Theodore Eliades
Editor

Research Methods in Orthodontics

A Guide to Understanding Orthodontic Research

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Foreword by T.M. Graber

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Foreword

Too often, the question is asked, "Are you a clinician or a researcher? Are you in the academic ivory tower or out in the everyday world, treating live patients?" This is an unfortunate "either-or" dichotomy that impugns the real status and integrity of our profession for the new millennium. For medicine, the hand and glove relationship of practice, individual research involvement and teaching is an essential fact—a "must for modern medicine." Most physicians are involved in both, at one time or another in their career. The public respects the essentiality of this intimate relationship. And so do the medical colleagues. Not so in dentistry. Why?

Too often in my career, I have heard the comment from fellow dentists, "If you can, you do! If you can't, you teach, or do research!" This attitude is still pervasive. There are probably a number of reasons for this unfortunate state of affairs. We in dentistry started out largely as a triple "R" challenge—Remove, Repair, Replace (the 3 Rs). Dental caries and periodontal problems, associated pain, and esthetic concerns provided the impetus for our existence as the medical profession had more general health challenges, involving life and death. Extracting or filling teeth, treating intractable periodontal problems was essentially a mechanical procedure. The importance of the oral cavity as a barometer for the rest of the body was not recognized. Other general health priorities with life and death implications were dominant considerations. Being largely a dental mechanic was less appealing to both the medical profession and the public.

Dentistry has, of course, progressed tremendously, and both the profession and public are now aware of the general health ramifications of oral disease. Yet, there persists the feeling among too many dentists, as well as patients, that dental service is still largely a mechanical service involving digital dexterity. Once mastered, there is relatively little need for an academic lifeline. We are not aware of the research continually being done, nor the proper modus operandi of research, judging the value of research being quoted in the literature. Orthodontics, with its degree of mechanical orientation and high level of recompense, does not inspire most clinicians to wade through professional journals replete with statistical tables, particularly when their training did not help them to understand the orthodontic literature. They do not have a current guide to orthodontic research.

The numbers of dentists returning to the ivory tower is distressingly small, whether it is for research, teaching, or continuing education. While recompense for private patient dental service is more than ample, this is surely not true in dental

schools and research careers. As medicine suffers more and more from restrictive regulations, from the malpractice suit attacks by vulturous hordes of trial lawyers and need for myriad medical protective stringencies, as doctors try to avoid the malpractice morass and multimillion dollar public lottery awards, with reduced medical expectations from a general lowered morale, with problems in hospitals, drug companies, and service organizations, dentists are more than happy not to face most of these problems in solo private practice.

Adding to the reluctance of dentists in private practice to teach or do research is the feeling of loss of independence when teaching, as well as the politics that exists in any institution. With continuing education now provided by dental societies and "online" via the Web, less and less dentists are opting for academic careers to update their knowledge and experience. We call this the "Google phenomenon," since we all can look up almost any question and get answers from our computer storehouses. The dental education situation is critical now. Research positions are underpaid and unfilled. Dental journals write about "The crisis in dental education" and justifiably so.

Fortunately, in America, we have more and more dental researchers and teachers from abroad filling the vacancies. I regard this as a highly positive development because we are truly one world of dentistry, and we learn from these colleagues from Greece, Italy, Germany, Scandinavia, China, Japan, South America, and Arabia, to mention a few. A survey of the faculty members at the University of Illinois shows almost a 50% non-American percentage. This also true of our advanced degree specialty programs. Superb, cutting edge research is now being done, much of it funded by NIH (National Institutes of Health), as well as private organizations. The language barrier is not prohibitive for teaching, doing research, or studying in America, as so many in dental research and education have English as a second language. Our outstanding computer and statistics training courses at the university have literally hundreds of students (and some faculty) from abroad. We all benefit. It is a win-win situation! It is increasingly important that we as dentists who are outside the ivory tower understand the *raison d'etre* and *modus operandi* of these research efforts and become able in assessing the value in clinical practice!

Yet, because of the lower academic salaries, American dentists are reluctant to give up their financial advantage and personal freedom, and they are missing something! The multifaceted research and advanced clinical opportunities advance dentistry beyond the "applied mechanical" aspects of private practice. With the new millennium, emphasis has been placed on evidence-based procedures in orthodontics and mechanics (appliances) became bio-mechanics. Biological possibilities and limitations became apparent and exciting as we extend the horizons of our service. Growth and development studies advance our treatment horizons well beyond the teeth themselves, involving the neuromusculature and growth guidance potential for abnormal jaw relationships and function.

At Illinois, fascinating research is being done by Thomas Diekwisch, Budi Kusnoto, Carla Evans, Guy Adami, and their faculty, staff, and students that will extend the treatment horizons of private practitioners. Those dentists doing this work at dental schools and research institutes are excited by their efforts, in contrast to the redundancy of repetitive mechanical procedures. No wonder so many dentists

in private practice retire early, bored by the day after day of performing mechanical tasks they know, but with little new challenge and excitement. Financial security is not everything. The academic environment can pay rich dividends in personal satisfaction and higher levels of patient service because of research in areas related to many aspects of clinical practice. As we understand the orthodontic literature more and can truly appreciate the work being done in the disciplines of materials science, craniofacial biology and the effectiveness of various treatment modalities (i.e., evidence-based orthodontics) not only will our patients benefit, but we will find clinical practice more challenging and exciting.

It was G.V. Black the eminent dental pioneer at Northwestern University at the turn of the last century who said, “The professional man (woman) cannot be other than a continuous student.” Hundreds of advances in *materia technica*, basic histological studies, long-term clinical techniques and appliances and their ultimate effectiveness, particularly in orthodontics, make us what we are today. Literally we are “applied biologists,” not only dental mechanics, and our patients get better service, far beyond the mechanical manipulations from our pioneers in the field. The public respects us more as true orofacial doctors, which augments our own enhancement of personal esteem as we truly earn the title of “doctor.”

My own personal experience of a lifetime of melding private practice, advanced study, research, teaching, writing, editing, lecturing, traveling, and being a continuous student is incredibly exciting. It is such a dream come true that I am afraid I might wake up to find it only a figment of the imagination! This team effort volume by Dr. Eliades reinvigorates me, and I am sure it will for my colleagues, also. This book fills a long-existing void in our beloved specialty.

T. M. Graber, DMD, MSD, Ph.D., M.D. (hc), Odont Dr (hc), DSc (hc), ScD (hc), FRCS, Dr Dent (hc)
Professor, University of Illinois at Chicago

T. M. Graber (*1917–2007*)

Dr. Tom M. Graber photographed on December 2005, during his visit to Thessaloniki, Greece, for the bestowment of the honorary Doctor of Dentistry degree by the Aristotle University of Thessaloniki (photo by T. Eliades)

Dr. Thomas M. Graber received the DMD degree from Washington University in 1940 and continued his postdoctoral training in Orthodontics and Anatomy at Northwestern University, where he received his MSD and PhD degrees and taught during the 1950s. In 1964, he founded the Kenilworth Dental Research Foundation. He was Professor and Chairman at the Orthodontic Department of the Pritzker School of Medicine, University of Chicago from 1969 to 1982 where he also held joint appointments in anatomy, anthropology, pediatrics, and orthopedics. During this time, he directed 18 master's theses and 4 doctorate dissertations. At that time, the University of Chicago was the first dental school to provide stipends to orthodontic residents, the funds coming primarily from the Kenilworth Research Foundation. Dr. Graber continued this tradition by fostering scholarships and endowed professorships at the University of Michigan. He held also the position of Clinical Professor of Orthodontics at the University of Illinois and was a Visiting Professor at universities around the world.

The list of honors and awards of T.M. Graber is unmatched for any professional in orthodontics: he received the American Association of Orthodontists' (AAO) Brophy Distinguished Service Award in 1970, American Board of Orthodontics' Albert H. Ketcham Memorial Award in 1975, and Jarabak Memorial International Teacher's and Researcher Award in 1994. He was named by the AAO to give the prestigious John Valentine Mershon Memorial Lecture in 1989 and Jacob A. Salzmann Lecture in 1988 and has given more lectures on the AAO annual programs than any other person in history.

In 1980, Dr Graber was appointed editor of the Reviews and Abstracts section of the *American Journal of Orthodontics*. In 1985, he took the reins as Editor-in-Chief of the same Journal and served in that capacity until 2000. Under his editorship, the journal was renamed *American Journal of Orthodontics and Dentofacial Orthopedics* to reflect the influence orthodontics might have on craniofacial growth.

Dr. Tom Graber and his associates provided orthodontic treatment to thousands of patients in a Chicago and North Suburban orthodontic practice that he started in 1946 and still bears the name Graber Orthodontics, through the practice of his son and granddaughter.

T.M. Graber's first article appeared in the *Washington University Dental Journal* in 1938 on tissue changes incident with orthodontic tooth movement, whereas his PhD thesis in 1950, the first to be awarded to a dentist in the state of Illinois, was

widely acclaimed. The result of his life-long dedication to research and writing amounts to over 20 textbooks, many of which were translated into multiple languages and served as standard texts in the field, 22 chapters in other textbooks, over 180 publications in journals, and 930 book and journal abstract reviews. Dr. Graber's lecture list includes cities of most countries on every continent, having taught more than 450 continuing education courses, aside from frequent lectures at conferences.

Dr. Graber was the first dentist to receive an honorary doctorate degree from the University of Michigan. He served on editorial boards of 15 journals, held honorary membership in 20 international orthodontic associations, and received citations and certificates from a long list of organizations, countries, and universities among which is the bestowing of 5 honorary doctorate degrees and the decoration of the Japanese Order of the Sacred Treasure.

Preface

Research in orthodontics is primarily structured around three axes corresponding to the following disciplines: materials science and mechanics of tissues and materials; craniofacial biology including biology of tooth movement, genetics, embryology, and anatomy; and clinical epidemiological research pertaining to evaluation of the effectiveness of various treatment modalities, with conventional cephalometrics and novel noninvasive imaging techniques. These areas can be divided in a number of subfields, which include the current trends in orthodontic research represented in the body of literature appearing in the relevant journals or textbooks.

In general, our understanding of the sequence of events associated with a variety of phenomena including craniofacial growth and adaptation, tooth movement, and materials performance in the intraoral environment has been substantially improved during the past two decades. This may be assigned to the advanced level of research contributed by collaborative efforts of scientists possessing diverse educational backgrounds, which range from materials science and engineering to molecular biology and anatomy/embryology. The results of the interactive efforts in the field have played a pivotal role in clarifying key issues in the relevant orthodontic literature, which had long been considered matters of dispute.

A study analyzing 3,000 articles published in the three major orthodontic journals over two 5-year intervals suggested that research papers as well as author affiliations from non-orthodontic sources were encountered more frequently in the second more recent interval. This trend implies that more research and more complicated techniques appear in the orthodontic periodicals.[1] The increased number of author affiliations observed to occur over time may be related to cross-appointed faculty who may serve in associated disciplines (sciences, engineering). The latter is indicative of an increased collaboration among orthodontic departments or between orthodontics and other sciences in response to the demands of the current research environment, which necessitates the interaction of different disciplines to effectively respond to research challenges.

On the other hand, incorporating basic science and engineering research techniques in orthodontic papers has complicated the process of keeping abreast of developments and following the literature because of the lack of familiarity of

[1] Kanavakis et al (2006) Orthodontic journals with impact factors in perspective: trends in the types of articles and authorship characteristics. Am J Orthod Dentofacial Orthop 130:516–522.

clinicians with the principles of techniques and associated background in basic and engineering sciences. Thus, there has been a need to introduce a source, which will guide the reader through the fundamental principles, applications, and limitations of the most commonly methods reported in orthodontic research. This necessity was long ago recognized in the broader biomedical literature, and similar books on research guides to molecular medicine (*Springer Verlag*) and molecular biology (*Humana Press*) have been published.

The main scope of this book is to comprehensively review the methods reported in the orthodontic literature including materials, clinical, and biological research protocols. The text emphasizes the role of appropriate experimental instrumental analysis in facilitating scientifically coherent evidence.

In summary, this book:

(a) Introduces the practicing professional to aspects of instrumental analysis and basic science research methods applied to orthodontic topics
(b) Reviews fundamental research methods utilized in the analysis of issues of orthodontic interest along with the limitations and misconceptions associated with their use
(c) Aids in formulating a background which facilitates an informed opinion on literature readings
(d) Analyzes the principles and mechanisms underlying the application of techniques employed in biological, materials science, and clinical research issues
(e) Establishes a reference guide providing information on preparation, selection of testing procedure, and appropriate treatment of biological or material samples utilized in orthodontic research

Throughout the lengthy course of the development of orthodontics as an applied scientific discipline, there are many individuals who have contributed to the departure of this specialty from the empirical stage of adopting anecdotal evidence as a substantiation of treatment protocols. The work published by these eminent figures resulted in a major shift of the interest in academic orthodontics from plain observation to establishing a research hypothesis, and integrating scientific approaches. To honor these pioneers, this book is dedicated to a person who, along with other prominent scholars, laid the foundation of this transformation. T.M. Graber achieved exceptional accomplishments, which reflect upon the recognition of orthodontics as a field. These extend from the bestowment of honorary degrees, fellowships, and awards from countries of almost all continents to publishing series of renowned textbooks, fostering research scholarships, and endowing chair professorships. The editor, who was fortunate to get acquainted with his brilliant journal editorship and visionary academic and research service, returns only a fragment of the debt he owes him for the collaboration he has enjoyed through the years, by acknowledging his legendary path in the field.

William A. Brantley and Christoph Bourauel from Ohio State and Bonn Universities, respectively, should be acknowledged for discussions and contribution to the fruition of this project.

Theodore Eliades

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Part I

Orthodontic Materials

1 An Overview of Research Methods on Orthodontic Alloys and Ceramics

Spiros Zinelis and William A. Brantley

1.1 Introduction

Four clinically popular archwire alloys (stainless steel, cobalt-chromium-nickel, beta-titanium, and nickel-titanium) are currently used with brackets for tooth movement, and metallic brackets are manufactured from stainless steel and titanium [1]. Single-crystal (monocrystalline) or polycrystalline aluminum oxide (alumina) brackets are often used in place of metallic brackets because of much better appearance match to tooth structure, and there has also been interest in zirconium oxide (zirconia) for brackets [1].

For successful clinical performance, it is essential that these orthodontic alloys and ceramics have the appropriate properties to withstand clinical manipulation and the oral environment. Consequently, the necessary in vivo physical and mechanical properties must be defined, and these materials must be characterized appropriately for assurance that their properties are suitable for clinical conditions.

The first portion of this chapter will summarize some important current research areas for orthodontic materials: mechanical testing of the wire alloys and ceramic brackets, evaluation of the corrosion resistance of the wire alloys, use of x-ray diffraction to determine the structure of the wire alloys, and thermal analysis techniques to determine the structure of nickel-titanium archwires. Although not discussed in this chapter for the sake of brevity, thermal analysis can also be profitably employed to characterize the elastomeric impression materials and polyurethane modules used in orthodontics [1].

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The second portion of this chapter will describe the preparation of orthodontic materials for microscopic examination and the important examination techniques. The materials are typically resin-embedded, then ground and polished, and sometimes chemically etched. The surfaces of these prepared materials, or other orthodontic materials in their bulk appliance form, are generally studied with the scanning electron microscope (SEM), which has largely replaced the optical microscope because of its vastly greater depth of focus and much wider range of magnifications available. Elemental compositions of orthodontic materials can be conveniently determined by energy-dispersive spectrometric analysis (EDS) with the SEM or by wavelength-dispersive spectrometric analysis with the electron microprobe. Recently, the technique of microtomography, which uses x-rays to create cross sections of objects at the microscopic level, has been employed to gain new insight into dental materials.

1.2 Mechanical Testing of Orthodontic Wires and Ceramic Brackets

The mechanical properties of orthodontic wires are best determined using the tension test and an electronically controlled universal mechanical testing machine with a straight archwire segment. An extensometer attached to the wire is employed to determine the change in a central gauge length (such as 1 in. or 10 mm), and the force developed within the wire during the tensile loading is sensed by a load cell. It is highly important that the load cell and extensometer be calibrated to obtain accurate results. Special wire grips must be employed, along with some strategy (such as surrounding the test specimen with metallographic abrasive paper) to prevent wire slippage within the grips. While not of clinical relevance, the wire segment can be loaded to failure, so that the ductility (percentage elongation) can be measured. Special breakaway extensometers are available for this purpose. Otherwise the extensometer should be removed from the test specimen after evident permanent deformation has occurred and the percentage elongation determined by fitting together the two halves of the fractured specimen.

Figure 1.1 shows tensile stress–strain curves for stainless steel, cobalt-chromium-nickel, Nitinol™ (3 M/Unitek), and Japanese NiTi [2] orthodontic wires, subsequently marketed as Sentinol™ or Sentalloy™ (Dentsply GAC International). The much lower elastic modulus of the nickel-titanium alloys is evident, along with the difference between elastic ranges of the nonsuperelastic Nitinol™ and superelastic Japanese NiTi. The latter has nonlinear elastic behavior, since stress and strain are not always linearly proportional below the elastic limit (end of upper superelastic plateau below point b) [2–4].

Determination of elastic modulus (E) and yield strength (YS) of a linearly elastic alloy from the tensile stress–strain plot is described in textbooks [3, 4], and reports of tensile properties of orthodontic wires are available [5, 6]. An important clinical property of an orthodontic wire is *springback*, which is defined as (YS/E) [7]. This relationship for springback is used to indicate the practical tensile elastic strain recovered

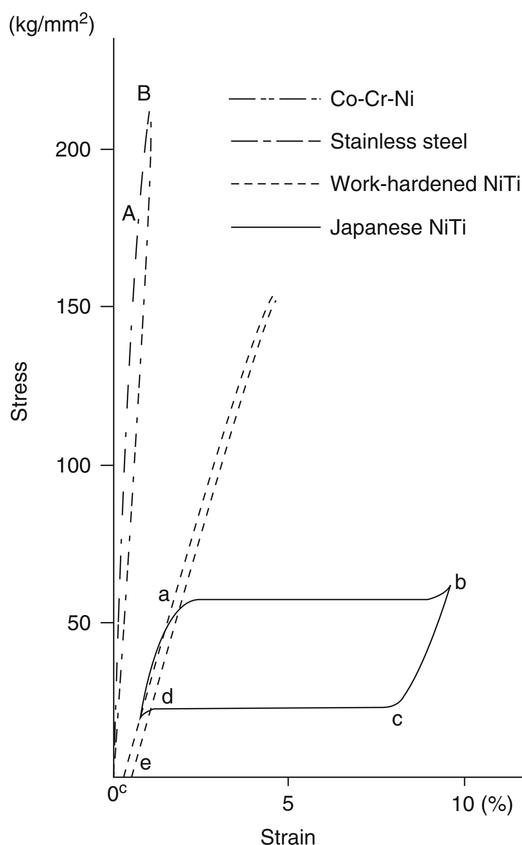

Fig. 1.1 Comparison of tensile stress–strain curves for 0.016 in. diameter stainless steel, Co-Cr-Ni, nonsuperelastic Nitinol™, and superelastic Japanese NiTi wires (From Ref. [2] and reproduced with permission from American Association of Orthodontists)

when the wire is unloaded, rather than (PL/E), where PL is the proportional limit, since orthodontists may activate wires into the permanent deformation range.

Mechanical properties of orthodontic wires are generally measured in the clinically relevant mode of bending, which is much more convenient to perform than the tension test. Figure 1.2 shows cantilever bending plots for stainless steel, Nitinol™, and Chinese NiTi (marketed as Ni-Ti™ by Sybron/Ormco) orthodontic wires, using 5 mm test spans appropriate for interbracket distances [8]. The effect of test span length on springback is pronounced, when comparing the original cantilever bending plots for Nitinol™ with 0.5 in. spans [9]. The Ni-Ti™ wire also exhibits superelastic behavior (and nonlinear elasticity), but the upper and lower superelastic plateaus evident with the tension test are not as sharply defined with the cantilever bending test [10], because of the variation in stress and strain over the wire cross section [1]. Springback in bending is much greater for superelastic NiTi wires than nonsuperelastic NiTi wires [10].

While the original version of American Dental Association Specification No. 32 had a cantilever bending test to evaluate mechanical properties of orthodontic wires [11], the current version [12] has three-point bending and tension tests. The specification

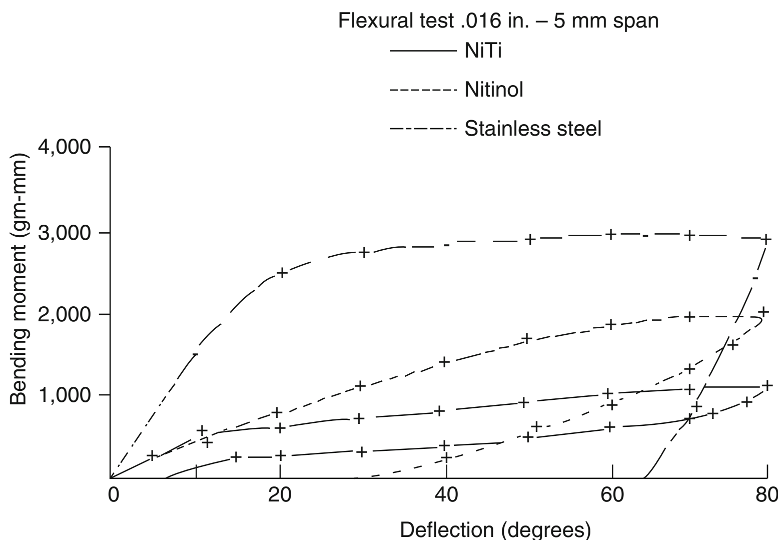

Fig. 1.2 Cantilever bending plots for 0.016 in. diameter stainless steel, Nitinol™, and Chinese NiTi orthodontic wires. For 5 mm test spans and bending deflection of 80°, springback is approximately 75° for Chinese NiTi and 50° for Nitinol™ (From Ref. [8] and reproduced with permission from American Association of Orthodontists)

testing mode depends on the manner in which the mechanical properties are given by the manufacturer. The wires are classified as type I or II, depending upon whether they exhibit linear or nonlinear elastic behavior, respectively, during unloading from temperatures up to 50°C. The bending test may be performed at room temperature (23 ± 2)°C for type I wires but must be performed at (36 ± 1)°C for type II wires. The test span between knife-edge supports is 10 mm, and the midspan deflection rate is 7.5 ± 2.5 mm/min. Type I wire specimens are bent to a permanent deflection of 0.1 mm, and the bending stiffness (units of N/mm) from the slope of the linear force–deflection plot and the bending force (N) for 0.1 mm permanent offset are determined. If the tension test is performed on a type I wire, the elastic modulus (GPa), 0.2 % offset yield strength (MPa), and percentage elongation are determined for a 20 ± 0.2 mm gauge length, using a crosshead speed between 0.5 and 2.0 mm/min. Type II wire specimens are tested only in three-point bending and are deflected to 3.1 mm. Forces during unloading are measured at deflections of 3.0, 2.0, 1.0, and 0.5 mm, along with the permanent deflection after unloading.

For ceramic brackets, it is essential to have sufficient strength to resist forces imposed during initial manipulation and bonding and the subsequent debonding. Such strength requires suitable resistance to fracture, since the ceramics used for brackets are brittle materials that fail by crack propagation, and ceramic brackets have historically shown a tendency for tie-wing fracture during debonding. The critical fundamental mechanical property is fracture toughness [13, 14], which is related to the force or energy for crack propagation. Fracture occurs with greater facility for single-crystal alumina brackets than polycrystalline alumina brackets where the irregular path of crack propagation is along grain boundaries [15].

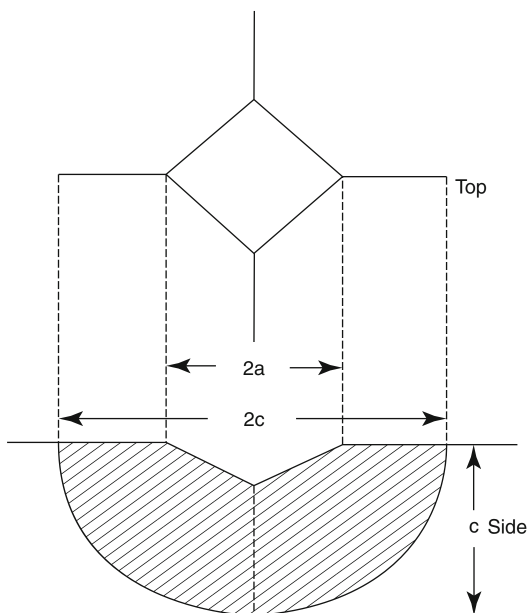

Fig. 1.3 Schematic illustration of Vickers hardness indentation and desired fracture pattern in ceramic material for determination of fracture toughness (From Ref. [21] and reproduced with permission from Academy of Dental Materials)

For convenience, the fracture toughness of dental ceramics is generally measured under plane-strain conditions as K_{Ic}, in which the tensile stress is applied perpendicular to the crack face in a crack-opening mode [4]. Several approaches have been used to measure the fracture toughness of bulk dental ceramics, including fractographic analysis, indentation methods, and bending tests with notched beams [16–21]. Figure 1.3 shows the idealized fracture pattern for the Vickers indentation technique, which is the only method that would permit measurement of fracture toughness for the very small ceramic brackets.

The Vickers indentation technique was used to obtain fracture toughness values of 3.60 and 5.22 MPa $m^{½}$ for two brands of polycrystalline alumina brackets [22]. However, these results were apparently obtained with radial cracks much shorter than the lengths of the diagonals for the indentations and thus may not be correct. Pham and colleagues [23, 24] found that the Vickers indentation technique did not yield appropriate long straight radial cracks emanating from the corners of Vickers indentations for five brands of polycrystalline alumina brackets, and the radial cracks followed the alumina grain boundaries in each ceramic. In contrast, Tilson and colleagues [25, 26] observed straight radial cracks in zirconia brackets (Hi-Brace™, Toray Ceram) with the Vickers indentation technique and obtained 3.92 ± 0.35 MPa $m^{½}$ for fracture toughness. However, SEM observations at high magnification revealed that the apparent straight radial cracks were the result of the very fine zirconia grain size, with crack propagation apparently still occurring along grain boundaries.

From these experimental results, it is recommended that future fracture toughness studies should focus on the bulk ceramic used to fabricate the brackets and that manufacturing efforts should be directed toward producing brackets of the highest

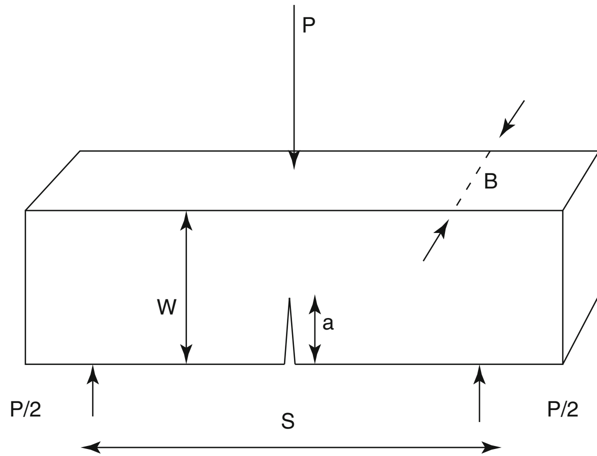

Fig. 1.4 Rectangular specimen geometry for single edge-notch technique used to measure fracture toughness. Appropriate specimen thickness or breadth (*B*) is necessary to have plane-strain conditions (From Ref. [28] and reproduced with permission from Quintessence Publishing Company)

purity ceramic with minimal surface cracks and porosity. Another approach for comparison of different ceramic brackets is to evaluate the force required to fracture the tie wings, which requires the use of a very small, well-controlled loading member [27]. A convenient technique for measuring the fracture toughness of bulk ceramic materials is the single edge-notch technique, schematically shown in Fig. 1.4 [28].

1.3 Corrosion Studies of Orthodontic Wire Alloys

Corrosion of orthodontic wires has become an active area of research, because of concern about biocompatibility. The major focus has been on nickel-titanium wires, which have potential to release nickel ions in vivo. A classic study, using potentiodynamic polarization [29], found that only the nickel-titanium alloy (Nitinol™) exhibited breakdown of passivity. X-ray analyses with the SEM suggested that loss of nickel could have occurred at the pitted regions. These results were consistent with clinical observations of stainless steel and Nitinol™ wires [30]. Long-term immersion of Nitinol™ was observed to have no effect on flexural properties and that occasional fracture was due to surface defects from manufacturing and not corrosion [31].

Figure 1.5 illustrates cyclic potentiodynamic polarization plots for three superelastic nickel-titanium wires, Nitinol SE™ (3 M/Unitek), Sentinol™, and Ni-Ti™ [1, 32, 33]. The directions of the forward and reverse scans are indicated by arrows. Similar results were found for nonsuperelastic Titanal™ (Lancer), Orthonol™ (RMO), and Nitinol™ wire alloys. The upper curve for each plot is the anodic region, and the lower curve is the cathodic region. These curves asymptotically approach each other at the zero-current potential [34]. The nickel-titanium wire alloys (and the other three orthodontic wire alloy types) have native surface oxide layers that provide corrosion resistance, and breakdown of the oxide layers (accom-

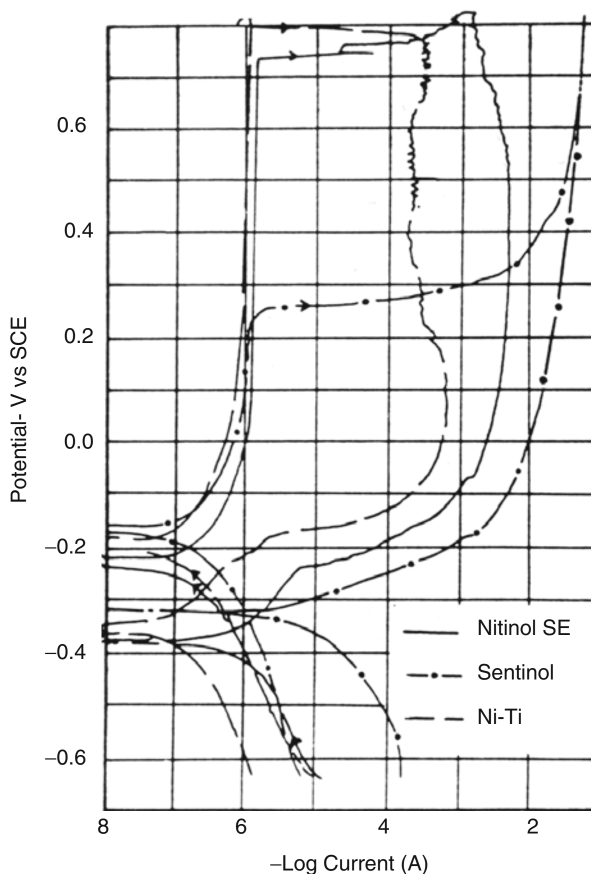

Fig. 1.5 Potentiodynamic polarization plots for three nickel-titanium orthodontic wire alloys [1, 32, 33] (From Ref. [1] and reproduced with permission from Georg Thieme Verlag (Stuttgart))

panied by pitting) can be seen in Fig. 1.5, where the current rapidly increases at sufficiently high anodic potentials. Because the wire surface has changed, the reverse scan does not follow the forward scan, and Fig. 1.5 shows that the zero-current potential has decreased for the reverse scan. The breakdown potential for the passive film depends on the surface roughness and oxide composition.

Corrosion studies have investigated breakdown potentials for oxide layers [35–37], ion release from wires [38–40], and galvanic coupling with brackets [41–44]. The in vitro corrosion of nickel-titanium wire products (including coated wires) has been compared [45–49] along with the potential of nickel-titanium wire for stress corrosion cracking [50]. An area of particularly active research interest has been effects of fluoride solutions (relevant to use of topical fluoride agents) on beta-titanium and nickel-titanium wires [51–59]. The beta-titanium wires, which are inherently the most biocompatible because of the absence of nickel, are susceptible to hydrogen absorption, leading to embrittlement and delayed fracture [51, 52]. The nickel-titanium wires also appear to be susceptible to hydrogen embrittlement [54]. The interested reader should consult the original articles for the diverse experimental procedures in these studies.

In closing this section, it is important to note that standardized in vitro corrosion tests may not adequately simulate the complex oral environment, where diverse chemical species and fluctuating stresses are present. This has been shown in studies of retrieved nickel-titanium archwires, where there was an apparent decrease in the grain size of the nickel-titanium wire [60] and an absence of significant nickel ion release in vivo [61]. Additional studies of this type are highly recommended for the future. Another very important related area for research is the cytotoxicity of the wire alloys. Studies generally show that the nickel-titanium alloys have low cytotoxicity [62] and that the surface quality of these alloys and the cobalt-chromium-nickel alloy Elgiloy [63] is an important factor.

1.4 X-Ray Diffraction

X-ray diffraction (XRD) is an important analytical tool in materials science for investigating the structure of metals. Orthodontic wires can be readily studied by conventional XRD, using a test specimen consisting of several adjacent segments of the wire and having a width greater than the incident x-ray beam size. Each crystalline phase has a characteristic XRD pattern, which is dependent upon crystal structure, lattice parameter(s), and atomic species in the unit cell. The principles and applications of XRD are discussed in a well-known textbook [64], and a succinct presentation of relevant information is also available [1] for people interested in orthodontic materials research.

For conventional XRD used to investigate orthodontic wire alloys, a beam of nearly monochromatic x-rays (typically Cu $K\alpha$) is used, and the XRD spectrum or pattern is recorded with a diffractometer. The relationship between the angle of incidence (θ) and wavelength for the x-ray beam and the lattice parameter(s) of the material being analyzed, in order to have a diffracted beam with x-ray waves in phase (yielding a strong peak), is given by Bragg's law. The diffraction angle recorded by the diffractometer is (2θ).

The crystal planes that yield the XRD peaks for a given material depend upon the structure factor. X-ray diffraction standards for powder specimens of materials, which have randomly oriented crystals, are maintained by the International Centre for Diffraction Data (ICDD). The standard for a material provides the relative intensities of the diffracting crystal planes and the corresponding interplanar spacings. These interplanar spacings are converted to the diffraction angles, using Bragg's law with the wavelength of the given characteristic x-rays from specific electron transitions in the source. When a material contains more than one phase, the XRD pattern contains the peaks from all of the phases.

X-ray diffraction analysis of several sizes of two stainless steel wire products showed that the 0.016 in. diameter and 0.017 in. × 0.025 in. wires of both products had a duplex austenite and martensite structure, rather than the completely austenitic structure [65]. It was found that heat treatment of one wire product converted this duplex structure to the austenitic structure, but the duplex structure persisted for the other product. The heat treatment responses were attributed to a difference in

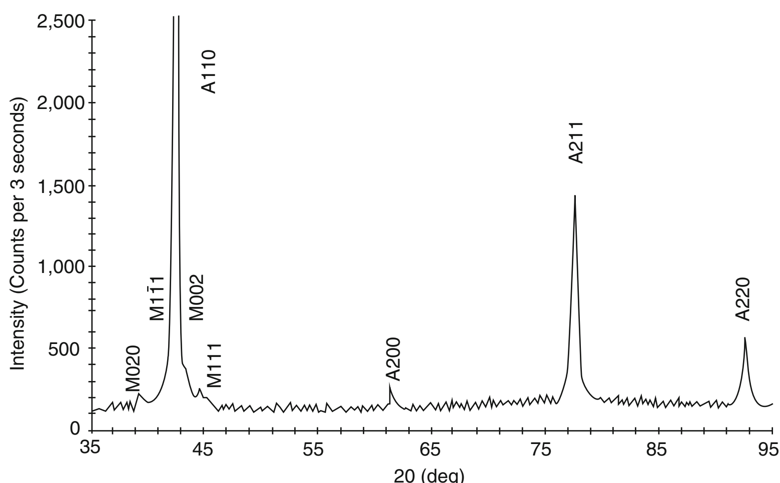

Fig. 1.6 X-ray diffraction pattern for 0.016 in. × 0.022 in. Neo Sentalloy™ (From Ref. [1] and reproduced with permission from Georg Thieme Verlag)

carbon content for the wire products from the two manufacturers. The XRD pattern for as-received beta-titanium wire (TMA™, Ormco) had peaks for the body-centered cubic beta-titanium structure, which were broad with preferred crystallographic orientation, as expected for the work-hardened wire [1, 66].

Conventional x-ray diffraction has been used extensively to study nickel-titanium wires [1, 32, 66–68]. Figure 1.6 shows the XRD pattern for Neo Sentalloy™ (GAC) [66], in which the crystal planes in the austenite and martensite structures have been indicated. Although this wire alloy has shape memory in the oral environment, when it is in the fully austenitic condition, the XRD pattern in Fig. 1.6 also shows peaks for martensite because the analysis was performed at room temperature, which is below the austenite finish (A_f) temperature for Neo Sentalloy™, as shown in the next section. The much weaker intensity of the USE 220 austenite peak, compared to the 211 peak, referring to the relatively intensities in the ICDD powder standard [1], is indicative of preferred orientation.

Figure 1.7 shows the XRD pattern for Ni-Ti™ (Ormco) after heat treatment for 2 h at 600°C [1, 32], which causes complete loss of the superelastic behavior for this wire alloy [10]. The wire has the completely austenitic structure, indicating that the martensite start temperature (M_s) is below room temperature after the heat treatment, so that minimal austenite transformed to martensite when the wire was cooled to room temperature for the XRD analysis [1, 32]. The sharp XRD peaks are indicative of substantial stress relief after heat treatment and perhaps recrystallization of the wire structure with new, stress-free grains. There is also strong preferred orientation. Referring to the ICDD standard, the peaks in Fig. 1.7 correspond to the 110, 200, 211, 220, and 310 reflections.

An exciting recent development is the use of micro-x-ray diffraction (micro-XRD) to investigate orthodontic wires [69–73]. With the micro-XRD technique,

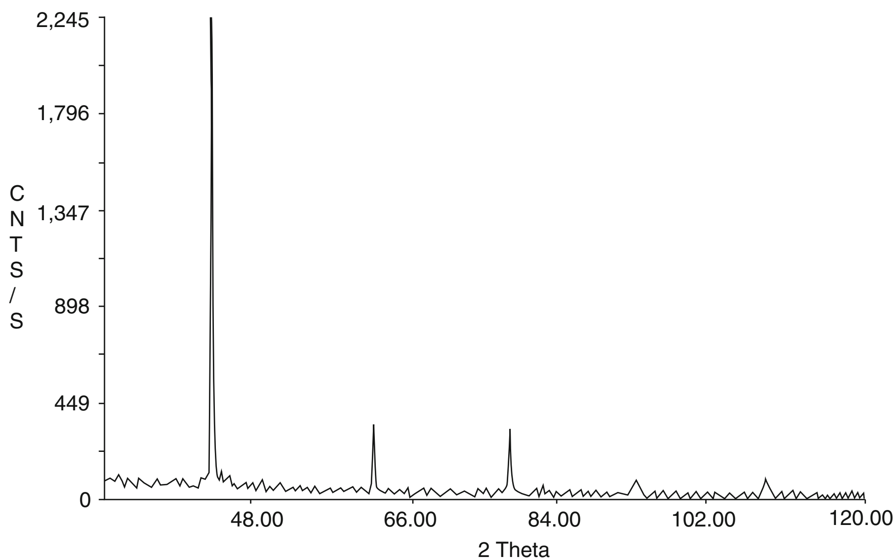

Fig. 1.7 X-ray diffraction pattern for 0.016 in. diameter Ni-Ti™, following heat treatment for 2 h at 600°C [1, 32] (From Ref. [1] and reproduced with permission from Georg Thieme Verlag)

which employs a relatively large tube current, the spot size for the incident x-ray beam can be as small as 100 μm. For example, analyses of tension and compression regions on bent wires, and of soldered and welded joints for orthodontic wires, become possible.

1.5 Differential Scanning Calorimetry (DSC) and Temperature-Modulated DSC

Conventional differential scanning calorimetry (DSC) has become the major analytical tool for study of the transformations of the NiTi microstructural phases with changes in temperature for the nickel-titanium orthodontic wires. Two classic articles [74, 75] describe the first reported use of DSC to investigate these wires, and there were several subsequent studies [76–78]. The current ANSI/ADA specification and ISO standard for orthodontic wires [12] stipulates the use of DSC to determine the A_f temperature for nickel-titanium wires. The advantages of DSC over XRD are its ability to determine the NiTi phases present at a given temperature, to investigate the phase transformation processes with changes in temperature, and to measure the enthalpy changes (ΔH) for these processes. The enthalpy changes provide insight into the transformation processes, which is not possible with x-ray diffraction or with the measurement of electrical resistivity changes. While the latter measurements can be readily performed over a range of temperatures, this is not generally possible with XRD apparatus.

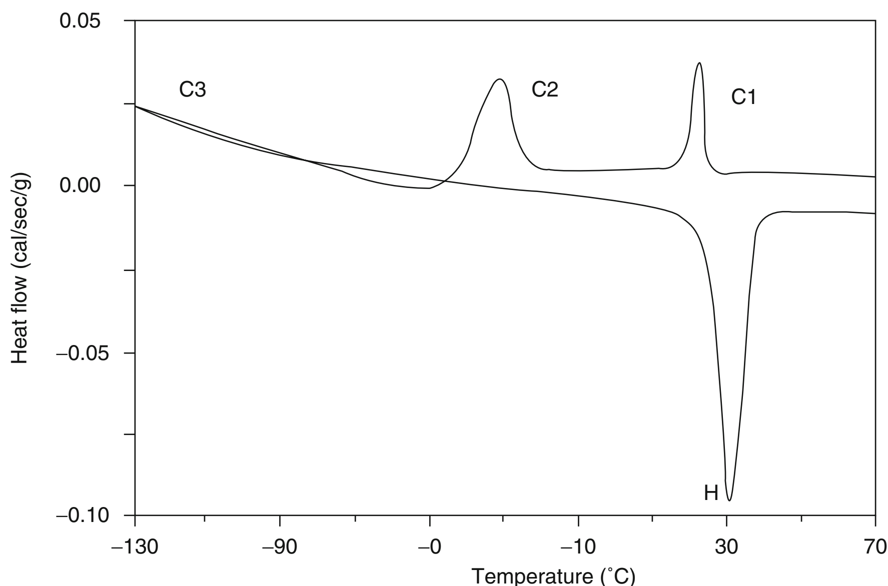

Fig. 1.8 Conventional DSC plot (thermogram) for Neo Sentalloy™ (From Ref. [76] and reproduced with permission from American Association of Orthodontists)

With conventional DSC, a small test sample of the experimental material (a few wire segments) is placed in one pan and an empty pan (typically aluminum) serves as the inert reference material [1]. Some investigators prefer to use indium as the reference material. Both pans are heated or cooled at a constant rate (typically 10°C/min), and the difference in heat flow (in units of cal/s/g or W/g) to the two pans to maintain the same temperature change for both pans is recorded as a function of temperature.

The conventional DSC plot for the shape memory wire Neo Sentalloy™ is shown in Fig. 1.8 [76]. The single peak (H) on the lower curve for the heating cycle suggests the direct transformation from martensite to austenite. The A_f temperature, at which the transformation to austenite has finished, is approximately 36°C. The upper curve for the cooling cycle has two peaks (C1 and C2), which correspond to transformations from austenite to R-phase and R-phase to martensite, respectively. (C3 was placed where low-temperature martensite peaks were previously found from measurements of electrical resistivity changes [79,80]).

Conventional DSC [76] shows that in vivo shape memory NiTi wire products have A_f temperatures lower than body temperature (37°C), whereas superelastic wire products have A_f temperatures that exceed body temperature (approximately 60°C for Nitinol SE™ and 40°C for Ni-Ti™). The nonsuperelastic Nitinol™ has A_f temperature of approximately 55°C and weak peaks (low ΔH values) [75, 76] corresponding to substantial quantities of stable work-hardened martensite in this wire.

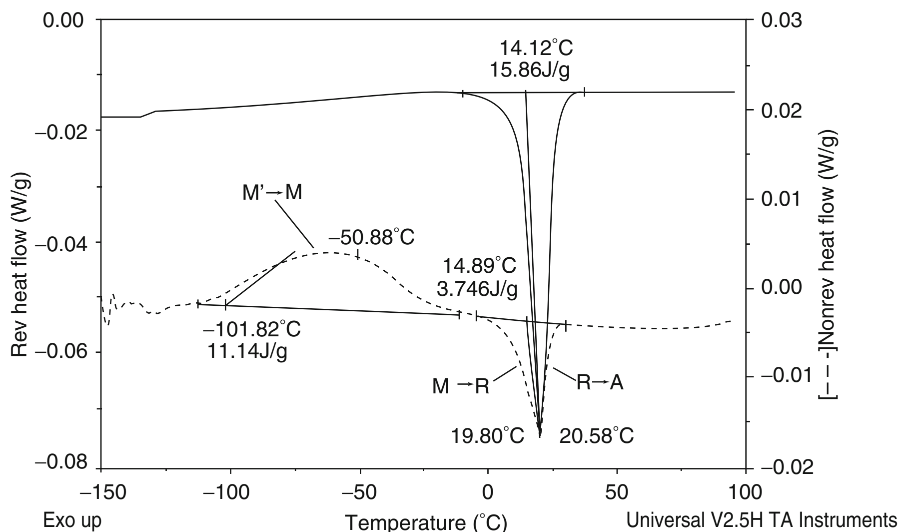

Fig. 1.9 TMDSC plots of reversing and nonreversing heat flow for heating cycle of Neo Sentalloy™, with phase transformation processes for peaks labeled (From Ref. [83] and reproduced with permission from American Association of Orthodontists)

The recently introduced technique of temperature-modulated differential scanning calorimetry (TMDSC) [81, 82] provides insight into phase transformation processes that is not possible with conventional DSC. (The generic term is TMDSC; use of the term *modulated differential scanning calorimetry* is specific to apparatus from TA Instruments.) With TMDSC, the linear heating or cooling rate is much slower (such as 2°C/min) to maintain a uniform temperature in the bulk specimen, and a small sinusoidal thermal oscillation (such as an amplitude of 0.318°C/min with a period of 60 s) is superimposed on the linear ramp. When selecting the thermal oscillation conditions, it is highly important to maintain a heating-only condition during the heating cycle and a cooling-only condition during the cooling cycle. Thin test specimens are needed, and helium is the preferred purge gas because of its high thermal conductivity, rather than nitrogen which is typically used with conventional DSC. Utilization of the small sinusoidal oscillation superimposed on the linear heating or cooling ramp allows mathematical subdivision of the total heat flow measured by conventional DSC into its reversing and nonreversing components with respect to temperature changes.

The advantages of TMDSC are evident in Fig. 1.9, which shows the heating cycle for Neo Sentalloy™ [83], compared to the heating cycle on the conventional DSC plot in Fig. 1.8. The dashed nonreversing heat flow curve in Fig. 1.9 shows that a two-step transformation from martensite to austenite occurs, which involves the intermediate R-phase. There is also a strong exothermic peak on the nonreversing heat flow curve arising from low-temperature transformation within martensite (designated as M′ → M for heating).

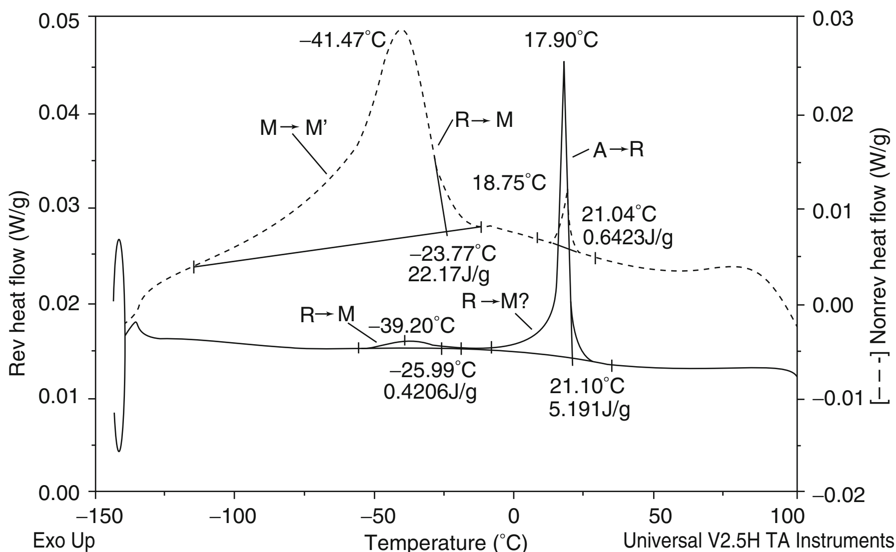

Fig. 1.10 Corresponding TMDSC plots of reversing and nonreversing heat flow for cooling cycle of Neo Sentalloy™ (From Ref. [83] and reproduced with permission from American Association of Orthodontists)

The corresponding TMDSC plots for the cooling cycle of Neo Sentalloy™ are shown in Fig. 1.10 [83]. The reversing heat flow curve has a large exothermic peak for the transformation from austenite to R-phase, which can be seen to have a small amount of nonreversing character. Possible transformations from R-phase to martensite are labeled on both the reversing and nonreversing heat flow curves. There is a large exothermic peak on the nonreversing heat flow curve (designated as M → M′ for cooling), which again corresponds to transformation within the martensite structure. As noted previously, low-temperature martensite transformations in NiTi orthodontic wires were originally reported from measurements of electrical resistivity changes [79]. Recent low-temperature transmission electron microscopy examination of 35°C Copper Ni-Ti™ wire has shown that twinning within martensite is the origin of this peak on the TMDSC nonreversing heat flow curves [84]. During both the heating and cooling cycles, the martensite structure undergoes twinning to relieve internal stresses in the microstructure. This low-temperature peak has been observed in all orthodontic wires examined by TMDSC in two studies [83, 85].

