 12.5). The activity of these two pharmaceutical compounds was to eliminate the virus and to reduce inflammation so that the lesions would heal more quickly. A placebo-controlled clinical trial of these compounds showed the combined topical drug therapy to be significantly more efficient at reducing the time of clinical signs of aphthous ulcers and pain from the lesions (Skulason et al. [25]).

Further studies of antimicrobial activity of monocaprin were aimed at evaluating its anti-candidal effect. While oral infections with *Candida albicans* can have numerous underlying causes, it is most commonly seen as an infection of the oral mucosa associated with patients wearing dentures (Figs. 12.6 and 12.7). This group of patients is increasingly elderly and do not have good denture hygiene. There may also be associated conditions and therapeutic products in these patients that promote denture stomatitis. As denture stomatitis has a considerable effect on the quality of life of this patient group, the research team thought that developing anti-candidal monocaprin products could be potentially highly beneficial. Consequently, monocaprin was incorporated into a denture adhesive material and also into a hydrogel, and these compounds were tested in clinical trials to measure the reduction of the counts of yeasts from standardized samples, the reduction of mucosal inflammation and the patients' opinions on the treatment outcomes [26–28]. The clinical trial was carried out in a residence for geriatric patients. Initial sampling from the fitting surfaces

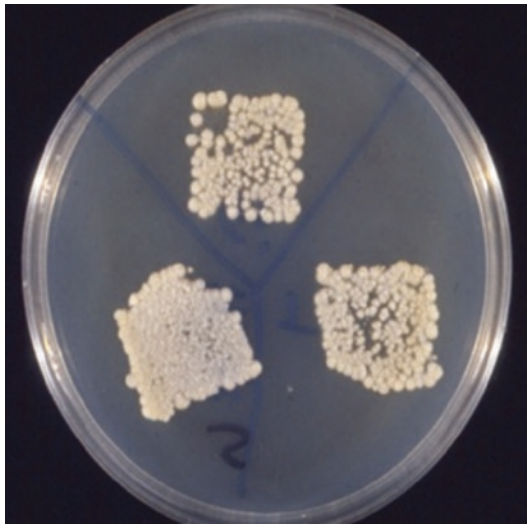

Fig. 12.7 *Candida* growth from samples collected with sterile sponges from the fitting surface of the denture, the lingual and palatal mucosa from a patient about to participate in a clinical trial of monocaprin (see Ref. 22)

of dentures, palatal and lingual mucosa, was carried out using sterile sponges that were moistened in sterile water and placed on the sampling surface and then transferred to Sabouraud agar plates for the culture of *Candida*. The study showed that 60% of subjects had dense growth of *Candida (> 50 cfu/cm*^{2}) from the denture and palatal samples. A double-blind placebo-controlled trial of a denture adhesive and hydrogel containing 3% monocaprin was then carried out among these patients over a 4-week period. Patients receiving the adhesive or gel that contained monocaprin had a rapid decline in the counts of *Candida* to <10 cfu/cm^2. No problematic side effects were noted by patients, but an improved function of their dentures was reported by several patients that received the monocaprin-containing products [26, 28]. This study, and an earlier study by this group evaluating the anti-candidal activity of monocaprin, found the beneficial effect of reducing counts of *Candida* to be short-lived, and the yeast counts began to rise again only a few days after the clinical trial had stopped. This reflects the environment under dentures, particularly in elderly patients, that will encourage the growth of *Candida*. Consequently a product containing monocaprin needs to be developed that is suitable for long-term use on the fitting surface of dentures especially for patients for whom denture cleaning is a problem.

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Ethics in Biomedical Research

13

Tuula Wahlman-Calderara and Jukka H. Meurman

Abstract

Scientific research on human subjects or animals calls for meticulous ethical scrutinizing and approval of the study plan before any such research can be commenced. Modern techniques such as genetic biomanipulation have brought unforeseen ethical problems calling for special attention. In this chapter we briefly discuss the philosophical and historical basis of ethics with focus on medical research ethics. The process of ethical approval is also being described.

13.1 Introduction

Since the traumatic aftermath of World War II, ethical issues have become an essential part of any research project whether clinical study on patients or good research practice in animal or laboratory studies. In this chapter we briefly review research ethical principles in general and, more specifically, regarding oral health-related research.

T. Wahlman-Calderara · J. H. Meurman (✉)
Department of Oral and Maxillofacial Diseases, Helsinki University Hospital, Helsinki, Finland

13.2 Ethics Is a Field of Philosophy

The word "ethics" has several meanings in English. In philosophy, it refers to philosophical ethics or moral philosophy, but in the ordinary language it merely can refer to common human ability to ponder ethical problems. Further, the word "ethics" may describe idiosyncratic principles or habits of a particular person. We first discuss the philosophical meaning of ethics and how the important values and principles influence the decision-making and actions in research.

Ethics as a branch of philosophy is interested in what is morally right or not. Typical questions are the following: "What is the best way for people to live?" and "What actions are right or wrong in particular situations or circumstances?" Ethics thus covers different ways of understanding and examining the moral life. In *metaethics* moral philosophers study abstract issues like the theoretical meaning and reference of moral propositions by asking how should truth values of moral propositions be determined, if ever it is possible to determine them at all. Other philosophers are more interested in the practical means of determining a moral course of action concentrating on the rightness and wrongness of actions. In this *normative ethics*, there are many traditions, for example, virtue ethics, deontology and

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consequentialism of which utilitarianism is a well-known example. *Applied ethics*, also called practical ethics, is the main concern in biomedical research. It aims to apply normative ethical theories to specific cases and situations of human life by telling what is right and what is wrong. Bioethics and professional ethics or moralities such as medical ethics are examples of applied ethics.

Ethics is pluralistic and what is ethical may differ in different societies. Philosophers may also give different answers to the same question. Even the concepts may be defined in divergent ways. Dissimilar and opposing views arise in philosophy which, in turn, lead to progress and improvement of ethical thinking. However, there is sufficient consensus about fundamental research ethics [1].

13.3 Ethics and Morality

The word "ethics" is often used interchangeably with "morality" [2], but in philosophy the meaning of the two words is not the same. Ethics is often defined as philosophical study of morality, and moral philosophy and ethics can indeed be used as synonyms. Ethics nevertheless has more to do with philosophical theories and can be defined as a matter of knowing, while morality is more connected with human character and acts as, for example, morally good and honest behaviour. Some philosophers argue that it would be possible to apply some kind of common morality to all persons in all places [3]. Moral philosophers debate whether or not there exists some or any kind of common morality, but this debate is not relevant in the context of research ethics. However, it is of utmost importance that every member of a research group, whether in laboratory, animal care unit or hospital, is familiar with research ethics. Since close collaboration between clinicians and researchers is an essential part of translational research, it is necessary to comprehend and share basic ethical principles.

13.4 Research Ethics

Research ethics is regulated in a different way than medical ethics [3]. Scientific research has for a long time been heavily regulated, while medical practice still is much less regulated. The physician's focus is on the best interests of the patient by relying on proven beneficial treatment choices with acceptable risk. Research on the other hand is hypothesis driven by investigating treatments and questioning diagnoses, which need to be confirmed. Principles like benevolence, beneficence and non-maleficence guide the physician's actions. Empathy should always be there in professional–patient relationship. For the researcher, however, the patient may primarily be research subject where the benefit cannot be guaranteed.

In all medical research, however, it is absolutely necessary to respect the basic ethical principles and protect the rights of the subjects. Furthermore, in order not to lose credibility, it is highly important to keep the confidence of the patients, research subjects and society. Understanding and following the codes and regulations of research ethics, and local legislation, also guarantee high quality of research in all areas.