1.6 Metallographic Preparation

1.6.1 Background

Metallographic preparation is extensively used in order to reveal the true bulk structure of solid materials. The distribution of pores, presence of cracks or other internal

defects, quality of joints, and grain size and shape are only a few of the features that can be qualitatively and quantitatively determined by the analytical techniques (i.e., optical and electron microscopy). Sectioning, mounting, grinding, polishing, and etching are the main steps of metallographic preparation although the latter is applied only when the grain structure and microstructural phases are the subject of research. As a standard operating procedure, metallographic preparation must be characterized by reproducibility and reliability. Reproducibility is associated with the ability of a method to provide the same results for the same material, every time it is carried out, while reliability represents the ability of the method to provide the true structure of the material free of possible structural alterations (e.g., deformation and smearing) and other artifacts.

1.6.2 Sectioning

The main goal of sectioning is the removal of a conventionally sized, representative specimen from a larger sample. Although sectioning is routinely used in metallographic preparation of specimens from various applications, this is not the case in orthodontics where the specimens are of very small dimensions. Orthodontic wires can be easily cut by pliers, avoiding the use of sophisticated sectioning machines. Complete details on sectioning are not included in this chapter, but the reader can find extensive information elsewhere [86].

1.6.3 Mounting

In general, mounting is considered optional, and it may not be necessary for some bulk specimens. However, a small or oddly shaped specimen (e.g., an orthodontic bracket) should be mounted in order to facilitate handling during metallographic preparation Fig. 1.11. Standard molds usually have diameters of 25, 32, or 38 mm. Caution should be exercised about the final thickness of mounted specimens, as very thin mounted specimens are difficult to handle, and the flatness of very thick ones is difficult to preserve during metallographic preparation.

The first criterion for the selection of the proper mounting material and technique is the protection and preservation of the sample. Delicate and fragile samples may be subjected to physical damage or microstructural alterations due to the heat and pressure required by some mounting materials. Ideally the mounting material should have similar grinding and polishing characteristics as those of the sample. Additionally, the mounting material should effectively resist physical distortion caused by the heat developed during grinding and polishing and withstand exposure to suspensions, lubricants, and etching solutions. The mounting material should have low viscosity so that it can easily penetrate crevices, pores, or other irregularities in the sample. In addition, the mounting material should be easily manipulated and stored and should present no health hazards to the operator. It should not be susceptible to the formation of any defects (e.g., cracks and pores) and should be

Fig. 1.11 Three pieces of orthodontic wire embedded in acrylic resin (*yellow cylinder*) reinforced by mineral fillers to provide good ability for achieving planar surface and good edge retention. Cylinder diameter is 25 mm

available for purchase at a reasonable cost. Sometimes an electrically conductive mount is desirable, such as for electrolytic polishing and scanning electron microscopy analysis. Currently, there is no mounting material that fulfills all of the aforementioned requirements, and a variety of materials and methods are available. Proper selection is considered the one that meets the most critical requirements for each combination of material and subsequent analytical technique.

The available mounting materials can be classified into two groups: (1) materials that require the application of heat and pressure and (2) materials that can be poured into a room temperature mold [86]. The technique used with the first group of materials is termed *hot mounting*, and the technique used with the second group is termed *cold mounting*. In the hot mounting technique, the specimen is placed in the mounting press, and after the pouring of resin, the mounted specimen is processed under high pressure and heat. The mounting materials used for this technique are (a) thermosetting resins which cure at elevated temperatures and (b) thermoplastic resins which soften or melt at high temperatures and harden during cooling to room temperature. Epoxy, acrylic, and polyester materials are used in cold mounting. Epoxies are characterized by low shrinkage and excellent adhesion to most materials but have relatively long curing times. Acrylics and polyesters are catalyzed systems and have short curing times.

1.6.4 Grinding and Polishing

The next steps of metallographic preparation after mounting are the grinding and polishing of the sample surface. The purpose of grinding is primarily to remove damaged or deformed material at the sample surface and to produce a plane surface that will be easily removed during the polishing step. Successive steps with finer abrasive particles are employed during this step to remove material from the sample surface until the required result is reached. Grinding and polishing are commonly

carried out in special designed metallographic machines in which the abrasive papers are placed on a rotating wheel and the sample surface is firmly placed sequentially against each abrasive paper. Significant heating of the surface during the initial coarse grinding step is avoided by using a copious amount of water or another liquid as a coolant. The aim of polishing is to remove the surface damage introduced during grinding, which is accomplished with several steps of successively finer abrasive particles.

During polishing, the abrasive particles are suspended in a liquid and retained among the fibers of special metallographic cloths. Diamond and aluminum oxide suspensions (slurries) are most commonly used for polishing. Generally, the final abrasive particle size is 0.5 μm, which is approximately half the wavelength of visible light, so that the polishing scratches on the sample surface cannot be seen visually or in the optical microscope. While the principles for grinding and polishing are straightforward, the reader should be aware that both procedures are much more complicated and that revealing the true microstructure is always a challenge for the metallographer. Selection of the appropriate metallographic technique and achieving an artifact-free sample surface are the two milestones of proper sample preparation. Decades of experience have led to grinding and polishing procedures that are appropriate for each material type [87]. For example, the preparation procedure for a bracket-adhesive resin-tooth interface requires a completely different procedure compared to that for an orthodontic Ni-Ti alloy, which is considerably different from the grinding and polishing procedure for a Co-Cr archwire. All these procedures have been summarized in an excellent reference book [87].

1.6.5 Etching

Although some microstructural features (e.g., pores, cracks, pits, and nonmetallic inclusions) may be observed in the as-polished condition, the microstructure is usually revealed only after etching. A well-polished sample surface will not reveal its microstructure because light is uniformly reflected, and the eye cannot distinguish small differences. Generally, only features that show greater differences than 10 % in reflectivity can be viewed without etching, such as features with large differences in hardness that cause surface relief formation or with strong color differences. In the vast majority of cases, image contrast must be produced.

Although metallographic contrasting methods include physical, optical, and electrochemical etching techniques, chemical etching is most extensively used to reveal the microstructures of samples. Alternative etching techniques have been developed for cases in which an effective etching solution cannot be found such as for chemically stable ceramics (e.g., ZrO_2 and Al_2O_3). Etching solutions are provided by the relevant literature in a form similar to cooking recipes. The metallographer has to produce the proper etching solution according to the material and the microstructural feature under study. The results of chemical etching are very sensitive to temperature, composition, and pH alterations of the etchant employed, and thus considerable care should be taken to follow the given instructions. Figure 1.12

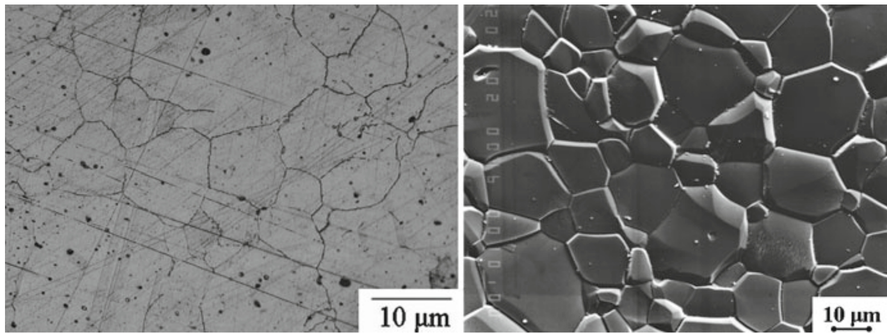

Fig. 1.12 *Left*: Microstructure of austenitic stainless steel alloy revealed after etching with solution composed of 1 g picric acid, 5 mL HCl, and 100 mL ethanol. *Straight lines* are scratches created during grinding and polishing steps (original magnification 200×). Photomicrograph was obtained with optical microscope. *Right*: Microstructure of alumina ceramic bracket after thermal etching. Backscattered electron image was obtained with scanning electron microscope (original magnification 100×)

(left) shows the microstructure of an austenitic stainless steel alloy used for the production of orthodontic brackets by the metal injection-molded (MIM) technique [88] and (right) the grain structure of a ceramic bracket.

It can be seen that metallographic preparation is a multistage procedure with a variety of materials and techniques potentially involved, and the metallographer should be extremely cautious in order to avoid artifacts and/or contamination during the preparation procedures that might significantly alter the true microstructure of the sample.

1.6.6 Light (Optical) Microscopy

Despite the evolution of advanced electron-optical metallographic methods, such as use of the scanning electron microscope (SEM) and transmission electron microscope (TEM), the optical microscope remains an important and easy technique for the study of microstructures. Polished or etched samples can be directly viewed in the optical microscope, whereas imaging with the SEM and TEM requires further preparation steps and advanced facilities. Specimen preparation can be particularly time-consuming for TEM, in which the extremely thin foil specimen must be transparent to the primary electron beam. (This advanced materials science technique will not be discussed in this chapter.) All examinations of microstructure should be done from the lowest (nominal magnification 50×) to highest magnifications (1,000×) to characterize the microstructural features. However, if some microstructural features are too fine to be identified with the optical microscope, other techniques that provide higher magnifications must be employed.

Both as-polished and etched surfaces can be easily investigated, although some features are more readily identified in the former condition because they are not

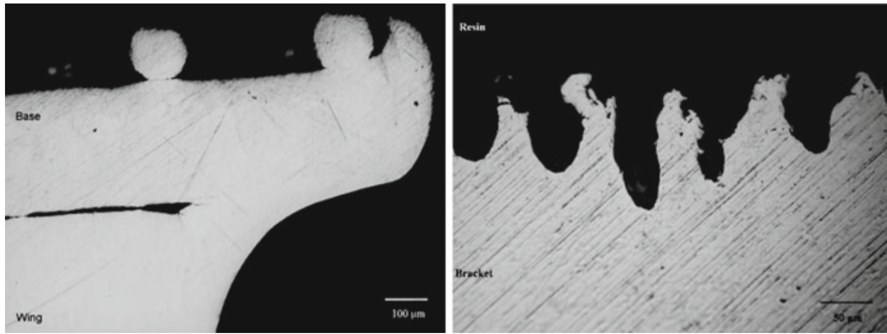

Fig. 1.13 Cross-sectional images obtained by bright-field optical microscopy. Large gaps are prominent along base-wing interface in *left image* (original magnification 100×). *Right image* shows cross section of single-piece bracket with laser-structured adhesive base (original magnification 200×)

obscured by etching detail. Optical microscopy has been extensively used to determine the microstructures of several metallic orthodontic brackets (Fig. 1.13) [88–90]. In addition to the use of conventional bright-field illumination, image contrast can be enhanced by oblique lighting, dark-field illumination, phase contrast, interference contrast, and examination under polarizing light. Image analysis software is available that can provide quantitative information about microstructural features such as percentage of porosity, shape and size of constituents, and interparticle distance, and such software can also be utilized for images obtained by other methods such as SEM and EPMA (electron probe microanalysis).

However, in some cases (e.g., broken parts such as debonded brackets from dental enamel) observation and photography at very low magnification should be performed. This macrophotography procedure can be performed using typical cameras, perhaps aided by use of a macrolens or close-up lens attachments. In the research laboratory, the use of a stereomicroscope equipped with cameras for photography is more common. Nominally, macrophotography uses magnifications from less than 1× to 50×. Correct lighting is critical to properly emphasize details on the sample being photographed and to provide adequate contrast and even illumination without reflection or glare. In orthodontics macrophotography is commonly used to investigate mesh-type brackets and characterize the fracture mode through the estimation of percentage retained resin after bracket debonding from enamel (Fig. 1.14).

1.6.7 Scanning Electron Microscopy (SEM)

The scanning electron microscope is probably the most widely used analytical technique in materials science worldwide. Useful magnifications start from very low (6×) and extend to 150,000×, thus closing the gap between optical microscopy and transmission electron microscopy. Additionally, compared to the optical microscope, the SEM provides higher resolution and depth of focus by one and two orders

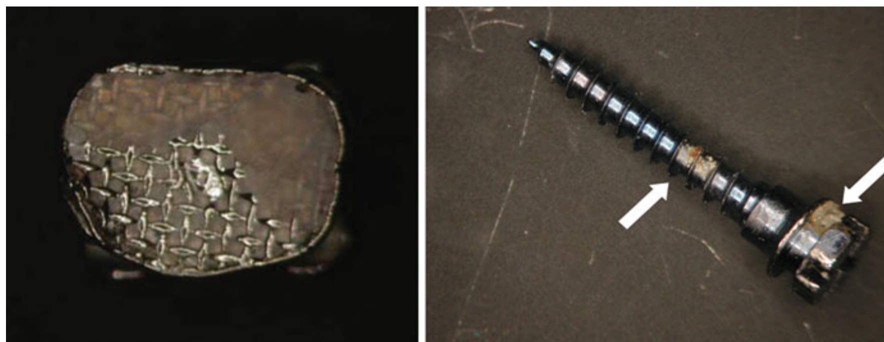

Fig. 1.14 Stereomicroscopic images of orthodontic appliances. *Left*: Surface of bracket debonded from enamel after shear bond strength test. Presence of retained resin is readily distinguished from bracket surface. *Right*: Retrieved orthodontic miniscrew implant showing intraoral aging-induced integuments (indicated by *arrows*)

of magnitude, respectively. Moreover, the SEM yields clear images of rough surfaces, polished surfaces, and etched surfaces, although focusing can be more difficult with very smooth surfaces. Elemental analysis with the SEM is carried out with the use of detectors that determine the energy or wavelength of the characteristic x-rays (created by the incident primary electron beam) that are emitted from each element in the sample. Good lateral resolution can be achieved. Given that the SEM images are built by the raster scanning of successive primary electron beams across the sample surface, individual line scans can be acquired that portray the distribution of elements of interest in the sample. While the conventional SEM is extensively used in a wide range of applications in materials research, recent advancements can provide imaging of nonconductive specimens (low-vacuum SEM) and wet samples at 99 % relative humidity (environmental SEM), dramatically expanding the capabilities of this method.

The SEM uses an electron beam emitted from a heated tungsten cathode filament and focused to a small diameter (approximately 5–15 nm) by a system of magnetic lenses. Accelerating voltages vary from 0.1 kV to maximum generally of 30 kV, while the beam current ranges from 10^{-7} to 10^{-8} A. The whole system is placed under high vacuum, and the incident primary electron beam raster-scans the specimen surface as in a cathode ray tube used for image formation on a television screen. The interaction between the primary electron beam and the specimen yields secondary and backscattered electrons along with other radiation that can be collected by the detectors to form images and provide analyses of elements in the overall sample or in the individual microstructural phases or constituents.

Secondary electron images (SEI) are formed by the secondary electrons emitted from the specimen surface. These electrons are created by interaction of the incident beam with loosely bound electrons in the elements comprising the sample. They are emitted from the uppermost layers of the material with an excitation depth up to 10 nm below the specimen surface, and thus they provide a very clear image of surface morphology. This property, along with the increased depth of focus of the

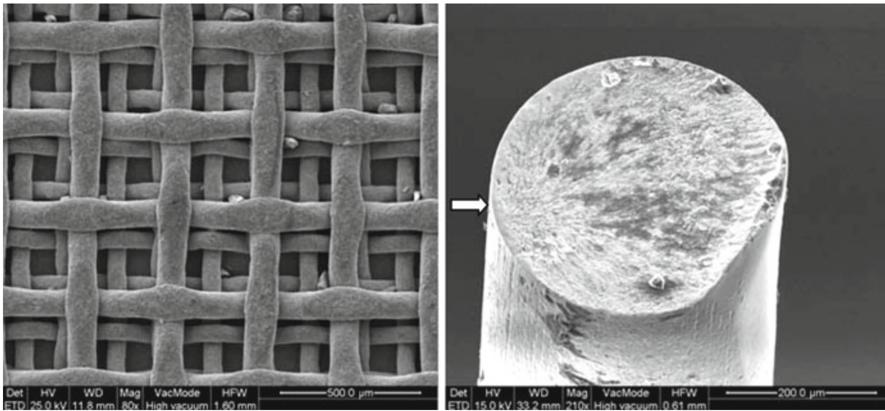

Fig. 1.15 *Left*: Multilevel foil mesh of contemporary metallic bracket. *Right*: Fracture surface of niti archwire that failed during clinical use. Fracture pattern is characterized by fibrous texture, and origin of fracture is indicated by *arrow*. Shear lip at right edge of wire was final region to undergo fracture

electron beam, can provide very clear images for specimens with multilevel surfaces. Examination of mesh patterns from different brands of orthodontic brackets and analysis of the fracture surfaces of orthodontic wires have been carried out using this type of imaging [88–91] (Fig. 1.15).

Backscattered electron images (BEI) provide atomic number (Z) contrast for the material, because the energy distribution of backscattered electrons created by the incident electron beam depends primarily on the atomic number of the material. Thus, regions composed of different elements, or more precisely regions with difference in mean atomic number, can be readily discriminated by this mode of SEM operation. Such contrast is useful for microstructural phase discrimination, and thus BEI are extensively used for the investigation of multiphase materials such as soldering alloys for stainless steel orthodontic brackets (Fig. 1.16) [92]. This type of imaging is especially suitable for quantitative evaluation of microstructural geometry that employs image analysis procedures.

Two more modes of SEM operation known as cathodoluminescence and thermal wave imaging have limited applications to orthodontic research, and they are not included in this section.

1.6.8 Electron Probe Microanalysis (EPMA)

In electron probe microanalysis (EPMA) also utilizes a primary electron beam of high intensity is accelerated at generally 5–30 kV and focused on a small area. The specimen microvolumes, which are again excited by scanning the beam over the target, produce two types of spectra [93]. The first type, commonly known as bremsstrahlung (“braking radiation”) and having a continuous spectrum, originates from the range of interactions between the electrons and atoms of the material

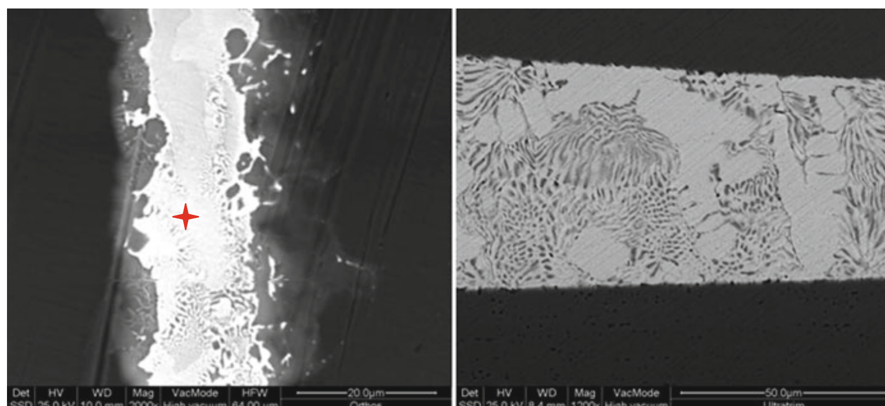

Fig. 1.16 Backscattered electron images from polished surfaces of soldered brackets. brazing zones are clearly distinguished from base (*left region*) and wing (*right region*) bracket components. *Red cross* identifies point for spot analysis described in next section on EPMA. The *left image* shows transition zone between brazing alloy and two brackets components (original magnification 2,000×). In contrast, *right image* shows prominent two-phase eutectic structure regions in brazing alloy and no transition zone between base and wing components of bracket (original magnification 1,200×)

through which they are passing. The second is a characteristic radiation for the particular element with a distinct line spectrum in each case. The inner shell electrons of the target atoms are primarily ionized by the incident electron beam and, upon relaxation to the ground state, emit characteristic x-rays that correspond to the energy loss for the particular transition in the excited element. Since these characteristic x-rays are independent of the chemical environment of the atoms, they are suitable for elemental analysis. When this emission occurs, these characteristic x-rays can be acquired by suitable detectors and analyzed qualitatively and quantitatively by WDS (wavelength-dispersive spectroscopy) or EDS (energy-dispersive spectroscopy) [93].

EPMA offers three analysis modes, which will be described in the following paragraphs.

1. Spot (point) analysis involves the acquisition of a spectrum at a selected point. This type of analysis is very convenient for the elemental analysis of specific regions, such as small regions of phases, foreign particles, and inclusions. Figure 1.17 illustrates a typical spectrum showing the quantitative results for the soldering alloy of Fig. 1.16.
2. Area scan analysis involves the presentation of the elemental distributions in the form of maps. This type of analysis is employed to identify the overall surface distribution of each element. Figure 1.18 shows the BEI of a soldered orthodontic bracket made from a stainless steel alloy in this presentation format. The base and wing components were soldered using a soldering alloy with higher mean atomic number. Elemental mapping reveals that Au is solely found in the soldering alloy, while Ni has maximum values at the transition zone with the base and wing components. In addition, some Fe diffusion is evident at the transition zone.

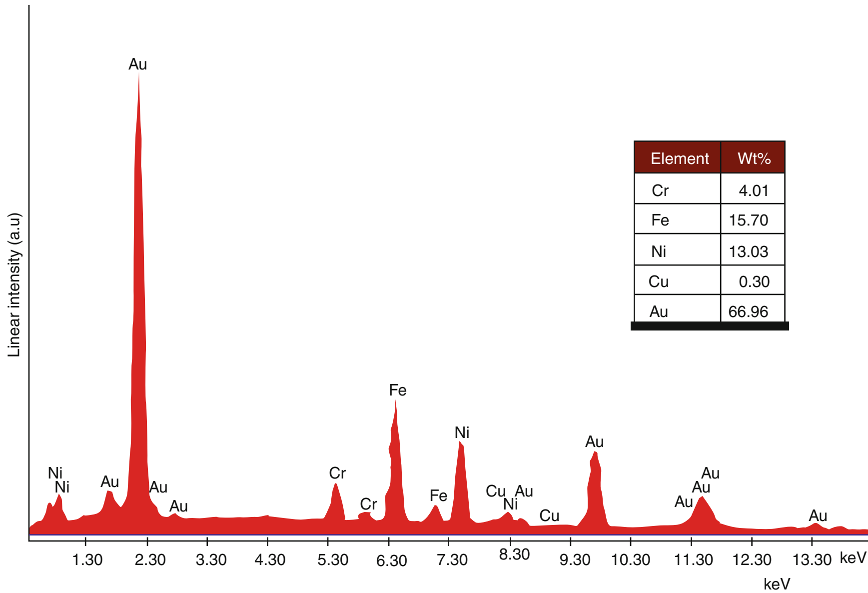

Fig. 1.17 Spectrum showing energy-dispersive x-ray microanalysis analysis of brazing filler material, acquired from *red spot* in Fig. 1.16. Table summarizes quantitative results of elemental weight percentages

3. Line scan analysis where the elemental gradients along a linear direction are measured. This analysis is very helpful to understand the variation in concentration of each element, information which is not so clear in area mapping. Figure 1.19 presents the concentration profiles of Au, Fe, Ni, and Cr for this example of a brazed orthodontic bracket.

Energy-dispersive spectrometric (EDS) analyses have been extensively used to analyze the elemental composition of orthodontic alloys [43, 44, 88, 89, 92], ionic release comparing the elemental compositions before and after intraoral aging [61, 90] of metallic orthodontic materials, and the distribution of intraoral integuments [60, 94].

1.6.9 Quantitative Image Analysis

The introduction of digital imaging and image processing has given a tremendous boost in the quantitative evaluation of microstructural features of materials. Generally, an image acquired with good resolution and contrast can be further enhanced by image analysis software for the quantitative evaluation of various features of interest (e.g., distance between specific points, interparticle distance, shape of phases or grains, percentage of porosity, percentage of area covered for the evaluation of fracture mode). In orthodontics, image analysis processing has also been used to characterize the fracture mode of brackets and the cohesive/adhesive nature of the interfacial failure process for different adhesive systems (Fig. 1.20).

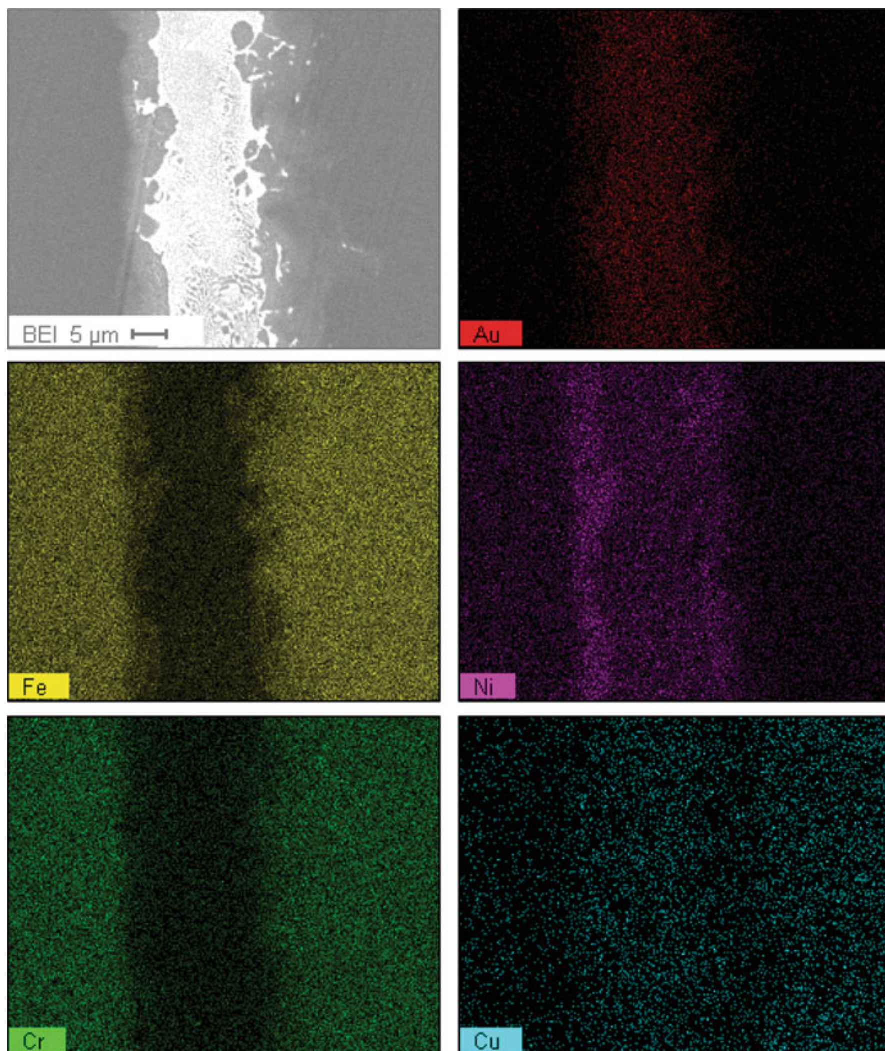

Fig. 1.18 Backscattered electron images for cross section of bracket consisting of two components (base and wing) joined together with brazing alloy. Maps show elemental distributions for Au, Fe, Ni, Cr, and Cu

1.6.10 Microcomputerized X-Ray Tomography (Micro-CT)

Computer tomography and image processing are extensively used in the medical field for the development of virtual models. In the last decade, new benchtop models for materials characterization were developed and are currently commercially available. These micro x-ray scanners employ the same principle as for medical tomography but with an isotropic resolution up to some hundreds of nanometers. A

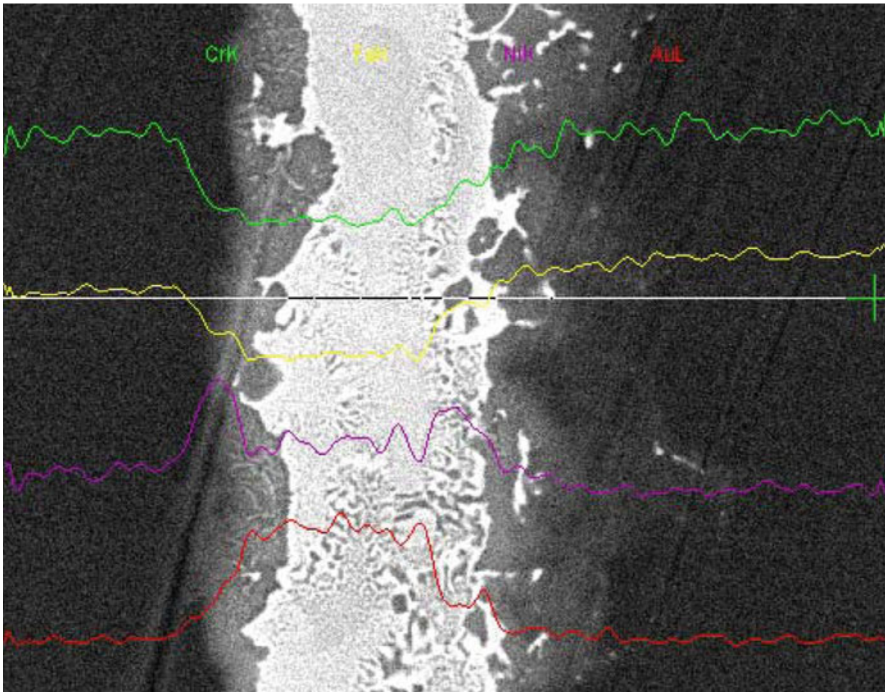

Fig. 1.19 Line scans demonstrating variation of four elements from base (*right*) to tie-wing (*slot*) area (*left*). *White horizontal line* shows specific location of linear region in which elemental concentration profiles of Cr, Fe, Ni, and Fe were determined

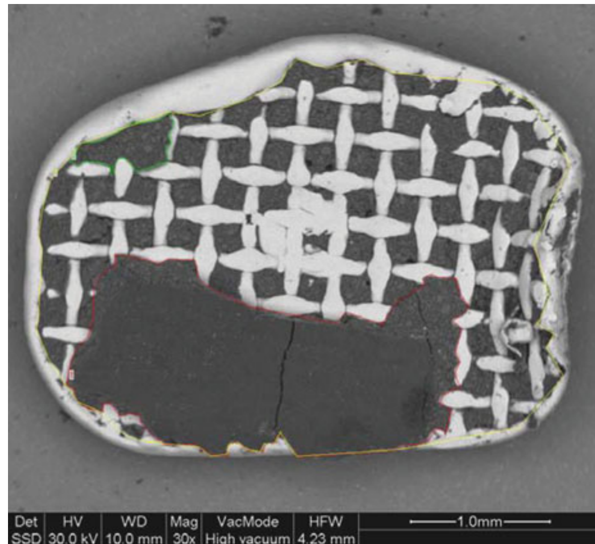

Fig. 1.20 Quantitative evaluation of fracture mode at bracket-resin interface. *Red* and *green lines* outline retained resin, while *yellow line* indicates whole interfacial surface. Areas of three surfaces are quantitatively estimated by image analysis software. Results showed that 34.5 % of total area is covered by retained resin, and thus fracture mode was characterized as 65.5 % adhesive at bracket-resin interface

Micro-CT system mainly consists of an x-ray radiation source, an x-ray detector, and a processing unit which reconstructs the image from the measurements. The latter uses alternatively two reconstruction algorithms commonly known as *iterative method* and *filtered back projection* [95]. The major advantage of this technique is that the bulk analysis is performed nondestructively, allowing the possibility for further analysis at a later time. This is very important when specimens must remain intact through the experimental analysis (e.g., archeological findings and forensics).

The Micro-CT scanning produces hundreds of horizontal slices of the tested sample, as shown in Fig. 1.21. These horizontal slices are used to reconstruct the entire structure, providing information about the geometric features and internal defects of the sample. Additional software can be employed in the development of three-dimensional models, pseudocoloring, and the quantitative analysis of geometrical features of irregular samples (e.g., the total surface area of an orthodontic bracket).

In orthodontics, the quality of soldering between base and wing components of a metallic bracket has been analyzed [96] by a Micro-CT scanner. Large empty spaces were located at the joint interface between the base and wing components (Fig. 1.21).

The clinical efficacy of orthodontic materials and devices is dominated by their mechanical, physical, and electrochemical properties. However, specific properties have distinct clinical implications.

- Modulus of elasticity determines the imposed elastic strain. The higher the modulus, the higher force demanded to achieve the same elastic strain. In alternative expression Ni-Ti alloy deforms the same extent with Co-Cr loaded with lower external forces.
- Clinicians should avoid the great differences in hardness between orthodontic archwires and brackets to minimize the fragment release due to wear between them.
- Fracture toughness is indicative for the resistance of materials to crack propagation. Orthodontic materials (i.e., aesthetics brackets made of various ceramics) are more prone to brittle fracture.
- Electrochemical properties of metallic orthodontic materials must be taken into account especially in order to avoid galvanic phenomena under clinical conditions.
- The majority of the methods described in this chapter can be effectively used to characterize retrieved orthodontic devices in order to describe the underlying aging mechanism providing fundamental information for further research and development of new materials and/or devices.

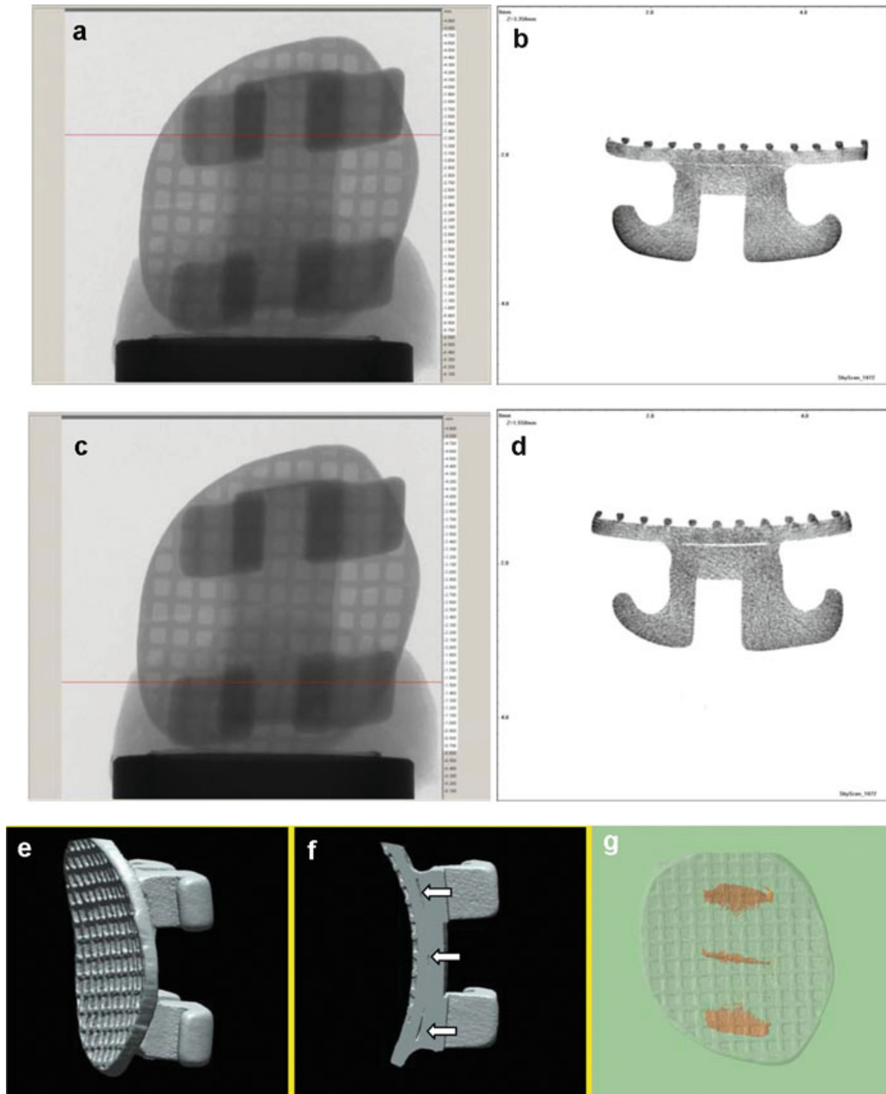

Fig. 1.21 Three-dimensional x-ray microtomographic images and three-dimensional model of metallic orthodontic bracket. (**a**, **c**) X-ray images of specimen. (**b**, **d**) Horizontal cross sections at level of *red line* in images (**a**) and (**c**), respectively. Wing and base components are readily distinguished. Gap between wing and the base is clearly shown in (**d**). (**e**) Three-dimensional model of scanned bracket. (**f**) Details of section of three-dimensional model at base-wing interface, showing presence of gaps (*arrows*). (**g**) Reconstructed image of empty spaces, showing gaps (*orange color*). Base component is also outlined

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Research on Orthodontic Polymers

2

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2.1 Introduction

Research on orthodontic polymers involves the study of a wide array of materials used in a spectrum of orthodontic applications. Table 2.1 summarizes these materials and applications.

The main contribution of the relevant articles to the broader orthodontic materials literature derives from the extended presence of bond strength studies employing

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Table 2.1 Classification of orthodontic polymers and relevant testing

Material	Tests	References
Adhesives	Bond strength (shear, torsion, and tensile)	[1–17]
	Degree of cure/monomer leaching	[18–24]
	Fluoride release/enamel fluoride uptake by adhesives	[25–27]
	Critical surface tension	[28–40]
	Bonding-induced enamel color alteration	[41, 42]
	In vivo determination of bonding-induced color alterations in orthodontics	[43–66]
Brackets	Morphology/structure	
	Torsional stiffness	
	Hardness	Chapter 1
	Wear	
	Roughness	
	Critical surface tension	[28–40]
	Retrieval analysis	Chapter 1
Elastomeric modules and chains	Creep and relaxation	[67–70]
	Tensile strength	[15]
	Fluoride release	[25–27]

a large number of materials and techniques. However, orthodontic polymers as a scientific subject involves also elastomers, which are basically polyurethane modules and chains, latex elastics, and polycarbonate or poly(oxy)methylene plastic brackets.

There is a considerable array of properties of clinical interest, which have attracted investigators: bond strength, degree of cure and fluoride release potential for adhesives; creep and relaxation for elastomeric modules; and hardness, wear, roughness, friction, light transmittance, and torsional stiffness for plastic brackets. Some topics may also be found in associated areas such as metallurgy (for the corresponding properties of interest of stainless steel or titanium brackets, i.e., friction, bond strength, stiffness), and the reader is referred to Chap. 1 for further details.

The purpose of this chapter is to overview the techniques used in these tests and provide a background for the application of these to specific materials.

2.2 Research on Polymeric Adhesives

Research in this area is mainly comprised of bond strength studies, probably because of the apparent simplicity of the experimental setup and the extrapolation of values, which supposedly project the efficacy of the bonding system or the bracket base design.

2.2.1 Bond Strength

2.2.1.1 Background

A common means of evaluating the potential clinical effectiveness of an adhesive orthodontic material is by measuring its bond strength to a tooth surface. In orthodontics, the bonding of brackets to tooth surfaces is temporary because the attachments are removed after the active treatment period.

Bond strengths are defined as a measure of interfacial adhesion between a substrate and the bonded material, sometimes mediated by an adhesive agent, and are calculated as fracture force divided by the bonded area [1]. In practice, the fractures do not always occur at the interface and often involve the bonded adhesive material and the substrate.

In orthodontic literature, many studies have evaluated the bond strengths *in vitro*, *ex vivo*, and *in vivo* [2]. Most of this research has been under *in vitro* conditions because it is difficult to expose the materials to and retrieve them from the oral environment without interfering with the environment itself or taxing the subjects' compliance.

An interesting issue, which has attracted much discussion in the orthodontic materials literature, pertains to the actual clinical requirement of bond strength based on the estimation of the magnitude of forces developed during treatment by the activated archwires. Most studies refer to a 1970s paper by Reynolds and von Fraunhofer [3], which proposed a value of 6–8 MPa. This number has been cited more than 150 times in the relevant literature as the minimum requirement for a clinically derived bond strength threshold value. However, this proposition may not be accurate for the following reasons: (a) It is conjectural since it is based on an assumed profile of the incompletely known load application during mechanotherapy and thus presents an undefined margin of safety. (b) It does not take into account the stresses developed during mastication of hard food or higher chewing velocities. (c) It fails to include aging of the polymeric adhesives and associated environmental stress-fatigue phenomena. Therefore, "threshold strengths" may not cover the requirements for a sound bond throughout the entire period of treatment, which may exceed 18 months.

2.2.1.2 Description

A variety of teeth have been used in orthodontic bonding experiments, including upper central incisors, premolars, and lower incisors. While premolar extraction may be an integral part of orthodontic therapy, facilitating the collection of those teeth, premolar crown contour variations may complicate the effort to have substrate surface consistency [4]. On the other hand, upper and lower incisors are mostly retrieved from periodontally involved dentitions. Use of such teeth introduces the complicating factor of the age of the average periodontal patient, since the fluoride content in the outermost surface layers has been documented to change with time. Perhaps etching patterns vary accordingly, although no evidence regarding this parameter has been presented [5]. In addition, possible adsorption of inorganic or proteinaceous species, as well as the consequences of various therapeutic

procedures and pharmaceutical agents administered to these patients, may modify the reactivity of the enamel surface layers with an undetermined impact on etching patterns. The extraction time and storage media have little if any influence on adhesive bond strength to enamel [6]. This critical review suggests that a storage time of 6 months may be used for normalization purposes among miscellaneous experimental protocols.

Often, experimental treatments of collected teeth include leveling of the prospective enamel surfaces by grinding in an attempt to standardize the topographic variants of the substrate [7]. The argument supporting this notion is related to the incongruities found in the profile contour and convexity of the labial enamel surface, particularly those of premolars. The latter induce a variable pertinent to adaptation of the adhesive layer to the tooth crown, inevitably modifying the composite resin thickness. Although this procedure is obviously inappropriate for clinical conditions, its major flaw is the profound alteration of the substratum. Apparently, surface layers of enamel possess properties dissimilar to those found in deeper zones, due to the higher fluoride content of the outermost 10 μm layer [8]. In addition, grinding of the enamel surfaces is performed ad libitum, using stones or silica disks of varying roughness, while the duration of this process is determined by visual inspection, being thus highly subjective [9]. Therefore, not only is there failure in constructing a simulated clinical analogue, but this method also introduces variability in enamel condition that precludes comparing results from different studies.

The procedure of adhesive application to the bracket base has raised the issues of the quantitative aspects of adhesive and force utilization during bonding. The methods in published studies involve either the application of a standardized quantity of adhesive or the use of an undetermined amount of composite resin [7]. Even though the first approach may normalize variables related to adhesive paste application, allowing for the estimation of reference material properties such as degree of conversion and monomer leaching, it lacks the essential element of simulating the typical clinical procedure employed by orthodontists. A method proposed to overcome this deficiency combines components from both approaches by having multiple pilot trials involving application of an adhesive to bracket bases for bonding by a trained orthodontist [10]. This approach allows an estimate of the weight range of adhesive used, which represents a standardized baseline amount for application to the bracket bases. A similar concern has been expressed about force application during bracket-adhesive attachment to enamel.

Figure 2.1 shows the bond strength cell in which a bracket bonded to the enamel surface of a tooth is placed and subjected to a tensile or shear force to cause bond failure. A value of bond strength is calculated as the quotient of the force at which debonding occurs (determined from the sudden load drop on the mechanical test machine) and the interfacial area of the adhesive or bracket base. Bond strengths have been measured under tensile, shear/peel, and torsional loading, and are reported in units of MPa.

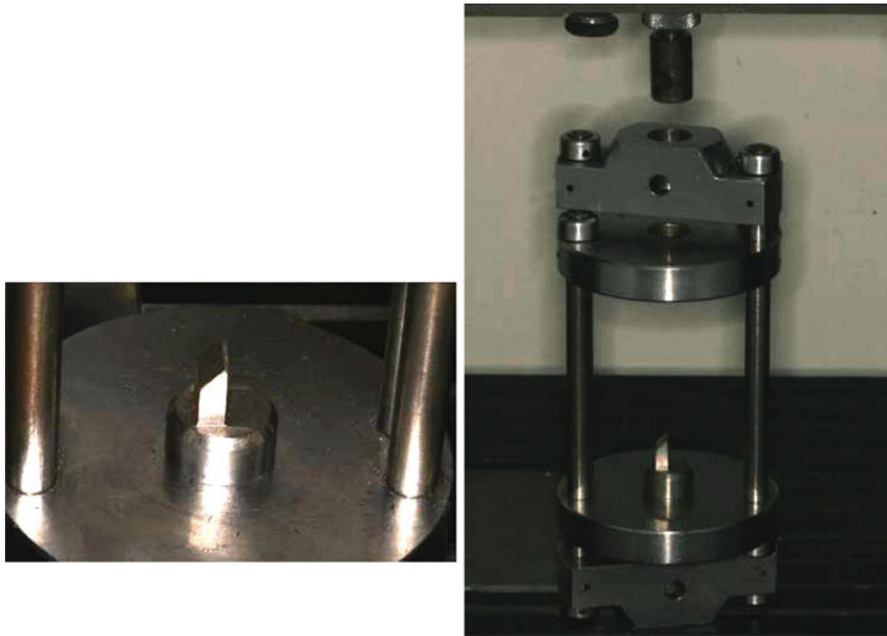

Fig. 2.1 Details of the testing machine cell used for bond strength tests

2.2.1.3 Limitations

As a rule, bond strength data interpretation should be limited to relative effectiveness of the adhesives included in the study, and thus the tactic of extrapolating absolute values and comparing them with a supposedly clinically derived, "gold standard" should be avoided. This is because the bond strength data derived from a given experiment are associated with the conditions, materials, procedures, and testing variables for this specific experiment and cannot be transferred to a different testing environment.

Although the orthodontic literature is saturated with a wide variety of bond strength papers, considerable variability can be seen among the results for the same materials, probably deriving from the multiplicity of test configurations, as well as the assumptions and approximations integrated in experimental methodologies. For example, it has been demonstrated that the variability among various manufacturers with respect to wing design or dimensions for brackets of a nominally identical prescription may contribute to the misalignment of loads during testing. Thus, the resultant bending moment may vary considerably, which impacts on the stresses developed at the interfaces of the bonding system (enamel adhesive and adhesive bracket). Additional important factors, which may vary among studies, include the method of load application, e.g., tension, shear/peel or torsion, and the crosshead speed of the testing machine, which is typically set to 1 mm/min but may range among studies from 0.5 to 10 mm/min.

The mode of load application and the instrumental configuration for bond strength testing have been discussed by Katona and Chen [11]. Finite element analysis has established that the stress distribution within the adhesive layer and the stresses generated in the brackets and enamel during testing are inhomogeneous, contradicting the uniform stress assumption that has been prevalent in the majority of *in vitro* experimental protocols. Evidence emphasizing the inappropriateness of comparing results derived from different loading modes (shear, tension, and torsion) was presented, and it was shown that the maximum stresses developed in the orthodontic bonding system under tensile loading may be five times greater than the reported average stress [12]. Hence, traditional bond strength studies substantially underestimate the probability of system failure. Moreover, failure analyses that are intended to provide inferences about the strength of individual components of the bonding system based on their prospective interfacial fracture characteristics should be questioned [13]. This is because the site of failure may arise from crack initiation caused by higher stresses compared with other areas, which is not taken into consideration in the traditional assumption of homogeneous stress.

The validity of comparing results of similar studies is affected by the experimental test configuration, as analyzed by Fox et al. [14]. The applied force may generate moments of various magnitudes, depending upon the distance of the point of force application from the bracket base surface. This parameter may complicate the extrapolation of conclusions regarding the anticipated failure events [15].

The reliability in simulating the strength of appliances during mastication and the occasional contact of brackets with opposing teeth may be questioned, since the standard rates employed in the literature are vastly smaller than the velocity at which teeth occlude during chewing, which may reach 2,000 mm/min [2]. This value greatly affects the risk of bond failure, because very high loading rates eliminate the viscoelastic response of the polymeric adhesive to the applied load, inducing a stiffer elastic response, which decreases bond strength [2].

In addition to the practical problems and standardization difficulties encountered in bond strength testing, a concern has arisen during recent years about the actual clinical relevance of *ex vivo* bond strength protocols. These studies fail to simulate the multifactorial intraoral aging of resin composites, which include pH fluctuation, complex cyclic loading, microbial attack, and enzymatic degradation [2].

In spite of the presumed appropriateness of simulating the *in vivo* milieu in laboratory testing, it is worth noting that the oral environment contains a number of parameters which are impossible to reconstruct in an *ex vivo* model. Some of these factors are the stresses arising from an activated archwire coupled with occlusal loads, extreme pH and temperature variations, and the presence of complex oral microflora and their by-products. This latter factor has been found capable of inducing substantial alterations in the structure and surface properties of orthodontic adhesives [16] and archwires in the oral cavity [17]. In particular, orthodontic adhesive degradation induced by microbial attack during treatment has been recently described by Matasa [16], who examined retrieved brackets intended for recycling.

In summary, some critical aspects of orthodontic bond strength protocols that affect the outcome of research trials may include the following:

1. The crosshead speed of the loading plate in shear testing is usually set at 0.5 mm/min for consistency, although this value lacks correspondence to clinical conditions. *In vivo* debonding incidents are expected to occur at much higher impact velocity where viscoelastic behavior of the adhesive, which may be important at low crosshead speeds, is largely absent.
2. In debonding procedures where the bracket is pulled with the use of a wire loop, the loop harness adaptation and frictional resistance may complicate interpretation of the results. Katona and Chen [11] propose that long, thin wires should be used in such an experimental model.
3. Bracket design may contribute to misalignment of load application, making the bonding system prone to failure, depending on the stress gradients generated. It has also been found that variability exists among manufacturers with respect to wing design or dimensions for brackets with a nominally identical prescription. This variability poses a substantial problem for the comparison of studies evaluating bracket bond strength.