13.5 From Hippocratic Oath to Bioethics

13.5.1 Hippocratic Tradition and Nuremberg Code

Throughout the history of mankind, medical practicing has been controlled. Both doctors themselves and sovereigns have regulated this action. The earliest written orders can be found from the Mesopotamian law code of Hammurabi, the king of Babylon. The code was written down in about 1750 BC, and it treats questions like the doctor's rights and duties and what kind of punishments should be prescribed for a doctor who due to negligence or unskillfulness caused death

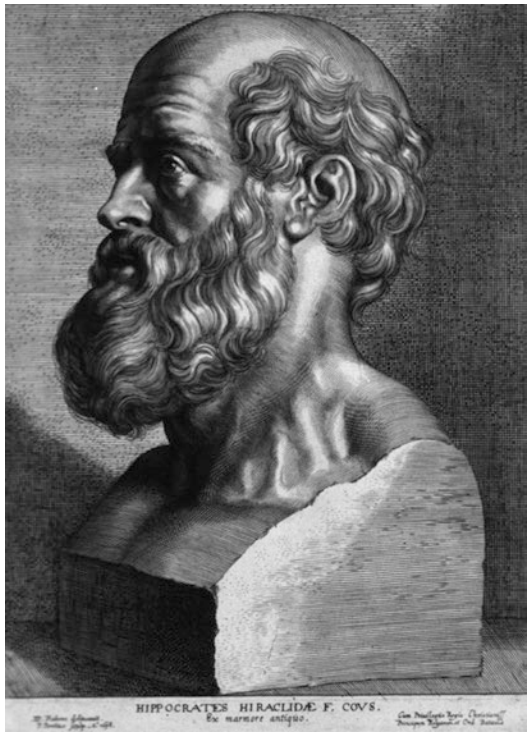

Fig. 13.1 Hippocrates 460–377 BC

of the patient or made other mistakes. Many religious scriptures also have regulations on practising medicine [4].

The Greek physician Hippocrates, who headed a medical school on the island of Cos around 500 BC, is regarded as the father of medicine and medical ethics (Fig. 13.1). The Hippocratic school mostly had writings about medicine, science and ethics [4]. It is thought that the concept of medicine as a profession comes from Hippocrates who emphasized that a physician must always place the interests of the patient above his own and that this promise must be made public: the *Hippocratic Oath* [5]. The Oath further demands collegiality, duty to render aid, refraining from causing harm or hurt and concealment. All these still are essential part of modern medical ethics. The Hippocratic Oath thus is the model of ethical codes in medicine, and it maintained its position until the middle of the twentieth century.

The need for something broader than the traditional Hippocratic medical ethics became apparent after World War II and the special tribunal at Nuremberg, Germany. Many Nazi physicians were tried and convicted because they had violated fundamental human rights in their research during the war. The trials began on December 9, 1946, and the verdict was delivered on August 20, 1947. During the trials ten important points in legitimate medical research, known as the *Nuremberg Code*, were compiled and were used as the basis of the judgement. The Nuremberg Code has been one of the fundamental documents of modern research ethics, and, for example, the request of voluntary informed consent is in the Code. Many are of the opinion that the Hippocratic tradition continues in the Nuremberg Code, which represents a new and expanded interpretation of the Hippocratic Oath endorsing the experimental approach to medicine but at the same time protecting the patient [6].

13.5.2 World Medical Association and the Declaration of Helsinki

In 1947, when the Nuremberg Code was set forth, the World Medical Association (WMA) was also established. A significant reason for establishing WMA was the need to ensure and reinforce the awareness of physicians of their ethical obligations after what had happened in Germany and some other countries during World War II. The WMA is "an international organization that seeks to represent all physicians, regardless of nationality or specialty". One of its roles is to establish general and globally applicable standards in medical ethics both for medical practice and research [5].