2.2.2 Degree of Conversion

2.2.2.1 Background

During the setting process, orthodontic adhesives are polymerized by a free radical mechanism, in which the functional dimethacrylate monomers are converted into a polymer network. However, the *in situ* polymerization does not always fully convert the monomer to a polymer. It is evident from many studies that all of the dimethacrylate monomers exhibit considerable residual unsaturation of the carbon-carbon double bonds (C=C) in the final product. The degree of conversion (DC) ranges from 45 to 60 % [18]. The unconverted methacrylate groups must reside in the polymer network either as residual monomer or as pendant side chains (in the majority of cases) which extend from the main chains by virtue of having reacted at only one end of the difunctional molecule.

The DC depends upon numerous factors: (a) the structure, composition, type, and polarity of the monomer molecules contained in adhesives; and (b) the exposure time, photoinitiator concentration, light intensity emitted by the curing unit at the peak absorbance wavelength of the photoinitiator, and filler volume fraction in light-cured adhesives [19].

In general, the DC of polymer adhesives modulates the physical and mechanical properties of the material, particularly solubility and degradation. This effect has a pivotal role in altering the biological performance of these materials, since an insufficiently dense polymer network arising from a decreased conversion of (C=C) bonds results in monomer leaching and release of substances, such as plasticizers, polymerization initiators, and inhibitors. Concurrently, the demands for the safety of the patients have strongly been emphasized in various legislations, because of the potential side effects arising from the action of various polymer

derivatives to induce allergic, mutagenic, and carcinogenic effects at the cell and tissue levels [20].

2.2.2.2 Description

FTIR (Fourier transform infrared) spectroscopy has been extensively used to study the degree of C=C conversion in dental dimethacrylate-based resins for various applications (sealants, adhesives, and restoratives) and to evaluate the extent of the acid–base reaction in glass-ionomer materials and their modifications. The basic principles of this technique are based on the fact that when a polymer sample is irradiated by infrared radiation (IR) at photon energies from about 0.05–0.5 eV, the molecules are excited into specific vibrational states due to resonance absorption at frequencies characteristic of the chemical bonds present. Thus, information about the structures of polymeric materials can be obtained. However, this is possible only when changes in dipole moment occur after irradiation. Most studies are performed at the mid IR region of 4,000–400 cm^{-1}.

A major use of FTIR spectroscopy is quantitative analysis. Matching of an unknown spectrum with a reference spectrum is the most positive form of identification. Otherwise band-by-band assignments should be made based on reference databases or custom-built spectral libraries. Subtraction techniques can be employed for the interpretation of spectra, but care should be exercised with internal reflectance and photoacoustic spectroscopy techniques where the depth of beam penetration depends on the wavelength. Several techniques have been introduced for interpretation of spectra that are based upon mathematical algorithms, which may assist quantitative analysis, such as factor analysis for determining the number of components in an array of mixtures or resolution-enhancement methods like derivative spectroscopy and peak deconvolution. Quantitative analysis is based upon Beer's law. Several techniques have been developed like the calibration curve method, the standard addition method, the absorbance ratio method, the internal standard method, and the coincidence band method. For quantitative analysis in internal reflectance spectroscopy, it is essential that the sample is always in intimate contact with the same surface area of the crystal.

An experimental procedure [21] for the measurement of DC is described in the following paragraphs. After setting, cellulose strips were removed from the bracket-bonded adhesive flat surfaces, which would correspond under clinical conditions to the material in contact with enamel. These surfaces were analyzed using micro-multiple internal reflectance FTIR (micro-MIR FTIR) and the apparatus shown in Fig. 2.2. The flat surfaces were pressed against the sampling surface of a KRS 5 minicrystal of the micro-MIR FTIR accessory. The accessory cell (Fig. 2.3) was then placed on a FTIR spectrometer that was interfaced with a data station. Spectra from each resin surface were recorded under the following conditions: 4,000–400 cm^{-1} wave number range, 1–4 cm^{-1} resolution, and 20–50 scans. The mean micro-MIR sampling depth was estimated as 3 μm at 1,000 cm^{-1}.

The DC of each specimen was estimated on a relative percentage basis with the two-frequency method and the tangent baseline technique [21]. Aliphatic (linear) C=C bond-stretching vibrations at 1,638 cm^{-1} were chosen as the

Fig. 2.2 Instrumentation for FTIR-ATR spectroscopy and FTIR microscopy

Fig. 2.3 FTIR accessory cell

analytical frequency, whereas the aromatic (C..C) bond-stretching vibrations at 1,605 cm^{-1}, which are not affected by the polymerization reaction, were selected as a reference frequency. In the cases where the adhesives contain no aromatic monomers, the C=O ester groups at 1,638 cm^{-1} or the N-H stretching vibration band at the region near 3,380 cm^{-1} was chosen as a reference frequency [21].

The % DC is obtained from the relationship that % DC = 100 (1 − RDB), where RDB is the fraction of residual C=C double bonds. The RDB can be estimated from an equation [21] containing four peak areas: (1) A_p (C=C), the net peak absorbance area for the set material at 1,638 cm^{-1}, (2) A_m (C=C), the net peak absorbance area for the unset material at 1,638 cm^{-1}, (3) A_p (C..C), the net peak absorbance area of the set material at 1,605 cm^{-1} and (4) A_m (C..C), the net peak absorbance area for the unset material at 1,605 cm^{-1}.

2.2.2.3 Limitations

The IR method is essentially a surface analytical technique with a mean sampling depth of 3 μm, and consequently the middle zone of minimum conversion in the adhesive cannot be analyzed. Thus, the DC has a poor predictive value for the biological properties of orthodontic adhesives owing to the unique setting characteristics of these materials, noted in the following paragraph, which differentiate them from restorative resins.

2.2.3 Leaching

2.2.3.1 Background

Besides the lack of available evidence on biological properties of adhesives, achievement of a consensus on their behavior is complicated by the unique mode of application of these materials, which involves thin layers with distinctive setting characteristics [22]. These factors, which differ substantially between orthodontic adhesives and restorative composite resins, may modify the qualitative and quantitative aspects of leaching. Therefore, evidence derived from associated disciplines such as restorative dentistry, where there is an abundance of data on the DC of composite resins, may be irrelevant to the orthodontic analogue.

There has been only limited assessment [23] of the biological properties of the constituent components of composite resins on an individual basis. Inconclusive evidence has been found because various material constituents tested in an isolated form may produce different effects compared to the material as a whole [24]. This may be attributed to alteration of the reactive status of some components by the simultaneous presence of other constituents and the interactions of these substances during aging.

Lastly, the cell type utilized in assaying the biological effects of resins is crucial for the interpretation of results. In the past, immortalized mouse cell lines or even cancer cells have been used in relevant biocompatibility testing for biomaterials. Obviously any extrapolation of the findings of these studies to the normal human tissue is highly problematic.

2.2.3.2 Description

Monomer leaching has usually been investigated with high pressure liquid chromatography (HPLC), which is one of the most powerful tools in analytical chemistry, with the ability to separate, identify, and provide quantitative information about the

compounds that are present in any sample that can be dissolved in a liquid. Today, trace concentrations of compounds, as low as parts per trillion (ppt), are easily obtained. HPLC can be applied to pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals, in addition to dental polymers.

With liquid chromatography, a material for analysis (analyte) passes down a chromatographic column under the action of gravity, and separated colored bands are observed. The bands correspond to different chemical species that were originally contained in the test sample. Separation is based on chemical attraction of some species or compounds to the particles of a stationary phase, which causes these species to move more slowly in the column, while other species are attracted to the solvent (mobile phase) in the column and move more rapidly. Thus, chemical species in the analyte are distributed or partitioned between the mobile phase and stationary phase, creating the separation into bands.

Liquid chromatography can be performed by three primary approaches. In all cases, the analyte must be dissolved into a liquid that is then transported by the solvent onto or into the chromatographic device:

1. The sample is "spotted" onto, and then flowed through, a thin layer of chromatographic particles fixed onto the surface of glass plates. The sample appears black, but is actually composed of yellow, red, and blue dyes. The bottom edge of the plate is placed in a solvent. The flow is created by solvent diffusion through the dry particle layer (capillary action) and movement up the glass plate. This is called thin layer chromatography (TLC).
2. The sample is "spotted" onto paper to which solvent is added to create flow. This is called paper chromatography.
3. In the most powerful approach, the sample passes through a column or device containing appropriate particles, which are called the chromatographic packing material, stationary phase, or adsorbent. Solvent flows continuously through the column. At a point in time, an injection of the sample solution is made into the solvent stream, which then carries the sample through the column. The compounds in the sample can then be separated because they travel at different individual speeds through the device. The designation of HPLC originally referred to the need for high pressure to generate the flow required for liquid chromatography in packed columns.

Specimen preparation is identical to that for DC specimens, with the exception of applying a cellulose strip to the bracket base and background surface so that recovery of the adhesive layer separately from the bracket is possible. Specimens are immersed in sterile tubes containing 50 mL of 0.9 % w/v saline solution and stored at 37°C for 2 months. During immersion, the solution is agitated twice daily. At the end of this period, 40 mL of eluent is removed from each solution, and the saline samples are processed for HPLC. The analysis is performed using methanol/water (4:1) mobile phase ratio, isocratic elution mode, 1 mL/min flow rate, and detection at 254 nm. The column is calibrated with known concentrations (standards) of TEGDMA and Bis-GMA solutions of each monomer in methanol.

Linear fittings of the calibration curves are used to calculate the concentration of monomers in the saline solution from the area of chromatographic peaks at the corresponding retention time.

2.2.4 Fluoride Release

2.2.4.1 Background

It is well known that the presence of orthodontic appliances impairs the efficacy of oral hygiene, increasing the incidence of demineralization of tooth surfaces. Loss of the mineral surface of the tooth results in clinically detectable white spots, which are most pronounced at the gingival region where a greater accumulation of plaque occurs.

To overcome this problem, protective measures such as oral hygiene instruction, mechanical removal of plaque, and the topical application of fluoride agents have designed [25]. However, the efficacy of those measures depends on patient cooperation and compliance. For this reason manufacturers incorporate fluoride into orthodontic adhesives in order to achieve high levels of fluoride release.

2.2.4.2 Description

The fluoride release is usually measured in a solution by using a fluoride ion-specific electrode. There are two ways to measure this release:

1. The test specimens of each adhesive are formed into disks of specific diameter and thickness by using a mold. All materials are handled according to the manufacturers' instructions. Each test specimen is immersed in deionized water in a sealed container stored in an incubator at 37°C. The bottom of each container has a raised center that ensures that the sample disk is tilted to expose all of its surfaces to the water. The fluoride levels in the solutions are measured, and the solutions are changed daily for the first 7 days, weekly for the next 3 weeks, and monthly for the next 4 months, followed by an additional 10 days of daily water changes and fluoride measurements. When the solution is changed, each specimen is rinsed with deionized water to minimize carry-over contamination, and the rinse water is added to the sample solution. The sample solution is allowed to reach room temperature before the fluoride level is measured. Fluoride levels in parts per million are obtained using a fluoride ion-specific electrode connected to an expandable ion analyzer. The total fluoride released in μg is calculated by multiplying the parts per million (1 ppm = 1 μg/mL) by the water sample volume. The total fluoride is then divided by the area of the sample disk to obtain the fluoride release in $\mu g/cm^2$. Weekly cumulative levels are divided by 7 to yield values in $\mu g/cm^2$/day, and an analogous procedure is used to obtain daily values from total monthly levels.
2. Brackets are cemented onto teeth with orthodontic adhesives. The teeth with brackets are immersed in deionized water in a sealed container and stored in an incubator at 37°C. The fluoride levels of the solutions are tested, and the solutions changed daily for 7 days, and then once a week for 3 weeks. The solutions are allowed to reach room temperature before testing for fluoride level. Fluoride analysis and

calibration are carried out in the same manner as for the disk solutions described previously. Fluoride release is recorded as parts per million and converted to μg/cm^2/day, based on an assumed width of 0.1 mm adhesive at the bracket base perimeter.

2.2.4.3 Limitations

There has been very little standardization of the protocol for fluoride release studies. Wide variations are found in the size and shape of the sample disks, the amount of storage water, the frequency of water changes, the timing of fluoride measurements, the length of the observation period, and the units of measurement [26].

The small exposed area of adhesive around the bracket base, combined with the relatively large quantity of water required to immerse the tooth, results in very low levels of fluoride that dropped to meaningless levels after only about 7 days. Future research should include multiple brackets on each tooth and removal of the tooth roots to enable the absolute minimum volume of storage water to be used. This, in turn, will result in higher concentrations of fluoride that allow monitoring the fluoride release over longer time periods [27]. The optimal amount and timing of fluoride release have not been confirmed because the method to correlate fluoride release levels from an in vitro study with *in vivo* fluoride release or clinical reduction in demineralization has not been determined. Further complicating the speculation of clinical benefits based on in vitro testing is the phenomenon of fluoride recharging, which may have considerable influence on protection from demineralization. The discrepancies observed in fluoride release from orthodontic adhesives, depending on the timing of water changes and between sample disks and bracketed teeth, suggest that caution must be exercised in evaluating existing and future fluoride release studies. Further research is required to increase the understanding of fluoride release from orthodontic adhesives, in particular, different water and recharging conditions. The relationship between bracketed teeth and sample disks also needs to be studied. These recommendations will help define in vitro test conditions that more closely mimic in vivo conditions which, in turn, will provide more meaningful clinical information.

2.2.5 Critical Surface Tension

2.2.5.1 Background

Clinical reports have shown that patients who receive orthodontic treatment are more susceptible to enamel white spot formation [28]. Orthodontic brackets have also been found to inflict ecologic changes in the oral environment, such as decreased pH and increased plaque accumulation [29]. In general, adhesion to surfaces is a result of specific reactions, electrostatic interactions, and van der Waals forces [30]. Although it is clear that initial attachment is an important factor governing further colonization, the mechanisms of the attachment and those of subsequent adhesion may differ significantly. A search of the relevant published reports reveals a strong trend toward investigation of the early attachment mechanism involved in microorganism colonization through the study of interfacial surface free energy, hydrophobicity, and zeta potential of interacting surfaces [31, 32]. Specifically, a significant

correlation between the surface free energy of a material and its plaque-retaining capacity has been established, with the higher energies showing a favorable effect on bacterial adherence [33]. For this reason, the study of the surface free energy of orthodontic appliances by evaluating the critical surface tension is of importance.

2.2.5.2 Description

Surface free energy and work of adhesion of selected materials are evaluated by contact angle measurements. For this purpose, rectangular specimens (20 mm × 10 mm × 2 mm) are prepared. The specimens are polished with 1 μm metallographic Al_2O_3 powder, which is removed in an ultrasonic bath with water, after cleansing with a detergent [34]. The contact angles (θ) of a homologous series of liquids on the specimen surface are measured by the sessile drop method.

Specifically, very small (20 μL) drops of each liquid are delivered from a 5 mm height by a microsyringe onto the surface of a horizontally leveled specimen under controlled temperature and humidity (usually 23°C and 50 % relative humidity). After 20 s of spreading time, the drop profile is photographed. The contact angle between the liquid drop and the plane surface of the solid substrate is calculated trigonometrically from the height and base dimensions of the meniscus formed. The cosine of the contact angle and the solid–liquid (γ_{SL}) and liquid–vapor (γ_{LV}) interfacial tensions for each liquid are used to determine the following parameters for each material: [33] (a) the critical surface tension (γ_c) and (b) the work of adhesion due to nonpolar or dispersion forces (W^D).

At equilibrium, the following condition must be satisfied: $\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos\theta$, where θ is the angle formed between the liquid drop and the plane surface of solid substrate on which the drop is resting (contact angle). This angle can be measured directly (in degrees) or obtained from linear measurements using the following equation, which becomes more accurate with increased wetting (lower θ):

$$\theta = 2\arctan\,(2h/H)$$

where h and H are the height and base of the liquid meniscus, respectively. The wetting phenomena on surfaces have a polar component that involves mainly hydrophilic interactions and a nonpolar component that includes van der Waals forces, Debye forces, Keesom electromagnetic interactions, and London dispersive forces [33–37]. Thus, $W^A = W^P + W^D$ and $\gamma_c = \gamma_C^P + \gamma_C^D$, where W^A is the total work of adhesion, and the P and D superscripts denote the polar and nonpolar or dispersion components of the measured variables.

The nonpolar component of the work of adhesion can be found as: $W^D = W^A - W^P$. Finally, the critical surface tension of the surface (γ_c) is given by the equation:

$$\gamma_c = \Sigma(\gamma_{LV}\cos\theta)/6$$

where the summation contains six terms, each of which has the appropriate value of γ_{LV} and θ for each of the six homologous Zisman liquids.

2.2.6 Limitations

Raw materials should be chosen to develop a standardized testing method, independent of topographic factors that may complicate the wetting and adsorption processes. This consideration is especially accentuated by the small sizes and complex configurations of orthodontic brackets that limit the selection of a surface suitable for testing the bracket base. Moreover, base morphologic factors may vary substantially. Furthermore, variations in composition (e.g., hydrophobic silane coating of base) strongly affect wetting characteristics and surface free energy, limiting applicability of the values derived to the bracket base exclusively. In addition, differences in surface roughness have been postulated to account for decreased contact angle values.

In general, a surface energy range of 30–50 erg/cm^2 has been empirically estimated to stimulate biologic adhesion. The surface tension of human saliva has been proposed to vary over a range of 53–60 dyn/cm [38]. However, these values can be strongly affected by methodologic approaches used [39]. Specifically, studies on the surface tension of biomaterials, with water or an aqueous saline solution as the exclusive indicator of wettability, fail to investigate the role of dispersion forces in the adhesion process, because of the highly polar nature of water [40]. Therefore extrapolation of surface energy values from these studies for comparison purposes is inappropriate. Since the nature of most biologic bonding mechanisms is still uncertain, such a restriction in testing parameters imposes a barrier to elucidating information about the adhesion mechanisms involved.

2.3 Research on Bonding-Induced Enamel Color Alteration

2.3.1 Background

In general, orthodontic bracket bonding and debonding have been shown to cause enamel loss. These alterations may also lead to decalcification in the form of white spot formation and discoloration. Enamel color alterations may also result from the irreversible penetration of resin tags into the enamel structure [41]. Since resin impregnation in the enamel structure cannot be reversed by debonding and cleaning procedures, enamel discoloration may occur as well through direct absorption of food colorants and products arising from the corrosion of the orthodontic appliance [42]. Alternatively, the invasive procedures of resin grinding following debonding may adversely affect the color variables of enamel by altering its surface properties.

2.3.2 Description

The most popular way to measure the color changes is the use of instrumental analysis because of its objective and quantifiable nature. The usual apparatus employed for color measurement is the colorimeter (Fig. 2.4).

Fig. 2.4 Color analysis device

The teeth used for color measurements must be free of caries, restorations, and coronal staining. On black rectangular pieces of adhesive tape, a round opening of 3-mm diameter is cut to match the size of the colorimeter window. The tape is applied to the middle third of the buccal surface of the tooth to provide a means to standardize the enamel surface area intended for analysis and is further secured on the crown with a cyanoacrylate glue, applied on the mesiodistal surfaces to avoid contamination of the prospective bonding buccal surface. The teeth are code-numbered for identification purposes, and the exposed enamel surfaces are colorimetrically evaluated according to the CIE Lab system [43] (CIE L^*, a^*, b^*), employing a repeated measures design. The CIE color L^* parameter corresponds to the value of degree of lightness in the Munsell system, whereas the a^* and b^* coordinates designate positions on red/green and yellow/blue axes, respectively.

The color measurements are performed initially before bonding (baseline), following debonding and cleaning, and after artificial aging. The color difference (ΔE) for each sample is calculated from the baseline measurement and any time interval using the following equation:

$$\Delta E = [\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}]^{1/2}$$

2.3.3 Methods for In Vivo Color Measurements

There are two common methods of analyzing in vivo the apparent tooth color: visual determination and instrumental measurement [43]. Visual determination by comparison of teeth and shade guides is considered highly subjective but still remains the most frequently applied method in dentistry for color communication [44]. However, several factors such as external light conditions, experience, age and fatigue of the human eye, and inherent limitations of the contemporary shade guides can influence consistency of visual color selection and specification [45, 46].

The general demand for objective color matching in dentistry, coupled with rapid advances in optical electronic sensors and computer technology, has made instrumental measurement devices a supplementary adjunct to visual tooth color evaluation [47]. Currently, a variety of commercial systems, including tristimulus colorimeters, spectroradiometers, spectrophotometers, and digital color analyzers, are used in clinical and research settings for objective color specification [48]. Color measurements are highly dependent on the design and optical elements of the color-measuring devices. In the majority of these instruments, color parameters are quantitatively determined using the CIE system, which enables color perception to be based on standard observer curves and standard illuminants [49, 50]. The influence of systematic and random errors on the quantitative evaluation of color, and subsequently the precision of various commercial instrumental devices, has been documented [51–55]. Systematic errors are inherent in all instruments and result from calibration techniques, fluorescence, metamerism, and variations in measurement geometry [56]. These errors are difficult to manage and can be expected to adversely affect instrument accuracy regardless of the degree of precision or control of environment [57]. The amount of uncertainty during the measuring process is associated primarily with random errors. Several methods have been suggested for reducing the detrimental effect of variability of color measurements, either with the use of multiple measurements and averaging or with better control of the methodological and environmental factors [56].

2.3.4 Tooth Color Alterations Associated with Orthodontic Treatment

Despite the extensive evidence available on enamel effects associated with orthodontic treatment using fixed appliances [58], the incidence of bonding-induced color alterations has not been thoroughly investigated. A search of the relevant literature revealed only two in vitro studies [59, 60] relating to the enamel color changes during bracket bonding and debonding. Although the results of these studies showed that some alterations in tooth color are inevitable, it must be emphasized that in vitro tests may not be a reliable reflection of the clinical situation [61]. Color determination of natural teeth is affected by many factors present in the oral cavity, such as lighting conditions of the surrounding environment, light scattered from adjacent perioral and gingival tissues [62, 63] and, therefore, the reflection characteristics of the underlying surfaces as a function of wavelength.

Tooth color changes associated with orthodontic treatment using fixed appliances were recently evaluated in vivo [64]. Two prospective clinical trials were conducted, aiming to assess the color alterations of natural teeth during the active and retention phases of treatment. In the first study, spectrophotometric color data from a standardized circular area on each tooth were recorded in vivo prior to and following active treatment. In the second study, color measurements were performed after debonding and cleaning procedures 1 year later. The reflectance spectrophotometer (SpectroShade™, MHT Optic Research AG, Zurich, Switzerland) was used in order to objectively assess these color alterations, and this instrument was found to provide precise measurements during longitudinal evaluation in vivo [65].

The results of both studies clearly showed that orthodontic treatment does indeed influence the CIE color parameters of natural teeth. Following active orthodontic treatment, all measured types of teeth demonstrated significant color changes ranging from 2 to 3.6 ΔE units. Smaller color differences, ranging from 1.48 to 2.11 ΔE units, were also observed during the first retention year. The clinical relevance of the color changes was addressed by comparing the color difference values with a standard limit of clinical detection, set to 3.7 ΔE units, beyond which the differences were considered as clinically unacceptable [66]. It was found that 13 and 4.6 % of the bonded teeth presented visible, clinically significant color changes at the end of active and retention phase, respectively. This outcome may be caused by the irreversible nature of modifications in enamel microstructure associated with bonding, debonding, and cleaning procedures that affect the color parameters of natural teeth, as well as the exogenous and endogenous discoloration of the remaining adhesive material following removal of orthodontic appliances.

2.4 Research on Force Relaxation of Elastomeric Chains

2.4.1 Background and Summary of Previous Experimental Methods

Elastomeric chains, generally manufactured from polyurethane, are in widespread clinical use to provide orthodontic force for closing spaces and correct rotations. Despite the abundance of data available on the force decay of elastomeric chains produced by various research efforts during the past three decades, a reliable and accurate method to study this parameter has yet to be proposed.

Table 2.2 presents a summary of the methods that have been employed, along with their advantages and disadvantages. It can be seen that the relevant literature lists basically three methods for the study the force relaxation of elastomeric chains.

The first method in Table 2.2 involves stretching of an elastomer by attaching its terminal modules to abutments placed on a frame [67, 68]. The choice of chain length is made so that the chain is extended up to 50–60 % of its original length. Periodically, the one end of the chain is detached from the abutment, and the force required to stretch the chain to the desired length is recorded with the commercial force-measurement apparatus. Although this method allows for the study of force fluctuations in a controlled environment, which is facilitated by the immersion of the whole frame in various media such as artificial saliva [69], it has two major weaknesses. The first relates to the lack of continuous data arising from the periodic recording of the force levels, since force measurements are initially obtained at intervals such as the first, sixth, and twelfth hours after extension, followed by weekly measurements thereafter for the period of study, which often extends to 3 weeks or longer. More importantly, the second major weakness is that this method induces incorporation of stresses arising from the laborious handling of the chains, because during each recording the elastomer must be relieved from the abutment, re-stretched by the force-delivery apparatus to 50–60 % of the original length to

Table 2.2 Summary of major methods for investigating force decay of elastomeric chains

Method	Advantages	Disadvantages
1. Stretching of elastomer across fixed abutments, removal of specimen, and record force with custom apparatus	Specimens can be immersed in various media to simulate oral environment	Recording of noncontinuous data; excessive handling of specimens induces fatigue; curve is constructed from multiple specimens to prevent same specimen from being used repeatedly
2. Same as above but force measured with tensometer	Same as above	Same as above
3. Specimen attached to tensometer and subjected to sequential loading and unloading cycles; force required for extension is recorded before and after cyclic loading	Accurate recording	Irrelevance to force relaxation
4. Attachment of specimen to tensometer; extension and suspension for specific time, followed by force removal and re-extension; force is recorded prior to and after suspension	Accurate recording	Irrelevance to force relaxation

measure the force, removed from this apparatus, and finally stretched again for reattachment to the abutment. Because such repeated stresses impose fatigue phenomena on the chain, it is expected the behavior of the elastomeric specimen would be substantially affected. Alternatively, some authors have used multiple specimens, with each chain tested only once for each time period to prevent the incorporation of fatigue. Although this method allows for decreased stresses to be experienced by a given specimen during testing, it employs multiple specimens for the construction of a single curve, which complicates the data analysis process.

The second and third methods in Table 2.2 employ mechanical testing machines to study force relaxation, using various methods to initiate strain of the elastomeric chain. It has been proposed that the attachment of each one of the terminal modules of a chain specimen to the fixed base and jig of a testing machine, respectively, followed by initiation of sequential loading and unloading cycles could be used for measuring relaxation. In this case, the force required to induce the original extension after one or more cycles is recorded and compared to the initial force [70]. It must be noted that while these methods utilize more technologically advanced instrumentation relative to the manual recording of the force with a conventional force-measurement apparatus, they too possess major deficiencies. Specifically, the use of loading-unloading cycles may by definition be irrelevant to both the force relaxation experiment and the clinical analogue. In addition, suspension of these elastomeric materials for periods of time as short as 30 min, which represents a fraction of the full term of their expected intraoral service, and reloading them afterwards is inappropriate and may not provide true insight into the performance of the material.

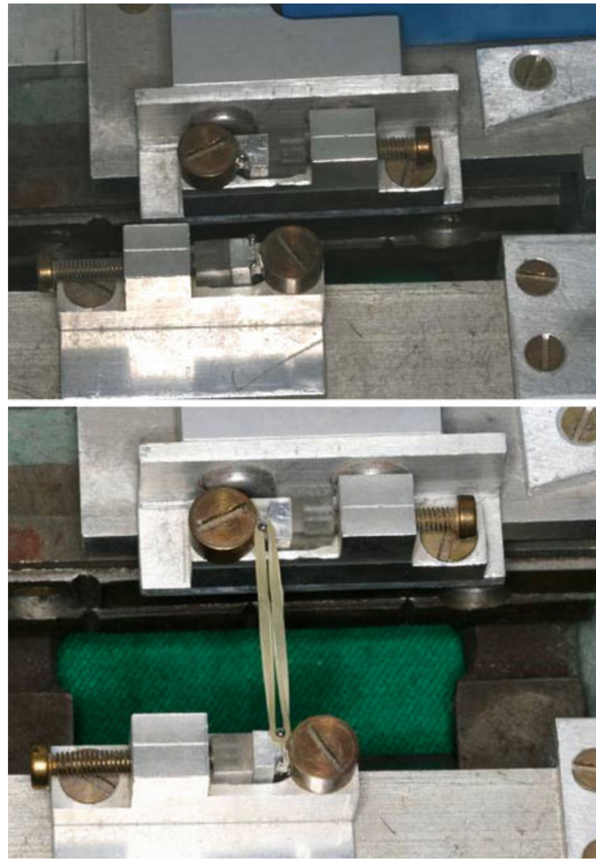

Fig. 2.5 Custom-made configuration for force relaxation testing

2.4.2 Description of New Experimental Method

The initial length of all specimens is measured with a digital caliper. For this purpose, the inner circular ends of the chain are taken as reference points, and the distance is measured in mm. Each specimen is extended 50 % of its original length. A portable test assembly is employed to monitor the force exerted from a stretched elastomeric chain during the testing period, in real time and with a continuous data collection mode (Fig. 2.5). The main components of this assembly are a 2-kg load cell connected with a strain gauge and an amplifier, a power source unit, and a machine vise. Upon extension of the chain to the desired length by opening the vise jaw, there is a change in the voltage, which is magnified by the amplifier and sensed by the strain gauge unit of the assembly. The assembly is connected to a signal-conditioning unit, linked to a computer with data-logging software via an ADC-16 multichannel data acquisition unit. Recording of force for the entire period of study is performed in real time, without any operator interferences and under a continuous mode, at 1-min intervals.

Because the method of recording voltages involves mV units possessing a negative sign, the data should be transformed to gain physical meaning, and this is

achieved by multiplying the voltages by −1. The calibration of force units (g) with mV units is performed by extending a chain specimen to increasing lengths (50–250 %) in order to produce various forces. These are determined with the force-measurement apparatus, and the selection of g as opposed to N units for force is based on the familiarity of clinicians with this unit.

2.4.2.1 Limitations

Further questioning of the methods employed for the estimation of the force decay may be provided by the clinical situation and the level of force required to induce tooth movement in reality. Since the earliest publications on the subject, the clinicians have found difficult to explain the paradox of moving teeth using a system, which presents a 50–70 % force loss during the first day, most of which occurs within the first hours. The literature suggests that for efficient movement of a tooth, i.e., canine, into the space of an extracted premolar, a force level of about 200–250 g is required. By adopting the force decay values proposed by some studies employing the methodology previously critiqued, it is expected that, immediately following application of the orthodontic biomechanical system, the force level would drop to 100–120 g, provided that the appropriate force level would be applied initially. Thus, a question arises to whether the force levels required to move teeth have been overvalued, or the force decay rates have been overestimated.

Bond Strength

Measure the bond strength to tooth surface *in vitro*.

The load application could be tension, shear/peel, or torsion, and the crosshead speed of the testing is usually set at 0.5 mm/min for consistency. This value lacks correspondence to clinical conditions. *In vivo* debonding incidents are expected to occur at much higher impact velocity.

The bond strength data are associated with the conditions, materials, procedures, and testing variables for this specific experiment, and could not extrapolated to the real clinical conditions.

Traditional bond strength studies substantially underestimate the probability of system failure.

The bond strength studies fail to simulate the multifactorial intraoral aging of resin composites, e.g., pH fluctuation, complex cyclic loading, microbial attack, and enzymatic degradation.

Degree of Conversion

The degree of conversion (DC) of resin adhesives ranges from 45 to 60 %.

The DC depends upon numerous factors as the type, structure, composition of the monomers contained in adhesive, and polymerization properties (the exposure time, photoinitiator concentration, light intensity)

DC of polymer adhesives modulates the physical, mechanical, and biological properties of the material.

Fourier transform infrared spectroscopy has been used to study the degree of C=C conversion, but this is essentially a surface analytical technique with a mean sampling depth of 3 μm, and consequently the middle zone of minimum conversion in the adhesive cannot be analyzed.

Degree of Cure Leaching

Monomer leaching influence the biocompatibility of the material and may arise adverse biological effects, i.e., allergic and hypersensitivity side effects.

Monomer leaching has usually been investigated with high pressure liquid chromatography.

In general, the lower the DC, the greater the monomer leaching.

The orthodontic adhesives involve thin layers with distinctive setting characteristics, but data for degree of cure leaching derived from restorative dentistry may be irrelevant to the orthodontic analogue.

Fluoride Release

Manufacturers incorporate fluoride into orthodontic adhesives to achieve high levels of fluoride release, in order to preserve tooth caries decalcification during the orthodontic treatment.

The fluoride release is usually measured in vitro in a solution by using a fluoride ion-specific electrode.

The amount of fluoride releasing depends on size and shape of the sample disks, the type and amount of storage solution, the frequency of water changes, the timing of fluoride measurements, and the length of the observation period.

The fluoride release does not mean that this amount of fluoride necessary would be incorporated into tooth tissues.

Critical Surface Tension

The critical surface tension estimates the surface free energy of orthodontic appliances influencing the microbial plaque-retaining capacity.

The surface free energy and work of adhesion of the selected materials are evaluated by contact angle measurements.

The small sizes and complex configurations of orthodontic brackets limit the selection of a surface suitable for testing the bracket base.

Bonding-Induced Enamel Color Alteration

Orthodontic bracket debonding causes to enamel loss and to the presence of resin adhesives remnants, leading thus to the white spot formation and discoloration.

Two common methods of analyzing in vivo the apparent tooth color: visual determination and instrumental measurement.

Visual determination by comparison of teeth and shade guides is considered highly subjective.

The usual instrumental apparatus employed for color measurement is the colorimeter.

The color is evaluated according to the CIE Lab system (CIE L*, a*, b*). The CIE color L* parameter corresponds to the value of degree of lightness in the Munsell system, whereas the a* and b* coordinates designate positions on red/green and yellow/blue axes, respectively. The color difference (DE) for each sample is calculated from the baseline measurement and any time interval using the following equation:

$$\Delta E = [\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}]^{1/2}$$

In clinical condition, color alteration over 3.7 DE units are considered as clinically unacceptable.

All studies show that orthodontic treatment influences the CIE color parameters of natural teeth.

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In Vitro Assessment of Biocompatibility for Orthodontic Materials

3

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3.1 Introduction

The use of biomaterials, i.e., synthetic or modified natural materials intended to interact with biological systems, has seen steady growth during the last decades. Consequently, biocompatibility testing is attracting increasing interest. Biocompatibility has been defined as "the ability of a material to function in a specific application in the presence of an appropriate host response" [1]. In this sense, it is the interaction of all three factors – (1) the material's properties, (2) the host, and (3) the conditions under which the material is meant to function – that has to be taken into account. For example, a material suitable for a specific application in one species can be detrimental to another, or materials used satisfactorily in orthopedics may be inappropriate for cardiovascular applications because they may be thrombogenic.

Testing the biocompatibility of materials used in dental practice is of primary importance, as it may raise profound ethical, social, technical, and legal issues [2]. It is not only a matter of the patient's safety but also of the safety of the dental staff [3], as well as of regulatory compliance issues and legal liability [2]. Many parameters are involved for biocompatibility, such as genotoxicity, mutagenicity, carcinogenicity, cytotoxicity, histocompatibility, and microbial effects [4]. Thus, a battery of various in vitro and in vivo tests is required for the biological characterization of the materials under study [4]. Over 35 years ago, a pyramid scheme was proposed for the complete evaluation of a new material, beginning with "unspecific toxicity" tests, followed by "specific toxicity" tests and concluding with clinical trials [5].

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The International Organization for Standardization (ISO) has implemented a similar pyramid concept regarding dental materials: materials have to pass the "initial tests" (general cytotoxicity, systemic toxicity in animals, and mutagenicity) in order to be considered for the "secondary tests" (allergy, mucosal irritation, and inflammation assays) and only if they succeed in these, too, are they subjected to the "usage tests", i.e., the clinical trials [6].

The above schemes express something more or less self-evident: the most relevant biocompatibility tests are clinical trials on humans. However, beyond their ethical and legal complexities, they are expensive, time-consuming, extraordinarily difficult to control and sometimes difficult to interpret [2]. So, they have to be used as the final criteria after some prerequisites are fulfilled. Animal models have always played an important role in these procedures, since the information obtained from such a system applies to the organism as a whole. On the other hand, there is an increasing realization of the limitations of animal models in relation to the human organism. Thus, the use of cell culture systems of human origin seems to be an advantageous choice, since they are experimentally controllable, repeatable, fast, and relatively simple. Moreover, the considerable financial advantages of in vitro testing over the in vivo experiments – multiplied by the increasing numbers of new materials awaiting assessment – make the impetus for the use of cell culture systems even greater. Finally, in vitro tests avoid the ethical and legal issues associated with the use of animals and humans for testing. Not surprisingly – particularly in fields where an overwhelming amount of novel active compounds is produced, such as that of cancer chemotherapy – the potential value of such systems for cytotoxicity and viability testing is now widely accepted.

3.2 Selection of the Appropriate Culture Type

The vast majority of in vitro cytotoxicity testing for dental materials has been conducted on mammalian cell lines, especially murine fibroblast strains, like Balb/c 3T3 [7–9] and L929 [10], and to a lesser extent human strains, such as the HeLa cancer cell line [4] or the normal embryonic lung fibroblasts WI-38 [11]. Established cell strains – which are nowadays commercially available from a number of sources – are very well characterized, and new data are continuously accumulating in the literature regarding their properties. Furthermore, they are available in sufficient amounts to perform a large number of assays, offering the possibility of comparing numerous compounds on exactly the same cell population. According to ISO 10993-5 [12], established cell lines, such as L929, Balb/c 3T3 A31, MRC-5, WI-38, and Vero, are preferred for the biological evaluation of medical devices, and it is furthermore recommended to obtain them from recognized repositories. However, for the choice of a suitable cell culture type, among the several parameters to be considered, the target tissue for the test material is of major importance. Primary cell cultures – i.e., cells taken from the tissue of interest and placed directly in culture [13] – are offering the advantage of better mimicking the in vivo conditions, avoiding selection procedures and the possibilities of differentiation or dedifferentiation

which arise from serial subculturing. However, cell-line selection is often disregarded, when the in vitro assay is viewed only as an initial test and usage tests are going to follow. Yet, given the problems due to species specificity mentioned earlier and the different properties of cells derived from various tissues, the use of an inappropriate cell strain may lead to false results [14]. Thus, it is obviously more accurate to use human cell strains originating from the target tissue, i.e., regarding dental materials, cells from the mouth cavity, such as dental pulp, gingival or periodontal ligament ones [15–17].

One has to mention separately the use of "engineered" cells – usually transfected with a gene in vitro or derived from a transgenic animal – as a powerful tool for the disclosure of the mechanism of action of the test compound. In the same vein, the underlying mechanisms can be revealed through careful selection of cells with certain properties, e.g. the parallel use of the estrogen-responsive cell line MCF-7 and the estrogen-insensitive MDA-MB-231 can show whether the test compound has estrogenic activity [18, 19]. In addition, in vitro immortalized cells [20–22] are very useful for overcoming the problem of the limited in vitro replicative capacity of normal cells [23].

3.3 In Vitro Studies of Cell Proliferation and Cell Death

An important parameter in tissue homeostasis is the balance between cell proliferation and cell death. Hence, the study of these fundamental cell functions is indispensable for the research in areas such as cancer, fibroproliferative disorders, aging, and age-related diseases, where tissue homeostasis is severely compromised. Furthermore, the contact of a biomaterial with living tissues may affect their homeostasis, which means that biocompatibility testing requires in vitro assessment of cell proliferation and/or cell survival. In some instances, a direct or indirect determination of the number of cells in a culture may be enough to draw a rough conclusion. For example, during a first screening for potential hazardous materials, the end point is whether or not the cell number is lower after the treatment, in comparison to the control culture [24]. However, this can be the result of (a) inhibition of cell proliferation, (b) cytotoxicity or (c) a combination of both. Hence, after the first screening, a more delicate method is required in order to distinguish among the possible effects of the potential drug.

3.3.1 Cell Proliferation Assays

The direct measurement of the cell number can be performed under the microscope. The cells can be fixed and stained with Giemsa or hematoxylin to facilitate counting or even better with a fluorescent dye, such as fluorescein diacetate (see Fig. 3.1). But this technique is tedious and is suitable only for a limited number of samples. Alternatively, cells can be counted by means of a haemocytometer [25] or by an electronic cell counter. The former also becomes tedious for a large number of samples

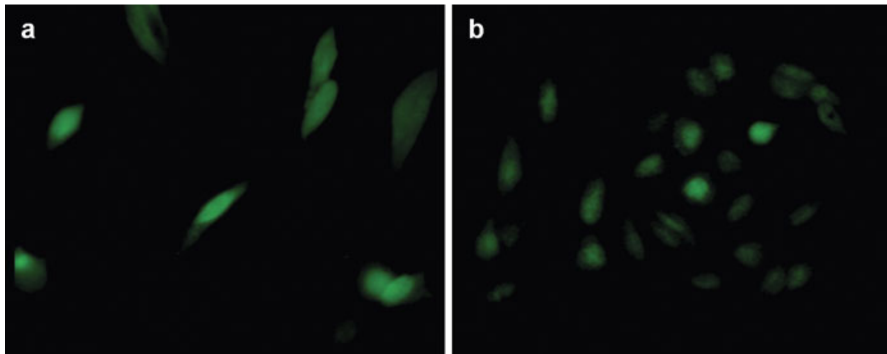
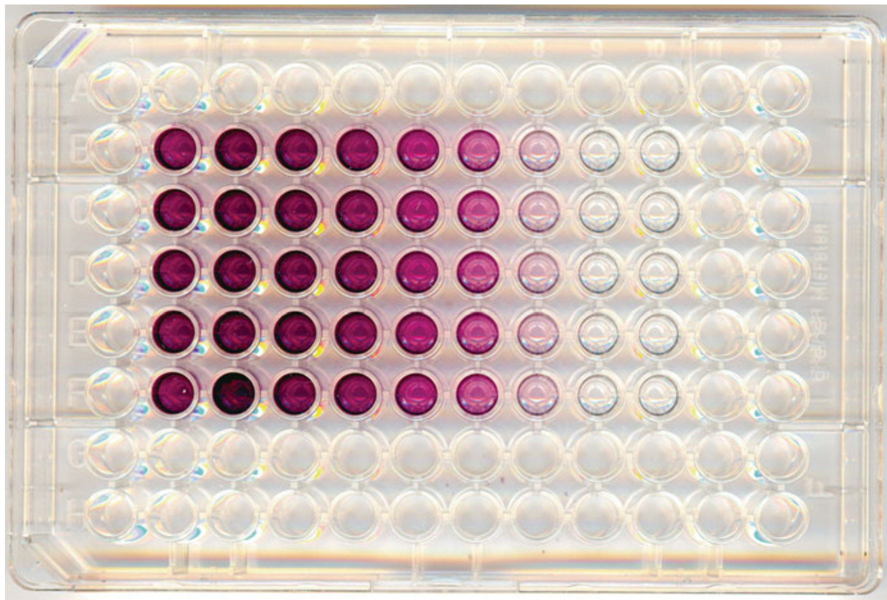


Fig. 3.1 Direct cell counting. Cells growing on two different substrata (**a** vs. **b**) were loaded with DCFH-DA for 24 h, and fixed with neutral formalin. More cells are to be counted in (**b**)

and is subject to error or bias, but enables judgements about the quality of the cell suspension, e.g., whether or not debris is included. On the other hand, the electronic cell counter is, in general, more reproducible; however, it requires larger samples and the quality of the suspension (e.g., the existence of cell clumps) may affect the result.

The indirect methods for the estimation of the cell number are mostly based on the absorption of dyes or fluorescent molecules in the cells, thus allowing comparisons of the cell numbers in the treated vs. the control cultures, by spectrophotometric or spectrofluorimetric techniques. Most of the dyes used are absorbed in the cells' proteins or in the cellular nucleic acids, and then they are solubilized by an appropriate solvent, so as to enable the spectrophotometric estimation of the amount of the absorbed dye. The most common dyes are the following: methylene blue [26–29], crystal violet [3, 30, 31], Coomassie blue [32], sulphorhodamine B [33], neutral red [24, 34–37], BCECF [2′-7′-biscarboxyethyl-5(6)-carboxyfluorescein] [38], PI [propidium iodide] [39, 40], Hoechst 33258 [41–43], alamar blue [44] and fluorescein diacetate [45]. Some of these, such as neutral red, BCECF or fluorescein diacetate, stain only living cells. They are called vital dyes, and they can be used to determine the viability of the cells, i.e., the percentage of living cells in the total cell culture. But the most widely used method for assessing viable cell numbers is the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [46], which is based on the reduction of this dye by the mitochondrial enzymes to its formazan product and the concomitant change in its absorption spectrum (Fig. 3.2). Many modifications and re-evaluations of the method have appeared [3, 16, 36, 47–50], and moreover, other tetrazolium salts have been developed, which can replace MTT [51–53].

Another way of indirect estimation of the cell number is by following the uptake of radiolabeled molecules necessary for the cell functions, such as amino acids [54, 55] or nucleotides, using either autoradiography or liquid scintillation counting [16, 56, 57]. Again these methods are dependent on the viability of the cell culture, since the radiolabeled precursors have to be metabolically incorporated in the cells, and not passively absorbed. Especially regarding incorporation of [^{3}H]-thymidine



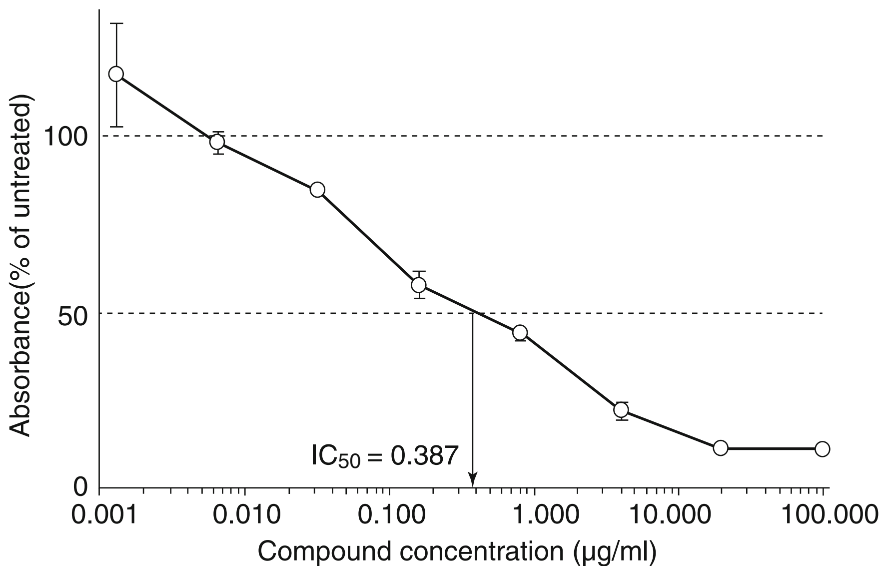


Fig. 3.2 Indirect assessment of viable cell number by the MTT method. A representative 96-well plate is shown after treatment of the cells with a cytotoxic agent for 48 h and the formation of MTT-formazan by the living cells (*upper panel*). After counting the absorbance of each well in a microplate reader, the concentration of the agent that eliminates 50 % of the cells (IC_{50}) can be estimated (*lower panel*)

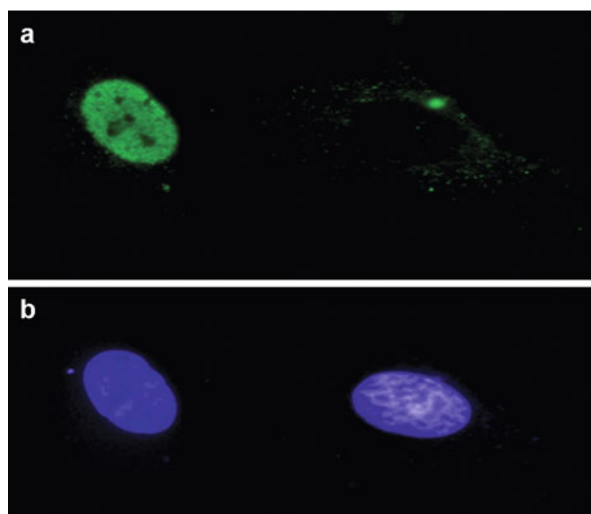


Fig. 3.3 Evaluation of BrdU incorporation by immunofluorescence. The nucleus of a cycling cell (*left*) incorporated BrdU in contrast to an arrested one (*right*), as shown using a monoclonal antibody against BrdU and FITC-conjugated anti-mouse-IgG (*panel a*). In *panel b*, the same nuclei were counterstained with DAPI (magnification 1,000×)

into newly synthesized DNA, there has been some criticism, since (1) changes in [^{3}H]-thymidine incorporation may relate to changes in the intracellular nucleotide pools or in the activities of key enzymes, such as thymidine kinase, rather than to changes in DNA synthesis; and (2) there are drugs, like 5-fluorouracil or methotrexate, which cause increased uptake of [^{3}H]-thymidine through the salvage pathway, although they inhibit DNA synthesis [58]. In spite of these drawbacks, this is perhaps the most widely used method for assessing the proliferative index of a culture. Furthermore, the method can be used also for the determination of the distribution of the cells in the various phases of the cell cycle, so it provides some clues on the mechanism of action of the test compound.

Nowadays, there is a tendency to replace radioactivity-based techniques with nonradioactive ones, since the former require specific set-up and licence, not available in all laboratories, and they pose potential risks for the personnel. In this vein, an alternative to [^{3}H]-thymidine incorporation for the estimation of DNA synthesis is the incorporation of the thymidine analog BrdU (5-bromodeoxyuridine) and its subsequent tracing in situ with the help of a specific antibody [59–61]. As shown in Fig. 3.3, the nuclei of the cells that progress through S phase incorporate BrdU and can be visualized by immunofluorescence. Although this method is much more reliable than [^{3}H]-thymidine incorporation for the estimation of the percentage of cells in the S-phase of the cell cycle, it is more laborious and, consequently, inappropriate for the screening of large amounts of substances.

Here, one should mention separately the flow cytometric techniques, which give fast and accurate results from large cell populations. Staining of the cells to be used for flow cytometry is accomplished as already mentioned above; however, the information obtained is at the single cell level and multi-parametric. So after staining of the DNA with propidium iodide (see above), the DNA content of each cell from a given cell population can be estimated, thus allowing determination of the distribution of the cells in the various phases of the cell cycle [62–66]. As an example, typical histograms

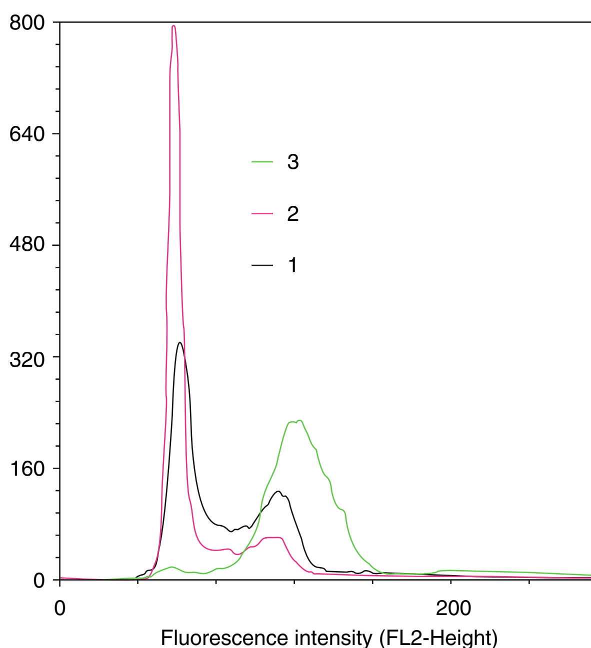


Fig. 3.4 Flow cytometric cell-cycle analysis using propidium iodide. The distribution of human cells in the various phases of cell cycle was assessed by flow cytometry after PI staining (*1* untreated cycling culture, *2* culture arrested at the G_0/G_1 phase, *3* culture arrested at the G_2/M phase)

of PI-fluorescence produced by a FACScalibur flow cytometer (Becton Dickinson) using the ModFit software (Verity) are shown in Fig. 3.4, where the left peak corresponds to the number of cells at G_0/G_1 phase and the right one to those at G_2/M. Using fluorescent antibodies and flow cytometry, the percentage of cells that are synthesizing DNA can also be estimated after BrdU incorporation (see above) [67]. Furthermore, the simultaneous detection of the expression of certain proteins, together with the above markers can give information on the mechanism of action of the test compound [68]. However, the high cost of flow cytometric techniques and the requirement of processing each sample individually are limiting their use for cases where relatively small sample numbers are being studied.

3.3.2 Cell Death Assays

Direct toxicity represents a major problem for the use of several biomaterials. For the determination of cell death there are stains special for dead cells, such as trypan blue, which was traditionally used due to its exclusion by cells with undamaged cytoplasmic membranes [25, 69, 70]. Other substances used in “dye exclusion tests” are fast green [71], erythrosin B [72], and dansyl-lysine [73]. On the other hand, one can assess the cytotoxicity of the materials under examination by estimating the percentage of the cells in the culture which are living, using the above mentioned vital stains, e.g., neutral red, Hoechst 33258, BCECF or fluorescein diacetate, always in comparison to a control culture.

In another approach, cells are preloaded with a radioactive compound and then assayed for leakage of radioactivity from the cells with damaged membranes. Traditionally, [^{51}Cr] has been used for such studies [74–77], but nowadays the trend is to substitute for it with the nonradioactive method of europium release [78]. A disadvantage of both the dye exclusion and the radioactive compound release methods is the underestimation of reproductively dead cells, i.e., those incapable of proliferation [78, 79]. A more simple method to obtain quantitative information about the cells with damaged cytoplasmic membranes is the spectrophotometric measurement of the released lactic acid dehydrogenase (LDH) in the culture supernatant [80].

Since there are two types of cell death – necrosis and apoptosis – it is important in several cases to identify which type of cell death is occurring in order to understand the mechanism of the cytotoxic action of a compound. Apoptosis or programmed cell death follows a predetermined series of events, during which the chromatin becomes fragmented and condensed, the organelles and the cell shrinks, and the cell surface blebs, leading to budding off of membrane-bound apoptotic bodies [81]. These morphological alterations allow monitoring of apoptosis in several ways. One common approach is to document the appearance of DNA laddering by gel electrophoresis [80, 82]. Since this method is not applicable in all cell types [83] and does not allow analysis of a large number of samples, the staining with nucleic acid fluorochromes, such as propidium iodide or DAPI (4,6-diamidino-2-phenylindole) is preferred, followed by microscopic inspection for the assessment of DNA fragmentation [84] (see Fig. 3.5a–b). Actually, this method can become quantitative only in combination with flow cytometry, so that one can determine the percentage of the cells with reduced DNA content, i.e., hypodiploid cells [63, 85] (Fig. 3.5c–d). Furthermore, DNA fragmentation can be visualized after addition of labeled nucleotides in the DNA breaks in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TUNEL method) either in situ by fluorescence microscopy or by flow cytometry [80, 86, 87]. On the other hand, alterations in membrane integrity which occur much earlier than DNA fragmentation in apoptotic cells can be detected after staining with annexin V [88, 89] or the viable dye merocyanine 540 [90]. Another early event in apoptosis is loss of the mitochondrial inner transmembrane potential [91, 92]. This can be monitored by using rhodamine 123 [93, 94], 3,3′-dihexylocarbocyanine iodide (DiOC6) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) [95], as well as MitoTracker Green FMTM or RedTM CMXRos [96] in conjunction with flow cytometry or confocal microscopy.

3.4 Other Assays

A common mechanism for the induction of cell death by several materials used in the dental practice is through oxidative stress [97]. Accordingly, many studies of the cytotoxicity of certain materials include their ability to act as oxidants and to promote the production of reactive oxygen species (ROS) inside the cell. Often the oxidative properties of the materials are initially evaluated in cell-free systems, e.g.,

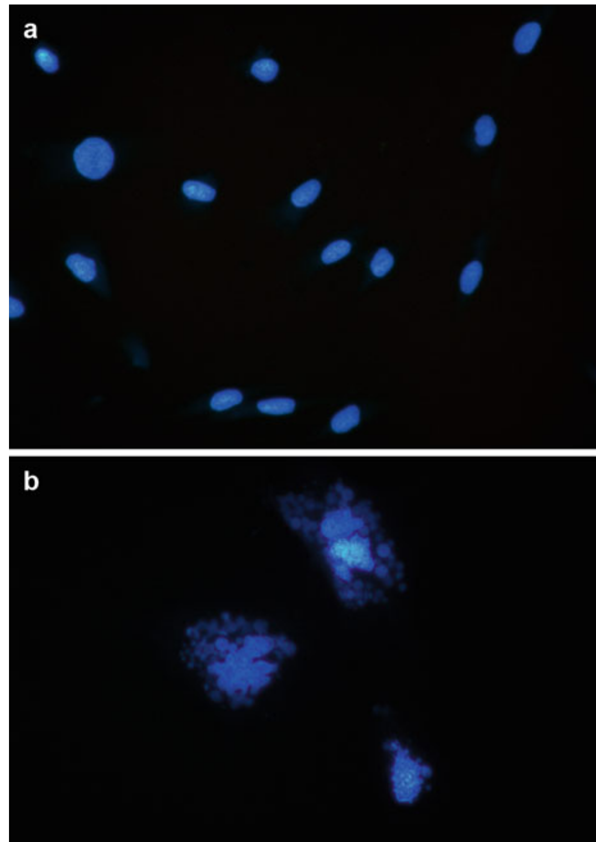


Fig. 3.5 Detection of apoptosis. The nuclei of control (**a**) and apoptotic (**b**) cells were stained with DAPI after fixation with neutral formalin and permeabilization with Triton X-100 (magnification 1,000×). Alternatively, the distribution of human cells in the various phases of cell cycle was assessed by flow cytometry as in Fig. 3.4 (**c**: control culture, **d**: culture undergoing apoptosis). The *arrow* indicates the sub-diploid peak due to apoptosis

by their interaction with the free radical diphenylpicrylhydrazyl (DPPH), followed by spectrophotometric monitoring [98, 99]. A more novel, cell-based technique is the evaluation of the material's ability to induce oxidative stress inside the cell using a convenient fluorochrome, i.e., 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable nonfluorescent probe, which is de-esterified intracellularly and turns to the highly fluorescent 2′,7′-dichlorofluorescin upon oxidation [98, 100–103]. Further evaluation of the oxidative status of the target cells may include monitoring of intracellular glutathione depletion [97, 104, 105] and glutathione transferase activity [106], but these methods are laborious and time-consuming, not allowing for the screening of extensive numbers of materials. Another parameter that needs to be tested in biomaterials under study is their genotoxic potential, i.e., their ability to provoke DNA damage. Single cell gel electrophoresis (comet assay) followed by labeling of the samples with a fluorophore such as DAPI can reveal chromatin fragmentation in the form of single- or double-strand DNA breaks [107, 108]. When visualized under a fluorescent microscope, damaged DNA appears as a comet tail that trails the comet head containing the undamaged nuclear DNA (Fig. 3.6).

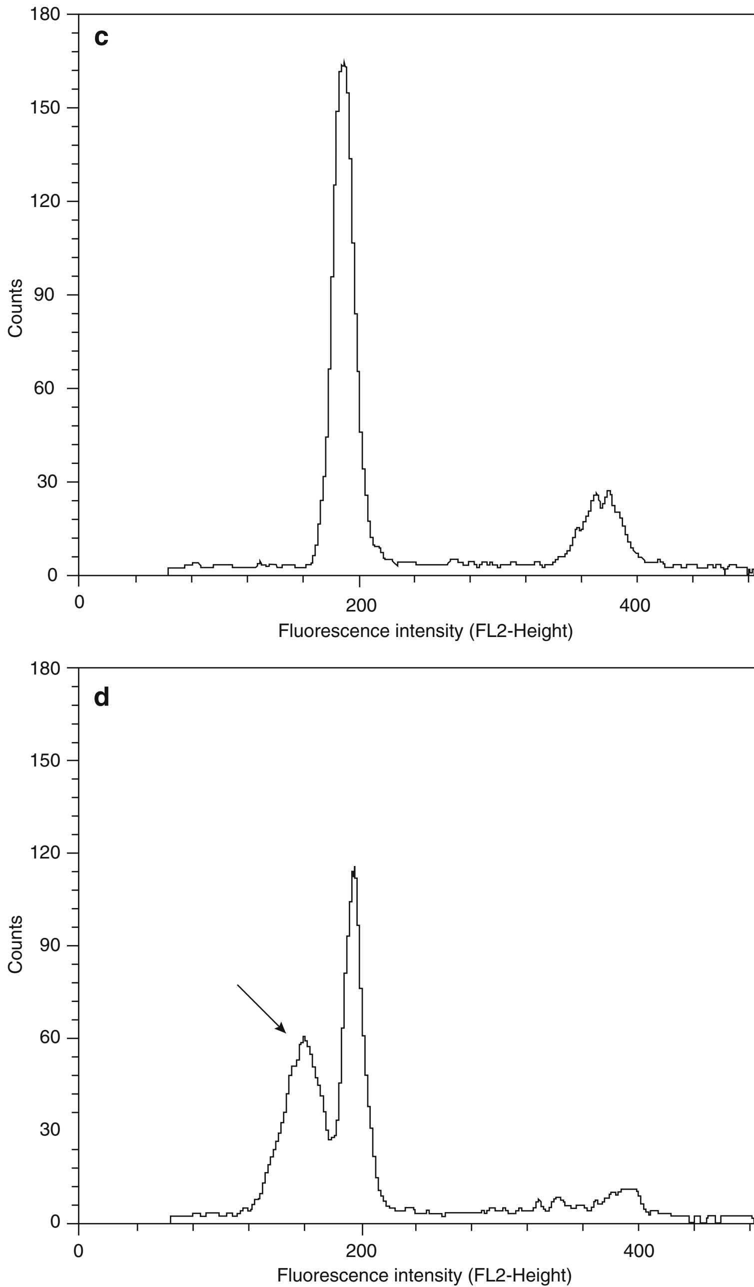


Fig. 3.5 (continued)

In general, after the evaluation of the possible cytotoxic, genotoxic and/or anti-proliferative activities of the test materials, more specific targets are chosen, usually to investigate the effects on certain cellular products. However, a detailed presentation

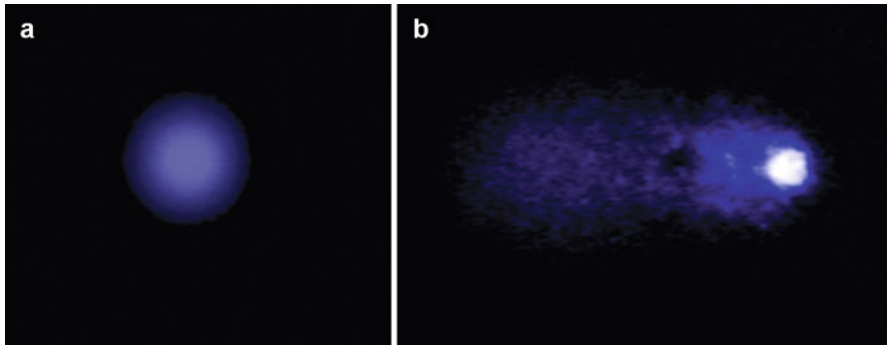


Fig. 3.6 Assessment of DNA integrity by single cell gel electrophoresis. Cells were detached, electrophoresed in low-melting point agarose and stained with DAPI. (**a**) Nucleus containing intact DNA; (**b**) nucleus with damaged DNA. Damaged DNA appears in the "tail" of the "comet"

of such methods is beyond the scope and the space limitations of the this chapter. Briefly, assays monitoring gene and protein expression are used, such as RT-PCR [109] or northern blotting [110] and western blotting, ELISA [111], immunofluorescence [60, 112] or flow cytometry [113, 114], respectively. Phosphorylation of specific intracellular substrates [115] or activity of certain transcription factors [21, 116] can also provide important information regarding the intracellular signaling mechanisms affected by the test compounds. Interestingly, novel technologies such as the use of DNA microarrays for gene expression profiling have also been added to the toolbox of the researchers nowadays [117, 118].

3.5 Important Parameters

In all of the in vitro assays described above, there are a number of common parameters, which are critical for the outcome of the assay. Perhaps the most important are the concentration spectrum of the evaluated compound, as well as the time of exposure. Usually, the concentrations used in the assay are dictated by the background knowledge on similar compounds and also by the application to which the test compound is aiming. Of course, the concentration range chosen should always result in a dose–response curve. It should be mentioned here that the term "concentration" is applicable only for soluble compounds; for certain solid materials, a different approach of testing is followed by growing the cell cultures in direct contact with the material [119–121], although such systems are suitable mostly for qualitative studies (see also alternative culture systems below).

For the choice of the exposure time, one should know exactly the duration of the cell cycle in the cultures used for the assay. Usually, exposure for a period of one to two cell cycles is enough for the manifestation of the proliferative, cytostatic, or cytotoxic action. However, there are cases where longer exposure times could be required, since prolonged incubation with a variety of cancer chemotherapeutic agents has been shown to result in gradually decreasing IC_{50} values, as exposure time increases [55]. Undoubtedly, only a prolonged exposure will demonstrate

whether a cell population remains clearly unaffected by the test compound. Especially for materials used in dental practice, the study of the possible slow release of putatively harmful agents due to material aging or corrosion requires the examination of lower concentrations for longer incubation times, thus simulating more closely the in vivo conditions. Furthermore, in some instances a recovery period after exposure to the test compound is included in the assay, either to allow for the occurrence of delayed effects or to permit the recovery of metabolic perturbations which could affect the index of the assay per se.

Other important issues may occur mainly from the nature of the test materials; for example, false positive results have been reported to arise due to release of unexpectedly volatile compounds from one extract affecting the neighboring cultures [122]. Precise knowledge of the physicochemical properties of the test materials is crucial for the design of each assay, as well as to avoid the wrong interpretation of the results obtained.

3.6 Alternative Culture Systems

While in conventional assay systems cells are cultured on plastic surfaces, there is now the trend to create three-dimensional cultures using extracellular matrix components, such as collagen gels [123, 124] or fibronectin-coated meshes [125–127]. In such more complex environments, one can take into account the mutual and organizational interactions between cells and their surrounding matrix, under conditions more closely simulating the tissue milieu [123]. In analogous approaches, it has been proposed to test orthodontic materials on reconstituted human oral epithelium produced by keratinocytes cultured on inert polycarbonate filters [128, 129], as well as dental restorative materials in the presence of a dentine barrier, since the interaction with the latter may affect the cytotoxicity of the materials [130, 131].

In conclusion, biocompatibility testing is fundamental for the development of novel biomaterials. In this chapter, we have presented a plethora of in vitro assays of vital cellular functions, such as cell proliferation or cell survival, which can provide a first clue for the biocompatibility of a given material. Innovative methods are continuously being developed, as well as improvements of the existing ones, although the basic principles of all of these assays remain generally the same as the ones presented here.

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Part II

Computers in Orthodontic Research

Computers in Orthodontic Research

4

Demetrios J. Halazonetis

4.1 Introduction

Orthodontic research is no exception to the widespread application of computers. Computers are used at almost all stages of a scientific research project, from submission of a proposal (word processing) to collection of data (measuring and statistical processing) to presentation at meetings and final publication (word processing and slide presentation). This chapter focuses on the more specialised uses of computers in orthodontic research, in an effort to reveal the advantages that they offer, but also to point out their limitations. Due to the continual advancement of hardware and software, the information presented here is certain to become outdated very quickly. Emphasis will therefore be placed on the basic principles, so that the reader will be able to appraise current techniques as well as new developments.

4.2 Measuring Diagnostic Records

Measuring is the basis of all scientific research. Orthodontics may well be the most measurement-preoccupied specialty in dentistry. Since the advent of cephalometrics in the early 1930s and its flourishing in subsequent decades, there are very few published clinically oriented papers that do not include some cephalometric measurements. These are currently performed mainly by computer software on scanned images or on digital radiographs. Any measurement potentially includes sources of error, and cephalometrics is no exception. Studies analysing cephalometric errors have been reported in the literature [1–8], but they mainly deal with human error, presuming that computers are mathematically accurate and precise and do not make

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mistakes. Although this may be true, it is only under the condition that computers are appropriately used. Unfortunately, unfamiliarity with the internal workings of computers, scanners and other devices is not uncommon and may lead to compromised results. Also, special features and capabilities of computer-aided measurement methods may not be appreciated and go underused. These topics are discussed below and apply both to cephalometric radiographs and other diagnostic records that can be measured in similar ways, such as facial photographs, dental casts, panoramic radiographs, animal radiographs and histological preparations.

Before discussing the above issues, it may be appropriate to review a few definitions pertaining to measurements and errors. The following terms will be used:

Precision or Repeatability: the degree to which the measurement has the same value when measured several times. It is assumed that the same examiner performs the measurements, so this is also known as intra-examiner reliability.

Reproducibility: the degree to which the measurement has the same value when measured by different operators, also known as inter-examiner reliability.

Reliability (inter- and intra-examiner) is assessed by repeated measurements. The variance of the repeated measurements shows the amount of random error. Possible bias between a first and second measurement is revealed by testing the difference between the replicated measurements.

Validity or Accuracy: the degree to which the measurement actually represents what it was intended to represent, that is, is close to the 'true' value. Note that validity in identification of cephalometric landmarks cannot be assessed by repeated tracings, either by one or more observers, because there is no "gold standard" against which to compare the observers' judgments. One method to test validity is to place markers on anatomical structures of skulls and compare their positions, as seen on a cephalogram, with the corresponding cephalometric points, as located without the markers. Such studies have reported validity errors for several commonly used points, both skeletal and dental [9, 10].

4.2.1 Digitisers

Computer-aided cephalometric software has been widely available since the introduction of personal computers. Early implementations relied on a digitiser to get the coordinates of the points directly from the radiograph or from its tracing, and only relatively recently have scanners or digital radiographs been introduced. Digitisers have now been almost completely phased out, so only a brief section will be devoted to them.

Digitisers are electromagnetic devices that detect the position of a cross hair pointer on a tablet by means of electromagnetic fields. For this reason, a certain amount of interference from external fields or metal objects is expected. Accuracy is reported to be approximately 0.1 mm, although values differ between various

models and manufacturers. Studies assessing digitiser accuracy [11, 12] have found both systematic and random errors, but the total errors are not large, at least when compared to more serious error sources, such as the error of landmark identification.

Digitisers can be used either directly on the radiograph or indirectly on a tracing of the radiograph. Direct use requires a digitiser with a translucent surface, so that the radiograph can be lit from underneath. This is the recommended procedure, as it is considered to exhibit smaller errors, because there is no need for the intermediate step of creating a tracing [3, 6].

The major benefit of using digitisers is that the radiograph is inspected directly by the human eye, thus taking advantage of the capabilities of this organ. A very important capability, when viewing a radiograph for landmark identification, is the power to discriminate between different shades of grey. The human eye has a rather limited power in this respect, being able to discern approximately 30 grey levels. However, this is coupled with an extraordinary ability to accommodate to a very wide range of light intensities (of the order of 10^{10}) by adaptation mechanisms, such as the opening and closing of the iris and the different sensitivities of the photoreceptors. At each adaptation level, there are limited intensities that can be discerned, but as the observer scans the radiograph, the eye changes its accommodation level, in effect extending the total range of grey levels detected [13]. To facilitate accommodation, careful control of the ambient light is essential when viewing radiographs. Also, masking around the region of interest is helpful, because it excludes light from neighbouring areas, which may affect the adaptation level.

4.2.2 Scanners

Scanners digitise an image by shining light on it and reading the reflected or transmitted light, using a light-sensitive element (usually a charge-coupled device, CCD). In the case of radiographs, the light source is typically placed on a cover above the radiograph (the light transparency unit), and the CCD scans the radiograph from underneath. The intensity of the transmitted light is converted to a digitised signal that constitutes the image of the radiograph. Scanners have certain characteristics that determine the quality of the image [14]. These are resolution, colour depth and optical density. Ensuring a good image is paramount to precise measurements.

4.2.2.1 Resolution

Resolution refers to the number of pixels of the resulting image. Resolution is usually measured in dots per inch (dpi) and can extend above 1,200 dpi. It is important to differentiate between the optical resolution of the scanner and the total resolution. The optical resolution is determined by the number of elements of the CCD and the step size of the motor that moves the CCD. These factors commonly allow resolutions of 600–1,200 dpi. Software interpolation can then be applied to increase the resolution to much higher values. However, this increase is, in essence, guesswork and does not represent actual information gathered from the image.

The optimum resolution for scanning cephalometric radiographs is an important consideration [15]. The decision is usually a compromise between the need for sufficient detail to identify landmarks and practical aspects of computer speed and storage. Conventional cephalometric measurements have many sources of error, the most significant being the error of point identification (see below), which is of the order of millimetres (mm). Therefore, a resolution that will give a few pixels per mm would be sufficient for most purposes. Simple calculations show that resolutions of 150–300 dpi result in 6–12 pixels/mm, which should be more than enough. No significant benefit has been shown for 600 dpi as compared to 300 dpi [16]. Indeed, resolutions as low as 75 dpi have been regarded as sufficient [17], but this conclusion was reached with an experimental setup that did not allow zooming of the image, thus negating the increased detail of higher resolutions (see below).

4.2.2.2 Colour Depth

Colour depth corresponds to the number of bits dedicated to each colour of the image. Computer colour images are composed of three primary colours (red, green and blue). These colours are mixed in different proportions (intensities) to produce all the different colours of the image. The intensity of each of the primary colours is described by a number. The computer usually assigns one byte (8 bits) to represent each primary colour, so most computer images are 24-bit images. A single byte can describe 256 different levels of intensity, so a 24-bit colour image can contain $256 \times 256 \times 256$ different colours (approximately 17 million). Note that shades of grey need equal amounts of the primary colours, so greyscale images can be represented by one byte instead of three and contain a maximum of 256 shades of grey. Higher-level machines and scanners can use more bits per colour (12 or 16 bits) to describe more shades of grey (4,096 or 65,536). However, most computer screens can display only 8 bits per colour, so even if the image is a 16-bit greyscale image, it will be converted into 8 bits in order to be displayed.

High-bit images (12 or 16 bits) seldom offer substantial benefits for orthodontic research purposes [18]. One reason for this is the inability of conventional monitors to display high colour depth. Another is the inability of the human eye to detect very fine gradations in light intensity. It should be pointed out that 12- or 16-bit greyscale images are used effectively in computed tomography (CT) images. In such images, either the greyscale range can be compressed to 8 bits for display of the whole dataset or only a part of the extended range can be selected. By choosing to view the high intensity pixels, the bony tissues are made visible, whereas the soft tissues can be displayed by selecting the lower range. This procedure is commonly known as windowing.

4.2.2.3 Optical Density

Optical density refers to the range of light intensities that a scanner can acquire. This depends on the capabilities of the CCD and the electronics of the scanner. It is expressed in a logarithmic scale and typically ranges from 2.4 to 4.2 for consumer scanners on the market.

Assume that a radiograph is being scanned. Light of a particular intensity falls on the radiograph, and part of this is transmitted through it and reaches the CCD. The ratio of transmitted light to incident light at a particular location of the radiograph is the transmittance. The inverse of this ratio is the opacity. The logarithm of opacity is the optical density of the radiograph:

$$\text{Density} = \log_{10}(\text{opacity}) = \log_{10}\left(\frac{\text{incident light}}{\text{transmitted light}}\right)$$

In the case of photographs, where the incident light is reflected, opacity is defined as the ratio of incident over reflected light.

Dark areas of a radiograph transmit very little of the incident light, so they have a large optical density, whereas light areas are almost completely transparent and have a small density. The difference between the smallest and largest density is the density range. The density range of a radiograph may extend from 3.4 to 4.2 or more, signifying that the intensity of light passing through the brighter areas is more than 15,000 times higher than the intensity of light going through the dark areas. In contrast, the density range of a printed image is much lower, because light is reflected and not transmitted through it. Photographs and printed images seldom have a density range that exceeds 2.4. Scanners can capture a limited density range. The lower-priced models have a range of approximately 2.4, which is adequate for photographs and other printed documents, but insufficient for slides or radiographs. When the density capabilities of the scanner are insufficient, the brighter part of the image is given priority, and the darker areas are captured as a uniform blackness. This results in obliteration of the detail in dark areas, such as, for example, the hyoid bone area in lateral cephalograms.

Density values should be at least 3.4–3.6 for adequate results, values achievable by high-end flatbed scanners. Figure 4.1 shows the same image acquired from two

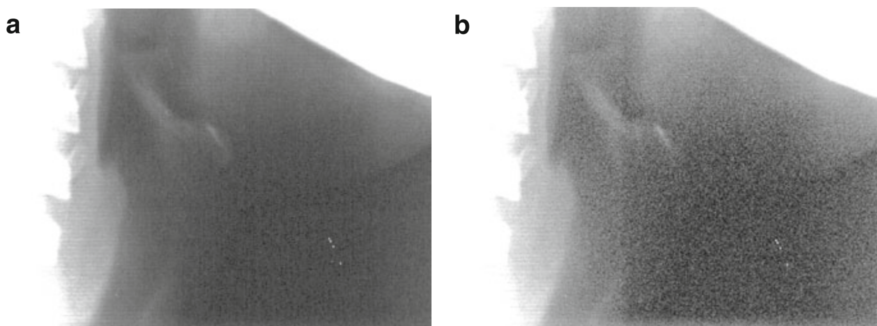

Fig. 4.1 The area of the hyoid bone scanned (**a**) with the Epson 1600 Pro (optical density 3.3) and (**b**) the Epson 1680 Pro (optical density 3.6). Note the reduced noise level and the significantly better detail capture from the scanner with the higher density. Both images have been processed by adjusting gamma so that dark areas are more apparent. For this reason, the mandible and vertebrae appear “washed out”

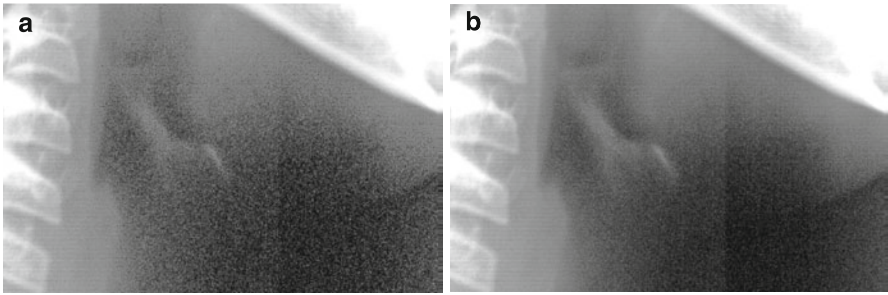

Fig. 4.2 (**a**) The hyoid bone area, scanned once, with a scanner of optical density 3.3. Notice amount of random noise. The image has been manipulated by changing the gamma value, so that dark areas are enhanced. (**b**) The same area, produced after scanning four times and merging the results

scanners, one with optical density of 3.3 and the other with optical density of 3.6. (Note that because of the logarithmic scale used in expressing optical density, the better of these two scanners can discern twice the range than the other one.) Both images have been enhanced by the same amount to show the difference in the detail of the dark areas. Optical density is perhaps the single most important factor that determines the quality of a scanned radiographic image.

4.2.2.4 Noise

Noise is another factor that degrades scanned images [19]. Noise appears as random variations in image intensity at the pixel level and is most evident in the dark areas of the image. Because noise is random, it can be reduced substantially by multiple scanning. This technique scans the radiograph more than once and averages the results. Due to the random nature of noise, the average of multiple scans will tend to cancel out noise and retain the true value. High-end scanners are capable of acquiring multiple values for each pixel and take the average during a single pass. If this feature is not available, it can be done by software, but then multiple passes are required. Figure 4.2 compares the same area scanned once and four times. Noise reduction is evident.

4.2.3 Digital Radiography

Digital radiographic machines register the intensity of the radiation transmitted though the patient's head by an electronic sensor and convert it directly to a digitised signal, thus eliminating the intermediate steps of developing a film and digitising it with a scanner. The sensor is a charge-coupled device (CCD). Most sensors have small dimensions and it is not possible to cover the whole area of interest. To solve this problem, the manufacturers use an array of sensors, arranged side by side. The array extends along the required width and is moved vertically during the exposure, in tandem with the x-ray source, to scan the whole head (horizontal scanning is also available). This method presents certain differences in comparison to the conventional approach:

1. A longer exposure is needed, because of the movement of the source and sensor array. This requires the patient to remain still for a number of seconds (exposure times differ between machines) and may introduce errors due to movement of the head or changes in posture of the soft tissues, including the tongue and soft palate. Some systems are capable of acquiring a cephalometric digital image without scanning ("one shot") and overcome the disadvantages of long exposure.
2. Even though a longer exposure is used, total radiation exposure is reduced, due to higher sensitivity of the sensor array and the fact that only a thin stripe is radiated at any one time.
3. Beam geometry may be different, resulting in a different magnification pattern. The conventional (non-digital) systems use a beam that diverges from a single point towards all directions, to produce a cone-shaped geometry. This results in symmetrical magnification of the exposed structures around the central beam. In contrast, some digital systems use a fan-shaped beam that scans the patient vertically (or horizontally) in a parallel fashion. Therefore, magnification is present only along the horizontal (or the vertical) axis [20]. This problem has been overcome in newer machines that incorporate rotational movement of the x-ray source, or movement of a slit diaphragm, thus producing comparable magnification to conventional systems.

Other digital systems use a hybrid method to acquire the image. A special phosphor storage plate (PSP), similar to a conventional film, is used to obtain the image, by means of conventional x-ray equipment. The plate is then inserted into a special scanner, and the captured image is converted to a digital file.

4.3 Advantages and Capabilities of Computer-Aided Cephalometrics

The advantages of using a computer for performing cephalometric measurements are so significant that probably no research is being conducted by manual methods any more. The obvious speed factor is especially important when a large number of diagnostic records need to be processed [21, 22]. Ease of use is also important, because it relieves the operator of fatigue. However, these are secondary benefits. Error control, a major problem in any investigation, is where computer-aided cephalometrics should focus. Reducing both random and systematic errors is not an easy task. Several methods are discussed below, but it should be noted that very limited data are available regarding their effectiveness. Most recommendations are based on logical deductions and assumptions, and further studies are needed for validation.

4.3.1 Error Control

Errors in cephalometric analysis have been extensively discussed in the orthodontic literature [1–8]. It is a common conclusion that error of point identification is the most significant source. Computers may help in reducing this error by the following methods:

4.3.1.1 Image Enhancement

Various image manipulations can be applied to make some areas more conspicuous and aid in visualisation. Contrast, brightness and gamma can be adjusted, and histogram techniques can be applied over the whole image or at specific areas [13], as shown in Fig. 4.3.

4.3.1.2 Multiple Digitisation

Multiple digitisation has been recommended as a method to reduce point identification error [2, 23]. Baumrind and Miller [23] reported that in order to reduce this error by half, each point should be digitised four times, and the average of the four attempts should be used as the location of the point. Although this may be excessive [4], even a double digitisation is not possible without a computer system. Cephalometric software allow multiple digitisation on screen (without showing the previous attempts, so as not to bias the user) and calculate the average position. The user can inspect the digitisations and delete outliers. Multiple digitisation may be more important in case reports than large-scale studies, where errors tend to average out [2].

4.3.1.3 Magnification of the Image and Precision Limitations

The scanned image can be magnified on-screen to almost any detail in order to facilitate point placement. The limiting factor here is the resolution at which the radiograph was scanned. As mentioned earlier, a resolution of 150 dpi will produce approximately 6 pixels per mm, which far exceeds the usual requirements of cephalometrics.

The precision with which points are located on-screen depends on a number of factors, such as scanning resolution, the zoom setting when digitising and the internal design of the software [15, 24]. The important thing to keep in mind is that the points are located using the mouse, so the movement of the mouse on screen is a major factor in determining the precision. For example, assume that a radiograph is displayed at approximately life size on a computer monitor. Assume a 15″ TFT monitor running at a resolution of 1,024 × 768 pixels with physical dimensions of 304 × 228 mm. The dimensions of a screen pixel are easily calculated as 0.297 × 0.297 mm. Since the mouse can only move from pixel to pixel on the screen, it becomes evident that it is not possible to digitise points with more precision than approximately one-third of a millimetre. If the radiograph is zoomed out to occupy less screen space, this value will worsen. If more detail is required, the software should allow zooming. Assume that the image is zoomed in so that 10 mm is now displayed at a size of 10 cm on screen. This should allow digitisation at an accuracy of 0.03 mm. However, two other factors come into play at this magnification. One is the resolution used during scanning. If this was relatively low, then the image pixels will be apparent, setting a limit to the effective precision; although we can place the mouse and digitise at sub-pixel positions of the image, there is no way to ensure that the digitised location is correct. The final limiting factor is the internal design of the software program, which may not allow unlimited precision.

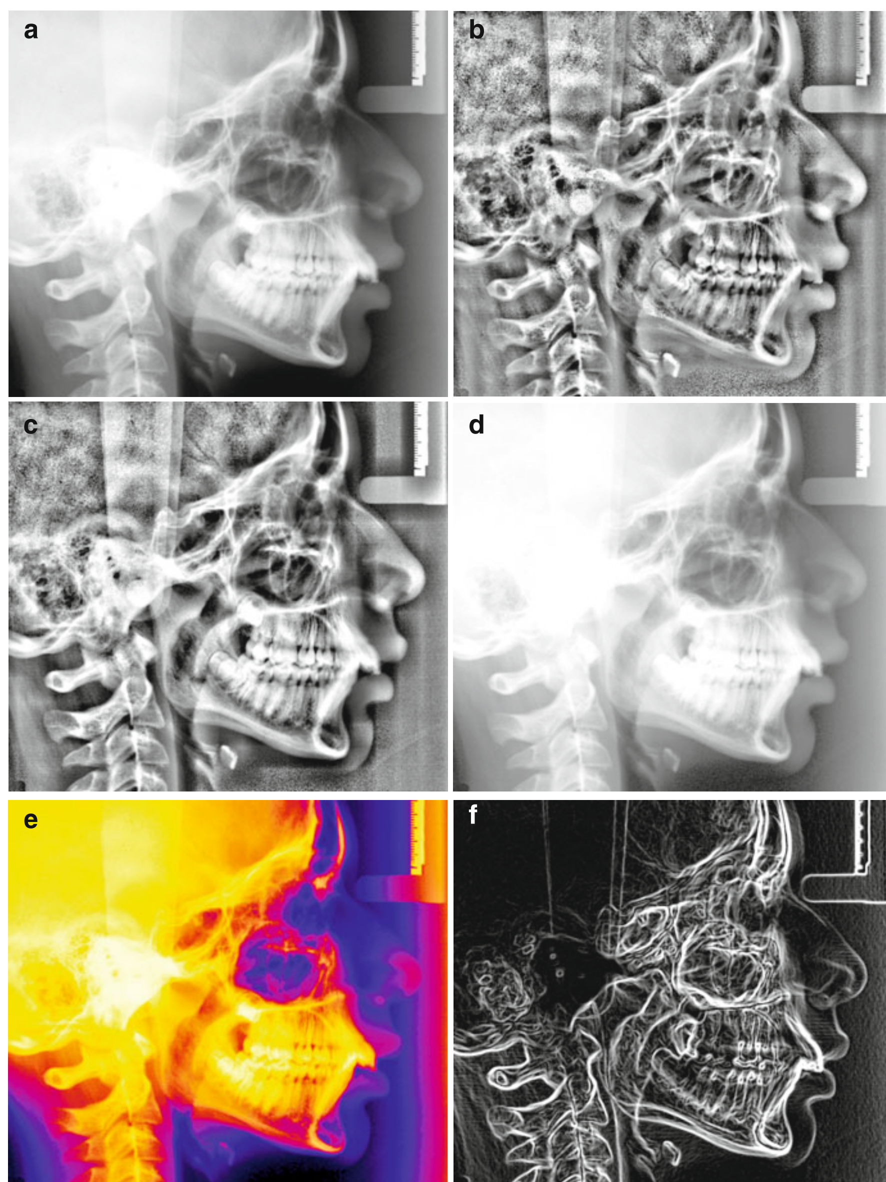

Fig. 4.3 Results of manipulation procedures: (**a**) original image, (**b**) adaptive histogram equalisation, (**c**) adaptive levels, (**d**) gamma adjustment, (**e**) colourisation and (**f**) edges

A practical method to calculate the maximum precision under specific conditions is the following:

1. Calculate the precision offered by the scanning procedure. This is equal to 25.4 divided by the resolution in dpi (1 in. is 25.4 mm). So a resolution of 150 will offer a maximum precision of approximately 0.17 mm.
2. Calculate the precision offered by the screen and zoom factor. First, divide the physical width of the computer screen by the number of pixels to get the size of each pixel. Then adjust by the zoom value. For example, if the screen is 304 mm in width and is running at a resolution of 1,024 × 768 pixels, the pixel size is 0.297 mm. This is the maximum precision if the radiograph is viewed at life size. For double than life size, divide the number by 2 to get 0.148 mm. For ten times life size, the precision is 0.0297 mm.
3. The final precision is the worse of the results calculated in steps 1 and 2 above. Check this with the data given by the software manufacturer, because the software itself may set limits, due to internal number representation or other design factors.

4.3.1.4 Automatic Point Location

One of the reasons that the error of point identification is high for points such as Gonion and Gnathion is that these points are located on curved osseous boundaries. The investigator has the task of locating the most extreme point along this boundary (e.g. the most inferior and posterior point, in the case of Gonion), not an easy task given the absence of anatomical markers. Geometrical constructions can be used as aids, but these may also introduce errors of their own. An alternative is to delegate the task to the computer. Software already exists that can assist in this respect. The user need only draw the outline of the boundary, and then the software automatically locates the points on this boundary following simple geometrical rules of point definition.

As an example, Fig. 4.4 shows the placement of points along the outline of the mandibular symphysis. The computer locates each point according to its definition; Pogonion is placed at the most anterior position, Menton at the most inferior, etc. The anteroposterior direction is defined by the Frankfurt horizontal plane, which has been digitised previously, thus circumventing errors caused by improper head orientation.

Automatic location of points on digitised outlines may remove some of the subjectivity, and therefore error, of point identification, but it may introduce a new source of error, that of tracing the outline. However, because the outline usually represents a well-defined brightness edge in the radiographic image, it is possible to use computer vision techniques to identify it. Edge-detection methods [13, 25] are among the first developed methods in computer vision, and, although not as reliable as one would like, they can be used to good effect. Such methods have only recently been introduced in cephalometric software, and their effectiveness in error reduction is beginning to be investigated [26].

4.3.2 Magnification Adjustment

A common problem with orthodontic research studies is that the cephalometric radiographs may have been acquired by different x-ray machines, each possessing a different magnification factor. Although angular measurements are not affected,

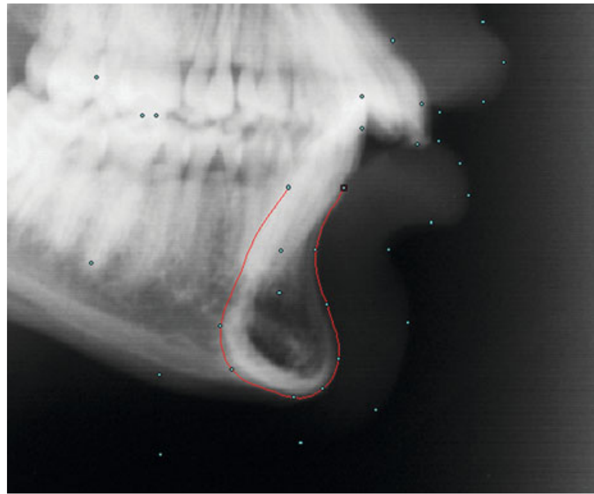

Fig. 4.4 Automatic placement of points at the mandibular symphysis. The user has drawn the outline of the symphysis (*red line*) and the computer automatically places each point (*cyan dots*) on this outline, according to predefined geometrical relationships. The Frankfurt horizontal plane (not shown) is used to establish the reference horizontal. Other digitised points are also shown

linear measurements need to be rescaled to a common magnification for proper comparison. The aspect of different magnification between different machines is also important when assessing linear measurements of a patient in comparison to published standards. Correction to natural size is recommended, in order to avoid confusion and obtain valid results [27].

Differences of magnification present larger problems in superimpositioning. If the original and final cephalometric radiographs of a patient have been taken on machines with different magnification, it is not possible to superimpose them manually, unless one resorts to such creative measures as enlargement or reduction of the tracings by photocopying. The use of computers can help in this respect. All tracings can be rescaled to the same magnification (or to life size), thus enabling correct superimpositioning.

4.3.3 Structural Superimposition

Superimposition of radiographs on internal osseous structures is recommended for assessing treatment or growth changes [28]. This is not easy to accomplish manually because a lot of structures need to be traced and precision may be compromised. Computers allow direct superimpositioning of radiographic images using different colours for each (Fig. 4.5). When two structures align, the colours are blended together to produce a different colour. This facilitates the procedure significantly. Automated methods that aim to superimpose anatomical structures so that the optimum alignment is achieved are currently in the experimental stage.

4.3.4 Morphometrics

The field of morphometrics [29–32] is relative new in biology and has only recently been applied to orthodontics [33–38]. Morphometrics aims to overcome some of the fundamental problems of conventional cephalometrics [39], such as the problem of

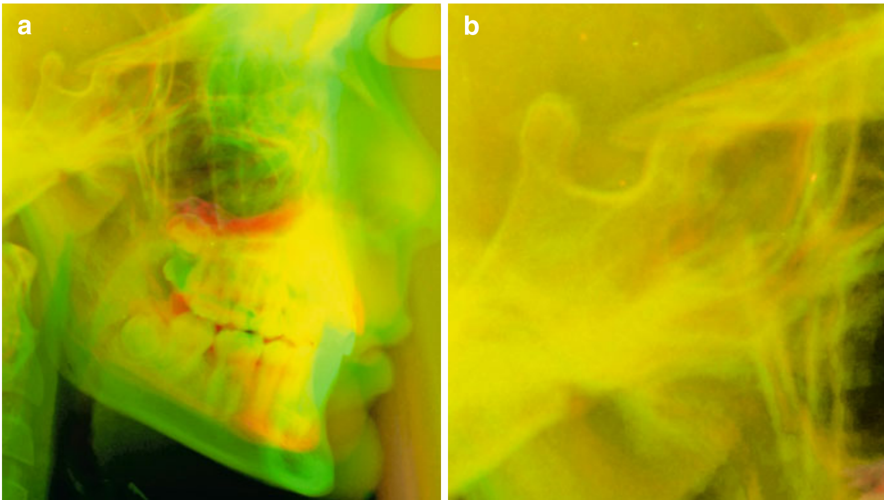

Fig. 4.5 (**a**) Structural superimposition of before and after radiographs on anterior cranial base. The radiographs have been colourised to *red* and *green*; aligning structures are yellow. (**b**) Detail of the sella area

separating size from shape, selecting an appropriate superimposition scheme and interpreting the results of the measurements. Morphometric methods require extensive calculations that are not feasible without computer assistance. Cephalometric software are now available that can perform Procrustes superimposition and calculate principal component analysis and other morphometric procedures. Detailed explanation of such methods is beyond the scope of this chapter.

4.3.5 Warping of Images

Computer graphics have advanced to the point that it is now easy to modify images by deforming them and blending them in a controlled manner to create realistic pictures of objects that do not exist. Such effects have been applied successfully in the movie industry and have also found application in orthodontics. Two terms are coined for such procedures [40, 41], but they are sometimes used interchangeably in the literature: "Warping" refers to the deformation of a single image, and "morphing" refers to the deformation of two images and the creation of a new image by blending the two warped images together. Morphing can be used to generate smooth transformations from one image to another and is most often used for the creation of animated sequences [41]. In orthodontics, such movies are used to show simulations of treatment, by depicting a smooth transition from the initial photograph of a case to the final result (Fig. 4.6). The impact to a prospective patient is significant, because treatment procedures can be explained and presented in a highly visual manner.

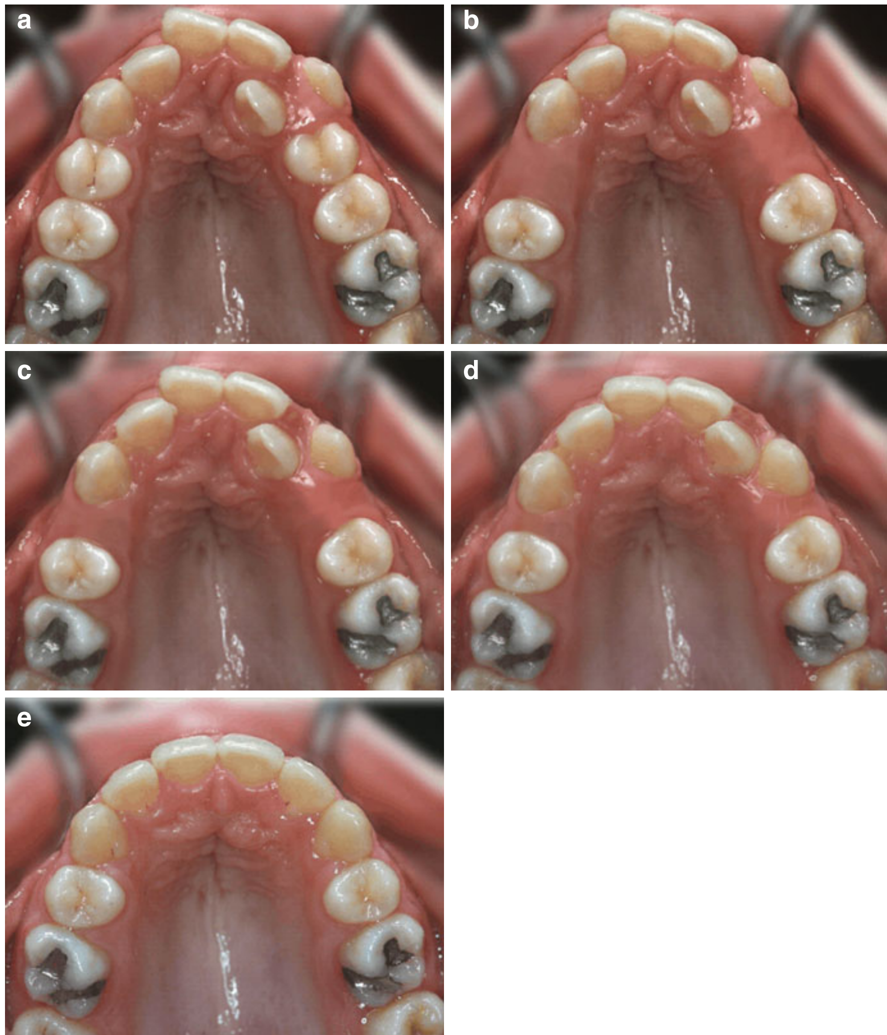

Fig. 4.6 Series of images used for the creation of a morphing movie that shows a simulation of orthodontic treatment. The only real images are the initial (**a**) and final (**e**) ones. Image (**b**) was created from (**a**) by digitally deleting the premolars. Intermediate images (**c**, **d**) were constructed by morphing between images (**b**, **e**)

The technique of warping is used for creating photorealistic treatment predictions of the patient's face. Instead of drawing a prediction tracing from a lateral cephalogram to show how the facial profile may look after a surgical procedure, it is now possible to use the pretreatment profile photograph of the patient as the starting point. The photograph is warped (deformed) so that the facial outline takes the shape of the predicted outline. Various mathematical procedures exist for such deformations [42, 43]. The result has been found to

enhance patient–doctor communication [44], but the diagnostic value is debatable, due to the following reasons:

1. The warped image is based on the cephalometric prediction of the facial outline. Thus, the final result is no more accurate than the cephalometric tracing.
2. The warped image is based on the initial pretreatment photograph. The photograph is warped so that the facial outline changes shape and becomes the same shape as the tracing prediction. In this process, the remainder of the face is also deformed, not according to any biological model, but based on mathematical algorithms. This deformation will not reflect the true changes that are produced by treatment, even if the final facial outline has been accurately predicted. Orthodontic and surgical treatments have effects on the lateral aspects of the face (cheeks, nose, mandibular outline, etc.) that cannot be predicted by this method.