Soon after its establishment, the WMA had two important tasks: to define the duties of the physician pointing out also the humanitarian aspects of medicine and to specify the essential principles in medical research. The *Declaration*

of Geneva [7] and the *Declaration of Helsinki* [8] are the result of these attempts and are the most important declarations of WMA. Both have been revised several times already. In January 2005, WMA launched the *Medical Ethics Manual*, which is a comprehensive presentation of ethics and its role in medicine dealing also with medical research ethics (Table 13.1). In addition, many research institutes and organizations have formulated ethical guidelines. For oral health researchers, the International Association for Dental Research (IADR) Code of Ethics is essential [9]. However, the principal guidelines in different documents are mainly the same, and most of them refer directly to the Declaration of Helsinki as the basic code of internationally accepted guidelines in biomedical research.

Table 13.1 World Medical Association's declarations and codes of medical and research ethics

Declaration of Geneva
• The "Physicians' Oath"
• Adopted at the World Medical Association (WMA) 2nd General Assembly in 1948, revised latest in 2017
• The physician declares his dedication to the humanitarian goals of medicine
International Code Of Medical Ethics
• Adopted by the WMA 3rd General Assembly in 1949 and amended three times since then
• Describes the duties of physicians in general, to patients and to colleagues
Declaration of Helsinki
• A concise summary of research ethics as it is defined by the WMA in 1964
• Revised many times to meet the requests of the developments in research and in the technologies; latest revision in 2013
• Regarded as the most important document on research ethics
The WMA Medical Ethics Manual
• Launched in January 2005, 3rd edition in 2015
• A comprehensive presentation of ethics and its role in medicine and research
• Intended also for the use of medical schools throughout the world; translated in many languages

13.6 Biomedical Ethics

13.6.1 Bioethics

As noted before, bioethics is a field of applied ethics. It is interested in ethical questions arising from life sciences, i.e. medicine, biology (including genetics), biochemistry, biotechnology, ecology and many others. Bioethics is inter- and multidisciplinary, and bioethicists have different backgrounds, not only in life sciences but also in disciplines such as sociology, philosophy, theology, history and law. Bioethics began to develop during the second half of the twentieth century with historical background in medical ethics. Modern bioethics is commonly understood to consist of three main subdisciplines, namely, medical ethics, animal ethics and environmental ethics [1]. The need for bioethics arouse because traditional medical ethics was not able to respond to the new questions and challenges caused by technological progress. There were no appropriate tools to treat subjects and issues such as privacy and research involving human subjects in the larger perspective. Bioethics had first the human aspect in forefront. Animal ethics and environmental ethics have then become subdisciplines of bioethics, when the issues such as the responsibility for the nature and environment have emerged. Today, there are established centres dealing with issues of bioethics (Table 13.2).

The rational approach to the ethical issues is through different ethical theories and approaches. Most important in biomedical ethics including biomedical research are virtue ethics, deontology, consequentialism (especially utilitarianism) and principlism. However, none of these can fully answer even to the most usual ethical questions [5]. Hence, comprehensive ethical discussion should include the best features of each of these approaches, briefly described in the following.

Virtue ethics focuses in particular on the character of decision-makers: "What kind of person should I be?" Moral goodness of an action

depends both on the right action and on the right motive. Since antiquity, wisdom, courage, self-control and justice have been the central virtues, that is, types of moral excellence. Virtuous qualities of a physician, for example, are compassion, honesty, prudence and dedication.

Deontology or *duty theories* consider that the basis of morality lies on specific, foundational principles of obligation. Well-founded rules could then serve as the basis for moral decisions. One well-known foundational principle of duty is the "categorical imperative" of the German philosopher Immanuel Kant (1724–1804): "Treat people as an end, and never as a means to an end". People should always be treated with dignity. It is noteworthy that deontology is not interested in the consequences of an action but in motives and intentions. Therefore, an act is thought to be morally good, if the motive or intention, which leads to that act, is good.

Table 13.2 Examples of centres for bioethics

The Hastings Center (originally *The Institute of Society, Ethics and the Life Sciences*), New York
• Founded by Daniel Callahan and Willard Gaylin in 1969
• *The Hastings Center Report*—explores ethical, legal and social issues in medicine, healthcare, public health and the life sciences, both in print and online
• Visiting Scholar Program in collaboration with Yale University
The Kennedy Institute of Ethics (originally *The Joseph and Rose Kennedy Center for the Study of Human Reproduction and Bioethics*), Washington, DC
• Established at Georgetown University in 1971, backed by the Kennedy Foundation
• *The Kennedy Institute of Ethics Journal* is an interdisciplinary journal dedicated to philosophical bioethics, online
• Visiting Researchers Program
Eubios Ethics Institute
• Founded by Darryl Macer in 1990 in Christchurch, New Zealand and in Tsukuba Science City, Japan—Bangkok, Thailand added to the network in 2005
• Nonprofit group aiming to stimulate the international discussion of ethical issues and how to use technology in ways consistent with "good life" (eu-bios)
• Cooperation with many individuals and groups, including, e.g. UNESCO, UNU and Asian Bioethics Association
The European Association of Centres of Medical Ethics (EACME)
• Founded in 1985
• Network of academic and nonacademic centres
• Aim is to promote research, education and consultation in the field of biomedical ethics

Consequentialist theories are, according to definition, interested in the consequences or outcomes of the choices and actions. One of the best-known examples of this is *utilitarianism* where the good is measured by means of utility and may be defined as "the greatest good for the greatest number". The action which produces the best outcome is then the right one. Outcome measures obviously vary and, for example, in healthcare decision-making cost-effectiveness and quality of life are important. QALYs (quality-adjusted life-years) and DALYs (disability-adjusted life-years) are typical examples. From the physician's daily practice, an example could be health and well-being or lack of pain and suffering of a patient. It is, however, important to notice that although the principle "the end justifies the means" is a plausible deduction from utilitarianism, it can never be approved in research. Thus, for example, it is morally unacceptable to sacrifice individual human rights to attain a social goal.

Principlism is currently one important trend in approaches of bioethics. Its origin is connected with the Belmont Report (1978/1979), which, according to definition, is "a statement of basic ethical principles and guidelines that should assist in resolving the ethical problems that surround the conduct of research with human subjects" [10]. The report defines three basic ethical principles in biomedical and behavioural research, namely, respect for persons, beneficence and justice [11]. Beauchamp and Childress have developed principlism further in the context of medical practice by adding to the list of principles also *non-maleficence*

("not doing wrong"), which in traditional medical ethics is usually expressed as the maxim primum non nocere ("above all do no harm"). Although principles indisputably play a central role in rational decision-making in biomedicine, principlism has been criticized for emphasizing the respect for autonomy over other principles, which is thought to reflect the Western liberal culture and hence not to be necessarily universal [3].

13.6.2 Principles in Applying Biomedical Ethics

Respecting human rights is the cornerstone of ethical assessment. This was proclaimed by the United Nations' Universal Declaration of Human Rights already in December 1948 and has ever since played an important role both in medical practice and in biomedical research. In the following we briefly describe the basic principles that should be taken into account when pondering biomedical issues of ethics.

Respect for autonomy. The word *autonomy* derives originally from Greek words *autos* ("self") and *nomos* ("rule", "governance"). In ancient Greece, it was connected to the independent city-states but now refers to individuals who should be treated as autonomous agents capable of deliberating their personal goals and acting accordingly. In medical context, a person or patient must be able to make his/her own decisions. This is the fundamental principle in biomedical research ethics and also prerequisite for an informed consent. Hence, particular attention is needed in situations where the research subject's autonomy is diminished, and he/she is unable to give consent (for details, see later).