In addition to clinical applications, warping and morphing have found increasing use in research [45–50]. Until recently, studies on facial attractiveness and investigations on facial symmetry, facial shape and skin texture have been limited, because they were conducted using drawings or silhouettes, and the investigation of the subtle effects of the many confounding variables was not possible. The computer opens up a large array of possibilities, enabling us, in an experimental setting, to control, manipulate and test each parameter individually. Starting from an original photograph, we can deform it or blend in other photographs and thus change the texture of the skin, the shape of the facial outline (in profile and frontal view), the shape of the internal facial components (e.g. the shape of the eyes, the mouth or the nose), the configurational arrangement of the internal components, their colours, the hair style and a number of other features [51]. This process leads to the creation of novel photorealistic faces that can be used for testing purposes or for education (Fig. 4.7). This is a young area of research but it is growing rapidly, because the questions addressed are not limited to orthodontics but extend to aesthetics, plastic surgery, facial perception and other fields.

4.4 Three-Dimensional Records

Although cephalometric radiographs are the main research source in orthodontics, other diagnostic records can be assessed in a similar manner. Facial photographs, photographs of dental casts, other radiographs (e.g. panoramics, hand/wrist) and animal records – anything that can be entered into the computer as an image – can be measured and analysed.

Three-dimensional records are utilised in increasing frequency, driven by advances in computer hardware and software and by the recognition that two-dimensional records are inherently limited in their ability to document the 3D craniofacial structures and the dentition. Since the beginning of orthodontics, the only 3D orthodontic records have been the study casts. The ideal goal in orthodontic diagnosis would be to replace all two-dimensional records (cephalometric and panoramic radiographs, facial and intraoral photographs) with three-dimensional ones. The diagnostic procedure and treatment planning (measurements and treatment prediction) would take

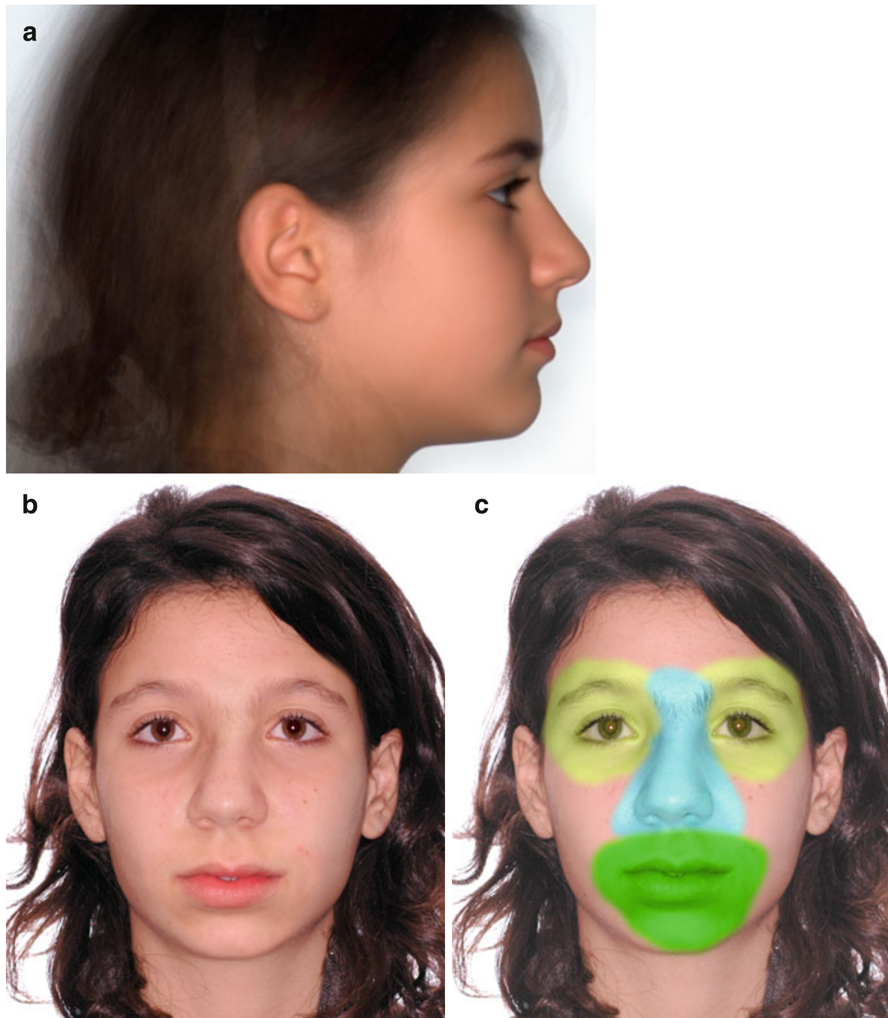

Fig. 4.7 (**a**) Average face created by merging the warped photographs of 20 patients. Photographs were warped to a common shape before merging so that corresponding facial features would superimpose closely. Warping was not applied to the hair area, and area is blurred. Average faces have been found to be highly attractive, when compared to the original faces from which they were created. (**b**) Virtual face created from a composite of face parts taken from four subjects. (**c**) Colourised areas show the parts that were taken from other subjects and merged with the underlying face. The left eye is identical to the right eye but flipped

place directly on the 3D records, thus circumventing many of the current limitations. Orthodontic 3D records aim at acquiring the geometry of three different parts of the craniofacial complex: the skeletal structures, the soft tissue surface of the face and the dentition. These components present different problems regarding methods of acquisition, because of differences in their material nature and the required accuracy.

4.4.1 Skeletal Structures

Acquisition of the 3D geometry of the craniofacial skeleton has been a mainstream application for several years now. The evolution of CT scanners and related software has enabled detailed imaging of the skeleton, but orthodontic applications have been limited to complex craniofacial problems that are seldom encountered in mainstream clinical practice. The main problems of CT scanning are the cost of the procedure and radiation exposure.

4.4.1.1 Radiation

Radiation risk is commonly assessed by the "effective dose". The effective dose is the sum of the doses to each organ exposed to the radiation, weighted by a coefficient, which represents the organ's sensitivity to radiation exposure. Organs such as the gonad and bone marrow have high weighting factors, whereas skin and neural tissue have low weighting factors. The unit of effective dose is the Sievert (Sv). For dental applications, where dose is low, the mSv (1,000 mSv = 1 Sv) and the μSv (1,000 μSv = 1 mSv) are used.

Due to exposure to natural radiation (from the earth minerals, from cosmic radiation, and from radiation within the human body), it is estimated that an individual receives about 3,000 μSv/year. A typical conventional CT scan of the whole head will incur an effective dose of about 2,000 μSv, which is equivalent to 8–12 months of background radiation. In comparison, a cephalometric radiograph entails a dose of about 5 μSv, and a panoramic gives approximately 25 μSv [52–56]. These values show that dental radiography involves low radiation exposure, but incorporation of 3D CT records as a routine orthodontic procedure would increase the dose substantially [57]. Cone-beam computed tomography (CBCT) results in a wide range of effective dosage to the patient, depending on the machine and the parameters of the examination, including field of view size and image resolution. Typical dosage ranges from 70 to 370 μSv [58]. Guidelines for CBCT imaging in dentistry have been established by national societies and a European initiative [59, 60].

4.4.1.2 Accuracy

The diagnostic value of 3D records is directly related to their validity and precision. The validity of CBCT records has been reported in a number of investigations, by comparing measurements taken from the 3D reconstructions of skulls or cadaver heads with direct measurements using callipers or 3D digitisers [61–70]. It is generally accepted that CBCT measurements are valid and accurate, but the threshold used for 3D reconstruction and demarcation of tissues may be a significant factor that needs attention.

The choice of the threshold for reconstruction and measurement is difficult because tissues may appear more or less dense than expected, depending on the area being examined and the effect of artefacts. Among the most significant artefacts in CBCT imaging are noise, the partial object effect and the partial volume averaging effect [71, 72]. Noise is present due to reduced x-ray energy, purposely set low on CBCT machines in order to reduce patient exposure. The partial object effect arises

because the field of view is smaller than the object under investigation; the parts of the patient's head that lie outside the field of view may significantly alter the density of the voxels, causing inconsistencies between tissue density and voxel density. These inconsistencies do not allow a reliable correspondence between voxel density and Hounsfield values and thus do not allow reliable differentiation between tissues, based solely on voxel values [73–75]. The partial volume averaging effect is evident when the voxels are large relative to the size of the object under investigation, resulting in multiple tissues occupying the space of a single voxel. In such cases, the value of the voxel will represent the average of the multiple tissues, giving a false impression of the voxel's composition.

From the above it is evident that a global threshold for tissue segmentation and measurement may not be appropriate [76]. Especially prone to systematic errors are thin structures, such as the alveolar covering of incisor roots. Due to reduced resolution of CBCT images [77], alveolar bone is consistently underestimated and dehiscences and fenestrations are significantly overestimated [78–81]. However, high-contrast globular structures, such as teeth, also show errors in measurement which may exceed 1 mm, even if images are taken under ideal conditions, mainly because of the inconsistency of voxel values due to the artefacts mentioned above [82].

Reliability of CBCT measurements is also a significant concern. As with conventional 2D cephalograms, point identification seems to be one of the largest sources of error. CBCT images, due to their 3D nature, present new challenges to the user, as multiple views of the dataset may be needed in order to identify a landmark, including 3D reconstructions and sections through the volume. Some points may even require new definitions; for example, the external auditory canal extends in depth and follows an oblique path, so Porion's traditional 2D definition needs updating [83]. Such difficulties reflect on repeatability; errors of measurement may exceed several degrees or mm, casting doubt on the capability of 3D measurements to reveal small treatment changes [84].

4.4.2 Soft Tissues

The geometry of the facial surface may be acquired from MRI or CT data if such data are available, but less invasive and costly methods that provide more accuracy and detail are recommended. Several techniques have been investigated, including laser scanning, structured lighting and photogrammetry [85, 86] (Fig. 4.8).

4.4.2.1 Laser Scanning

Laser scanning for 3D measurement and analysis of facial shape were introduced in orthodontics by the group of Moss in England [87–90]. Laser scanning of the head involves projecting a stripe of laser light and registering the shape of the stripe as it reflects from the three-dimensional surface. The stripe is usually projected as a vertical line and rotated around the face so that it scans the head from one side to the other. Scanning can be performed by movement of the laser and camera assembly

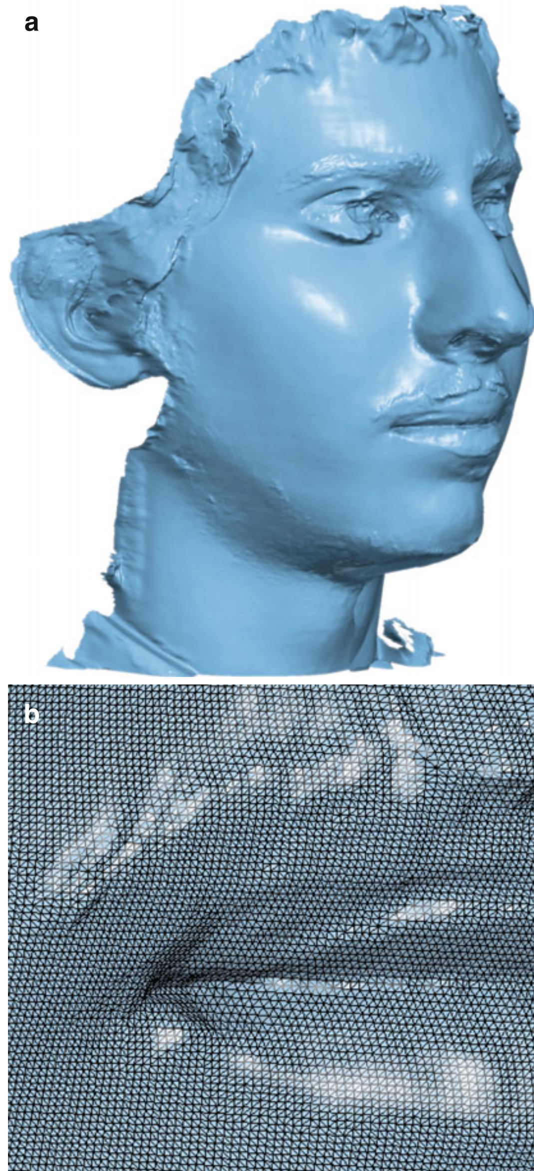

Fig. 4.8 (**a**) Facial surface as acquired by passive photogrammetry. (**b**) Detail of the corner of the mouth, showing size of triangular elements that constitute the surface

or by rotation of the patient seated on a motorised chair. Typical scans of the face may take 10–20 s. Other manufacturers use a horizontal laser stripe that scans the head vertically. This system is much faster than the rotational scan (less than 1 s) but requires multiple shots to acquire the whole face.

4.4.2.2 Structured Lighting

Structured lighting methods do not use a single laser stripe but project a complex light pattern over the whole object and acquire the 3D information from a single image. The main advantage of this method is the reduced exposure time, making it possible to register dynamic movements and facial expressions.

4.4.2.3 Photogrammetry

Photogrammetry uses two or more digital cameras to photograph the subject from different viewpoints. If the precise position of the cameras is known, then the location of each point of the subject's face in 3D space can be computed by simple triangulation, using data of the point's location on the images. The difficult part of this method is to identify corresponding points on the two images. Fortunately, skin has texture that allows matching between images, provided resolution and clarity are high enough. If texture is not sufficient, a random pattern can be projected on the face in order to facilitate correspondence search ('active' photogrammetry).

4.4.3 Dentition

Three-dimensional computer models of dental casts have many potential advantages. The reduced storage requirements may have been the first incentive to develop a 3D substitute of dental casts, but other benefits may prove more important. Models in electronic format can be retrieved faster than conventional ones and can be viewed on computer screens together with other electronic records. They allow transmission through the Internet for viewing at out-of-office locations and for sharing between doctors [91]. They enable virtual setups for treatment planning and can be used for fabrication of indirect bonding trays or other appliances and for guidance during bracket positioning [92, 93].

4.4.3.1 Hardware

Three-dimensional models can be created with a variety of methods, such as laser scanning, structured lighting, destructive scanning and CT scanning [94–96]. Differences between the methods relate to the accuracy and resolution, the time needed for a complete scan, the cost and simplicity of the procedure and the investment in equipment.

Laser scanning and structured lighting systems are relatively inexpensive and can be accomplished in a private office setting [96]. Both systems rely on projecting light (a laser beam or a pattern of dots or stripes) on the model and registering the reflected pattern by a single camera or two cameras. A major disadvantage is that multiple scans are usually required in order to acquire areas that are not visible to the projected light or the cameras from a single viewpoint. The individual scans then have to be "stitched" together by software in order to produce the final model, a process that may be time consuming. Another problem is that each model has to be processed individually and the method does not scale well to mass production. Intraoral scanning is an exciting possibility of this technology, as no impressions are

needed and view "stitching" is done automatically. The time factor is still an issue, as a complete scan may take several minutes.

Destructive scanning entails cutting thin slices of the models and scanning those. The 3D model is constructed by electronically stacking the individual slices. This method can give very detailed results and can be used for concurrent scanning of more than one model, but it is time consuming and the original model is destroyed in the process.

Use of CT scanning promises to cure most of the above shortcomings, but it is a process that requires expensive equipment. The level of detail may be lower than with some of the other methods, but progress in CT scanners may alleviate this problem. A major advantage is the simultaneous processing of many models and the possibility of scanning both plaster models and impressions.

4.4.3.2 Software

The final result of scanning is a computer file that contains the model of the dental casts as a collection of points in 3D space or as a triangular mesh surface. The utility of virtual dental casts depends heavily on the software for viewing and manipulating these surfaces. Current software enables viewing from any angle and at various zoom levels. Upper and lower dental casts can be viewed individually or in occlusion, and measurements of the teeth can be taken by marking points on the screen with the mouse. More sophisticated software allow isolation of individual teeth and their movement for the creation of virtual treatment setups (Fig. 4.9).

4.4.3.3 Clinical Use

A concern that has been raised in relation to the use of virtual models in place of the plaster ones is that it may be more difficult to perceive the anatomy from an image than from a true 3D object. Although software allows viewing from any angle and zooming to high detail, manipulation of the models is not as convenient as done by hand. Also, because haptic feedback is absent, it is not possible to assess the intercuspation between upper and lower teeth and get a feeling of occlusal contacts. Current computer interfaces do not seem to offer much hope for a satisfactory solution to this problem. Force-feedback devices are not sophisticated enough to simulate the complex contact between two plaster models, so the only present solution seems to be the adaptation of the orthodontist to the new medium. Currently available research indicates that treatment planning is not affected by the use of digital models [97].

Incentives to become accustomed to virtual models are the advantages that were mentioned above, including the creation of virtual diagnostic setups that are especially time consuming when performed on plaster casts. Virtual models are a practical necessity for construction of aligners for orthodontic treatment without brackets. They are also used for constructing transfer trays for indirect bonding, for automated wire bending and for feedback during direct bonding [93].

4.4.3.4 Accuracy

Accuracy of 3D digital models has been assessed by comparing measurements between plaster models and their digital counterparts. In general, digital models

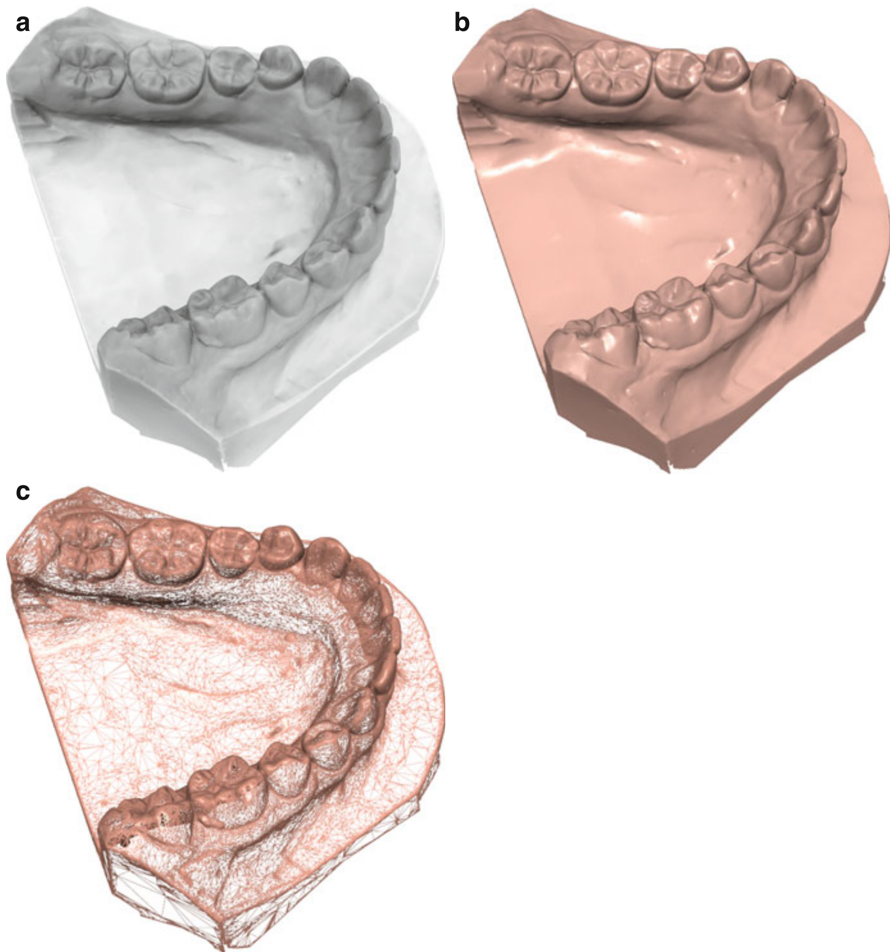

Fig. 4.9 Virtual models of dental casts rendered using different techniques. (**a**) Shading using ambient occlusion information, (**b**) shading using specular highlights to simulate glossy surface and (**c**) wireframe rendering to show level of detail

have been found to exhibit less accuracy and reproducibility [94, 98–100]. Some investigators reported systematic error, the digital models being smaller by about half a millimetre in all measurements [101], but this may have been because of alginate shrinkage during shipping or due to software problems [99]. Other systems have been found to be more accurate [94], but most investigators agree that any detected differences are not clinically significant for diagnosis and treatment planning [102]. In research settings, however, digital models need to be considered with caution, especially those produced by CT methods [98–100].

4.5 Simulation

Computer simulation is a rapidly expanding field in science. Simulation entails the construction of mathematical models that resemble their real counterparts, regarding their behaviour in specific circumstances. Simulation can be used for predicting the response of the real system, based on the calculations of a theoretical model that is known to apply, or for testing and refining theories, by comparing the behaviour of the model to the real data. In orthodontics, computer simulations can be used for research, education, treatment planning and patient information. Some of the current applications of computer simulation are discussed below.

4.5.1 Facial Soft Tissue Simulation

Facial simulation is a fast-moving research area, motivated partly by medical applications and partly by the film industry [103–108]. Various approaches are under development, and the details are mathematically and computationally rather involved and beyond the scope of this book. The basic principles are described in the following paragraphs.

Facial soft tissue simulation aims at constructing a computer model of the human face, including muscles and skin, so that simulation and prediction of orthodontic or surgical treatment is possible. This approach is different from the traditional predictions based on lateral cephalometric tracings in the following respects: (a) The computer model is three-dimensional, so it can be photorealistically rendered on the screen, with the skin having the texture and colour of the patient's face, and it can be rotated and viewed from whatever direction desired. (b) The response of the soft tissues to the surgical movement of the underlying hard tissues is not based on average ratios determined from previously studied patients. Instead, it is based on the physical properties of the soft tissues. (c) Muscles are also simulated, thus making it possible to view the face under varying expressions.

The model is composed of three parts, the skeletal unit, the muscles and the soft tissue covering. The skeletal unit is a model of the skeleton of the patient and is acquired from a CT scan. The skeletal unit is covered by a layer of simulated soft tissue, the outer surface of which is configured by data from a laser scan of the patient's face. Laser scanning equipment can digitise a large number of points on the skin surface in three dimensions and construct a representation of the face. The soft tissue layer is given the physical properties of actual soft tissues, so that it can respond to changes in the underlying skeletal unit or to pulling by simulated muscles embedded in it.

A simple model for simulating soft tissue is the mass-spring model. In this model, the soft tissue is represented as a collection of point masses connected by springs (Fig. 4.10). Some of the points are anchored on the skeletal unit, and they transfer any skeletal movements to the other points through the action of the springs. The outer points constitute the skin covering. Muscles are modelled as springs that connect skeletal units to soft tissue points or soft tissue points to soft tissue points and can contract and pull their endpoint towards each other.

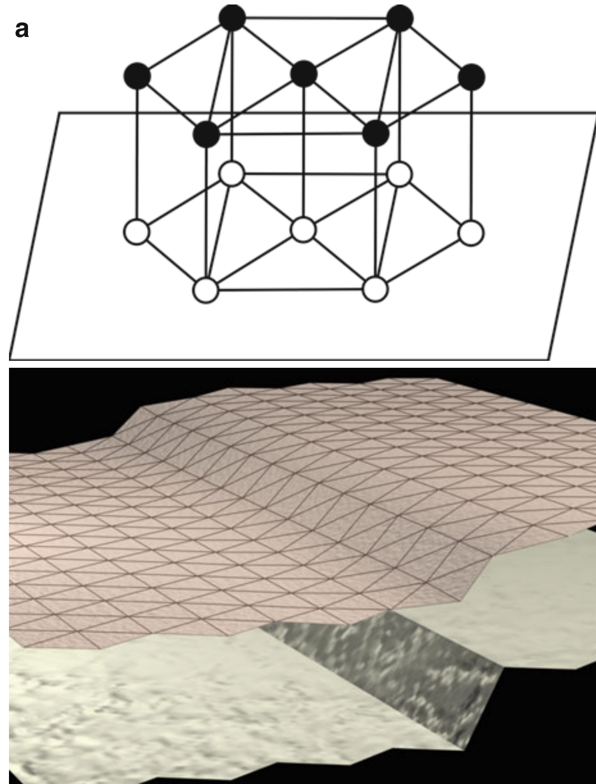

Fig. 4.10 Simulation model. (**a**) The spheres represent point masses that are connected by springs (only some are shown, as *straight lines*). *White spheres* are anchored to the osseous surface. *Black spheres* constitute the soft tissue surface. (b) Response of the model to simulated surgical movement of the skeletal surface. Even though the osseous surface has a sharp step, the overlying "skin" deforms smoothly, due to the elastic properties of the model. The grid on the "skin" shows the position of the individual triangular model elements, which extend down to the bone surface, but have been omitted for clarity

The main advantage of using such models of the soft tissues is that any changes in the underlying hard tissues should automatically result in accurate changes of the external facial shape. The model should be able to simulate changes due to functional mandibular movements (e.g. mouth opening and closing), orthodontic movements (e.g. incisor retraction) and surgical procedures. The 3D nature of the model makes it especially useful for predicting changes that cannot be modelled by the traditional 2D cephalometric methods, such as asymmetry cases and changes in the transverse dimension. Furthermore, because the model is physically based and simulates such behaviour of the soft tissues as elasticity and incompressibility, it should predict subtle effects such as soft tissue sagging and lip competence. The incorporation of muscle simulation opens the way for realistic prediction of facial dynamics [105, 109]. This should make it possible to predict the posttreatment aesthetics, not only in the neutral relaxed posture but during a smile or any other facial expression.

Facial simulation for surgical or orthodontic procedures is still in the experimental stages. Mainstream application in the orthodontic practice needs to overcome several obstacles. These include the added cost and radiation concerns of a CT scan for obtaining the underlying 3D skeletal geometry, the cost of obtaining a 3D laser scan of the facial surface and the development of easy to use software that is both

accurate enough for reliable predictions and fast enough for practical use. If these problems are overcome, then surgical planning should be able to provide significantly more help to the patient and doctor than current techniques.

4.5.2 Tooth – Alveolar – Periodontal Simulation

Simulation of teeth and periodontium is used for studying the response of these structures to loading, the pattern and magnitude of the generated stresses, the position of the centre of resistance and centre of rotation, the mechanical properties of the periodontal ligament and other variables. Most of the simulations use the technique of finite element (FE) analysis, where the simulated tooth and periodontium are subdivided into a large number of small units (elements). Each of these elements has simple mechanical properties requiring simple mathematical calculations, but taken together they can simulate complex geometric structures with nonlinear responses to applied forces. The literature contains several studies that have used this approach [110–116].

4.5.3 Wire Simulation: Biomechanics

Simulation of orthodontic wires is another field that has found application in orthodontic research and education. The behaviour of orthodontic loops under different conditions of activation is a subject of considerable interest. Laboratory testing is both time consuming and difficult [117], so testing on computer simulations provides valuable help. Computer simulations can be used for designing new loops, for investigating the force and moment properties of loops and for educational purposes.

Orthodontic wire simulation requires special considerations, because we are interested in large deformations. Therefore, equations from beam theory cannot be applied directly [118]. Finite element techniques, as used for the simulation of teeth and supporting structures, and other mathematical methods have been employed [119–122]. Surprisingly, although the theoretical considerations of orthodontic wire simulation are not especially challenging, very few software applications are currently available. Research into the biomechanical properties of loop designs and continuous archwires continues to be based on laboratory setups of force and moment sensors [123–128]. The most probable explanation is the lack of incentive from the clinical setting, due to the ever-increasing use of superelastic wires, and the related unpopularity of wire bending. This is unfortunate because reliable wire simulation methods could be coupled with periodontal simulation to construct a 3D "electronic typodont" that could be used both for research and educational purposes.

Figure 4.11 shows examples of activated loops and the resulting forces and moments, as predicted by a simulation program [120, 121]. The software allows the

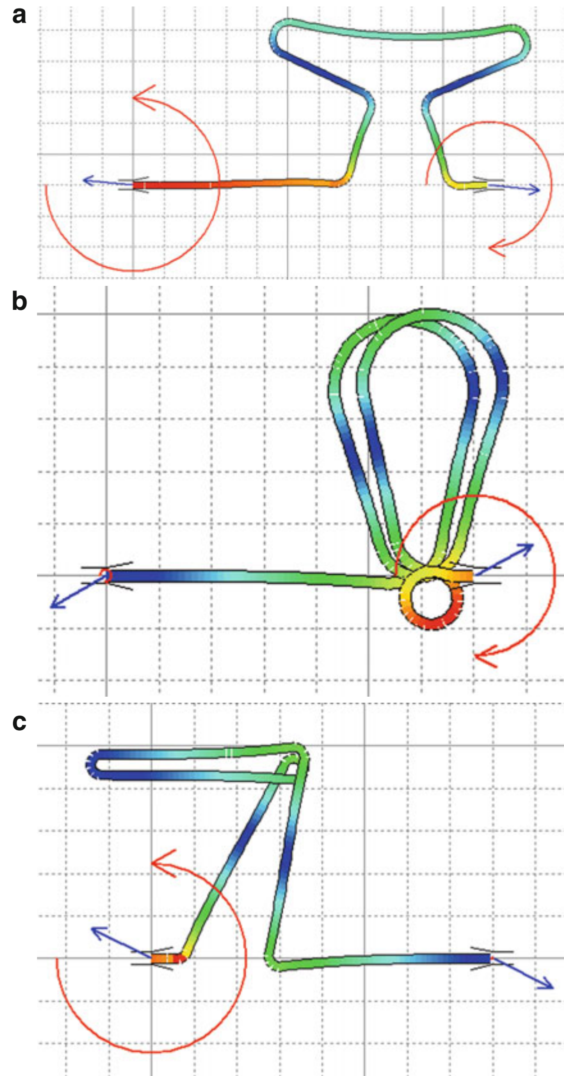

Fig. 4.11 Simulation of orthodontic loops. The loops are shown in the activation position, together with the forces and moments that are required for remaining at this configuration. Opposite forces and moments are applied by the loops on the two bracket positions. The loops are colour coded to show areas of low and high internal stress. The loops shown are (**a**) the T-loop, (**b**) the Gjessing loop [129] and (**c**) the Opus loop [127]

construction and evaluation of any loop design, but it is restricted to one plane of space, being a two-dimensional simulation. Therefore, the simulated loop is flat and cannot fully represent the designs that are used clinically. For example, in cases of canine retraction, the anti-rotation bends that are placed at the two ends of the loop cannot be simulated. Similarly, torquing moments resulting from twisting of the wire cannot be calculated. Otherwise, the software is easy to use and can be applied as a research and educational tool.

Conclusions

Computers have not only become ubiquitous but indispensable as well. Orthodontics, being a measurement-preoccupied specialty, is to benefit a lot from the application of computer-aided methods in clinical practice and research. For the time being, cephalometrics and imaging have been the most intense areas of development, but significant advances and explosive growth are shortly expected in 3D diagnostic systems and simulation. Knowledge in computer theory and practice is necessary for exploitation of the new methods. Postgraduate orthodontic programs will need to incorporate computer courses in their curriculum, in order to ensure that future researchers will be capable and proficient.

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Part III

Clinical Orthodontic Research

Clinical Orthodontic Research

5

Kevin O'Brien

5.1 Introduction

One of the great buzz phrases over the last few years is evidenced-based care. However, while this is easy to promote as a concept, we have to carry out the research that is going to provide us with the evidence that will either reinforce or make us change the care that we provide. At this point it is relevant for us to consider the type of investigation or research that is going to give the highest level of evidence, and this is always the randomized controlled trial or systematic review. All other types of study, such as case report, retrospective study, or comparison of case series, may not give us adequate information. As a result, I will confine this chapter to a description of how to carry out a randomized controlled trial.

If you are considering carrying out a trial, it is very important to be systematic in your planning, and the first stage of this is to prepare a protocol, and I will outline the steps that are necessary in preparing this important document. It is not my intention to be prescriptive in the outline; I am simply providing guidelines for the potential researcher who hopes to carry out a research project. None of these ideas are my own; they are a condensation of those derived from several readily available sources. Throughout this chapter I will provide examples from a hypothetical project that aims to investigate the influence of functional appliances upon facial growth.

5.2 The Research Protocol and Its Sections

A protocol is a document that explicitly states the reasoning behind and structure of a research project. The preparation of a protocol is the most important stage in the research process and is carried out for the following reasons:

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1. It encourages you to plan the project in detail, before you start.
2. It allows you to see the total process of your project.
3. It acts as a guide for all personnel involved in the project
4. It acts as a "reminder" to you and your supervisor (or coworkers) of the initial structure and aims of the project.
5. It enables you to monitor the progress of the project.
6. It is necessary if you need to apply for funding or ethical approval.

All protocols are divided into two main sections: (1) the problem to be investigated and (2) the method of investigation. These sections may be further subdivided as follows:

5.2.1 The Problem to Be Investigated

- Project title
- The research problem
- Background (including the literature review)
- The aims
- The hypothesis

5.2.2 Method of Investigation

- Plan of the investigation (including sample size calculation and statistical methods)
- Project milestones
- Dissemination of the results
- Resources required

5.2.3 The Problem to Be Investigated

5.2.3.1 The Project Title

The project title is one of the most important features of the protocol because it attracts the attention of the potential reader. It is, therefore, necessary to make it as short and to the point as possible. Let us consider two possible examples:

(a) An investigation to evaluate the effectiveness of functional appliances for the treatment of Class II skeletal growth anomalies.

This title is overlong and states the obvious in a rather "wordy" way. It goes without saying that because it is the title of a research protocol, it is an investigation that will evaluate something. A preferable approach may be:

(b) Do functional appliances modify facial growth?

The second title comes straight to the point without stating the obvious. It not only attracts the attention of a reader but it immediately tunes them into the subject matter.

5.2.3.2 The Research Problem

Before you start to develop a research project of your own, you must first identify a research problem. This is a difficult step particularly if you are an inexperienced researcher. Research problems are explanatory devices; they are carefully designed sentences about what you intend to find out.

It is difficult to design a problem statement and you should give it a great deal of careful thought. When you write the problem statement, your words must show an understanding of the research phenomena and should explicitly reveal your purpose.

You should go directly to the problem in the first sentence of page 1. Resist the temptation to give background or set the stage for the problem. When the protocol is ready, the readers will want to know the purpose of the study immediately. They will not want to search through several pages of text to discover what the protocol is about. To be effective your opening words should be clear and demand attention, for example:

(a) In this study I intend to find evidence that facial growth can be modified by functional appliances. If I can show that this occurs, this will be an important finding for orthodontic care.

(b) This will be an investigation to evaluate the effect of functional appliances upon facial growth.

If we examine the two statements above, statement (a) is easier to read because it is in the first person. This should be your preferred writing style as opposed to the use of passive voice (example b). You should, however, be careful that the first person is not overused and that your protocol does not read like a "letter to mum."

Avoid the look-around approach to a research problem. It is very important to avoid the "let's start a project and see what happens" approach. This will inevitably lead to a poorly coordinated and cumbersome project that drifts and may not have a well-defined ending. As a result, the statement of the problem should be explicit.

5.2.3.3 Background (Including the Literature Review)

The most important feature of the background to the project is that it should be brief and direct to the point. For a research protocol the background should be no longer than two single-spaced pages of A4 paper. In this section you should concisely review the literature that is relevant to the problem that you are trying to solve and is current. While it may be tempting to include all the literature, particularly classic papers, you should carefully consider whether to include research that is published more than 5 years before your project. In this respect it is probably good practice to limit the number of papers quoted to less than 20.

When you write the review, you should draw attention to the good points and the deficiencies of the studies quoted. You should also remember that it does not always mean that if a study has been published in a journal, it is flawless in its methodology and conclusion. Nevertheless, you should not be too critical of previous investigators because research technology and understanding of data analysis is a fast-moving field. Remember, if your study is published and it is considered state of the art today, it could be torn to shreds by neophyte researchers in 10 years' time!

In terms of writing style it is good practice to make your writing flow. There is a tendency to introduce concepts and previous studies by simply going through a shopping list of papers; for example,

> McNamara has shown that the Frankel appliance produces an increase in mandibular length of 3 mm. Pancherz (1979) used Herbst appliances and showed an effect of 3 mm increase in mandibular length; this is in agreement with a study by Hansen (1984). However, Tulloch et al. (1990) have suggested that it is not possible to come to any conclusions concerning the effect of functional appliances.

It is better to take the following approach:

> There have been many retrospective investigations that have concluded that either fixed or removable functional appliance have a growth-modifying influence on the mandible (McNamara 1984; Pancherz 1986; Hansen 1991). However, Tulloch et al. (1990) in a review of this literature have been critical of past research, and concluded that most studies are characterized by weak designs. As a result, it is not possible to come to any conclusions concerning the effect of functional appliances.

The literature review should logically lead to the statement of the aims of the proposed project.

5.2.3.4 The Aims

The aims of the project should be explicitly stated. These should be confined to the intention of the project, and they should arise from the literature review which is likely to show where more research is needed.

5.2.3.5 The Hypothesis

A hypothesis that you are trying to prove should be stated in the simplest form possible. It is considered good practice that hypotheses are stated in the null form, because they have their basis in inferential statistics. That is, you assume that there is no relation between variables and statistics are then used to calculate the probability that this relationship does exist. As a result, for our theoretical growth modification project, the null hypothesis will be: *Functional appliances do not have an influence upon the growth of the mandible*.

5.2.4 Method of Investigation

5.2.4.1 Plan of the Investigation

This is a description of the tactics of the research and is probably the easiest part of a research protocol to prepare. If you want to make the method easy to read, it is better to use the active voice, instead of the passive; for example,

> We will randomly allocate the subjects to the functional appliance and no-treatment groups, stratifying on age and sex. This is easier to read than: The subjects will be randomly allocated to the functional appliance and no-treatment groups, stratified on age and sex.

In a study protocol the method should be stated in the future tense. The method should be structured using the following subheadings: (1) subjects, (2) design, (3) experimental procedure, (4) materials, measurements and apparatus used, (5) sample size calculation, and finally (6) the statistical methods that you are going to use.

5.2.4.1.1 The Subjects

Many studies analyze information derived from patients that have been or are going to be treated. It is very easy for us to lapse into clinical language and state that the population under investigation is the "patients." This is patently not so when we have an untreated control group. It is therefore better practice to refer to the study population as subjects. When you describe the subjects of a study, you should report the following information:

1. The population the subjects will be drawn from.
2. The total number and the number in any subgroups within the investigation.
3. All aspects of subject selection that will provide information on the removal or minimization of bias.
4. The inclusion and exclusion criteria for the subjects

5.2.4.1.2 The Design

At this stage of the protocol, your readers should be beginning to understand the general design of your project. In this part of the method, they will learn exactly how you are going to do the study. The best way to approach this is to describe exactly how the total subject pool is to be divided into comparison groups.

> Subjects will be eligible for inclusion in the study if they satisfy the following criteria: (i) they have an overjet of greater than 6 mm (ii) they are still growing. Subjects with congenital clefts, or who have suspected or identifiable syndromes, will be excluded from enrollment. All subjects who are eligible for inclusion will be interviewed, and the purpose of the trial will be outlined. If informed consent is obtained, the operator will contact the central trial coordinator and provide details of the subject. The subject will then be randomly allocated, stratifying for operator and sex, to the experimental (treatment) or control (no-treatment) groups.

5.2.4.1.3 The Procedure

This will describe exactly what you are going to do with the subjects. This includes details of (1) the treatment to be provided to the experimental group and (2) the method to be used to collect data. For example,

> We will provide treatment to the subjects in the experimental group using the Twin Block appliance. Our treatment protocols will be those usually used in the department (more detail may be given here). After the subjects have received treatment for 12 months any changes will be evaluated. The control group will be observed only and evaluated 12 months after enrollment.

5.2.4.1.4 Measurement Used

Describe the materials and the method of measurement to be used in the study. For example, "In order to evaluate the effect of the treatment we will use the Pancherz

cephalometric analysis. This divides any reduction in overjet into dental and skeletal components. We will take cephalometric radiographs at the end of 12 months for both groups. The radiographs will be traced by one operator, and a sample of 30 will be retraced to calculate error."

5.2.4.1.5 Sample Size Calculation

I do not intend to describe the various methods for calculating the sample sizes to be used in an investigation. This is adequately covered in most statistical textbooks. Nevertheless, I should emphasize that this is an essential part of all protocols. If the sample size is too small, there is a considerable risk that the study may not be sufficiently powerful to detect a difference between the groups, if a true difference exists. The study would, therefore, be worthless, and a great deal of effort will be wasted.

5.2.4.1.6 Statistical Methods to Be Used

It is also essential that the statistical methods to be used in the investigation are outlined in detail. It is not sufficient to merely state the names of the tests to be used; you should describe the rationale for your choice of statistical tests. For example,

> The research question is concerned with the comparison of two groups (functional vs. control). The dependent variable will be the proportion of overjet reduction that is due to skeletal change. The independent variables will be study group, sex, age, compliance, and initial skeletal discrepancy. Because the influence of several, possibly interrelated independent variables, will be evaluated, we will use multiple linear regression analysis.

5.2.4.2 Project Milestones

This section is not essential. Nevertheless, it does provide a guide (and reminder!) for you and your supervisor to inform if you are ahead or behind schedule with your project.

5.2.4.3 Method of Dissemination of Findings

Again, this is not always essential, but it does let the reader know what you intend to do with the results of the study. It is occasionally possible to list the potential titles and publication strategy of the investigators. However, this can sometimes be considered an overly optimistic approach.

5.2.4.4 Resources Required

Finally, you should make a list of all the resources that you are likely to require to successfully complete your investigation. If these resources have cost implications, you should also note the potential cost of the investigation.

Preparing and presenting a protocol is one of the most difficult parts of carrying out a research project. It can also be the most interesting and satisfying. The result of this process should be a short (no more that 2,500 words) document that clearly outlines your research project. If the protocol is poorly prepared and not adhered to, it is unlikely that the project will yield the information that you hope for. At the worst the project may become unwieldy as you aimlessly drift through the research process, discovering little except disenchantment. On the other hand, if the protocol

is correctly structured and tight, your research will be an exciting experience that you and others will enjoy, and you will, hopefully, add to knowledge.

This has been a fairly generic description of the first part of carrying out a research project. Now I would like to consider the planning of a randomized controlled trial, which is an investigation that will provide us with the highest level of scientific evidence.

5.3 Planning a Randomized Controlled Trial

The randomized controlled trial (RCT) is one of the most simple and powerful research tools in which people are allocated at random to receive one of several clinical interventions. Before we consider the mechanics of how to carry out an RCT, we should consider the advantages of this type of research over other study methods. These are:

1. The RCT is prospective. As a result, the subjects and the data are under the direct control of the investigator.
2. The treatment or intervention is randomly allocated. Therefore, the perceptions of the investigator on the value of a particular treatment should not influence treatment allocation.
3. The experiment is planned before the data are collected. This is the important distinction between the RCT and the retrospective investigation and results in a minimization of bias that is inherent in the retrospective study.

While these advantages are clearly important and make the case for carrying out RCTs compelling, the orthodontic scientific community has shown remarkable reticence in adopting this method of research. The reasons for this are not important, and we hope that this simple guide will stimulate efforts to carry out randomized trials of some of our treatment methods.

When we considered a framework for this chapter, we decided to use the CONSORT guidelines (www.consort-statement.org). These are a set of guidelines that have been formulated to aid the reporting of RCTs, but they also help with study design. We will also use this paper to plan a theoretical investigation into the different methods of orthodontic space closure, as an example of the type of study that can be carried out. This study is an example of an effectiveness or "real world" trial evaluating the effects of an intervention under everyday clinical conditions.

The first stage in planning a trial is the generation of a question. In this example, our question could be concerned with whether nickel-titanium coils springs are more effective than power modules in space closure. The null hypothesis is therefore "There is no difference in the rate of space closure with Ni-Ti springs when compared to power modules."

5.3.1 The Study Population and Site of the Study

We now need to consider our study population. This is an important step because this population is relevant to both the question that we hope to answer and to the

provision of orthodontic treatment. It is fairly obvious that the study population for this example is easily defined and may be confined to children under 16 who are wearing the same type of appliance with extraction spaces that require closure. But this is not as simple as it sounds, as we have to make it clear that we are only going to include patients who have had first premolars extracted, as there may be a difference between space closure depending upon the tooth extracted. It is tempting to include all patients who have had an extraction, but this complicates, or "confounds," the study by introducing the additional variable of "tooth extracted." Furthermore, we should ensure that all patients are being treated with the same appliance type and prescription, as this is another confounder. Generally, it is best to produce a list of inclusion and exclusion criteria for the study. For our study the inclusion criteria are:

- Children under 16 years old at the start of treatment.
- Appliances will be MBT brackets.
- Teeth extracted will be first premolars.
- Space closure will start 1 month after placement of 019 × 025 stainless steel archwires.

While we need to consider the study population, we must not forget to pay some attention to the operators in the study. It is important that the findings of the study have generality, and the results are relevant to current orthodontic practice in the setting of care that most treatment is provided. This, however, is not always possible, especially when the RCT is investigating a new method of treatment. As a result, most orthodontic RCTs have been carried out in dental schools. This has the advantage of being able to keep close control of the operators and patients in the study. However, the trade-off for this control is the potential lack of generality. In our planned study, we would like to make the study results applicable to current practice, and the operators will be selected from local specialist orthodontic practitioners.

5.3.2 The Intervention

This is the treatment of interest in the study, and it is vital for the success of the study that this is clearly stated. Furthermore, it is essential that the existing literature does not already strongly suggest that one intervention is "better" or more effective than another. Finally, the operators in the study should not have a preference for any of the interventions that are being tested. This is termed *equipoise*. Importantly, if there is no equipoise, it cannot be ethical to randomize people to different interventions (or to intervention vs. control) because we already "know" the answer to the question that you are trying to investigate. This is a difficult situation, but in orthodontics it can be approached by considering the level of the evidence that the perceptions of any operator are based upon. If this is based on "evidence" from retrospective studies or more commonly expert opinion, this may also be considered to be unethical, and perhaps equipoise is the best place to be. Furthermore, if the operators have a preference, this may influence the way that they enter patients into

the study and could lead to bias. In our hypothetical study, the interventions may be clearly stated as:

- Nickel-titanium coil springs or
- Berman ligatures

Alternatively, an RCT may have a treatment compared with a "no treatment" or a control group. Ethically, it may not always be possible to randomize a control group and not provide treatment to some patients. Therefore, most RCTs in orthodontics will compare two or more treatments or interventions.

5.3.3 Patient Registration

Once ethical committee approval has been obtained, the next stage is patient recruitment. This may be considered in several stages:

5.3.3.1 Patient Requires Treatment and Is Eligible

It is important to ensure that patients entered into a trial are representative of the population. This is achieved by the operator considering that all patients who he or she sees with the entry criteria are eligible for the study. The clinician should not be selective.

5.3.3.2 Agreement to Randomize

The clinician should be in equipoise for a patient who is eligible for the trial, and he or she should be willing to accept the randomization.

5.3.3.3 Patient Consent

The patient should give written consent to take part in the study and agree to accept the randomization of any treatment. Importantly, the patient should be informed of the theoretical risks and benefits of the interventions under test. This allows fully informed consent that should be obtained in writing before the patient is registered and randomized. Ideally, patients should first be given the trial information and then given at least 24 h to consider whether they would like to participate.

5.3.3.4 Formal Entry

Details of the patient are then entered onto a logsheet of the trial or more commonly onto a computer database. The information that is collected is frequently the patient's name, hospital number, date of birth, and institution (if the trial is multicenter). The reason for this step is that the trial organizers need know about every patient entered. This enables them to obtain information on trial dropouts and patients who are not entered, and guards against the deviant investigators who do not give the randomized treatment.

The method of registration depends upon the setting of the trial. In a multicenter trial this is usually carried out by the clinician making contact with a central registration office by telephone. In a single-center study, this may be carried out by a person who is not a participating clinician. However, if the trial only has one

investigator, then patient registration can be left to the investigating clinician. In this case it is important that care is taken that no bias is introduced, such as, for example, through the investigating clinician having access to the randomization, which may influence whether they approach particular patients.

5.3.3.5 Random Assignment

It is only after these steps have been carried out does the operator learn to which treatment the patient has been assigned.

5.3.4 Randomization

This stage is central to the findings of the trial, because by allocating participants randomly, the characteristics are likely to be similar across the groups at the start. By keeping the groups balanced at baseline, the outcomes can be attributed to the intervention with minimal effects from other factors that may influence the treatments.