Non-maleficence, "do no harm", was emphasized already in the Hippocrates era. Researches thus have an obligation to avoid causing harm to patients and research subjects. Careful risk assessment is thus a prerequisite in research planning.

Beneficence. While non-maleficence is merely refraining from doing, the principle of beneficence demands more, literally "doing good". Although some authors define the good end in medicine almost exclusively as healing, beneficence is usually thought to have broader sense. It means acting to the best interest of the patient or research subject which may represent different things, for example, development of new treatments, better understanding human physiology, etc.

Justice. The concept of justice is explicated with the terms fairness, entitlement and "what is deserved". In healthcare, it means that individuals and groups are treated in a fair manner which, in turn, may lead to complex questions when, for example, individual and community interests are taken into account. The importance of social value as a criterion, when deciding if a research project should be approved or not, has become more significant. Consequently, it is thought that justice demands that the results of the research should benefit the population in which the research in question is being carried out. Hence it is not fair that research subjects undergo risks and feel discomfort in one place while the beneficiaries are patients elsewhere [5].

13.7 Practical Principles in Science and in Medical Research Ethics

Clinical research on human subjects is necessary for the progress of medicine. Furthermore, animal studies are often regarded as an essential part of research, especially in translational health research. Many different local, national and international codes, rules and policies on research ethics exist both for research on human subjects and for animal experimentation (Fig. 13.2).

13.7.1 Research Ethics Committees

The researcher has not been left alone with the challenging issues of ethics. The Declaration of Helsinki describes the role and the responsibilities of research ethics committees (Table 13.3).

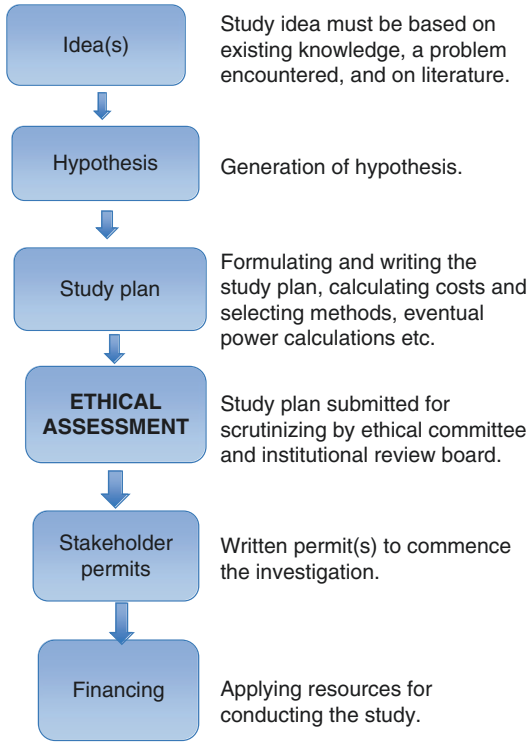

Fig. 13.2 Main steps for attaining permit(s) for scientific investigation. In life sciences approval by ethical committee has a central role

The committee's approval is necessary already before the study may begin, and its task continues in monitoring ongoing projects. The members of the committee should represent expert knowledge of different professions. The researchers have to demonstrate to the committee why that particular study is worthwhile and that they have enough competence to conduct it. Among other things, the committee reviews the research project's justifiability on scientific grounds and estimates the assessment of the risks and benefits made by the researcher. The researcher has to prove that the protection of potential research subjects is appropriately secured. Although the researcher may sometimes feel that the committee's only task is to delay the advances of science, the review and approval of independent research ethics committee actually guarantees the quality of research and is a necessity for the later publication process in any respectable forum.