The method of randomization should be decided before the trial starts. There are many methods of randomization, and we will not go into detail, but interested readers should refer to the two excellent texts listed at the end of the chapter that consider this in detail. In brief, the object of randomization is to allocate one or more interventions (or control) in a manner that ensures the samples that you are going to compare are similar in every respect apart from the intervention. In most trials a randomization list has been prepared in advance using random numbers. The next stage is the method by which the operator finds out which treatment the patient has been assigned to. It is essential that the operator does not know what the assignment will be in advance, and there are several methods of concealing this. One popular method is to transfer the randomization list to a series of sealed envelopes with each containing the allocation on a card. The clinician then opens the next envelope in the series when the patient formally enters the trial. This method is particularly relevant to when the clinician registers his/her own patients. However, care needs to be taken to ensure that the clinician does not reseal the envelope after having discovered that the allocation was not what he/she was hoping!

The best method of randomization is to make use of a central registration office. In this method the treatment assignment is read from a prepared list and given to the investigator while he or she is still on the phone, following the registration of the patient. While this method is more expensive and requires more preparation than using envelopes, it does provide an almost foolproof method of allocation.

5.3.5 Blinding for Orthodontic Studies?

One important concept of medical studies is blinding. This is important because we should consider that if a patient or operator knows the identity of the treatment, the results of the study could be distorted. The effect of this is minimized by concealing

the identity of the treatments and by the use of placebos. Blinding may occur in many ways, for example, blinding the patient, the operator, the investigator who measures the outcomes, and the statisticians. However, when we consider the nature of orthodontic treatment, it is impossible to blind treatment allocation to both the operator and patient. As a result, the only type of blinding that we can practice is blinding of the person who records and analyzes the data. This is important because, for example, if the evaluator knows that a group of patients have had a new treatment, then he or she may record this data in a favorable manner. This can be done by concealing the identity of the patient and the treatment allocation by using numbers or having the data recorded by a different person from those who are going to analyze the data. If handled carefully, the space closure RCT in our example could be a single-blind study where the patient does not know the treatment method allocated.

5.3.6 Monitoring Progress

So now you have set up your trial and you think that you can just sit back and the trial will run, and all you have to do is to collect and analyze the data. Unfortunately, this is not the case! It is essential that the progress of the trial is closely monitored. Several areas should be evaluated as part of this process. The first of these is protocol compliance. You need to check that the study protocols are being followed by the operator(s) in the study. The easiest way of doing this for an orthodontic study is to periodically look at the records of the patients in the study and check for protocol deviations that are recorded. You should also check for adverse effects. While these are unlikely for an orthodontic study, you could find, for example, that a new type of archwire is constantly fracturing and patients are beginning to complain about this and are withdrawing from the trial.

Another error is to allow the patient records to pile up so that there is no organized check on trial progress. It is far better to record the data as the trial progresses, and this allows you to identify any problems with your outcome measures or even your method of data collection.

Finally, a careful record of all study withdrawals or dropouts should be made and as much baseline data as possible recorded. This will ensure that a statistical check can be made to discover whether the dropouts were similar to those people who remained in the trial.

5.3.7 Interim Data Analysis

An area of controversy is the analysis of the interim results of the trial. It is very tempting, particularly, in a study that is lengthy, to run an interim analysis and "have a look to see how things are doing." This is a common occurrence if the trial is attracting a degree of attention, and you need some data to present at a conference, etc. The problem with this is that the patients who are analyzed first may not be representative of the trial population, and any conclusions that are released are incorrect.

However, the counterargument to this is that it is necessary to run an interim analysis to check that the treatments are not causing harm, and this is important for the ethics of the trial. While this may be essential in some medical trials, again for orthodontics this should not be necessary. Importantly, if an interim analysis is done for this reason, then the results should not be published.

5.3.8 Treatment Intervention and Stopping Rules

It is important that only the treatment intervention of interest is carried out during the trial. In our study the treatment effect of the different space closing mechanics may be complicated by the operator wishing to use inter-arch elastics. In this example, inter-arch elastics should not be used for the trial duration or their use must be accounted for in the statistical analysis.

Stopping rules are defined at the start of the trial to ensure that there is a "safety valve." If, for example, it becomes obvious during a trial that one or more treatments are significantly worse or better than another, then the trial should be stopped.

5.3.9 Data Analysis

Methods of data analysis for RCTs do not markedly differ from other orthodontic studies, and these shall not be discussed in this chapter. However, it is important to consider the difficult question of how to handle data from patients who dropped out of the investigation.

When this occurs we are left with several choices. The first is to report the number of patients who withdrew from the investigation and emphasize that the two interventions under investigation had certain success and failure rates. Alternatively, the data analysis should include the results of the treatments on all the patients who entered the study, regardless of successful compliance or completion of the treatment. This is termed an intention to treat analysis. This type of approach results in a measure of the true effectiveness of the treatment and should be attempted wherever possible. One possible drawback of this approach with orthodontic treatment is that we may not have collected data on the patients who dropped out of the investigation as they may not have returned to the clinic. One solution to this is to statistically input data to compensate for the lost data. Several statistical packages have the ability to be programmed to carry out this type of analysis.

References

These two books provide excellent and detailed overviews of randomized controlled trials:

1. Jadad A (1998) Randomised controlled trials: a users guide. BMJ Books, London
2. Friedman LM, Furberg CD, DeMets DL (1998) Fundamentals of clinical trials. Springer, New York

Meta-analyses and Evidence-Based Orthodontic Practice

6

Moschos A. Papadopoulos

6.1 Introduction

Evidence-based medicine has been defined by Rosenberg as the process of "systematically finding, appraising and using contemporary research as the basis for clinical practice" [1]. This definition can also be applied to dentistry and, in turn, to orthodontics [2]. According to Bader [3], "Evidence-based Dentistry is not simply a new name for an old practice. The process is designed to answer specific questions, and it includes systematic and qualitative search of all available evidence."

The scientific community perceived the need for evidence-based data, and many attempts were made to produce evidence-based conclusions when conducting studies. The research method used in a study is primarily dependent upon the specific question(s) the study is addressing. For any posed clinical question, specific research designs can be used in order to provide information that is more valid than others [4, 5]. Clinical decisions, which are based on evidence-based conclusions, are made with regard to a classification of the quality of the various study designs [6, 7]. A hierarchy of quality of evidence of the various study designs is presented in Table 6.1. Randomized controlled clinical trials (RCTs) can produce very strong evidence [8, 9] in contrary to other study designs, because with their use the effectiveness of a treatment intervention can be better evaluated [10], and it is feasible to assess if one treatment intervention is better than another [7]. Further, systematic reviews (SRs) of RCTs should be credited with the production of even more strong evidence of treatment effects, because the source studies are precisely selected, and after appropriate evaluation, the outcomes are qualitatively synthesized following a specific protocol [7, 11–14]. Finally, in addition to the argument about which study design should be placed at the top of the pyramidal hierarchy of

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Table 6.1 Evidence of clinical effectiveness: a hierarchy of quality of evidence of the various study designs (*in descending order*)

Types of study designs
• Meta-analysis
• Systematic review of randomized controlled clinical trials
• Randomized controlled clinical trial
• Prospective controlled cohort study
• Retrospective controlled cohort study
• Prospective uncontrolled cohort study
• Retrospective uncontrolled cohort study
• Case–control observational study
• Case series without a control
• Cross-sectional survey
• Case report
• Anecdotal case report
• Unsupported opinion of expert

evidence, meta-analysis (MA) is regarded as the highest level of analysis, in which conclusions are made by quantitatively synthesizing the source data of several studies, such as of RCTs, providing already strong evidence, and therefore the evidence produced from MAs should be considered as the strongest possible [15]. All these study designs create a challenging environment of knowledge which could result in wrong conclusions if the appropriate attention is not given. For this reason, the main challenge for the orthodontist is the necessity of integrating the gathered evidence into clinical practice [16].

The aim of this chapter is to discuss the basic principles of MAs and their conduct and reporting, as well as to investigate the evidence currently existing in the orthodontic literature derived from high-quality studies, such as MAs. The results from these studies will be critically presented and discussed according the specific fields of orthodontics that were investigated. Lastly, the meta-analytic procedures used to obtain conclusions that can be used for practicing evidence-based orthodontics will be evaluated.

6.2 Meta-analysis as a Tool for Evidence-Based Practice

6.2.1 Background

The introduction of MA in medical research has an important positive impact on research synthesis in reopening the question of how best to summarize the results of many separate studies, using statistical procedures for computation of effect size in order to evaluate treatment effects. By combining the data from individual studies, a MA increases the overall sample size to a great extent, which in turn increases the statistical power of the analysis, as well as the precision for estimating the treatment effects. Because the "data" used for MAs are derived from original studies published in scientific journals, the quality of the MAs depends heavily on the quality of these studies. Therefore, issues such as how well these studies were conducted,

how well the findings were reported, and how they came to the attention of the meta-analyst are of great importance. Well-performed MAs allow a more objective appraisal of the evidence than traditional narrative reviews, provide a more precise estimate of a treatment effect, and may explain the heterogeneity between the results of individual studies [17]. In contrast, imprecisely conducted MAs may be biased, mainly because of the exclusion of relevant studies or the inclusion of inadequate studies [18]. Clearly, MAs present several advantages over conventional narrative reviews and may constitute a very powerful tool in clinical research. It is, unfortunately, not a perfect tool. Any statistical procedure or analytic approach can be misused or abused. Most of the criticisms of quantitative approaches attempting to reviewing literature are objections to the misuse or abuse of MAs.

6.2.2 Definitions

The term "meta-analysis" was first proposed in 1976 by Glass, a psychologist [15]. He identified three levels of analysis and thereby established a meaningful context for meta-analytic statistical procedures. According to this classification, *primary analysis* refers to the original statistical analysis of the data as it is collected by the researcher, *secondary analysis* refers to analysis of this data by someone other than the researcher who collected the original data (possibly for purposes or with analytic strategies other than those of the original researcher), and *meta-analysis* refers to analysis of the data of several independent studies.

Meta-analyses are statistical procedures that integrate the results of several independent studies considered to be "combinable" [19]. In other words, a MA provides a logical framework to a research review. This means that similar measures from comparable studies are listed systematically and the available effect-measures are combined, where possible [20]. Trying to define more precisely the term "meta-analysis," Kassirer [21] stated that "Meta-analyses are studies of studies ….".

Sometimes MAs are called "statistical overviews" or "systematic reviews", although the later term should be used only when quantitative data synthesis is not possible. In detail, with a SR a qualitative synthesis of the available data without any statistical analysis is performed when the data derived from the original studies are not similar and they cannot be combined, while with a MA a quantitative data synthesis with specific statistical meta-analytic procedures is attempted when there is data similar enough to be combined. In other words, a MA is a SR with statistical analysis.

6.2.3 Meta-analysis vs. Systematic or Narrative Review Articles

Meta-analyses are similar to SRs because they are based on thorough reviews of the literature about a single research topic. Nevertheless, they differ from these articles, in that they *statistically* combine the results of several studies into a single outcome measure. In addition, a significant problem among the narrative review articles is that, although they may start with the same basic aims and may also use the same material in the literature, different conclusions might be reached. Perhaps even more important than the disagreement of conclusions derived from different narrative

reviews is the disagreement in conclusions between a narrative review and a MA or a SR of the same research field. For example, Cooper and Rosenthal [22] performed a study with 41 faculty and graduate students who read and integrated the results of the same seven studies of gender differences in task persistence. According to the results of this study, it was found that the traditional reviewers were significantly more likely to conclude that there was no support for the hypothesis tested, in contrast to the generally more accurate meta-analytic reviewers.

6.2.4 Purposes of Meta-analyses

Meta-analyses may have several purposes, such as (a) to summarize a large and complex amount of literature on a topic, thereby resolving conflicting reports, (b) to clarify or quantifying the strengths and weaknesses of various studies on a specific research field, (c) to avoid the time and expense of conducting a clinical trial or in contrary to the need for a major clinical trial, and (d) to increase the statistical power of the analysis by combining data from many smaller studies. By performing such studies, the precision of an estimated treatment effect can be improved, variations in treatment effects through subgroup analysis can be investigated, and the generalizability of known treatment effects can be improved.

6.2.5 Limitations and Strengths of Meta-analyses

Meta-analyses are not without controversies. The benefits and risks of the more complicated procedures of MAs continue to be debated in the medical research community [23–29]. Controversial issues include (a) the ability of the researchers to combine studies that differ in important aspects, such as study populations, experimental designs, and quality controls, (b) the possibility that publication or selection bias could exist when conducting such studies, and (c) the fact that sometimes the results of MAs on the same research topic have been contradictory [20, 30]. Meta-analyses present some limitations as well as strengths and advantages. The following paragraphs provide a summary of these aspects [31].

6.2.5.1 Limitations

Meta-analyses have been accused of oversimplifying the results of a research area by focusing mainly on overall effects while downplaying mediating or interaction effects, and they been criticized that sometimes they are mixing studies that measure “apples” with those that measure “oranges” [32]; ultimately, no meaningful results can be obtained. Some researchers support the idea that MAs ignore the possible impact of study quality and risk of bias on the results of a review. By assessing the quality and risk of bias of the source studies included in the MA, this problem could be eliminated. When conducting a MA, there could be some mistakes in the classification of studies or in calculating the effect sizes because of the complicated coding system used, as well as because many studies do not provide all the necessary information for inclusion in the analyses. A further weakness of MAs can arise from situations when the available studies for a particular research area are few in number

or they are of low quality, their data are heterogeneous [33], and thus the corresponding results should be interpreted with great caution.

6.2.5.2 Strengths

The procedures employed in a MA permit quantitative synthesis of the literature that addresses specific research areas. A MA is likely to be more objective than a traditional extensive literature review, and therefore its use is more efficient to summarize large bodies of literature [34]. By means of MA, it is possible to reach stronger conclusions because more studies can be analyzed objectively with specific statistical methods as opposed to traditional narrative literature reviews. Furthermore, it should be mentioned that a MA is also very helpful in examining lack of evidence in a specific research field, providing insight into new directions for research, as well as finding mediating or interactional relationships or trends that they are either too minimal to be observed or they cannot be hypothesized and tested in individual studies [34, 35].

6.2.6 Indications and Contraindications of Meta-analyses

When conducting MAs, misleading results could generally be avoided if some basic principles are defined at the outset and carefully followed throughout the investigation. First, the need for performing such a study must be examined, and this need must be later justified in the publication. Victor [36] proposed some indications and contraindications that should be seriously taken into consideration for a MA. According to him, a MA is indicated when: (a) there is a necessity of an urgent decision and because of the lack of time, the performance of a new trial is impossible, (b) there is research on the safety aspects of drugs and other therapies and especially the evaluation of side effects, (c) there are many non-conclusive studies on a specific treatment, where small effects are important and when target-reaching trials are unrealistic in view of needed sample size and time, and (d) there are contradicting results of studies or effects, which vary too much among different types of subjects.

In contrast, MAs are contraindicated when (a) they are performed as the basis of drug approval and registration or (b) they are conducted in an attempt to make an irrelevant or unimportant effect significant by combining numerous insignificant studies showing small effects. In addition, it is also a misuse of MA when a researcher uses it to avoid the time and hard work of conducting a study of his or her own. Some basic principles to overcome these, as well as other, controversial issues are discussed below.

6.2.7 Conducting Meta-analyses

The most important issue before conducting a MA is to define a protocol to work with. This protocol, which will also help to reduce bias, should be precisely followed during the study and should include (a) the definition of the response variables, (b) the methods of literature searching for the individual studies to be included in the analysis, (c) the measures taken to reduce and identify publication bias, (d) the inclusion and exclusion criteria for the studies to be included in the analysis (selection bias), (e) how the data will be extracted from the studies, (f) how the quality

(risk of bias) of the source studies will be assessed, and (h) how the data will be analyzed statistically, including among others information concerning the definition of the effect size, the use of fixed effect or random effects model, heterogeneity assessment, as well as subgroup and sensitivity analysis).

6.2.7.1 Definition of the Response Variables

The definition of the response variables that are intended to be examined is significant, because most of the times different definitions of the same variables (as they are described in the original studies included in the analysis), are not suitable for combining. Furthermore, there is more need to present the minimum difference in the response variable that is considered to be clinically important and not only the statistically significant differences. This way, bias in interpreting the results of the analysis could be avoided, and research is mainly focused on clinical importance.

6.2.7.2 Methods of Literature Searching

First of all, the period of time covered by the literature search during which the desired studies have taken place must be mentioned, because this places the MA in perspective with developments in medicine that may precede, coincide with, or follow the performed study, and allow other researchers to replicate the study if needed. Nevertheless, the most important issue is a detailed description of the information sources and search strategies that were used to locate the studies to be included in the MA. In MAs, the "material" to be studied consists of individual source studies of identical or similar research fields. It is very important to identify as many of these studies as possible, so that the "sample" used in the MA will be as large and as representative as possible. An incomplete search can lead to "selection bias" by failing to identify important studies. In order to avoid selection bias when conducting a MA, the literature should be searched thoroughly and systematically. It is recommended that these search methods should include: (a) Keyword (index terms) searches of computerized bibliographic databases (such as MEDLINE), which will be described later in this chapter [37]. All additional information about these searches should also be presented, such as the databases searched, the dates covered by the search, and whether or not the search was conducted by a professional medical librarian. (b) Cross-checking citations to appropriate studies, either through reviewing the bibliographies of published articles that have been already identified or through indexing and citation services, such as the Science Citation Index. (c) Checking investigators, granting agencies, and industries or pharmaceutical companies for information on published or unpublished studies. (d) Searching trial registries of pertinent studies, such as The Cochrane Library Databases and the Oxford Database of Perinatal Trials. (e) Journal hand-searching.

In order to identify all possible studies to be included in a MA, not only one but several search strategies should be performed, and computer literature searches should not be the only strategy used to identify studies [20, 38, 39]. Another question that needs to be answered is if non-English language studies should be included in the analysis [38]. Unfortunately, MAs published in the English language journals restrict their search to papers which were also published in English, although they usually claim a very thorough search to retrieve every paper dealing with the topic to be investigated, which is rather questionable.

6.2.7.3 Measures to Reduce Publication Bias

The tendency for increased publication rates among studies that show a statistically significant effect of a specific treatment has been already documented [40–42]. Publication bias is correlated to the fact that studies with statistically significant results are more likely to be published than those without statistically significant results [43], and therefore measures should be taken to identify and reduce publication bias.

The inclusion of unpublished data in SRs or MAs is still controversial [20, 21, 44, 45]. However, before conducting a search for a MA, a decision should be made whether unpublished data that have not undergone a formal peer review process, including dissertations or conference presentations, should be included [21, 39, 42, 46].

Finally, publication and selection biases in MAs are more likely to affect small studies, which also tend to be of lower methodological quality. This may lead to "small-study effects," where the smaller studies in a MA show larger treatment effects. Small-study effects may also arise because of between-trial heterogeneity. Thus, it is generally considered that studies with large sample sizes are less likely to be affected by publication bias than studies with smaller sample sizes, because larger studies are more likely to be reported even when they present negative or not-significant results [38, 39].

A very useful graphic method that can be utilized to identify and present possible publication bias in MAs in an association between treatment effect and study size is the "*funnel plot*" [20, 38, 39]. This is a scatter plot of the treatment effects calculated from the individual studies on the horizontal axis, against study size or standard error on the vertical axis [47]. Funnel plots take advantage of the well-known "law of large numbers," i.e., the larger the sample size, the more probable it is that the sample mean is a better estimate of the population mean. This suggests that the precision that results from estimating the underlying treatment effect will increase as the sample size of the component studies increases. Thus, large studies appear at the top of the graph and tend to cluster near the mean effect size. Smaller studies appear towards the bottom of the graph and tend to be dispersed across a range of values.

In the absence of bias the studies are expected to be symmetrically distributed about the pooled effect size (Fig. 6.1), and thus visual inspection of a funnel plot may give a clear indication of publication bias. In the presence of bias, the plot's lower part can be expected to show a higher study concentration on one side of the plot than the other. In case of funnel plot asymmetry, additional statistical tests can be used to further assess the publication bias, such as the Begg & Mazumdar's rank correlation test [48], the Egger's test of the intercept [18], the Failsafe N or "file-drawer number" approach [49, 50], and the Duval and Tweedie's "trim and fill" method [51].

6.2.7.4 Selection Bias

Selection bias is a systematic error that results from the way the subjects are selected into the study or because there are selective losses of subjects before data analysis. Selection bias can occur in any kind of epidemiological study [52] and is one of the main reasons for divergent results among MAs, [39]. Thus, there is significant need to define the specific inclusion and exclusion criteria used for the studies to be included in the MA. Another aspect of selection bias is the populations utilized in the papers to be included in the MA. As MAs combine results of many different

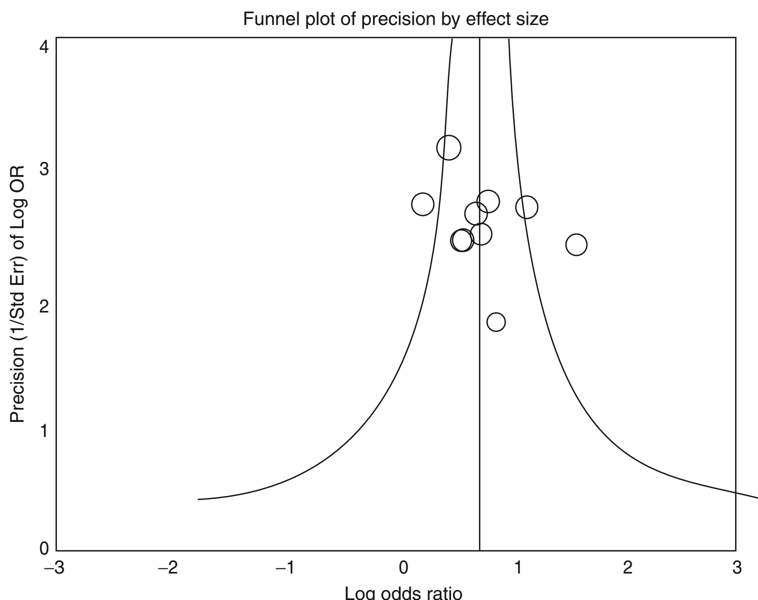

Fig. 6.1 Hypothetical funnel plot. A symmetrical plot indicates the absence of bias (*smaller circles* indicate smaller studies). The *circle* (study) on the right outside the *curve* indicates the possible existence of bias

studies, the populations involved in these studies may be many and diverse, and there is a need to describe these populations and to which the results are to be generalized. The variability in the populations included in the MA may make interpreting the results difficult.

The inclusion and exclusion criteria of the source studies should be as specific as possible in order to be able to compare only compatible and relevant studies of suitable quality. According to Kassirer [21], the studies included in a MA should meet the following criteria: (a) The included studies should test the same hypothesis [46] and should therefore have the same outcome or end point. (b) These studies should compare similar patients or similar interventions [38, 46]. For example, a study testing a drug against a placebo should not be compared with one that tests it against a competing drug. (c) The source studies should present some characteristics of scientific quality, such as adequate sample size, random assignment between treatment and control groups, masking of patients and examiners, quality controls on data collection and management, and finally formal statistical analyses. At least two reviewers should individually perform the selection of studies to be included in the analysis in a blind procedure, and then the inter-reviewer agreement should be objectively evaluated (i.e., by calculating the Cohen's kappa).

6.2.7.5 Quality (Risk of Bias) Assessment

Currently the term "risk of bias" assessment is widely used for evaluating each included study in a SR or a MA, while formerly, the authors tended to use the term "quality" analysis. When carrying out a SR or a MA it is important to distinguish between quality and risk of bias and to focus on evaluating and reporting the latter.

Assessing the risk of bias should be part of the conduct and reporting of any SR or a MA. In all situations, systematic reviewers are encouraged to think ahead carefully about what risks of bias (methodological and clinical) may have a bearing on the results of their SRs. There are three main ways to assess risk of bias: (a) scales, (b) checklists, (c) and individual component approaches. Scales that are commonly used for the assessment of risk of bias include: the Downs-Blacks scale [53], the Jadad scale [54], and the Newcastle-Ottawa Scale (NOS) as suggested by the Cochrane Handbook for Systematic Reviews of Interventions [55]. Checklists are less frequently used and potentially have the same problems as scales. The most commonly used is the PRISMA checklist [56]. The use of a component approach and one that is based on domains for which there is good empirical evidence and perhaps strong clinical grounds is advocated. The new Cochrane Risk of Bias tool is one such component approach [57].

Characterization of the quality of the studies allows researchers to use more specific inclusion or exclusion criteria and sometimes to assign weights to studies of different quality when combining them in the analysis. At least two reviewers should individually evaluate the quality of each study in a blind procedure, and then the differences among them should be objectively evaluated [38, 39] (i.e., by calculating the Cohen's kappa).

6.2.7.6 Data Extraction

The criteria used to extract data from various studies included in the MA should be reported and must be specified in advance. In other words, the "who, what, and how" of the extraction process should be precisely described. When the extraction criteria are too general, data extraction could be a subjective procedure, which may introduce bias into the process. Similar to quality assessment of the source studies, data extraction should be performed by two different researchers, and the inter-examiner reliability to establish the consistency of extraction should be evaluated by comparing the results for agreement (i.e., by calculating the Cohen's kappa).

6.2.7.7 Statistical Procedures

The effect of statistically pooling the results through a meta-analytic procedure is to increase the sample size. Consequently, the statistical power of the MA is stronger than that of the individual studies. Differences in statistical methods can result in different results, and therefore careful attention should be directed to choosing the statistical procedures for conducting MAs.

There are some basic differences in the statistical procedures used in the source studies and those used in MAs. The most significant difference is the unit of analysis, which in the individual source studies is the subject (e.g., patients, students, and observational entities), while in a MA, it is the results of a study.

Another important difference is the application of appropriate statistical techniques. Some meta-analysts simply apply the usual techniques used for primary analysis also in MAs [58]. As the hypothesis tested in a MA is based on differing sample sizes, these statistical tests will have different sampling variances and will probably violate the assumption of the homogeneity of these variances. Therefore, statistical procedures that are specially developed for MAs seem to be more appropriate for the quantification of the results of the individual studies [59–62].

Despite the existence of these specially designed procedures, some simple statistical methods for combining the results of individual source studies include various counting or summation procedures, such as the "*head counting*" or "*vote counting*" *approach* [32, 35, 63, 64] or simply combining significance tests of the source studies [35]. Researchers should be very careful in choosing these simple procedures for statistical analysis, which are actually not recommended. In the first procedure, the highest number of positive or negative studies determines the results of the analysis. In the second procedure, the results in a MA are summarized by mathematically combining the *p*-values of each of the studies into a single *p*-value. The latter approach is thus based entirely on *p*-values and because, as mentioned above, studies with nonsignificant results are published less often, this method will be associated with publication bias.

6.2.7.7.1 Effect Size

Before conducting a MA, an "effect size"of the variable under investigation should be initially estimated, which combines the differences and standard deviations of the response variables into standardized units across the studies. An effect size is a measure of the strength of the relationship between two variables in a statistical population, or a sample-based estimate of that quantity, and it is calculated from the source data. It is a descriptive statistic that conveys the estimated magnitude of a relationship without making any statement about whether the apparent relationship in the data reflects a true relationship in the population. In that way, effect sizes complement inferential statistics such as p-values. The most commonly encountered effect sizes (a) for continuous data include the Mean difference, the Standardized mean difference, and the Weighted mean difference, (b) for dichotomous data the Odds ratio, the Risk ratio, and the Risk difference, and (c) for censored or survival data the Hazard ratio.

6.2.7.7.2 Fixed-Effect and Random-Effects Models

The specific statistical method used depends on several factors, such as the hypothesis being studied and the nature of the response variable. The statistical methods may also involve one of the two types of models, which should be also reported. A "*fixed-effect*" model makes more assumptions about the variability/heterogeneity in the analysis, while a "*random-effects*" model makes fewer assumptions about the variability/heterogeneity in the analysis, is more conservative, and thus it may be a better summary of the effects reported in the individual studies of the MA.

6.2.7.7.3 Forest plot

A very useful way to report the results of MAs is to present a summary measure of the estimated size and direction of the effect of the treatment with the corresponding confidence intervals by means for example of the odds ratio [65]. This ratio corresponds to the likelihood of an outcome occurring in the treatment group divided by the likelihood that the same outcome will occur in the control group. The procedure can be very clear if it is presented by means of a table and/or a "*forest plot of odds ratio*" and its *95% confidence intervals* of each study included in the analysis (Fig. 6.2). The studies may be arranged in several ways to reveal certain features of the findings, such as publication date, sample size, and quality of the study. An odds ratio greater than 1 indicates an increased risk in the treatment group, while an odds

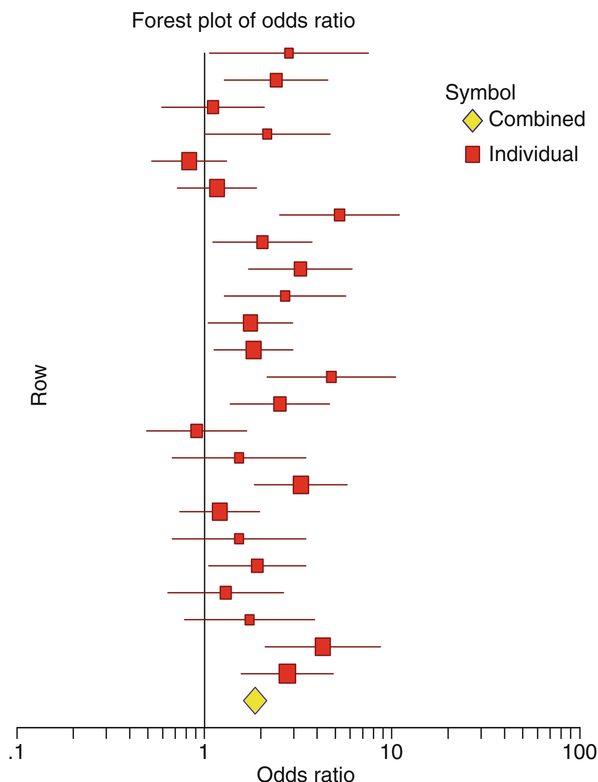

Fig. 6.2 A "forest plot of odds ratio." The estimated mean odds ratio and 95% confidence limits are shown for each study. An odds ratio of 1 means that the treatment neither increases nor decreases the risk of the outcome of interest

ratio less than 1 indicates a decreased risk. A ratio of 1 indicates no difference in risk. This means that the outcome is as likely to occur in the treatment group as it is in the control group, and therefore the treatment can be regarded neither as harmful nor as protective.

6.2.7.7.4 Heterogeneity or Homogeneity of the Data of the Source Studies

As a MA combines the results of several individual studies, the degree of disagreement or agreement among them (heterogeneity or homogeneity) can also affect the results of the MA. When the source studies present homogeneous data, they can be more easily interpreted; in contrast, heterogeneous data are more difficult to explain. Sometimes, the heterogeneity among the data of the individual source studies is due to the fact that these source studies are conducted without similar protocols [66].

Heterogeneity (and possible causes for it) can be initially assessed by means of visual inspection of the forest plots. However, for a more precise estimate, the Q statistic and the corresponding P-value, as well as the I^2 index, which is an indicator of true heterogeneity in percentages, should be used. A I^2 index value of 0% indicates no observed heterogeneity, while larger values show increasing heterogeneity (with 25% indicating "low", 50% "moderate" and 75% "high" heterogeneity) [58].

Another more useful way to examine and report the degree of heterogeneity between source studies and to identify outliers is the *L'Abbé scatter plot* (Fig. 6.3) [68]. This plot is a representation of event rates in the treated group against those in

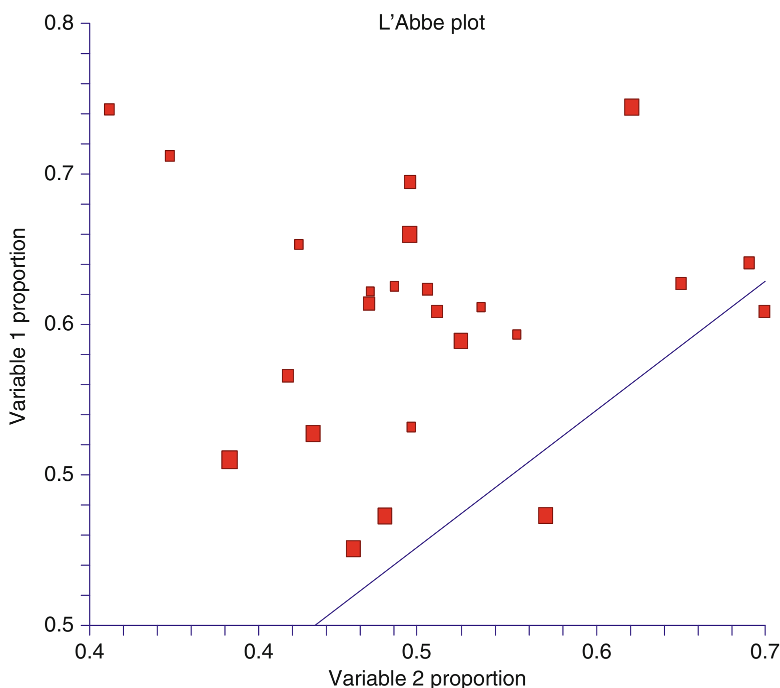

Fig. 6.3 The L'Abbé scatter plot shows the event rates in the treated group ("variable 1 proportion") against those in the control group ("variable 2 proportion"). If the treatment is beneficial, studies will fall to the right of the line of identity (the no-effect line), which is not the case in the current diagram. In addition, because the set of studies do not scatter around the line, which corresponds to the combined treatment effect, it could be concluded that this set of studies is heterogeneous

the control group. If the treatment is beneficial, studies will fall to the right of the line of identity (the no-effect line). A homogenous set of studies will scatter around a parallel line that corresponds to the combined treatment effect.

6.2.7.7.5 Sensitivity Analysis

When important choices and assumptions are made, there is a need to test them with a sensitivity analysis to determine whether their impact on the results is warranted. In a sensitivity analysis, some studies are excluded to determine how their exclusion affects the results. If the effect is great, the studies may have an excessive impact on the results. If the effect is small, the results may be more representative of all the studies.

6.2.7.7.6 Software for Meta-analysis

Over the past few years, computer software entirely designed for MAs has increasingly become available, while meta-analytic procedures have been introduced in general statistical software packages. These can be differentiated in commercial or freely available packages and are presented in detail in Tables 6.2a and 6.2b.

Table 6.2a Commercial software entirely designed for meta-analysis

Software	FAST* PRO	STATA	True Epistat	DSTAT	Comprehensive meta-analysis	MetaWin
Operating system	MS-DOS	Windows	MS-DOS	MS-DOS	Windows	Windows
Distributor	Academic Press, 24–28 Oval Road, London NW1 7DX UK	Timberlake Consultants, 47 Hartfield Crescent, West Wickham, Kent BR4 9DW UK	Epistat Services, 2011 Cap Rock Circle, Richardson TX 75080–3417 USA	Lawrence Erlbaum Associates, 365 Broadway, Hillsdale NJ 07642 USA	Biostat, 14 North Dean Street, Englewood, NJ 07631 USA	Sinauer Associates, 23 Plumtree Road, Sunderland, MA 01375–0407 USA
Internet address		www.stata.com			www.Meta-analysis.com	www.metawin-soft.com

Table 6.2b Freely available software entirely designed for meta-analysis

Software	RevMan	Easy MA	Meta-analyst
Operating system	Windows	MS-DOS	MS-DOS
Distributor	The Cochrane Collaboration	Dr. Michel Cucherat, Department of Clinical Pharmacology, 162 Avenue Lacassagne, F-69003 Lyon, France	Dr. Joseph Lau, New England Medical Center, Box 63, 750 Washington Street, Boston, MA 02111 USA
Internet address	http://ims.cochrane.org/revman	www.spc.univ-lyon1.fr/~mcu/easyma	Available by e-mail: joseph.lau@es.nemc.org

6.2.8 General Suggestions

In summary, when conducting a MA, there are some general rules that should be followed. Investigators are advised to:

- Do everything for the right cause and be prepared to defend it.
- Perform the procedures of statistical analysis in more than one way, whenever it is possible.
- Report everything (also in the form of tables or diagrams) in order to provide readers, reviewers, or editors with all the information they would need to evaluate the meta-analytic procedures followed.
- Finally, do everything to minimize mistakes and/or biases.

6.3 Meta-analysis in Orthodontic Literature

The first MA assessing the effect of a therapeutic intervention was published in 1955 and evaluated a placebo treatment [69]. Subsequently, the development of more sophisticated statistical techniques took place in the social sciences [70–73]. Meta-analysis was rediscovered by medical researchers for use mainly of the RCT research, particularly in the fields of cardiovascular disease [74–76], oncology [77, 78], and perinatal care [79]. Furthermore, MAs of observational studies [80] and "cross-design synthesis" [81, 82] have also been performed.

The number of papers published utilizing MAs in medical research has increased sharply during the last 10 years, and in the year 2003 represented 0.23 % of all published studies (Fig. 6.4 and Table 6.3). Despite the large number of MAs in medical research only 18 such papers were published (as cited in MEDLINE) in the field of orthodontics until the year 2003 (Tables 6.3 and 6.4). However, it must be emphasized that MAs are difficult to conduct in orthodontics because of the nature of the available literature [83].

Since MAs are considered, if not the best, one of the three most appropriate study designs that could provide the strongest level of evidence, and since there are very few of these type of studies investigating orthodontic issues, we can assume that

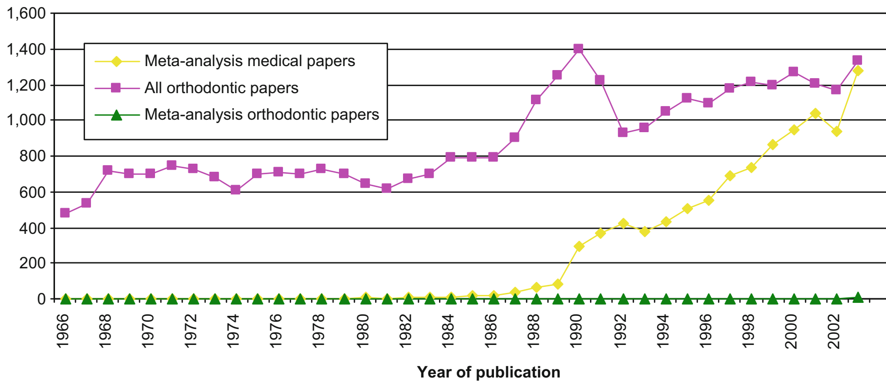

Fig. 6.4 Diagram presenting the number of meta-analyses indexed in MEDLINE during the period 1966–2002 (Results from MEDLINE search using title word or medical subject heading [publication type] "meta-analysis")

only some questions that concern the orthodontic community have been addressed satisfactorily so far. Furthermore, meta-analytic procedures are very complicated, and it is questionable whether the proper approaches have been used by the researchers when conducting these studies.

The aims therefore of this second part of the chapter are to investigate the evidence currently existing in the orthodontic literature that is derived from MAs, to critically present and discuss their results regarding the specific fields of orthodontics that they are dealing with, and to evaluate the methods used in these studies, in order to obtain conclusions that can be used for practicing evidence-based orthodontics.

For the identification of MAs, detailed search strategies were developed for the following databases: (a) MEDLINE (1966–2004), (b) the Database of Abstracts of Reviews of Effects (DARE) of the Centre for Reviews and Dissemination (CRD), and (c) the abstracts of Cochrane Reviews and the Cochrane Oral Health Group Trials Register. All electronic searches were conducted on February 25, 2004

The search strategy for MEDLINE was conducted after appropriate changes in the vocabulary and according to the syntax rules for each database, using 61 terms related to orthodontics (such as *orthodont**, *malocclusion*, *crossbite*, *openbite*, *prognath**, *retrognath**, etc.) and the term *meta-anal**. The search strategy implemented in the Database of Abstracts of Reviews of Effects (DARE) of the Centre for Reviews and Dissemination (CRD) and in the abstracts of Cochrane Reviews and the Cochrane Oral Health Group Trials Register, was performed using the term "*orthodontic*." Hand-searching was also conducted from the reference lists of the studies initially selected, in order to identify more articles to be included in this evaluation. No language restriction was applied during the whole identification process of the studies. The criteria for considering studies to be included in this MA were: (a) The studies should not only have been indexed as MA by the corresponding

Table 6.3 Number of meta-analyses as indexed in the MEDLINE database during the period 1966–2002

Year	All papers indexed	Publications about meta-analysis[a] (%)		Orthodontic publications[b]	Orthodontic publications about meta-analysis[a,b]
2003	568,232	1,279	0.23	1,336	5
2002	506,395	937	0.19	1,166	2
2001	516,499	1,039	0.20	1,207	2
2000	510,653	951	0.19	1,268	0
1999	471,569	865	0.18	1,199	2
1998	455,432	732	0.16	1,215	2
1997	437,230	690	0.16	1,175	1
1996	440,734	551	0.13	1,090	1
1995	430,932	509	0.12	1,119	0
1994	420,457	433	0.10	1,049	1
1993	410,592	374	0.09	955	0
1992	402,381	424	0.11	932	1
1991	399,002	368	0.09	1,220	1
1990	397,165	293	0.07	1,396	0
1989	390,099	86	0.02	1,247	0
1988	374,047	63	0.02	1,109	0
1987	355,824	34	0.01	898	0
1986	337,942	17	0.01	789	0
1985	324,870	16	0.00	795	0
1984	307,900	6	0.00	788	0
1983	299,009	13	0.00	698	0
1982	285,175	8	0.00	667	0
1981	274,284	3	0.00	612	0
1980	272,255	5	0.00	644	0
1979	274,001	0	0.00	703	0
1978	265,279	0	0.00	725	0
1977	255,556	1	0.00	702	0
1976	248,864	0	0.00	709	0
1975	244,272	0	0.00	703	0
1974	230,052	0	0.00	609	0
1973	226,714	0	0.00	680	0
1972	222,933	0	0.00	723	0
1971	218,695	0	0.00	745	0
1970	213,298	0	0.00	703	0
1969	211,202	0	0.00	699	0
1968	203,961	0	0.00	717	0
1967	186,620	0	0.00	534	0
1966	174,605	0	0.00	474	0
Sum	*1,276,4730*	*9,697*	*0.08*	*34,000*	*18*

Results from MEDLINE search using *a*, “meta-analysis” as *title word* or *publication type* and *b*, “orthod*” as *text word* or “cephalometry” as *medical subject heading term*

Table 6.4 Meta-analyses in the field of orthodontics as indexed in MEDLINE during the period 1966–2003

Number	Author
1.	Petren et al. [84]
2.	Papadopoulos [85]
3.	Popowich et al. [86]
4.	Bergstrand and Twetman [87]
5.	Ren et al. [88]
6.	Chen et al. [89]
7.	Kim et al. [90]
8.	Schiffman and Tuncay [91]
9.	Jager et al. [92]
10.	Nguyen et al. [93]
11.	Kim et al. [94]
12.	Orthlieb et al. [95]
13.	Burke et al. [96]
14.	Trpkova et al. [97]
15.	Miles et al. [98]
16.	Fox et al. [99]
17.	Tuncay and Tulloch [100]
18.	Mills [101]

Results from MEDLINE search using "meta-analysis" *as title word or publication type* and "orthod*" *as text word* or "cephalometry" *as medical subject heading term*

databases but they should also have performed statistical evaluation of the source data, since many SRs are indexed in MEDLINE as MAs although they do not perform quantitative synthesis of data.) (b) The research papers should have discussed a subject related to orthodontics.

Following the utilization of the search strategy in MEDLINE, 57 studies were initially retrieved. Forty-eight of them were excluded because either their subject was not related to orthodontics or, following evaluation of the methodology used, it was found that statistical methods were not applied (most of these studies were actually just SRs) or that the ones used were not appropriate. Only nine papers met the inclusion criteria and remained for further evaluation. The search strategy of the abstracts of Cochrane Reviews and the Cochrane Oral Health Group Trials Register revealed four studies. Two were SRs, one was not related to orthodontics, and only one study was a MA. The search strategy of the Database of Abstracts of Reviews of Effects (DARE) revealed initially 40 studies. Thirty-seven of them were excluded, while only three met the inclusion criteria described above. All three studies were also included in MEDLINE, while one was also included in the abstracts of Cochrane Reviews. Finally, one further study was retrieved with the hand-searching process. In total, only 11 studies met the inclusion criteria as set above and remained for further evaluation; they are presented in Table 6.5. These MAs investigated the following subjects: (a) functional appliances in Class II treatment, (b) maxillary

Table 6.5 Meta-analyses included in current evaluation, their subjects, and the corresponding statistical procedures for data analysis

Subject	Author	Statistical analysis of the primary data
Functional appliances in Class II treatment		
Effects of functional appliances on skeletal pattern	Mills [101]	Student's *t*-test, Mann–Whitney *U*-test
Efficacy of functional appliances on mandibular growth	Chen et al. [89]	ANOVA, Student's *t*-test for paired data, 95% confidence intervals
Maxillary protraction in class III treatment		
Effectiveness of protraction face mask therapy	Kim et al. [94]	Summaries of means and standard deviations and graphic representations
Effects of maxillary protraction in patients with Class III malocclusion	Jager et al. [92]	Standardized treatment effect variable "d," composite effect $d_{composite}$ and 95% confidence intervals, homogeneity analysis
Treatment of transversal problems		
Mandibular intercanine width in treatment and post-retention	Burke et al. [96]	Paired two-tail *t*-test, 95% confidence intervals
Maxillary expansion	Schiffman and Tuncay [91]	Funnel plot analysis (heterogeneity analysis), Student's *t*-test
Treatment of posterior crossbites	Harrison and Ashby [102]	Odds ratio, relative risk, relative risk reduction, absolute risk reduction for event data, weighted mean difference for continuous data
Orthodontics and temporomandibular disorders		
Orthodontics and temporomandibular disorder	Kim et al. [90]	Parametric homogeneity tests, probabilities of homogeneity, odds on parametric homogeneity
Cephalometric landmarks identification		
Cephalometric landmarks identification and reproducibility	Trpkova et al. [97]	Weighted average of the estimates, one-way analysis of covariance, 95% confidence intervals
Overjet size in relation to traumatic dental injuries		
Relationship between overjet size and traumatic dental injuries	Nguyen et al. [93]	Odds ratio with 95% confidence intervals, homogeneity tests
Obstructive sleep apnea syndrome		
Craniofacial structure and obstructive sleep apnea	Miles et al. [98]	Combined means and standard deviations for effect size, Z-scores, receiver operating characteristic (ROC) curves

protraction in Class III treatment, (c) treatment of transversal problems, (d) orthodontics and temporomandibular disorders (TMD), (e) cephalometric landmarks identification, (f) overjet size in relation to traumatic dental injuries, and (g) obstructive sleep apnea syndrome (OSAS).

6.3.1 Functional Appliances in Class II Treatment

In 1991, Mills [101] reported the effect of functional appliances on the skeletal pattern following treatment of Class II, division 1 patients. He reviewed the findings of 26 papers dealing with results of Andresen and Frankel appliances, as they were retrieved from cephalometric radiographs. These findings presented a high degree of consistency regardless of the backgrounds and the countries of the corresponding patient samples. In order to gain in statistical significance, the findings of the primary studies were combined to produce larger samples, and they were also compared with a control group produced from reports of untreated Class II, division 1 individuals. To accomplish this task, Mills [101] combined the means and the given standard deviations of the primary studies, using Student's *t*-test for the full figures and the Mann–Whitney *U*-test for the annual results. The author concluded that (a) there was no appreciable restraining effect on the forward growth of the maxilla in either group (functional appliances group and control group), (b) a slight mean increase in mandibular growth could be observed, mainly in a vertical direction, (c) no change in the position of the glenoid fossa was evident, and (d) there was a wide individual response, and average changes were rarely observed in a patient.

Regarding the methodology used in this study, although there was no language bias, no multiple publication bias, and no citation bias, the meta-analytic procedure followed by the author presented some problems: (a) No reference was made about the search method performed and whether databases were investigated or not, which could result in an incomplete inclusion of studies for the MA. (b) Statistical tests for primary analysis of studies (Student's *t*-test and Mann–Whitney *U*-test) were used and not specially designed tests for MA. Although some primary studies did not report standard deviations, nevertheless they stated that measurements did not vary in great extent between samples. The author assumed that the overall measurements given were a reasonable estimate. (c) The treatment durations of the patients included in the primary studies were different, and the control group consisted of cases with rather milder symptoms, which could lead to selection bias. For these reasons, the MA performed by Mills [101] cannot be regarded as done in an appropriate way, and therefore the corresponding conclusions stated by the author should be viewed with caution.

More recently, Chen et al. [89] investigated the efficacy of functional appliances on mandibular growth in patients with Class II malocclusion, aiming to test the hypothesis that functional appliances enhance mandibular growth when they are used for the treatment of Class II malocclusions. In order to identify the corresponding studies, they performed an electronic search in MEDLINE for the years 1966–1999. Six articles meeting validity standards were evaluated for 12 cephalometric variables. The data of the studies included in the MA were evaluated using ANOVA, Student's *t*-test for paired data, and 95% confidence intervals. For the variables Co-Pg, Co-Gn, SNB, LIA, and other measurements, the authors found no significant difference between the untreated control group and the group treated with functional appliances. However, for the variables Ar-Pg and the variables Ar-Gn, there was a significant difference between the control and the treated groups. These results

showed that it was not easy to reach definite conclusions about the effectiveness of the appliances used for the treatment of Class II malocclusion and suggested the need to reevaluate the use of functional appliances for mandibular growth enhancement. This was due to the many inconsistencies in measuring treatment results and to the different treatment durations.