Table 13.3 The role and responsibilities of an ethical committee

• The research protocol must be submitted for consideration, comment, guidance and approval to the concerned research ethics committee before the study begins
• The committee must be transparent in its functioning; must be independent of the researcher, the sponsor and any other undue influence; and must be duly qualified
• The committee must take into consideration the laws and regulations of the country or countries in which the research is to be performed as well as applicable international norms and standards, but these must not be allowed to reduce or eliminate any of the protections for research subjects set forth in the declaration
• The committee must have the right to monitor ongoing studies
• The researcher must provide monitoring information to the committee, especially information about any serious adverse events
• No amendment to the protocol may be made without consideration and approval by the committee
• After the end of the study, the researchers must submit a final report to the committee containing a summary of the study's findings and conclusions

13.7.2 The Concept of Informed Consent

According to the Nuremberg Code, "The voluntary consent of the human subject is absolutely essential". This indeed is the first requirement for research on human subjects. The person involved in a research as human subject must be capable of giving informed consent, and the participation in research needs be voluntary. The consent should be in written form and signed by the research subject. There are special guidelines for cases where subjects with diminished capability are not able to give consent, such as children, mentally handicapped or unconscious patients. There are three important elements in consent: information, comprehension and voluntariness. The research object has to be informed about the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail, post-study provi-

sions and any other relevant aspects of the study (see Declaration of Helsinki). It is the responsibility of the researcher to ensure that the subject has understood the information. Voluntariness means that the subject has the right to refuse to participate and also to withdraw consent at any moment. Withdrawal must not affect the treatment of the patient. The ethics committee reviews also the "consent form" to secure its legibility.

13.7.3 Physician's Role as a Researcher

One possible ethical problem, which needs attention and which the research group should bear in mind, is the potential dual role of a physician or dentist as a researcher. The role in the physician–patient relationship differs from the role as a researcher in the researcher–research subject relationship. While the researcher is primarily interested in generation of knowledge, the physician's responsibility is always the health and well-being of the patient. The WMA states clearly that if there is conflict between the two roles, "the physician role must take precedence over the researcher". Similarly, a conflict of interest is another potential ethical problem, which a physician–researcher might encounter. One example of this are the rewards offered for the participating physicians. They may be remarkable causing obvious conflicts of interest. Again, the WMA's viewpoint is clear: when the physician follows the basic rules of research ethics, there will be no inherent conflicts since "the ethical values of the physician – compassion, competence, autonomy – apply to the medical researcher as well" [5]. Table 13.4 summarizes the ethical principles for biomedical research on humans here discussed.

Table 13.4 Central ethical principles in research

Respect
• Human dignity, privacy and autonomy must be guaranteed
• Collegiality, teaching and mentoring students and other individuals associated with the research are important to keep in mind
• Discrimination on the basis of sex, race, ethnicity or other factors not related to scientific competence and integrity is forbidden
• International codes of ethics must be literally followed
• Relevant national laws and institutional and governmental policies need to be followed
Confidentiality
• Confidential communications, personnel records, trade or military secrets and patient records must be protected
Do not harm
• Protect research subjects; take special precautions with vulnerable populations
• Carefully avoid errors and negligence, and keep good records of research activities
• Researcher is responsible of proper respect and care for animals used in studies—avoid unnecessary or poorly designed animal experiments
• Maintain competence and expertise in profession—lifelong education and learning is necessary; be open to criticism and new ideas; openness in sharing data, results, ideas, tools and resources promotes the important aims in science
• Remember social responsibility; strive to contribute to social good and prevent or mitigate social harms through research; strive to distribute the benefits and burdens of research fairly
Do not steal
• Honour patents, copyrights and other forms of intellectual property
• Do not use unpublished data, methods or results without permission
• Give proper acknowledgement or credit for all contributions to research
• Never plagiarize
Do not lie
• Strive for honesty in all scientific communications from reporting data, results, methods and procedures; fabricating, falsifying or misrepresenting data is not permitted
• Do not deceive colleagues, research sponsors or the public—capability of being objective is an obvious requirement in science, but maintaining objectivity in the competitive world of science may from time to time imply careful deliberation

Modified from Shamoo and Resnik [12]

13.8 Ethical Issues with Animal Experiments

It was not before the nineteenth century that animal experimentation in research became common. For a long time, it was thought that animals were more or less like machines and did not feel pain, as presented by the French philosopher, mathematician and scientist René Descartes (1596–1650). Since his days, animal behaviour studies have shown that not only animal species feel pain but also that at least some vertebrate animals have abilities to intend, understand and communicate. During the late twentieth century, the animal rights movement emerged, and this together with the new scientific discoveries on animals has given to the public cause for concern about the treatment of animals in research [13, 14].