Although there was no citation bias and multiple publication bias, some problems could be observed in the methodology used by the authors, such as: (a) the lack of searching databases additionally to MEDLINE, (b) the limitation of the language of the studies to English, (c) the statistical tests used for primary analysis of the studies (Student's *t*-test and ANOVA) and lack of a specially designed test for MA, (d) no mention of control and treated group sizes in the main text, although the results of the analysis were given in box plots, and (e) the differences in the age of patients at the start of treatment as well as differences in treatment duration in the various studies included in the analysis, which could indicate the possible existence of selection bias. Consequently, the MA performed by Chen et al. [78] presents some weaknesses. However, if more detailed inclusion and exclusion criteria had been applied, most probably the conclusions would have been more indistinct.

To conclude, even if some positive effects of functional appliances on the skeletal pattern and especially on mandibular growth can be assumed as reported by Mills [101], there is still no strong evidence that actually could confirm these findings.

6.3.2 Maxillary Protraction in Class III Treatment

In the first study, an attempt was made to evaluate the effectiveness of maxillary protraction with orthopedic appliances in Class III patients by Kim et al. [94], aiming to determine whether a consensus exists regarding controversial issues, such as the timing of treatment and the use of adjunctive intraoral appliances. In order to identify the corresponding studies, they performed an electronic search in MEDLINE for the years 1966–1996 and reviewed the abstracts and summaries of these articles by hand-searching. Fourteen studies met the selection criteria. In order to combine the data in the primary papers, Kim et al. [94] performed an analysis by summarizing the means and standard deviations of the primary studies and by graphically representing these results.

The statistical analysis of the changes following treatment in selected cephalometric landmarks showed no distinct differences between the palatal expansion group and the non-expansion group except for one variable, which increased to a greater degree in the non-expansion group. Examination of the effect of age revealed greater treatment changes in the younger group of patients. These results indicated that protraction face mask therapy is effective in patients who are growing, but to a lesser degree in patients who are older than 10 years of age, and that protraction in combination with an initial period of expansion may provide more significant skeletal effects.

Although there was no language bias evident in this study, some problems of the meta-analytic procedures that weaken the results should be mentioned. These

include the following: (a) use of common statistical approaches for the primary analysis of studies (summaries of the means and standard deviations along with graphic representations) and not specially designed tests for MA, (b) limitation of the literature search conducted by the authors to identify the corresponding studies only to MEDLINE without investigating other databases, (c) the ethnic maturation differential that may well exist in the primary studies and should also be taken into consideration, (d) the lack of a matched control group, and (e) the lack of standardization of the design of the various studies. For these reasons, the results of this MA should be regarded with caution.

Maxillary protraction was also the subject of a MA conducted by Jager et al. [92]. Their aim was to quantitatively assess the published results concerning the treatment effects of maxillary protraction on craniofacial growth of patients with Angle Class III malocclusion using a MA. In order to identify the corresponding studies, they performed an electronic search in MEDLINE for the years 1966–1998. In addition, the reference lists of the studies collected in the database search were surveyed for further information. Twelve studies remained for subsequent evaluation after implementation of the inclusion criteria. In order to combine the data of the primary papers, the results of different cephalometric measurements were reviewed by the Dstat 1.10 software in order to calculate a standardized treatment effect variable. The homogeneity of the variances of the different effect variables as well as a composite effect was calculated. The results of this analysis demonstrated that a significant composite effect of the protraction treatment on some craniofacial skeletal and dental components was evident. In conclusion, maxillary protraction was shown to have a significant treatment effect. However, several of the individual effect variables demonstrated a significant lack of homogeneity. Study characteristics that might possibly account for this variability were the patients' ages at the treatment start and the combination of maxillary protraction with rapid maxillary expansion. Regarding the statistical procedures used, the fact that a specially designed analysis was performed adds to the strengths of this study, while the limitation of literature searching in only one database (MEDLINE) could be regarded as a weakness.

To summarize, maxillary protraction could modify the skeletal and dental components of the face. Thus, it seems that the protraction treatment is effective in patients who are growing but to a lesser degree in patients who are older than 10 years of age. Moreover, protraction in combination with an initial period of expansion may provide more significant skeletal effects.

6.3.3 Treatment of Transversal Problems

The subject of the first study conducted by Burke et al. [96] was a MA of the changes of mandibular intercanine width during and after treatment and post-retention. In order to identify the corresponding studies, they performed an electronic search in MEDLINE. The reference lists of the studies collected through this search were reviewed, and recent issues of relevant journals were also hand-searched. Twenty-six

studies that assessed the longitudinal stability of post-retention mandibular intercanine width were evaluated. For the statistical analysis, weighted averages and standard deviations for the means were compared for linear changes in intercanine transverse dimensions during treatment (T1), immediately after treatment (T2), and after removal of all retention (T3). Paired two-tail *t*-tests were performed between T3 and T1 means on all groups, and the corresponding 95% confidence intervals were computed. Following the above meta-analytic procedure, the conclusions reached by the authors were the following: (a) Regardless of patient diagnostic and treatment modalities or whether treatment was extraction or non-extraction, mandibular intercanine width tends to expand during treatment of approximately 1–2 mm, to contract post-retention to approximately the original dimension, and to show a net change in post-retention between 0.5 mm expansion and 0.6 mm constriction. (b) While statistically significant differences could be demonstrated within various groups, the magnitudes of the differences were not considered clinically important. (c) The net change in mandibular intercanine width that was calculated from the sum of cases included in the MA was approximately zero, which supports the concept of maintenance of the initial intercanine width in orthodontic treatment.

Regarding the methodology used by the authors to evaluate the primary data, the following problems should be mentioned: (a) The authors used statistical tests for primary analysis of studies (paired two-tail *t*-tests) and not specially designed tests for MAs. (b) No control group size was mentioned. (c) The literature search was limited only to MEDLINE, and no other databases were investigated. (d) The post-retention period varied from 4 months to 12 years, which could indicate the existence of selection bias. (e) No test for homogeneity was demonstrated. Consequently, it is questionable if the results of this MA are strongly supported by the methodology followed by the authors, and they should be considered with caution.

The second study performed by Schiffman and Tuncay [91] evaluated existing trials on maxillary expansion in order to understand the appropriateness and stability of this procedure. The authors searched MEDLINE from 1978 to 1999, and additional hand-searching was also performed. The evaluation of the primary data consisted of coding and scoring each study with respect to preestablished characteristics. Following this evaluation, only six studies remained in the final analysis. An overall effect size was computed, and aspects of the study design were analyzed. The results of this study were the following: (a) The mean expansion after appropriate adjustment was 6.00 mm, with a standard deviation of 1.29 mm. Of the 6 mm average, 4.89 mm was maintained while wearing retainers. (b) The 6 mm average expansion with retention in the short-term (less than 1 year) yielded a 4.71 mm residual expansion, which subsequently was reduced to 3.88 mm during the short-term post-retention period. (c) In the long-term post-retention study period, only 2.4 mm of the residual expansion remained, which was no greater than what has been documented as normal growth. The authors concluded that there was inadequate evidence to support the opinion that the expansion achieved beyond what is expected from normal development of the maxilla could be retained.

Although selection bias was not present in the study, it should be mentioned that: (a) the literature search included only one electronic database (MEDLINE). No

other databases were investigated, and this search was conducted only for the period from 1979 to 1999. (b) There was a language restriction, since only English language publications were included in the study. (c) The authors did not present any *p*-values for the measurements given in their analysis, while on the other hand, they stated that the significance level was set at $p = 0.10$ (instead of the commonly accepted value in the medical community of $p = 0.05$). Therefore, this meta-analysis presents a few weaknesses, and the derived conclusions should be regarded with some caution.

More recently, Harrison and Ashby [102] performed a meta-analysis aiming to evaluate orthodontic treatment procedures used to expand the maxillary dentition and correct posterior crossbites. In order to identify relevant studies, the Cochrane Controlled Trials Register and MEDLINE were searched for all RCTs and controlled clinical trials (CCTs). In addition, hand-searching was performed. In total, 5 RCTs and 8 CCTs were included in the study. For the statistical analysis of the primary studies, the odds ratio, the relative risk, the relative risk reduction, the absolute risk reduction, the number needed to treat, and the corresponding 95% confidence intervals were calculated for event data. The weighted mean difference and the corresponding 95% confidence intervals were calculated for continuous data. The conclusions made from this MA supported that: (a) occlusal grinding in the primary dentition with/without the addition of an upper removable expansion plate was shown to be effective in preventing a posterior crossbite present in the primary dentition from being perpetuated to the mixed and permanent dentitions, (b) no evidence of a difference in treatment effect (molar and canine expansion) between the test and control intervention was found in the trials that compared banded versus bonded and two-point versus four-point rapid maxillary expansion, banded versus bonded slow maxillary expansion, transpalatal arch with/without buccal root torque, or upper removable expansion appliance versus quad-helix, and (c) insufficient data were provided regarding two-point versus four-point rapid maxillary expansion to allow a formal analysis.

Although the procedures used in this study were well performed and followed the guidelines for undertaking MAs [85], some trials included small sample sizes, and they were inadequately powered. Therefore, the authors concluded that further studies with appropriate sample sizes would be required to assess the relative effectiveness of these interventions.

To summarize, from the results of the studies that evaluated the treatment of transversal problems, it could be concluded that regarding mandibular expansion, the net change in mandibular intercanine width following treatment and retention was approximately zero, which supports the concept of maintenance of the initial intercanine width in orthodontic treatment. Thus, there was inadequate evidence to support the opinion that the maxillary expansion achieved beyond what is expected from normal development of the maxilla could be retained, while occlusal grinding in the primary dentition, with or without the addition of an upper removable expansion plate, was shown to be effective in preventing a posterior crossbite present in the primary dentition from being perpetuated to the mixed and permanent dentitions. Some weaknesses and problems in the methodology used (i.e., language bias,

publication bias, and lack of control group size) were present in the above studies, with the exception of the third study which could be considered as an illuminating example to other meta-analysts for the methodology followed by the authors.

6.3.4 Orthodontics and Temporomandibular Disorders

The study by Kim et al. [90] investigated the relationship between orthodontic treatment and temporomandibular disorders (TMD) in patients following completion of orthodontic therapy. The authors conducted a computerized search in MEDLINE, while additional material was retrieved by the reference lists of the articles found and from a list of published and unpublished studies. For the statistical analysis, the authors divided and extracted data from 31 articles according to study designs, symptoms, signs, or indexes. To test whether all primary studies attempted to estimate or observed the same true effect and whether variability between results of the studies was due to random error only, a statistical test for the hypothesis of parametric homogeneity (H) was conducted. In addition, probabilities of homogeneity *P*(H) and odds on parametric homogeneity (H) were calculated. The authors could not deliver a definitive conclusion regarding the question about the relationships between orthodontic treatment and temporomandibular disorders (TMD) because of the severe heterogeneity of the data of the primary studies. In addition, they stated that the data included in their MA do not indicate that traditional orthodontic treatment increased the prevalence of TMD and that a reliable and valid diagnostic classification system for TMD is needed for future research.

Despite the extreme heterogeneity of the data in the primary studies, the following parameters regarding the methodology used weakened even more the results of this investigation: (a) Only one electronic database was investigated. (b) Only English language papers were identified, which could lead to language bias. (c) Not all of the primary studies included in this attempt to conduct a MA were of the same quality. (d) The inclusion of studies regardless of the age of the patients, the large number of different types of appliances that were evaluated in the study, and the different durations of the treatment could suggest selection bias. To summarize, it was not possible for the authors of the above study [90] to produce evidence on the relationships between the prevalence of temporomandibular dysfunction and orthodontic treatment.

6.3.5 Identification of Cephalometric Landmarks

In this MA Trpkova et al. [97] tried to assess the magnitude of identification error for 15 lateral cephalometric landmarks. In order to identify the corresponding studies to be included in the MA the authors performed a computerized search in the MEDLINE database from 1966 to 1995 along with hand-searching. In total, six studies were evaluated. The statistical analysis of the primary data included a weighted average of the estimates in order to combine studies reporting means and

standard error, and one-way analysis of covariance in order to combine studies reporting standard deviations. The results of this study were a measure of the systematic and random errors involved when locating landmarks on lateral head films, and they were presented as standard mean errors with the corresponding 95% confidence intervals for the repeatability and reproducibility of the 15 cephalometric landmarks. According to this investigation, the authors concluded that 0.59 mm of total error for the x-coordinate and 0.56 mm for the y-coordinate are acceptable levels of accuracy, and that only the landmarks B, A, Ptm, S, and Go on the x-coordinate and Ptm, A, and S on the y-coordinate presented an insignificant mean error and a small value for total error. Therefore, these landmarks may be considered reliable for cephalometric analysis of lateral radiographs. For these reasons, the authors emphasized the importance of critical interpretation of cephalometric measurements and careful selection of landmarks for cephalometric analysis and suggested that a separate analysis to estimate the identification errors of landmarks with dubious reliability should be a prerequisite both for research purposes and in clinical practice.

Regarding the methodology used when conducting this MA, the following limitations that could weaken its strength should be mentioned: (a)The literature search included only one electronic database (MEDLINE), and no other databases were investigated. (b) No information was given if a language restriction was applied during the identification process of the papers to be included in the analysis. (c) No homogeneity test of the primary data was presented in the study. (d) No information was given regarding the ages of the patients in the primary studies. Consequently, the methodology followed by the authors weakens the evidence produced by this MA, and therefore the above-mentioned results should be treated with some caution.

6.3.6 Overjet Size in Relation to Traumatic Dental Injuries

In the only study where traumatic dental injuries were examined, Nguyen et al. [93] investigated the risk of traumatic injuries of the anterior teeth due to overjet. In order to identify relevant studies, they performed a literature search of MEDLINE (1966–1996) and Excerpta Medica (1985–1996) databases. Eleven articles were included and evaluated in their study. In order to qualitatively assess these articles, a methodological checklist for observational studies was developed. The relative risk of overjet, compared with a reference, was expressed as an odds ratio. For each primary study, the odds ratio and the corresponding 95% confidence intervals were computed, and subsequently these odds ratios were pooled across the studies. Finally, the influence of the quality of the studies on the pooled odds ratio was addressed. From the results of this analysis, the authors concluded that children with an overjet larger than 3 mm are approximately twice as much at risk of traumatic injury on anterior teeth than children with an overjet less than 3 mm and that the effect of overjet on the risk of dental injury is less for boys than for girls in the same overjet group. In addition, the risk of anterior teeth injuries tends to increase with increasing overjet size.

Regarding the meta-analytic procedures used in this study and despite the fact that a possibility of publication bias cannot be excluded, some additional problems should also be mentioned: (a) The studies selected in this MA showed data heterogeneity with the exception of the subgroup between the ages 6 and 18. (b) There was no reference to the number of the control group size in any of the studies selected to be analyzed. (c) The authors did not mention if they included studies reported in other languages than English, which possibly could suggest an English language bias. Therefore, although this MA was performed adequately well, the above-mentioned conclusions should be appraised with some caution due to the limitations of the source studies and the heterogeneity of the primary data implemented in the analysis.

6.3.7 Obstructive Sleep Apnea Syndrome

The MA study conducted by Miles et al. [98] investigated possible significant differences between the cephalometric variables describing the craniofacial skeletal or soft tissue morphology of individuals with and without obstructive sleep apnea syndrome (OSAS). In order to identify relevant studies, a MEDLINE search for the years 1966–1993 and hand-searching were conducted. Subsequently, a hierarchical analysis was performed to examine the quality of evidence within this body of literature. The meta-analytic procedures employed in the study were: (a) combined means and standard deviations for the OSAS and control groups in order to examine the distribution and consistency of outcomes across studies; (b) Z-scores for statistical significance testing between groups; and (c) the potential diagnostic accuracy of the variables, represented by the area under the receiver operating characteristic (ROC) curves. Following this evaluation the authors concluded that: (a) The literature is characterized by several methodological deficiencies, and therefore it is equivocal regarding a causal association between craniofacial structure and OSAS. (b) Evidence for a direct causal relationship between craniofacial structure and OSAS is unsupported by the literature, both qualitatively and quantitatively. (c) The rationale for OSAS treatments based on morphologic criteria remain unsubstantiated. (d) The two most consistent, strong effect sizes with the highest potential diagnostic accuracies were variables related to mandibular structure (Sn/MPA, Go-Gn). (e) Although mandibular body length (Go-Gn) appears to be an associated factor, this does not support causality. (f) More standardization of research methods and data presentation is required.

Regarding the methodology followed by the authors, although no publication biases existed, the studies included in the MA were limited to those published in the English language, which implies the presence of language bias. Furthermore, only one study met all the inclusion criteria set by the authors, while none of the efficacy treatment studies met the inclusion criteria. Consequently, the methodology followed by the authors did not result in strong evidence, mainly due to the small number of the original articles that was included in the MA.

6.4 Concluding Remarks

In summary, according to the results obtained from the MAs related to orthodontic subjects discussed in the aforementioned pages it can be concluded that: (a) There is not enough evidence to reach definite conclusions about the effectiveness of functional appliances used for the treatment of Class II malocclusion. (b) Maxillary protraction treatment is effective in patients who are growing, especially in those who are older than 10 years of age, and when it is performed in combination with an initial period of expansion may provide more significant skeletal effects. (c) Regarding mandibular expansion, the concept of maintenance of the initial intercanine width during orthodontic treatment remains unchanged. (d) Regarding maxillary expansion, there is inadequate evidence to support the opinion that the expansion achieved beyond what is expected from normal development of the maxilla could be retained, while occlusal grinding in the primary dentition with or without the addition of an upper removable expansion plate was shown to be effective in preventing a posterior crossbite. (e) There is not enough evidence concerning the relationships between the prevalence of temporomandibular dysfunction and orthodontic treatment. (f) Only the landmarks B, A, Ptm, S, and Go on the x-coordinate and Ptm, A, and S on the y-coordinate may be considered to be reliable for cephalometric analysis of lateral radiographs. (g) The risk of anterior teeth injuries may increase with increasing overjet size. (h) There is no causal association between craniofacial structure and obstructive sleep apnea syndrome.

The discussion of the methodology of meta-analytic procedures employed in the various studies revealed some problems and limitations, which weaken the evidence produced by these studies, and therefore a more critical appraisal of the findings should be undertaken. The main problems and limitations that were found were the following: (a) bias in the procedure of identifying and selecting the primary studies, and language bias or publication bias were either present or the possible measures taken (or not) to avoid these biases were not reported by the authors; (b) the lack of the homogeneity evaluation of the primary data, and if done the existence of heterogeneity in many studies; (c) the lack of information regarding the sample and control group sizes, which eliminates the possibility for other researchers to repeat the MA using the same information and data provided or to re-evaluate the scientific progress by applying a cumulative MA; (d) limitations in the treatment groups, such as the existing differences of the age of the patients, the analysis of treatment groups with developmental differences, and the comparison of different types of interventions weaken the conclusions produced from the meta-analytic statistical procedures; and (e) the small number of the original articles possible for inclusion in almost all the meta-analytic procedures, due to the lack of high-quality original research articles in orthodontic literature.

For all these reasons, it is obvious that more high-quality research papers in orthodontic-related subjects are needed in order to produce strong evidence concerning the effectiveness of the various treatment approaches used in our everyday clinical practice.

Key Issues of Clinical Interest

- Evidence-based orthodontics gains with the time the attention of researchers and clinicians exponentially.
- Only a few study designs can provide sound evidence for clinical decisions in medicine and consequently in dentistry and orthodontics.
- Among all available study designs, meta-analyses are considered to provide the strongest evidence.
- Although the quantity of meta-analyses in orthodontics is still low, their number is constantly increasing during the last years.
- The meta-analyses with orthodontic-related subjects conducted so far provided only limited evidence, mainly due to methodological inconsistencies and the low number of original studies appropriate to be included in the analyses.
- There is not enough evidence to reach definite conclusions about the effectiveness of functional appliances used for the treatment of class II malocclusion.
- Maxillary protraction treatment is effective in patients who are growing, and when it is performed in combination with an initial period of expansion may provide more significant skeletal effects.
- The concept of maintenance of the initial intercanine width during mandibular expansion remains unchanged.
- There is inadequate evidence to support the opinion that the maxillary expansion achieved beyond what is expected from normal development of the maxilla could be retained.
- Occlusal grinding in the primary dentition with or without the addition of an upper removable expansion plate was shown to be effective in preventing a posterior crossbite.
- There is not enough evidence concerning the relationships between the prevalence of temporomandibular dysfunction and orthodontic treatment.
- The risk of anterior teeth injuries may increase with increasing overjet size.
- There is no causal association between craniofacial structure and obstructive sleep apnea syndrome.

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Key Issues in Designing Epidemiologic and Clinical Studies in Orthodontics

7

Argy Polychronopoulou

7.1 Introduction

This chapter addresses key issues related to the design of epidemiologic studies as they apply to research in orthodontics. First, the fundamental measures of epidemiology and measures derived from them to quantify causal effects are presented. In addition, basic epidemiologic study design strategies as well as their strengths and limitations are explored. Lastly, sources of error both in epidemiologic study design and methods of error control and evaluation are described. Emphasis is placed on epidemiologic principles and concepts without resorting to mathematical notation.

Epidemiology is the study of the distribution and determinants of health-related states or events in specified populations, and the application of this study in controlling health problems. The application of epidemiological principles and methods to the practice of clinical medicine/dentistry is defined as *clinical epidemiology* [1, 2].

According to the above definition, two regions of epidemiologic research are determined: *descriptive epidemiology*, which is related to the study of the distribution and development of diseases, and *etiologic or analytic epidemiology*, which is related to the investigation of likely factors that form these distributions. Consequently, the study and the calculation of measures of occurrence of illness or any outcome of interest as well as effect indicators of illnesses constitute the central activity in epidemiology [3].

Before the basic indicators that are used in epidemiology are described, some information should be provided on the *meaning of population*. Populations are at

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the center of epidemiologic research because epidemiologists are concerned about disease occurrence in groups of people rather than individuals. A population can be defined as a group of people with a common characteristic such as age, gender, residence, life event, and use of medical/dental services. Moreover, the population can be fixed or dynamic/open. In the fixed population, no new individuals are added. Membership is based on an event and is permanent. On the contrary, in the open population, new individuals are added and removed. Membership is based on a condition and is transitory.

7.2 Measures of Disease Occurrence

The need for the use of indicators arose from the fact that absolute numbers in epidemiology do not provide useful information. The basic indicators that are used for the measurement of frequency of the appearance of illness are: (a) *prevalence* and (b) *incidence* [1].

Prevalence measures the probability of having a disease or any outcome of interest, whereas incidence measures the probability of getting a disease. Incidence indicators always concern new cases of illness (newly appeared or newly diagnosed). There are two incidence indicators: *incidence proportion* and *incidence rate* [4, 5].

Epidemiologic indicators can be calculated for the entire population but also for parts of the population on the basis of any characteristic. In the first case, they are called "crude" and in the second case, "characteristic specific."

7.2.1 Prevalence

Prevalence describes the proportion of people in a population who have a particular disease or attribute. There are two types of prevalence measures, *point* and *period prevalence*, that relate prevalence to different time amounts [3].

The calculation formula of point prevalence is:

$$\text{Point prevalence} = \frac{\text{number of existing cases of disease at a point in time}}{\text{total population at risk at that point in time}}$$

while the calculation formula of period prevalence is:

$$\text{Period prevalence} = \frac{\text{number of existing cases of disease in a specific period}}{\text{total population at risk during that period}}$$

Prevalence is a proportion. It is dimensionless and can only take a numeric value in the range of zero to one (Table 7.1). *Point prevalence* is used to get a "snapshot" look at the population with regard to a disease. *Period prevalence* describes how much of a particular disease is present over a longer period that can be a week, month, or any other specified time interval.

Table 7.1 Comparison of measures of disease occurrence

Measure	Interpretation	Range	Unit	Cases	Source population	Uses
Prevalence	Probability	0–1	None	Existing	At risk of becoming a case	Planning
Incidence proportion	Probability	0–1	None	New	At risk of becoming a case	Etiologic studies
Incidence rate	Inverse of waiting time	0 to ∞	1/Time	New	At risk of becoming a case	Etiologic studies

Thus, a study group with an orthodontic treatment needing prevalence of 0.45 shows that 45 % of the subjects require some type of treatment at the time of the examination.

7.2.2 Incidence Proportion

Incidence proportion is defined as the proportion of a population that becomes diseased or experiences an event over a period of time [5].

The calculation formula of incidence proportion is:

$$\text{Incidence proportion} = \frac{\text{the number of people who got the illness in the duration of a specific time period}}{\text{number of people in the population who are in danger of becoming ill at the beginning of the period}}$$

Incidence proportion is dimensionless and can only take numeric value in the range of zero to one (Table 7.1). Additionally, it is always referred to in the specific time period of being observed. Incidence proportion is also called cumulative incidence, average risk, or risk. Both the numerator and the denominator include only those individuals who, in the beginning of the time period, were free of illness and were thus susceptible to developing it. Therefore, cumulative incidence refers to those individuals who went from being "free of illness" at the beginning of the time period to being "sick" during that particular time period. Consequently, cumulative incidence can evaluate the average danger for individuals of the population to develop the illness during this time period. Cumulative incidence is mainly used for fixed populations when there are small or no losses to follow up. The length of time of monitoring observation directly influences the cumulative incidence: the longer the time period, the bigger the cumulative incidence. Thus, a 2-year incidence proportion of 0.20 indicates that an individual at risk has a 20 % chance of developing the outcome over 2 years.

A useful complementary measure to cumulative incidence is the *survival proportion* [5]. Survival is described as the proportion of a closed population at risk that

Person	Time (2000–2006)	Person Years accrued
A	2000 → 2002 (Diagnosed with outcome/disease)	2
B	2001 → 2005 (Moves away/lost)	4
C	2001 → 2006 (End of follow-up)	5
D	2000 → 2005 (Diagnosed with outcome/disease)	5
E	2000 → 2004 (Moves away/lost)	4
Total		20

Fig. 7.1 Person-time measurement in a hypothetical population, individual follow-up times are known

does not become diseased within a given period of time and is the inverse of incidence proportion. Incidence and survival proportion are related using the following equation:

$$\text{Survival proportion} = 1 - \text{incidence proportion or}$$
$$\text{Incidence proportion} = 1 - \text{survival proportion}$$

7.2.3 Incidence Rate

Incidence rate is defined as the relative rate at which new cases or outcomes develop in a population [3]. The mathematic formula of incidence rate is:

$$\text{Incidence rate} = \frac{\text{number of illnesses that are expressed in a population for the duration of a period of time}}{\text{sum, for each individual of the population, the time observed in which he / she is at risk of developing the illness}}$$

The sum of the time periods in the denominator is often measured in years and is referred to as "person-years," "person-time," or "time at risk." For each individual of the population, the time at risk is the time during which this individual is found in danger of developing the outcome under investigation. These individual time periods are added up for all the individuals (Fig. 7.1).

Logic follows that the total number of individuals who change from being healthy to being sick, in the duration of each time period, is the product of three factors: the size of the population, the length of the time period, and the "strength of the

unhealthiness" that acts upon the population. Dynamic incidence measures this specific strength of unhealthiness. The entry and exit of individuals from the population during the study period for reasons such as immigration, fatality from other causes, or any other competing risks are automatically taken into account. Therefore, including the time of danger in the definition, the incidence compensates for the main disadvantages that come into question from the calculation of the incidence proportion. The dynamic incidence is not a percentage, like the two previous indicators, since the numerator is the number of incidents and the denominator is the number of person-time units. The size of incidence rate is always bigger or equal to zero and can go to infinity (Table 7.1).

Thus, using the data presented in Fig. 7.1, we can estimate the following incidence rate: 2 cases/20 person-years = 0.1 cases/person-year, that indicates that for every 10 person-years of follow-up, 1 new case will develop.

7.2.4 Relationship Between Incidence and Prevalence

Among the indicators, various mathematic relations have been formulated, taking, however, certain acknowledgements into consideration [2, 3]. The equation that connects prevalence with incidence rate is:

$$\frac{\text{Prevalence}}{1-\text{Prevalence}} = \text{Incidence rate} \times \text{Average duration of the disease}$$

That is to say, prevalence depends on incidence as much as the duration of the illness. This is in effect when concerning a steady state, where the incidence of illness and the duration of illness remain constant with the passage of time. If the frequency of disease is rare, that is, less than 10 %, then the equation simplifies to:

$$\text{Prevalence} \approx \text{Incidence rate} \times \text{Average duration of the disease}$$

7.3 Measures of Causal Effects

A central point in epidemiologic research is the study of the cause of illnesses. For this reason, in epidemiologic studies, the frequency of becoming ill, among individuals that have a certain characteristic, is generally compared to the corresponding frequency among those that do not have this characteristic. The compared teams are often referred to as "exposed" and "not exposed." Exposure refers to the explanatory variable, that is, any agent, host, or environment that could have an effect on health. The *effect indicators* are useful in order for us to determine if exposure to one factor becomes the cause of illness, to determine the relation between a factor and an illness, and to measure the effect of the exposure on the factor [3, 5, 6].

Table 7.2 Organization of prevalence and incidence proportion data

		Disease		
Exposure		Yes	No	Total
	Yes	a	b	a + b
	No	c	d	c + d
	Total	a + c	b + d	a + b + c + d

Where: $a + b + c + d$ total number in the study, $a + b$ total number exposed, $c + d$ total number not exposed, $a + c$ total number diseased, $b + d$ total number not diseased, a number exposed and diseased, b number exposed but not diseased, c number not exposed but diseased, d number neither exposed nor diseased

Table 7.3 Organization of incidence rate data

		Disease		
Exposure		Yes	No	Person-time
	Yes	a	–	Person-time exposed
	No	c	–	Person-time unexposed
	Total	a + c	–	Total person-time

Comparing measures of disease occurrence in either absolute or relative terms is facilitated by organizing the data into *a fourfold* Table [7]. For example, the recorded data for the estimation of the prevalence and incidence proportion can be seen in Table 7.2.

While the presentation of facts for the estimation of the dynamic incidence can be seen in Table 7.3.

7.3.1 Absolute Measures of Effect

Absolute effects are differences in prevalences, incidence proportions, or incidence rates, whereas relative effects involve ratios of these measures. The absolute comparisons are based on the difference of frequency of illness between the two teams, those exposed and not exposed [7]. This difference of frequency of illness between the exposed and not exposed individuals is called risk or rate difference, but also attributable risk or excess risk and is calculated using the following mathematical formula:

$$\begin{aligned}\text{Absolute effect} &= [\text{measure of disease occurrence among exposed}] \\ &\quad - [\text{measure of disease occurrence among nonexposed}]\end{aligned}$$

The difference measure gives us information on the absolute effect of exposure on the measure of disease occurrence, the difference in the risk to the exposed population, compared to those not exposed, and the public incidence of the exposure. The risk or rate difference has the same dimensions and units as the indicator which

Table 7.4 Comparison of effect measures

Measure	Type	Range	Unit	Interpretation
Risk difference	Absolute	−1 to +1	None	Excess risk of disease among exposed population
Risk ratio	Relative	0 to +∞	None	Strength of relationship between exposure and disease, the number of times the risk of exposed subjects is higher or lower than the risk of nonexposed
Incidence rate difference	Absolute	−∞ to +∞	1/time	Excess rate of disease among exposed population
Incidence rate ratio	Relative	0 to +∞	None	Strength of relationship between exposure and disease, the number of times the rate of exposed subjects is higher or lower than the rate of nonexposed

is used for its calculation (Table 7.4). The difference refers to the part of the illness in the exposed individuals that can be attributed to their exposure to the factor that is being examined, with the condition that the relation that was determined has an etiologic status. Thus, a positive difference indicates excess risk due to the exposure, whereas a negative difference indicates a protective effect of the exposure.

7.3.2 Relative Measures of Effect

On the contrary, the relative measures of effect are based on the ratio of the measure of disease occurrence among exposed and not exposed [1]. This measure is generally called the risk ratio, rate ratio, relative risk, or relative rate. That is to say, the relative effect is the quotient of the measure of disease occurrence among exposed by the measure of disease occurrence among nonexposed.

$$\text{Relative effect} = \frac{[\text{measure of disease occurrence among exposed}]}{[\text{measure of disease occurrence among nonexposed}]}$$

The relative comparisons are more suitable for scientific intentions [2, 3]. The relative risk gives us information on how many times higher or lower is the risk of somebody becoming ill. It also presents the strength of association and is used in the investigation of etiologic relations. The main reason is that the importance of difference in the measure of disease occurrence among two populations cannot be interpreted comprehensibly except in relation to a basic level of disease occurrence. The relative risk is a clean number.

If the risk ratio is equal to 1, the risk in exposed persons equals the risk in the nonexposed. If the risk ratio is greater than 1, there is evidence of a positive association, and the risk in exposed persons is greater than the risk in nonexposed persons.

Table 7.5 Strength of association

Relative risk/rate	Relationship interpretation
1.1–1.3	Weak
1.4–1.7	Modest
1.8–3.0	Moderate
3.0–8.0	Strong
8.0–16	Very strong
16–40	Dramatic
40+	Overwhelming

If the risk ratio is less than 1, there is evidence for a negative association and possibly protective effect; the risk in exposed persons is less than the risk in nonexposed. A relative risk of 2.0 indicates a doubling of risk among the exposed compared to the nonexposed. The power of the relative risk or relative rate can be accredited according to Table 7.5.

We have examined the most commonly used measures in epidemiologic research that serve as tools to quantify exposure-disease relationships. The following section presents the different designs that can utilize these measures to formulate and test hypothesis.

7.4 Study Design

Epidemiologic studies can be characterized as measurement exercises undertaken to get estimates of epidemiologic measures. Simple study designs intend to estimate risk, whereas more complicated study designs intend to compare measures of disease occurrence and specify disease causality or preventive/therapeutic measures' effectiveness. Epidemiologic studies can be divided into two categories: (a) descriptive and (b) etiologic or analytic studies [2, 7, 8].

7.4.1 Descriptive Studies

Descriptive studies have several roles in medical/dental research. They are used for the description of occurrence, distribution, and diachronic development of diseases. In other words, they are based on the study of the characteristics of individuals who are infected by some disease and the particularities of their time-place distribution.

Descriptive studies consist of two major groups: those involving individuals and those that deal with populations [7]. Studies that involve individual-level data are mainly *cross-sectional studies*. A cross-sectional study that estimates prevalence is called a prevalence study. Usually, exposure and outcome are ascertained at the same time, so that different exposure groups can be compared with respect to their disease prevalence or any other outcome of interest prevalence. Because associations are examined at one point in time, the temporal sequence is often impossible to work out. Another disadvantage is that in cross-sectional studies, cases with long

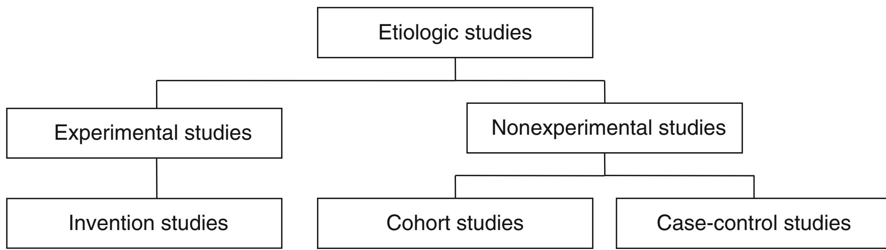

Fig. 7.2 Types of etiologic studies

duration of illness are overrepresented whereas cases with short duration of illness are underrepresented.

Studies where the unit of observation is a group of people rather than an individual are *ecological studies*. Because the data are measurements averaged over individuals, the degree of association between exposure and outcome might not correspond to individual-level associations.

Descriptive studies are often the first tentative approach to a new condition. Common problems with these studies include the absence of a clear, specific, and reproducible case definition and interpretation that oversteps the data. Descriptive studies are inexpensive and efficient to use. Trend analysis, health-care planning, and hypothesis generating are the main uses of descriptive design.

7.4.2 Etiologic Studies

The basic objective in epidemiologic science is the search for etiologic relations between various factors of exposure and various diseases. For this reason, etiologic studies are used (Fig. 7.2). It is about studies, experimental and not, which aim to investigate the etiology of a disease or to evaluate a preventive/therapeutic measure, through documentation of the association of a disease and a likely etiologic or preventive/therapeutic factor on an individual basis. Etiologic studies are distinguished in experimental (intervention studies) and in nonexperimental (observational studies). Nonexperimental studies are distinguished in cohort studies and in case-control studies [2, 3, 9].

7.5 Nonexperimental Studies

7.5.1 Cohort Studies

Cohort studies are also called follow-up studies, longitudinal studies, or incidence studies. The choice of individuals on which the study is based is made with the exposure or nonexposure on that factor as a criterion, for which its etiologic contribution to the illness is being investigated. These groups are defined as study cohorts.

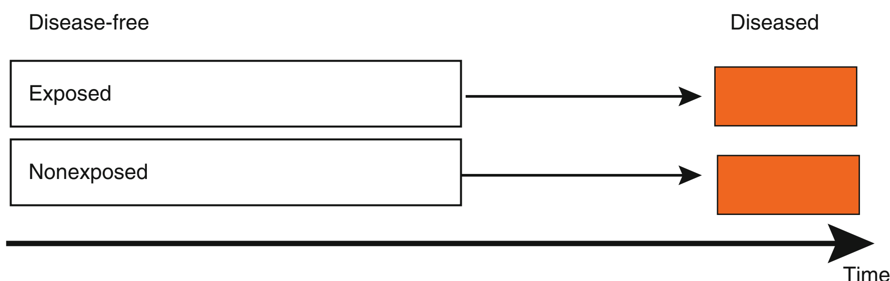

Fig. 7.3 Design of a cohort study

All participants must be at risk of developing the outcome. The individuals of the study are followed for a set period of observation, which is usually long, and all the new cases of the illness being studied are identified. Comparisons of disease experience are made within the study cohorts. It should be specified that the population studied is not free of all diseases but is free of the disease being studied at the beginning of follow-up (Fig. 7.3) [1, 3, 10].

There are two types of cohort that can be defined according to the characteristics of the population from which the cohort members are derived. The *closed cohort* is one with fixed membership. Groups are followed from a defined starting point to a defined ending point. Once follow-up begins, no one is added to the closed cohorts. The *open cohort*, or dynamic cohort, is one that can include new members as time passes; members come and go, and losses may occur.

The choice of the *exposed group* depends on the etiologic case, the exposure frequency, and the practical difficulties of the study, such as record availability or ease to follow up. The *nonexposed group* intends to provide us with information on the incidence of illness that would be expected in the exposed group if the exposure being studied did not influence the frequency of illness. Therefore, the nonexposed group is chosen in such a way as to be similar to the exposed group, in regard to the other risk factors of the illness being studied. It would be ideal for the exposure factor to constitute the only difference among the compared populations.

The exp osure is divided into two types: common and infrequent (increased frequency in certain populations). If the factor being studied is common enough in the general population, then it is possible to select a sample from the general population and then to separate the individuals in teams exposed to a varied degree and/or to nonexposed in the factor that is being examined. These studies are defined as *general-population cohort studies*. Cohort studies that focus on people that share a particular uncommon exposure are defined as *special-exposure cohort studies*. The researcher identifies a specific cohort that has the exposure of interest and compares their outcome experience to that of a cohort of people without the exposure.

The cohort studies that are based on information on the exposure and the illness that has been collected from preexisting sources in the past are called *retrospective cohort studies* (Fig. 7.4). The authenticity, however, of such a study depends on the thoroughness of the certification of the illness in the files on the population and for

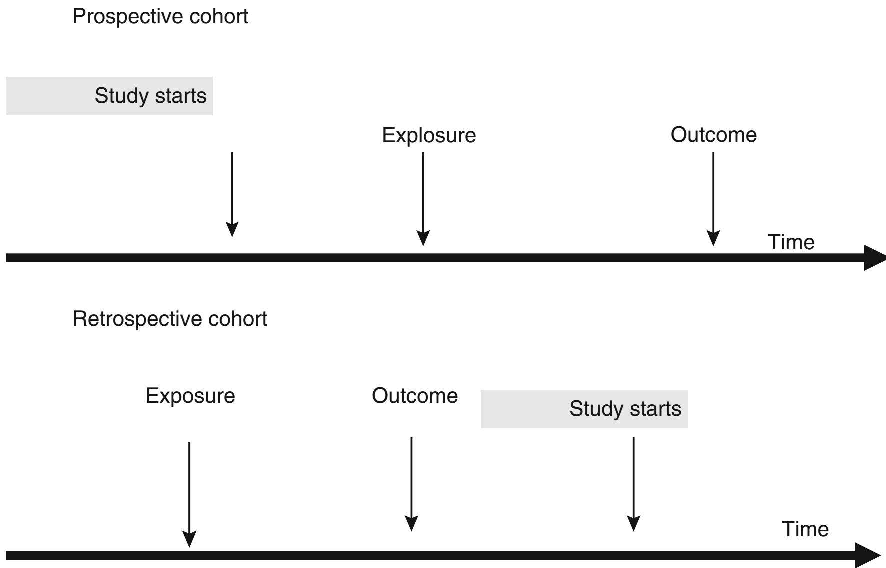

Fig. 7.4 Prospective and retrospective cohort study design

the time period being studied. Moreover, information on the relative confounding factors may not be available from such sources.

During the analysis of data, the frequency of illness is calculated (cumulative or dynamic incidence) in exposed and nonexposed individuals in accordance with the available data. With the use of a fourfold table, presentation and treatment of data take place. The relative risk or rate is calculated so as to determine the strength of the association and so that there exists the possibility of calculating the risk or rate difference.

Advantages and Disadvantages of Cohort Studies: Cohort studies are optimal for the investigation of rare exposures and can examine multiple effects of a single exposure. Since study subjects are disease-free at the time of exposure ascertainment, the temporal relationship between exposure and disease can be more easily elucidated. Cohort studies allow direct measurement of incidence rates or risks and their differences and ratios. However, prospective cohort studies can be expensive and time consuming, whereas retrospective cohort studies require the availability of adequate records. The validity of this design can be threatened by losses to follow-up.

7.5.2 Case-Control Studies

In prospective studies, a large number of individuals must be examined for their exposure conditions and must be followed up for a long time period so that a satisfactory number of outcomes are acquired. Such a study is not often practical or feasible.

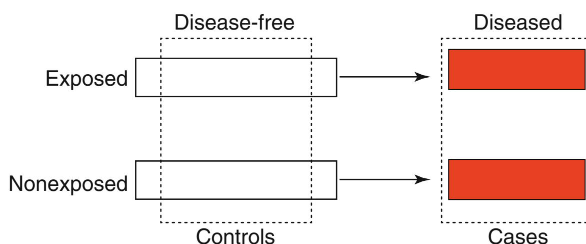

Fig. 7.5 Design of a case-control study

Table 7.6 Organization of case-control study data

		Disease		
Exposure		Yes	No	Total
	Yes	a	b	?
	No	c	d	?

This problem can be dealt with by using a study plan such as *case-control* (patient-witness). This design aims at achieving the same goal as the cohort study more efficiently using sampling. These studies have a characteristic methodology, during which a sample of the population being studied, featuring the common characteristic that they are cases, is used. Simultaneously, controls free from that particular disease are chosen as a representative sample of the population being studied. Ideally, the control group represents the exposure distribution in the source population that produced the cases. Then, exposure information is collected both for the patients and for the controls. Data are analyzed to determine whether exposure patterns are different between cases and controls (Fig. 7.5) [1, 3, 11].

The basic characteristics of case-control type studies are that the selection of individuals is made based on the criterion that they have or have not been infected by the illness being examined. There are many potential sources of *cases*, such as those derived from hospitals or clinics and those identified in disease registries or through screening programs. For causal research, incident disease cases rather than prevalent disease cases are preferable. Basic criteria in the choice of the group of cases are that they must constitute a relatively homogeneous group from an etiologic point of view and the facts about the illness must come from reliable sources.

Controls are a sample of the population that represent the cases and provide the background exposure expected in the case group. In many cases, the use of more than one witness group is necessary. Several sources are available for identifying controls including the general population (using random sampling), hospital- or clinic-based controls, and relatives and friends of cases. Persons with disease known or suspected to be related to the study exposure should be excluded from being used as controls. In Table 7.6, the organization of facts in a case-control study are described.

In these studies, it is not possible to calculate the frequency and effect indicators that we have known up to this point. This is owed to the fact that the size of the group of controls is arbitrary and is determined by the researcher. In these studies, we can use the odds ratio, which constitutes a very good estimate of the strength of association

of exposure-illness (relative risk) [12]. The odds ratio is defined as the ratio of the odds of being a case among the exposed (a/b) divided by the odds of being a case among the unexposed (c/d). Thus, the odds ratio is calculated as follows:

$$OR = \frac{a / b}{c / d} = ad / bc$$

The odds ratio is interpreted in the same way as the relative risk. An odds ratio of 1 indicates no association; an odds ratio greater than 1 indicates a positive association. Thus, exposure is positively related to outcome. If exposure is negatively related to the disease in a protective association, the odds ratio will be less than 1.

Advantages and Disadvantages of Case-control Studies: Case-control studies are cheaper and easier to conduct than cohort or experimental studies and are the method of choice for investigating rare diseases. In addition, case-control studies offer the opportunity to investigate multiple etiologic factors, simultaneously. However, case-control studies are not efficient designs for the evaluation of a rare exposure unless the study is very large or the exposure is common among those with the disease. In some situations, the temporal relationship between exposure and disease may be difficult to establish. In addition, incidence rates of disease in exposed and nonexposed individuals cannot be estimated in most instances. Case-control studies are very prone to selection and recall bias.

7.6 Experimental Studies

7.6.1 Intervention Studies

Intervention studies, commonly known as trials, are experimental investigations. They are follow-up studies where the researcher assigns the exposure study subjects. They differ from the nonexperimental studies in that the condition under which the study takes place is controlled.

Intervention studies are used for the evaluation of the effectiveness of preventive and therapeutic measures and services. In the first case, they are called preventive trials and, in the second case, therapeutic trials. Preventive trials are conducted among disease-free individuals, whereas therapeutic trials involve testing treatment modalities among diseased individuals. Additionally, two types of trials are determined: individual trials, in which treatment is allocated to individual persons, and community trials, in which treatment is allocated to an entire community. Epidemiologic studies of different treatments for patients who have some type of disease establish a broad subcategory, namely, *clinical trials*. The aim of clinical trials is to investigate a potential cure for disease or the prevention of a sequel [5, 13].

The individuals who participate in intervention studies come from a more general group, for which the results of the research should be in effect. This group is called a *reference population or a target population*. Once those who cannot

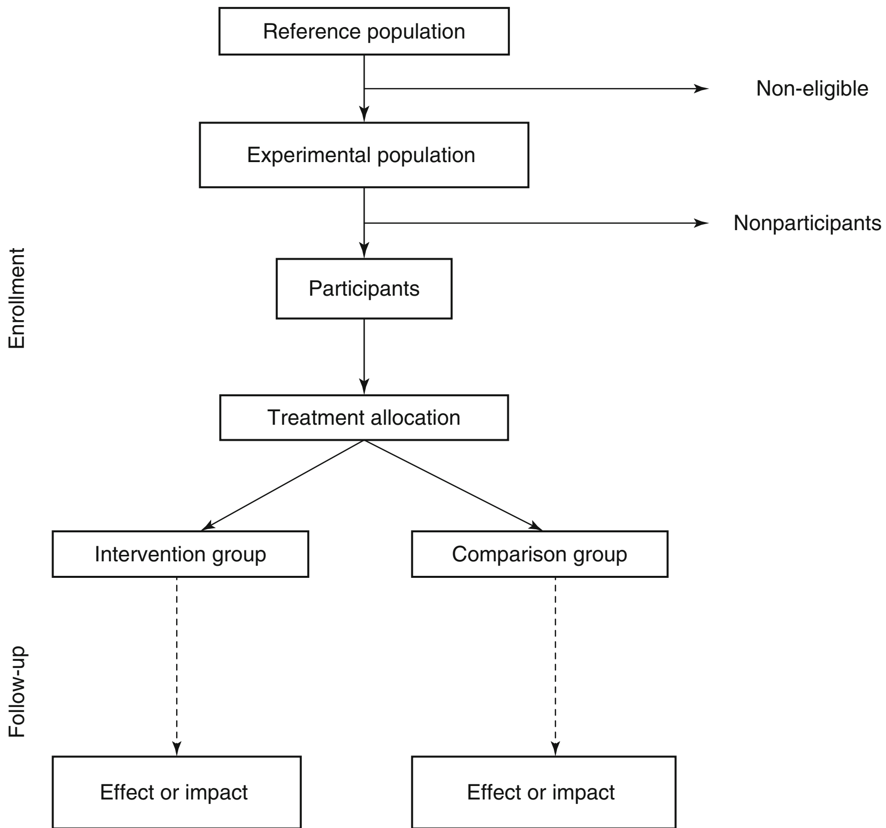

Fig. 7.6 Flowchart of an intervention study

participate in the study are excluded, those remaining, who are all likely candidates for the study, make up the *experimental population*. Once the potential group of subjects is determined, it is essential to get informed consent from the participants. Subjects unwilling to give consent should be removed. The eligible and willing subjects are then allocated into two main groups: (a) *basic-intervention/experiment group* and (b) *comparison group*. The study subjects are followed for a specified period of time under strict conditions, and the effects/outcomes are carefully documented and compared between the groups. Below, there is a flowchart of an intervention study (Fig. 7.6).

In the nonexperimental research (cohort and case-control), the known confounding factors can be monitored either as a choice of the compared groups or during the analysis of the data, but there is no sufficient ability to control the unknown confounding factors. On the contrary, in intervention research, it is possible to methodologically check both the known and the unknown confounding factors with the application of randomization. With *randomization*, each individual has the same

probability of receiving or not receiving the preventive or therapeutic measure being studied. Randomization of treatment allocation, if done properly, reduces the risk that unknown confounders will be seriously unbalanced in the study groups [14].

A particular problem in therapeutic intervention studies is *confounding by indication*, a bias that results from differences in prognosis between patients given different therapeutic schemes. Random treatment assignment ensures that prognostic factors are balanced between groups receiving the different treatments under investigation [15].