The debate on the moral status of animals and how to justify their use as research objects is an ongoing process. Nevertheless, it is still difficult to think about biomedical research without animal studies. Many efforts have been made to limit the use of animals and to minimize the harm caused for them. Several countries have national laboratory animal protection laws, and there seems to be a general agreement that the housing conditions and care of captive animals need to reach humane standards. Wherever possible, the degree of animal pain and suffering must be minimized. Furthermore, the laboratory personnel should be competent in recognizing and alleviating pain in animals. It is recommendable that persons other than the investigator concerned should review the proposed experiments. One example of this is the monitoring committees, which serve to ensure legal compliance of the project. Their approval is needed for the research, and they can eventually modify the project to improve the animals' welfare [13–15].

Principles of refinement, reduction and replacement, referred as *The Three Rs*, are of concern regarding animal experiments (Table 13.5). These principles were first suggested by Russel and Burch [16]. *The Three Rs Declaration of Bologna*, signed in the Third World Congress on Alternatives and Animal Use on the Life Sciences in 1999, strongly reaffirms "the vigorous promotion and application of the Three Rs" [13, 17]. The principles have thereafter been successfully applied in many countries resulting in decline in the numbers of animals used in scientific experiments. Nevertheless, in recent years this downward trend seems to have been ceased especially because the use of genetically modified animals has increased [18, 19].

Table 13.5 The three "Rs" that guide animal experiments

Refinement
• Refining the experimental procedures to lessen the degree of pain or distress
Reduction
• The numbers of the animals used should be reduced as low as possible (without compromising the reliability of the research)
Replacement
• Animal studies should be replaced with non-animal methods wherever possible

The *Basel Declaration* is another important attempt in promoting the well-being of animals used in experiments. The Basel Declaration from October 2011 was been adopted by the Basel Declaration Society founded by biomedical researchers from both industry and academia. The Society emphasizes the importance of the three Rs and the transparency when using animals in biomedical research. It aims at bringing the scientific community together to further advance the implementation of ethical principles and trust for animal experiments. The Society strives for establishing the position of the Basel Declaration as a leading document in animal experiments [20].

From an individual researcher's point of view, planning animal experiments calls for corresponding procedures, applications and approvals as with human subjects. Both international and local guidelines need to be known.

13.9 Ethical Challenges Now and in the Future

Advances in research in life sciences have brought unforeseen ethical questions earlier generations could not dream about (Table 13.6). Cloning and stem cell techniques combined with gene manipulation in general, such as the CRIPSR-Cas9 method, opens up totally new avenues of research. Manipulating human germ cells and constructing totally new organisms are examples where science will take us. Ethical

Table 13.6 Current and future potential areas where ethical issues may arise

• Biomanipulation
• Stem cells
• Germ cells
• Biobanks
• Children's rights
• Population studies
• Animal rights
• Data management
• Financing and sponsoring
• Local, national and world politics

debate is absolutely necessary for formulating guidelines and international declarations for research with these and, in the future, even more advanced techniques. It is the responsibility of the research community to be transparent and provide accurate and legible information to political decision-makers and other stakeholders. At the same time the public must have trust on researchers and this trust can only be lost once. We nevertheless believe that the ancient principles of human ethics provide the best guidance also for future ethical problems—whatever they might be—as they have principally guided mankind more than the past 2000 years.

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Conflicts of Interest

The authors declare no conflicts of interest.

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