Techniques that are used for the achievement of randomization are simple, for example, flip of a coin, use of random number tables, or computer random number generator or complex, such as stratified randomization. But perhaps in these studies, a nonrandomized trial may not be possible and, consequently, the adjustment toward all the factors that become confounded. This can be corrected in part since we can eliminate differences in the phase of the analysis of the data. Thus, the way a nonrandomized intervention study is done and analyzed resembles that of a cohort study.

The functionalism of such a study can, to a large degree, be influenced by the fact that the participants and the researcher know the group the members of the study belong to. Knowledge of the treatment might influence the evaluation of the outcome. The solution, then, is *blinding* [3, 5]. It should be noted that blinding is desirable; however, it is not always feasible or necessary; for example, in orthodontic appliance research, because appliances differ in appearance, the researcher or even the participant could be aware of the intervention received.

There exist three types of *blinding*: (a) simple blinding: the evaluator assessing the outcome knows the assigned treatment, the participant does not; (b) double blinding: neither the evaluator nor the participant knows; and (c) triple blinding: neither the evaluator nor the participant knows, and the person who administers the treatment does not know which treatment is being assigned either.

Placebo treatment for the comparison group is often used to facilitate blinding. The placebo is inactive, morphologically similar with the tried therapeutic or preventive measure, medicine that is applied to the comparison group (when no other measure with documented effectiveness exists). When intervention studies involve procedures rather than pills, sham procedures take place to match the experience of the treatment and comparison groups as close as possible. The beneficial effect produced by an inactive pill or sham procedure is reported as the *placebo effect* and is attributed to the power of suggestion.

The results of the experimental studies can be organized in a fourfold table, and measures of disease frequency and association can be estimated. Although experimental study data analysis is straightforward, two issues should be kept in mind. Application of the *intention-to-treat principle* states that all randomized participants should be analyzed in order to preserve the goals of randomization. All subjects assigned to treatment should be analyzed regardless of whether they receive the treatment or complete the treatment. In addition, analysis of nonrandom subgroups threatens study validity and is not universally acceptable [3].

Ethical considerations are intrinsic to the design and conduct of intervention studies. No trial should be conducted without due consideration to ethical issues. These studies should be reviewed and approved by an ethics committee.

Advantages and Disadvantages of Intervention Studies: Well-designed intervention studies often provide the strongest support for a cause-effect relationship. Confounding factors that may have led to the subjects being exposed in cohort studies are not a problem here as researchers decide on who will be exposed. However, many research questions cannot be tested in trials, for example, if the exposure is fixed or if the outcome is rare. Intervention studies can also be more difficult to design than nonexperimental studies due to their unique problems of ethics and cost.

In this section, various design strategies have been considered for epidemiologic and clinical research. Table 7.7 provides key features and examples of different etiologic research methods in orthodontics. Any study is an effort to estimate an epidemiologic measure although this estimate could differ from the correct value. Steps that researchers can take to reduce errors are presented in the next section.

7.7 Sources of Error in Study Design

In order for an epidemiologic study to be considered credible, what is being measured, either frequency of appearance of an illness or the result of some report on the frequency of an illness, has to be authentic (accuracy in the measurement of a parameter). Accuracy is a general term denoting the absence of error. Authenticity depends on two factors: (a) precision and (b) validity [7, 8].

Precision is the repetitiveness of the result of a study, that is to say the degree of similarity between its results if it were to be repeated under similar conditions. Loss of precision is reported as a random error. *Validity* is the extent in which the study measures that which it alleges that it measures. Loss of validity is referred to as systematic error or bias. The terms validity and precision are often explained with the help of an objective (Fig. 7.7).

High validity corresponds to the average number of shots hitting near in the center. High precision corresponds to the shots grouping together in a small region.

7.8 Systematic Error or Bias

Bias can occur in all types of epidemiologic studies. Systematic error is usually the result of bad methodology, which leads to the creation of fictitious increases or reductions, differences or effects, the extent of which cannot be easily limited. Systematic error is unaffected by sample size. A study can be biased because of the way study subjects have been selected, the way measurements or classifications are conducted, or some confounding factor resulting from unfair comparisons. Thus, systematic error can be divided into three types: (a) selection bias, (b) information bias, and (c) confounding bias (Table 7.8) [5, 19, 20].

Table 7.7 Etiologic study designs and examples of questions addressed in orthodontic research

Study design	Key feature	Example of study question	Source
Intervention study	Evaluating the effectiveness of an intervention	To evaluate the effectiveness of preoperative ibuprofen in reducing the incidence and the severity of pain after orthodontic separator placement	Steen Law et al. [16]
	Randomization		
	Blinding		
Cohort study	Measuring incidence of disease or outcome	To evaluate the dental and psychological status of individuals who receive and do not receive orthodontic treatment	Kenealy et al. [17]
	Examining causes of disease or outcome		
	Longitudinal		
Case-control study	Examining causes of disease or outcome	To evaluate whether the amount or the structure of mandibular bone affects post-orthodontic mandibular incisor relapse	Rothe et al. [18]

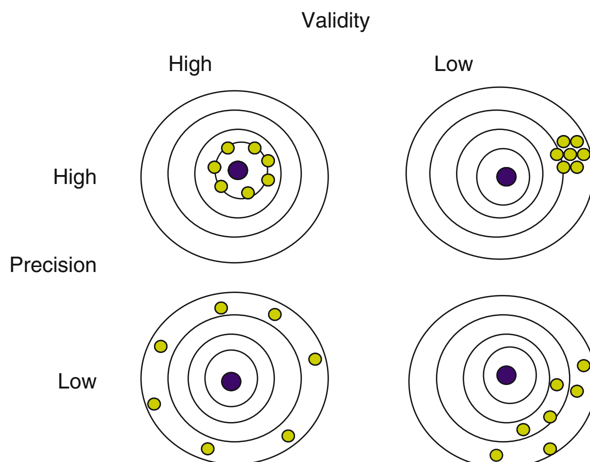

Fig. 7.7 Validity and precision

Table 7.8 Facts about bias

An alternative explanation for an association
Can be of a small, moderate, or large amount
Can overestimate or underestimate the correct value (positive/negative bias)
Is avoided when the study is carefully designed and conducted

7.8.1 Selection Bias

Selection bias results from the processes that are used for the selection of the members of the study and to factors that influence participation in the study [3, 7]. It is caused when the association of exposure-illness differs among those who participate and those who do not participate in the study. This type of bias can more often be found in case-control studies or in cohort studies of retrospective character because exposure to the factor and the illness has already occurred by the time the study has begun.

Selection bias can occur in different ways such as differential surveillance, diagnosis, or referral of study participants according to exposure and disease status; differential unavailability due to illness or migration (selective survival), or refusal (nonresponse bias); and inappropriate control group selection (control selection bias).

Because the selection bias cannot be corrected, it should be avoided. This is possible with correct and careful planning of the study and its proper conduct.

7.8.2 Information Bias

Information bias is the result of the method in which information is collected which concerns the exposure as well as the illness of the individuals who participate in the

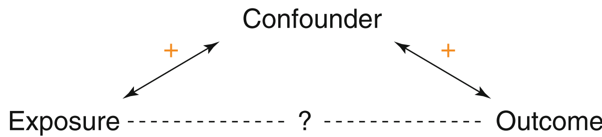

Fig. 7.8 The confounder is associated both with the exposure and outcome

study [7, 19]. It occurs when information that is collected for or by the individuals that participate in the research is erroneous.

Information bias leads to the placement of certain members of the study in the wrong category (misclassification) regarding the exposure, the illness, or both. Misclassification is divided into differential and non-differential. Differential is when misclassification regarding the exposure (or the illness) is different among those that are ill (or are exposed) and those that are not ill (or are not exposed). Non-differential is when misclassification regarding the exposure (illness) is independent from the appearance of the illness (exposure).

There are various types of information bias: recall bias, where the individuals that are ill refer to the exposure differently in relation to those that are not ill; interviewer bias, which is the fault of the researcher and is caused during the recording and interpretation of information of the exposure and illness; and follow-up bias, which results from the fact that those individuals who are not monitored for the duration of the study differ from those that remain until the end of the study.

Information bias can be avoided by carefully designing the study questionnaire, training interviewers, obtaining accurate exposure and disease data, and employing methods to successfully trace study subjects.

7.8.3 Confounding

Confounding is a result of the fact that the relation between the exposure and the illness is influenced by other factors so that it is led to confounding due to a mixture of these effects [2, 3, 20]. As a result, it constitutes an alternative explanation of the relation between exposure-illness, which is different from the actual explanation. The result of the exposure, therefore, is different from what would have resulted if no confounding factors existed, and only the factor being examined was influencing the individual (exposure). That is to say, the relation of the exposure and the illness is disturbed because it is mixed with the effect of some other factor which relates to the illness and exposure being examined. Thus, a change of the real picture is brought on, moving in the direction of either the undervaluation or the overestimation of the situation.

A confounding variable has three features: it is an independent cause or predictor of the disease, it is associated with the exposure, and it is not an effect of the exposure (Fig. 7.8).

There are three methods to prevent confounding in the design phase of a study: randomization, restriction, and matching. Random assignment of study participants

Table 7.9 Commonly used statistical tests for group comparison

Type of data	Number of groups	Independent	Paired
Continuous-normal	2	Student *t*-test	Paired *t*-test
	>2	Analysis of variance	repeated measures analysis of variance
Continuous-non-normal or ordinal	2	Mann-Whitney *U* test	Wilcoxon signed-rank test
	>2	Kruskal-Wallis test	Friedman test
Nominal	2	Chi-square test	McNemar test
	>2	Chi-square test	Repeated measures logistic regression
Survival	≥2	Long rank test	Conditional logistic regression

to experimental groups controls both known and unknown confounders, but can only be used in intervention studies. Restriction involves selection of study subjects that have the same value of a variable that could be a confounder. Restriction cannot control for unknown risk factors. Matching involves constraining the unexposed (cohort studies) or control group (case-control studies) such that the confounders' distribution within these groups is similar to the corresponding distribution of the index group. Once data has been collected, there are two options for confounding control: stratified analysis and multivariate regression models.

7.9 Random Error

The error that remains after systematic error elimination is defined as *random error*. Random error is most easily conceptualized as sampling variability and can be reduced by increasing sample size. *Precision* is the opposite of random error and is a desirable attribute of measurement and estimation. *Hypothesis testing* is commonly used to assess the role of random error and to make statistical inferences [3, 5, 21].

Statistical hypothesis testing focuses on the null and alternative hypotheses. The null hypothesis is the formulation of a non-relation or non-difference among variables that are being investigated (exposure-illness). That is to say, the two compared groups do not differ between themselves as per the size being examined (more than random sampling allows). The control of the null hypothesis takes place with the *statistical test* [21].

Commonly used tests include the student *t*-test and chi-square test depending on the nature of the data under investigation (Table 7.9). The test statistic provides a *p*-value, which expresses the level of statistical importance. The *p-value* is the probability of a result, like the one being observed or a larger one, to be found by chance when the null hypothesis is in effect (it shows the probability of the result that is being observed to occur, if the null situation is real). The researcher wishes for his/her results not be explained by random error, and consequently, he/she wishes the smallest possible *p*-value for the results. If the *p*-value is smaller than 5 % (level of

Table 7.10 Relationship between confidence interval and *p*-value for measures of effect

Measure of effect	No effect value	Confidence interval	*p*-value
Risk or rate difference	0	95 % CI includes 0	$p > 0.05$, nonsignificant
Risk or rate difference	0	95 % CI does not include 0	$p < 0.05$, significant
Relative risk, relative ratio or odds ratio	1	95 % CI includes 1	$p > 0.05$, nonsignificant
Relative risk, relative ratio or odds ratio	1	95 % CI does not include 1	$p < 0.05$, significant

statistical importance more often used), then the null situation has the probability of being less than 5 %, and it is rejected. When the null situation is not guaranteed, the alternative situation is adopted according to which the two compared groups differ between them, as to the size being tested (more than a chance sampling allows). The *p*-value depends on the strength of the association and the size of the sample. As a result, we may have a large sample in which, even a slight increase/reduction in risk, may seem statistically important or a small sample where large increases/reductions do not achieve statistical importance.

Many researchers prefer to use *confidence intervals* to quantify random error [2]. The confidence interval is calculated around a point estimate and quantifies the variability around the point estimate. The narrower the confidence interval, the more precise the estimate. The confidence interval is defined as follows: it is the breadth of values in which the real extent of the effect is found with given probability or specific degree of certainty (usually 95 %). That is to say, it is the breadth of values in which the real value is found with precise certainty. The confidence interval is calculated with the same equations that are used to calculate the *p*-value and may also be used to determine if results are statistically significant (Table 7.10). For example, if the interval does not include the null value, the results are considered statistically significant. However, the confidence interval conveys more information than the *p*-value. It provides the magnitude of the effect as well as the variability around the estimate, whereas the *p*-value provides the extent to which the null hypothesis is compatible with the data and nothing about the magnitude of effect and its variability.

As mentioned earlier, the primary way to increase precision is to enlarge the study size. *Sample size calculations* based on conventional statistical formulas are often used when a study is being planned [5, 21]. These formulas relate study size to the study design, study population, and desired power or precision. However, these formulas fail to account for the value of the information gained from the study, the balance between precision and cost, and many social, political, and biological factors that are almost never quantified. Thus, study size decision in the design phase can be aided by formula use; however, this determination should take into account unquantified practical constraints and implications of various study sizes. Calculation of study size after study completion is controversial and discouraged.

Deductively, there are two broad types of error affecting epidemiologic studies: systematic and random error. In designing a study, an effort should be made to reduce both types of error.

Conclusion

Understanding disease frequency and causal effects measures is a prerequisite to conducting epidemiologic studies. Strategy designs include experimental and nonexperimental studies, whereas causal effects can only be evaluated in studies with comparison groups. All research designs are susceptible to invalid conclusions due to systematic errors. Study outcomes should preferably be reported with confidence intervals.

Disease Occurrence

- Incidence and prevalence are measures of disease frequency.
- Prevalence provides the proportion of a population that has a disease at a particular point in time.
- Incidence measures the transition from health to disease status.
- Cumulative incidence provides the proportion of the population that becomes diseased over a period of time.
- Incidence rate provides the occurrence of new cases of the disease during person-time of observation.
- Measures of disease frequency are compared relatively or absolutely.
- Absolute measures of effect are based in the difference between measures and include the rate or risk difference.
- Relative measures of effect are based on the ratio of two measures and include the rate or risk ratio.
- Comparing measures of disease occurrence is facilitated by organizing data into a fourfold table.

Study Design

- Epidemiologic studies are divided into two categories: (1) descriptive studies and (2) etiologic or analytic studies.
- Descriptive studies consist of two major groups: those involving individuals (cross-sectional studies) and those that deal with populations (ecological studies).
- Etiologic studies are distinguished in experimental (intervention studies) and in nonexperimental (observational studies).
- Nonexperimental studies are distinguished in cohort and in case-control studies.
- In a cohort study, subjects are defined according to exposure status and are followed for disease occurrence.
- In a case-control study, cases of disease and controls are defined, and their exposure history is assessed and compared.
- Experimental studies are follow-up investigations where the researcher assigns exposure to study subjects.

- Well-designed experimental studies (trials) provide the strongest support for a cause-effect relationship.

Bias

- Bias or systematic error can occur in all types of epidemiologic studies and results in an incorrect estimate of the measure of effect.
- Bias is divided into three types: (1) selection bias (2) information bias and (3) confounding bias.
- Selection bias results from systematic differences in selecting the groups of study.
- Information bias results from systematic differences in the way that exposure and disease information are collected from groups of study.
- Confounding is a result of the fact that the relation between the exposure and illness is influenced by other factors so that it is led to confounding due to a mixture of these effects.

Random error

- Random error is the error that remains after systematic error elimination.
- Random error is most easily conceptualized as sampling variability.
- Increasing sample size reduces random error.
- Hypothesis testing is used to assess the role of random error and to make statistical inferences.
- Many researchers prefer to use confidence intervals to quantify random error.

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Part IV

Biological Research in Orthodontics

Basic Science Research Methods in Orthodontics

8

Dionysios J. Papachristou and Efthimia K. Basdra

8.1 Introduction

Orthodontics developed as a specialty of dentistry almost a century ago. Historical proofs clearly demonstrate that the history of orthodontics extends back to the ancient years. It seems that man very early realized the need for orthodontic treatment in order to accomplish correct function and to improve esthetics of the stomatognathic system and more importantly of the whole face.

The great Edward Angle, the father of modern orthodontics, categorized malocclusion and introduced his treatment principles. These principles were subsequently improved by other great figures in orthodontics, Charles Tweed, Raymond Begg, Joseph Jarabak, and Robert Strang, just to mention a few.

Orthodontic treatment at the beginning was more or less empirical, focusing mainly on the technicalities of tooth movement. Scientific evidence in the form of

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histological findings and tissue reactions to orthodontic tooth movement was developed later with the classic work of Kaare Reitan from Norway, who introduced the tension and compression theory based on histological findings [1, 2].

In Europe, orthodontics took a different route, that of dentofacial orthopedics. Later, the concept of orthopedic treatment of dentofacial anomalies found substantiation in Moss's functional matrix theory [3]. Function affects the structure and form of the jaws and face, and treatment based on a functional approach helps correct certain forms of skeletal anomalies. This orthopedic approach found its scientific support in the classical histologic work of James McNamara. The scientific impact of this work was extreme, and its publication was the turning point for orthodontics worldwide.

One of the most important researchers, teachers, and visionaries in the history of orthodontics and at that time the editor of the prestigious *American Journal of Orthodontics*, Thomas M. Graber, to whom this book is dedicated, added the words Dentofacial Orthopedics to the title of the journal, putting orthodontics on a route toward the twenty-first century.

In recent years, the field of orthodontics and dentofacial orthopedics follows closely the scientific advances in medical biology, mainly bone biology. Many complicated biochemical techniques are now being used in order to identify specific tissue reactions to orthodontic tooth movement, or more clearly to force-induced alveolar bone remodeling. Moreover, histology and molecular biology provided us with the tools to identify the biological events that follow the application of external mechanical stimulation/loading to alveolar bone and cartilage tissue. Complete elucidation of the biochemical bone tissue response will greatly improve our diagnostics, treatment planning, and outcome.

In the following sections, the main histological and histochemical protocols, as well as the major osteoblast and osteoclast cell tissue techniques, are presented. In addition, techniques and systems for external mechanical force application are described.

8.2 Histological Methods

8.2.1 Decalcification

The study of bone and cartilage cell morphology is of paramount importance for understanding the function of these cells. However, the physical rigidity of these tissues poses significant difficulties for the cutting of sections. These difficulties are mainly due to the intimate mixture of hard (bone and teeth) and soft tissues (osteoid, cartilage, and fat) within the same biopsy sample [4]. In order to obtain adequate sections, the embedded material should undergo a specific *softening* procedure, called decalcification. A more accurate term for this process should be *demineralization* since, except for calcium, other minerals are also removed. In general, decalcification methods are divided into two categories: acid (mainly nitric, hydrochloric, and formic) and neutral (*e*thylene*d*iamine*t*etraacetic acid—EDTA). Mineral (nitric and hydrochloric) and organic (formic) acids are preferable for routine

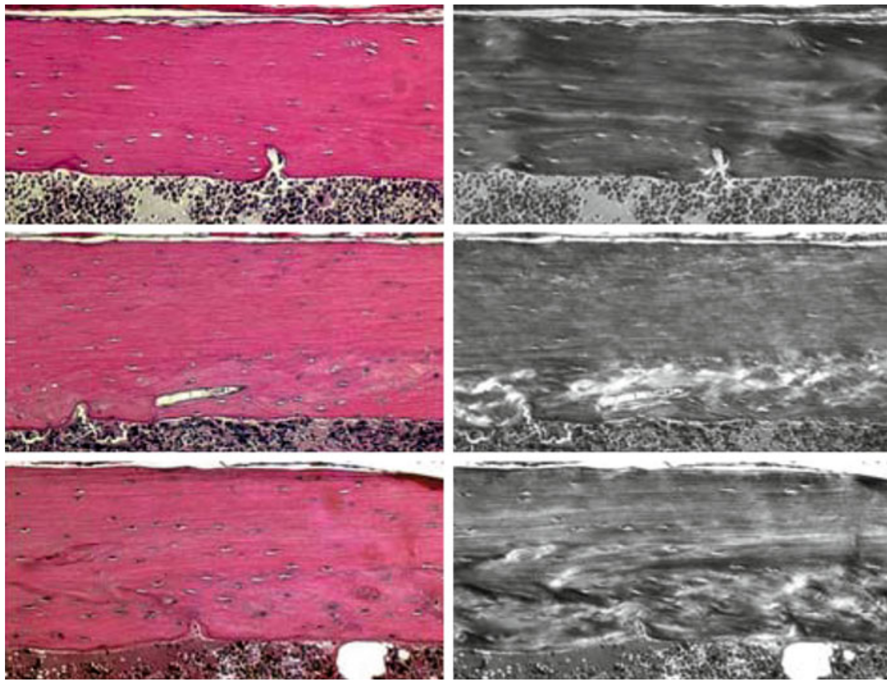

Fig. 8.1 Hematoxylin/eosin staining (*left panels*) and polarized light (*right panels*) micrographs of cortical bone of mouse proximal tibia (original magnification ×4)

decalcification because they remove large quantities of calcium at a rapid rate. However, they may damage cellular morphology, and therefore they are not recommended for small samples of hard tissue. On the other hand, neutral decalcification is the method of choice for small quantities of tissue since it preserves perfectly the cellular characteristics. Nevertheless, it penetrates tissue very slowly and is comparatively expensive when large amounts are used. A series of studies have shown that EDTA decalcification preserves proteins and nucleic acids for immunohistochemical, FISH, ISH, and CGH analyses [4–6]. However, some investigators believe that this procedure reduces enzyme activity and affects DNA and RNA function [7–9]. Therefore, they suggest that fresh-frozen specimens must be processed in an undecalcified way and sectioned with technologically advanced cryotomes (such as the CryoJane® Tape-Transfer System), which unfortunately are not available in most histology laboratories [10, 11].

8.2.2 Histochemical Methods

Hematoxylin and eosin (H&E) stain is an excellent method for visualizing the cell nucleus and cytoplasm, especially after tissue decalcification. The hematoxylin solution highlights nuclei, whereas eosin is bound on proteins and thus stains

Fig. 8.2 Tartrate-resistant acid phosphatase (TRAP) labeling of mouse femur. Note densely colored areas that represent bone-resorbing osteoclasts (original magnification ×4)

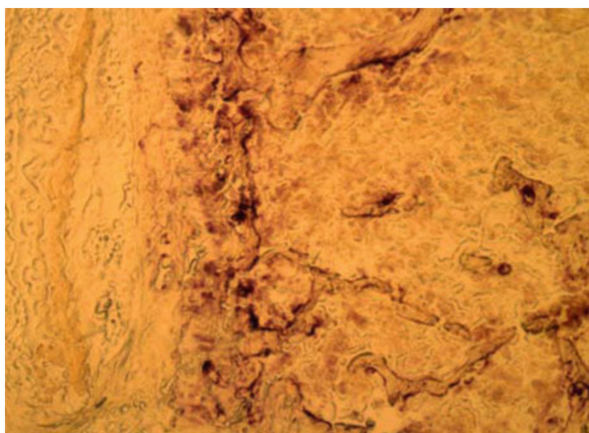

Fig. 8.3 Higher-magnification micrograph of area shown in Fig. 8.1, highlighting TRAP-positive osteoclasts (original magnification ×20)

primarily the cytoplasm (Fig. 8.1). However, the study of bone biology requires more accurate detection and characterization of the cells involved in bone metabolism. Therefore, special histochemical stains have been developed.

Cells of mononuclear origin express the band 5 isoenzymes of tartrate-resistant acid phosphatase (TRAP) [12, 13]. This enzyme is characterized by cathodal electrophoretic mobility at pH 4 and by resistance to inhibition by L(+)-tartate [14]. In mammals, TRAP has been detected in several tissue systems as a minor acid phosphatase isoenzyme [15, 16]. Nonetheless, it is primarily expressed in bone-resorbing multinucleated osteoclasts of the skeleton [12, 17]. The function of TRAP remains obscure. Several studies have proposed that in resorbing osteoclasts, TRAP is localized in the ruffled border or in the Howship lacunae [18]. However, in vitro and immunoelectron microscopy studies have documented that TRAP is also located in large transcytotic vesicles [19, 20]. Therefore, degradation of extracellular matrix proteins (namely, bone sialoproteins, osteopontin, and osteonectin) occurs in both the Howship lacunae

and the intracellular transcytotic vesicles [21]. TRAP can function as an excellent, highly specific osteoclast marker, which can be easily detected by commercially available histochemical kits. The histochemical TRAP staining results can be evaluated under light microscopy. The TRAP-positive osteoclast surface and the TRAP-positive osteoclast number can be calculated either manually or with the use of proper software [22, 23]. This stain can be applied on both decalcified and non-decalcified tissues [12, 17, 23]. The development of more sophisticated TRAP protocols, such as fluorescent-based TRAP stains, holds promise for better visual results and can be combined with other immunofluorescent, as well as immunohistochemical methods [24]. Example of TRAP labeling is shown in Figs. 8.2 and 8.3.

A commonly used marker of osteogenic development is alkaline phosphatase, an enzyme that was discovered by Robison in 1923 [25]. The term *ALP* was subsequently introduced in 1979 by McComb and colleagues [26]. Four distinct genes that encode for 4 different ALP isoenzymes have been discovered in humans: intestinal, placental germ-like, and tissue-non-specific (TNS). TNSALP is expressed in liver, bone, and kidney [27, 28]. The biological role of this protein is largely unknown. Within bone tissue and teeth, ALP is produced by osteoblasts and is involved in the process of osteoid mineralization [29]. This function is facilitated by local elevation of inorganic phosphate and destruction of inhibitors of hydroxyapatite crystal growth, phosphate transportation, and ATPase or tyrosine-specific phosphoprotein phosphatase activity [30]. Numerous studies of bone and cartilage development have highlighted the importance of measurement of ALP for the evaluation of osteoblastic activity. The identification of ALP activity in tissue sections is made primarily by histochemical methods [31, 32]. These methods are applied mainly to non-decalcified, frozen bone, and cartilage tissues. Indeed, ALP is sensitive to decalcification procedures since they remove the zinc and magnesium ions that are essential for ALP activity [31]. ALP is expressed in stimulated osteoblasts, bone-lining cells, and some newly formed osteocytes as well as in pre-apoptotic chondroblasts. Recently, histochemical methods that can be applied to decalcified, paraffin-embedded skeletal tissue have been developed [33, 34]. These methods are relatively easy, cheap, and reproducible and can be used in conventional pathology/histology laboratories.

In addition to histochemisty, immunohistochemical methods have been developed for the detection of ALP activity and localization [32, 35], using polyclonal antibodies against TNSAP or tissue-specific monoclonal antibodies against the bone isoform [32, 36, 37]. Histochemical and immunohistochemical approaches provide an in situ estimation of ALP localization and function, as they reveal differential localization of the examined enzyme during the different steps of bone and cartilage development and maturation.

Another commonly used assay for the study of bone maturation and mineralization was described in 1901 by von Kossa [38]. An example of this assay is shown in Fig. 8.4.

The von Kossa assay is an excellent method for the detection of calcium depositions. Notably, this stain does not react with calcium but with phosphate and carbonate ions in the presence of acid material [39]. More specifically, this method is based upon the principle that cationic silver ions can be removed from solution by

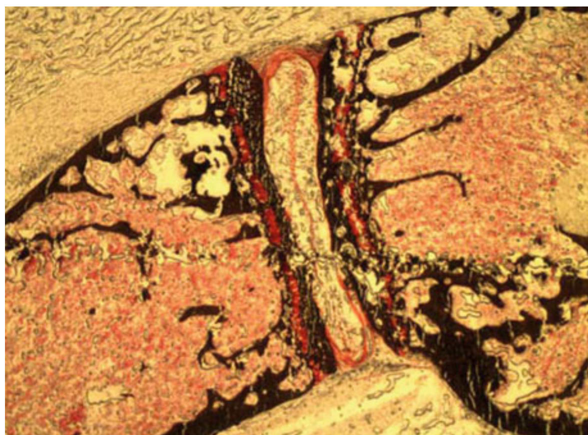

Fig. 8.4 Von Kossa assay: micrograph of mouse femur. *Black areas* represent sites of calcium deposition, whereas *red areas* correspond to osteoid (non-calcified bone) and collagen (original magnification ×4)

carbonate or phosphate ions because of their respective positions in the electrochemical series. When undecalcified tissue sections are treated with 5 % silver nitrate solution, cationic silver replaces calcium in the original salt and forms a silver salt that can be displayed by a reduction to metallic silver. This reaction is photochemical, and the activation energy is supplied from strong violet or ultraviolet light [40, 41]. Silver ions are associated with phosphate ions, and therefore they are considered to indirectly uncover calcium deposits. After the treatment with silver nitrate and aqueous sodium thiosulfate, counterstaining is required. For this purpose, van Gieson and hematoxylin-eosin stains are recommended. It can be seen in Fig. 8.4 that when the von Kossa assay is completed, sites of calcium deposition are stained black, and the osteoid and collagen are stained red, whereas fibrous tissue and red blood cells are stained yellow.

In addition, the H&E stain highlights the cellular components of the examined sections, providing important information regarding the histology of the examined tissues. The von Kossa assay is a very simple, accurate, and inexpensive technique for the study of bone maturation and mineralization. Furthermore, von Kossa-stained sections can be used for histomorphometric analyses. The purpose of histomorphometry is the evaluation of the structural integrity of the skeleton, the degree of bone formation and mineralization, and the rate of bone resorption. The tested parameters that reflect skeleton structural integrity are the total bone volume, the volume of cancellous bone, and the amount of trabecular osteoid. The parameters that are associated with bone formation and mineralization are the surface of the trabecular osteoid, the surface of mineralization, the distance between two tetracycline-pulse labels per day (see Sect. 8.2.3), and the mineralization lag time. Finally, the factors that indicate osteoclast-resorption function are: the trabecular, cortical, and periosteal resorptive surfaces; the trabecular osteoclast count (number of osteoclasts per area); and the cortical porosity (percentage of the cortex that contains pores without osteoclasts) [42, 43]. Histomorphometry is a method of choice for the study of conditions such as metabolic bone diseases, neoplasias, bone remodeling, and fracture repair, as well as bone-cartilage response to biomechanical stress [43].

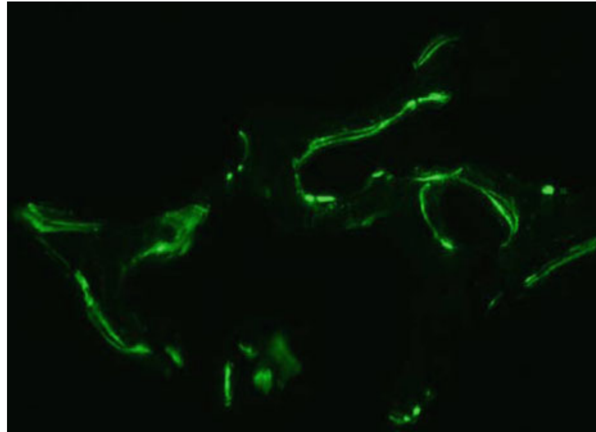

Fig. 8.5 Epifluorescence images of vertebral body from mouse following administration of two calcein labels, 3 days apart (original magnification ×10)

8.2.3 Fluorescent Labeling

Bone tissue continually undergoes shape and structure changes. Accretions in length and thickness, modeling, and drift activities lead to morphologic alterations that determine the relative position between various skeletal parts. Metabolic bone diseases, bone repair processes, mechanical stress, and aging create new functional demands and are responsible for the subsequent structural adaptations. An accurate method for the detection of such structural/functional modifications is fluorescent labeling [44]. Fluorescent double-labeling is used to calculate kinetic data on bone turnover. The fluorochromes are administrated systematically and form long-lasting chelate complexes with apatite, via their active iminodiacetic acid groups. Hence, they can serve as markers that allow the identification of mineralized tissues [45]. Different types of fluorochromes, such as yellow tetracyclines, xylenol orange, alizarin red derivatives, or green fluorescein derivatives like calcein (Fig. 8.5) or DCAF, which produce different colors, are available [46, 47]. The first dose of fluorescent dye is incorporated in the newly formed bone at the bone-osteoid interface, where it appears as a linear fluorescence under UV light microscopy. The second dose is administrated 3–14 days after the first. The amount of bone that has been synthesized during this time period can be calculated by measuring the width between the two lines of fluorescence. Dosing of the tetracycline is dependent upon the model individual, such as human, rat, or rabbit.

8.2.4 Immunohistochemistry (IHC)

Immunohistochemistry is used in everyday practice at pathology and histology laboratories. It is a relatively simple method for the in situ detection of proteins. IHC is based on the principle that specific intra- or extracellular antigens are bound to monoclonal or polyclonal antibodies that are associated with specific enzymes. The detection of the antigen-antibody complex is achieved with the use of chromogens.

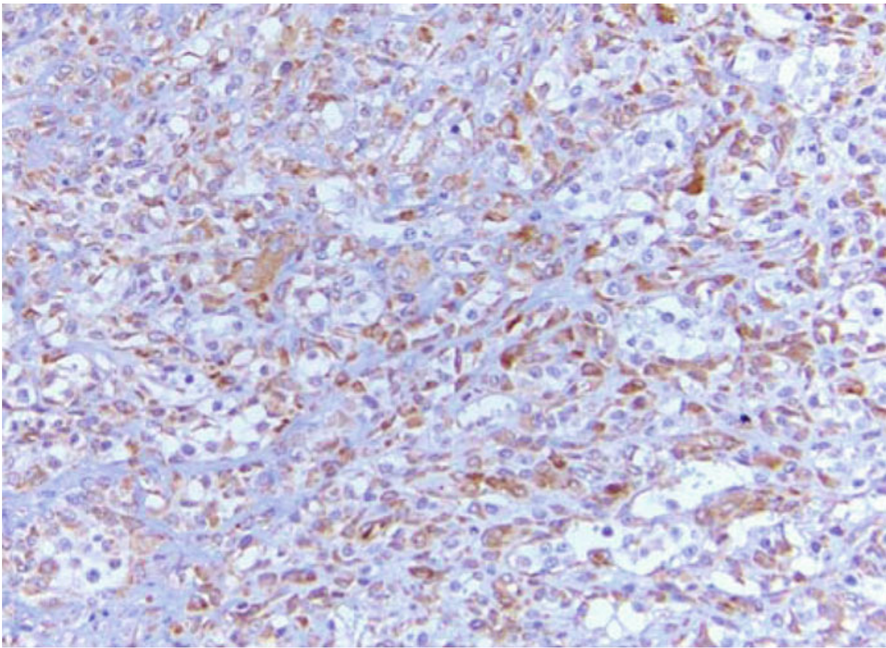

Fig. 8.6 Immunohistochemical method for detection of osteonectin that highlights the osteoid in high-grade osteogenic sarcoma (original magnification ×10)

The principal enzyme that facilitates the antibody detection is peroxydase, and the chromogen that is most commonly used is 3,3′-diaminobenzidine tetrahydrochloride (DAB). There are two major categories of IHC methods: direct and indirect. Indirect IHC methods include peroxydase-antiperoxydase (PAP), avidin-biotin complex (ABC), and biotin-streptavidin assay (B-SA), which is the most popular [48]. The B-SA IHC method relies on the non-immunologic binding of biotin to streptavidin (a 60-kD protein), produced by *Streptomyces avidinii*. Three reagents are used: (a) the primary antibody, which is specific for the antigen of interest; (b) the secondary (biotinylated) antibody that binds the first one; and (c) the streptavidin-peroxidase reagent that is associated to the secondary antibody. IHC provides an in situ approach to investigate the expression and activation status of the examined proteins.

Furthermore, this is a useful method for bone biology studies since it detects the expression levels of several proteins implicated in bone development and growth. Among them, osteonectin (ON) and osteocalcin (OC) are the most significant. Examples are shown in Figs. 8.6 and 8.7. ON is a 35–45-kD protein that has the ability to bind to Ca^{+2} , hydroxyapatite, and collagen [49]. Among its structural features, the two EF-hand high-affinity calcium-binding sites are functionally the most significant. ON mediates the deposition of hydroxyapatite and is involved in the regulation of the osteoblastic cell cycle and bone maturation. OC (also called *bone gla protein*) is a 5-kD protein that belongs to a family of extracellular matrix

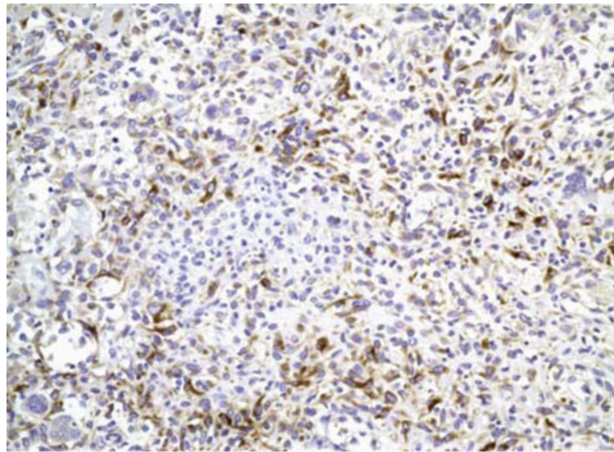

Fig. 8.7 Same tumor shown in Fig. 8.6, now immunostained for bone-specific marker osteocalcin (original magnification ×10)

proteins named *gla proteins*. OC possesses one disulfide bridge, and the gla residues are located in α helical region. A large volume of in vitro and in vivo studies have documented that OC plays a central role in bone remodeling and skeletal development. More specifically, it activates osteoclasts and recruits their precursors, determining the transition from bone resorption to bone formation [50–53]. Immunohistochemical detection of ON characterizes early stages of bone development, whereas OC typically determines later steps of skeletal growth and osteoid mineralization. Other proteins such as Cbfa1/Runx2 and AP-1 transcription factors have also been found to participate in chondroblastic/osteoblastic differentiation and maturation [54, 55]. Very recent IHC data on rat TMJs have shown that these proteins are selectively expressed in bone and cartilage tissue and that their differential expression highlights different maturation levels during the process of chondro-osteogenesis [56, 57]. Therefore, they support the notion that Runx2 and AP-1 (c-Jun/c-Fos heterodimer) can be used as bone/cartilage markers and indicators of chondro-osteoblastic maturation.

8.2.5 In Situ Hybridization

In situ hybridization (ISH) is a valuable molecular method in the field of bone research and diagnosis since it detects the localization of specific nucleic acids at the level of individual cells or complex tissue sections, combining histochemistry with recombinant DNA technology [58–60]. It was first described in 1969 and is based on the specific binding of a labeled nucleotide probe to target DNA or RNA sequences [61, 62]. Probes for ISH (double-stranded DNA, single-stranded antisense RNA, single-stranded DNA probes generated by polymerase chain reaction procedure, synthetic oligodeoxynucleotides, or oligoprobes) are usually 50–300 bases long. Originally, they were labeled with radioisotopes that limited ISH utility for research and diagnostic purposes [60]. Nonetheless, the introduction of

non-isotopic labels and development of detection methods based on classical histochemical and immunohistochemical assays reduced the background and improved the signal resolution, expanding the range of ISH applications. In the fields of orthopedic and orthodontic research, ISH can be applicable for both cytogenetic and archival preparations [23, 63–67]. A key advantage of histologic sections is that the examined cells are evaluated in their native architecture and localization. This is often essential, for example, in the study of conditions such as response to stress or metabolic bone diseases, where differential localization of cells with distinct molecular and biochemical properties determines the degree and the quality of bone growth. Furthermore, since locus-specific ISH can be detected by nonfluorescence reagents, it can be easily visualized with bright-field microscopy. The role of fixation is of great importance for ISH since it ensures the integrity of the nucleic acids and the preservation of tissue morphology. Cross-linking fixatives such as formalin and glutaraldehyde seem to provide the best results [68–70]. The degree of DNA/RNA damage caused by decalcification procedures is controversial [64, 65]. However, the combination of EDTA decalcification with formalin fixation seems to be efficient. After fixation and decalcification, unstained sections are transferred onto coated glass slides with advanced adherence properties that prevent tissue floating during the ISH process. Afterward, tissues are treated with pepsin and proteinase K that digest cellular proteins and facilitate probe access to targeted nucleic acids. Optimization of tissue preparation is a major technical challenge. The optimal protein digestion conditions are basically determined empirically for each tissue and probe combination. In order to evaluate whether the signal visualized by ISH is specific for the target DNA or RNA sequence, the use of controls (hybridization of samples from the same tissue with a probe complementary to the assessed probe and identical to the targeted sequence that does not generate any signal) is essential.

The applications of ISH in the areas of bone and dental research are numerous. For instance, it can be performed in order to determine the spatial and temporal distributions of specific mRNA sequences, especially in cases where the gene products are below the threshold of IHC detection. Indeed, by using ISH, the role of genes (namely, Ihh, PTHrP, TRAP, OC, ON, OPN, collagen type II, and Runx1, -2, -3) that are involved in cartilage-bone growth, development, and remodeling has been investigated. Furthermore, ISH has proved to be a valuable tool in the study of pathologic conditions such as metabolic and inflammatory bone and teeth diseases and neoplasias [23, 63, 66, 67, 71, 72]. In situ end-labeling (ISEL) is an ISH-related method that is performed on formalin-fixed, paraffin-embedded tissues for the identification of cells that undergo programmed cell death. ISEL detects the presence of DNA strand breaks that are generated by activated endogenous nucleases during apoptosis [73–75]. More specifically, in the presence of DNA polymerase, the DNA strand breaks are hybridized with non-isotopic reporter molecules, which can be detected with IHC methods. The ISEL assay can be applied as a corollary to the TdT-mediated dUTP-dioxigenin nick-end-labeling (TUNEL) method, which specifically labels the 3′-hydroxyl terminal of DNA strand breaks. Apoptotic cells are recognized by their dark nuclei (TUNEL-positive reaction). During the process of endochondral bone formation, chondrocyts and osteocytes progressively mature

and undergo programmed cell death. Osteoclasts are also susceptible to apoptosis in the absence of trophic and growth-stimulating factors, such as M-CSF and RANKL [75, 76]. Obviously, skeletal conditions that induce apoptosis (such as mechanical or biochemical stress and inflammatory or neoplastic diseases) are under intense scrutiny. ISEL and TUNEL are the methods of choice for the investigation of these apoptotic phenomena.

8.3 Polymerase Chain Reaction (PCR) and Reverse Transcriptase PCR (RT-PCR)

The polymerase chain reaction (PCR) assay is a recently developed molecular method for the detection, amplification, and quantization of nucleic acids [77]. Karry Mullis first described it in 1985 [78, 79], winning a Nobel Prize for his achievement. The reagents that are required for PCR include: (a) the dsDNA (template DNA); (b) two PCR primers, which are oligonucleotide sequences of single-stranded DNA that match the sequences at either end of the targeted DNA segment; (c) a thermostable enzyme to synthesize DNA copies; (The most commonly used enzyme is Taq polymerase from the heat-resistant bacterium, *Thermus aquaticus*.); (d) a pool of four deoxynucleotides (dATP, dCTP, dGTP, dTTP) that will be consumed by polymerase for the synthesis of the new DNA; and (e) buffers containing magnesium that are necessary for the function of Taq polymerase. A single PCR cycle is composed of three sequential steps: denaturation of the dsDNA at 92–96°C, primer annealing or hybridization at 55–72°C, and the synthesis or extension step at 72°C. During the denaturation step, the high temperature facilitates the breaking of hydrogen bonds between complementary bases, resulting in the separation of the dsDNA into two single strands. During the annealing step, the temperature drops, allowing the primers to hybridize to the complementary sequences on the template strands. Usually the primers are 20–30 bases long. The longer the primer, the more specific the binding on the target sequence. During the final step, new DNA strands are produced by Taq polymerase at 72°C. The elongation of the new DNA strands begins by using the oligonucleotide primers as starting points. DNA synthesis progresses from 5′ to 3′ for both new strands. Taq polymerase has the ability to synthesize approximately 1,000 base pairs per minute. The aforementioned three-step procedure is repeated from 30 to 50 times, leading to the synthesis of more than 1×10^9 copies of the original DNA template sequence.

The use of RNA, such as messenger RNA (mRNA), as a template for PCR amplification is accomplished by a modified PCR assay, named reverse transcriptase PCR (RT-PCR) [60, 80]. In a typical RT-PCR, mRNA is extracted from tissue samples or cells and then is copied into DNA (complementary DNA—cDNA) via reverse transcription, which is facilitated by the function of an enzyme called reverse transcriptase. This step is fundamental since Taq polymerase cannot use RNA as a template for the synthesis of PCR products. Several primers (such as random hexamers and oligo-dT) can be used for the reverse transcription step of PCR. The cDNA that is produced serves as the substrate for a classic PCR, as described

earlier. In contrast to classic PCR that amplifies genomic DNA (which contains introns and exons), RT-PCR amplifies cDNA (which contains only exons) and therefore only useful genetic information.

The evaluation and analysis of PCR and RT-PCR products are usually made by gel electrophoresis. Electrophoresis is used for the separation of negatively charged nucleic acids, which are mobilized through a liquid or solid matrix by an electric field. Separation is based either on their molecule size or on their three-dimensional conformation. Agarose gel permits the separation of large DNA fragments (1–20 kb), whereas acrylamide gel is optimal for smaller (up to several base pairs) fragments. DNA molecules are visualized by ethidium bromide. The application of PCR in the fields of orthopedics and orthodontics is numerous. More specifically, in the area of bone pathology and oncology, this method can be used for the detection of mutations, polymorphisms, and other genomic alterations in oncogenes and tumor suppressor genes that are involved in tumorigenesis (such as p53, Rb1, p16, HER2/neu, EGF-R, and EXT2) [60]. Regarding skeletal biology, PCR can be used for the detection and quantitative assessment of genes and growth factors that are implicated in several molecular processes, including metabolic bone diseases, tumors, and cellular response to stress factors [81, 82]. Technologic advantages, such as real-time PCR and real-time quantitative TaqMan RT-PCR, are very sensitive, accurate, and highly reproducible methods for the study of gene expression and precise quantification of PCR products [60, 83, 84]. In addition, the development of the in situ PCR assay is an ideal combination of PCR and in situ hybridization that permits the selective amplification and evaluation of specific genetic loci within intact cells [85]. This method can be applied to cells, frozen sections, and sections from archived paraffin-embedded material and has the unique advantage of detecting specific genes within their native environment.

8.4 Microdissection Techniques

Formalin-fixed, paraffin-embedded tissues (FFPET) are some of the most widely available and quality-controlled materials for clinical and basic science studies. However, FFPET are complex, three-dimensional structures, composed of different cell populations with distinct functions. In bone biology, a large volume of studies is focused on the morphologic characteristics and the functional interactions between different cell populations (osteoblasts—osteocytes—chondroblasts). Profoundly, cellular heterogeneity constitutes a major drawback for molecular genetic analyses. Tissue microdissection (TM) represents a reliable method to isolate morphologically well-defined cells and obtain relatively pure cellular populations [81, 83, 84, 86]. DNA or RNA extracted from these populations can be amplified by PCR and then undergo molecular studies for the detection of genetic characteristics and the quantification of genomic alterations. TM can be performed by several different techniques ranging from simple, inexpensive manual TM to more sophisticated (but significantly more expensive) methods such as laser-captured microdissection (LCM).

Manual TM is performed under direct optical visualization of the tissue sample with the use of a stereomicroscope. The target cells are identified on 5-μm thick tissue sections and then dissected with sharp and accurate instruments such as a 30-gauge needle or surgical blade. For better results, tissue sections should undergo deparaffinization prior to microdissection [86]. Tissue fragments are collected in tubes and prepared for nucleic acid extraction and PCR analysis. The primers should be designed to generate small PCR products (<200 bp), since PCR with larger targets may fail. MTM is a fast, cost-effective method that can be applied to any tissue and does not require expensive instrumentation. Nonetheless, neighboring tissues and cells, such as lymphocytes and red blood cells, can very easily contaminate the cell population of interest. In order to obtain uncontaminated cell populations, LCM is the method of choice. LCM was first described by Emmert-Buck in 1999 [87, 88], and several commercially available microdissection systems that use laser technology were developed soon after. The major components of an LCM system are an inverted microscope, an infrared laser, a control unit for the laser, a control mechanism for the microscope stage, a digital camera, and a monitor [89]. LCM can be applied on both frozen and FFPE tissues. Importantly, deparaffinization is required prior to microdissection [90]. The major disadvantage of LCM is the prerequisite of very expensive equipment and well-trained technical stuff. TM is one of the most promising FFPET-based techniques that bridge the gap between morphology and molecular/genetic characteristics.

8.5 Culture of Osteoclasts and Osteoblasts

8.5.1 Osteoclastic Cell Lineage

Bone resorption and bone synthesis are fundamental processes that determine normal bone morphology, skeletal mass, and calcium homeostasis. Any disturbances of this finely tuned *interplay* result in pathologic conditions such as osteoporosis, osteopetrosis, metabolic bone diseases, fractures, and malignant hypercalcemia. The cells that are specialized to carry out bone resorption are the osteoclasts. Osteoclasts are derived from the pluripotential hematopoietic progenitor CFU-GM (colony-forming unit—granulocyte and macrophage), which also gives rise to monocytes and macrophage-committed precursors [91, 92]. The human mononuclear osteoclast precursor circulates in the monocyte fraction of the peripheral blood. It expresses the monocyte/macrophage integrins CD11b-c and the lipopolysaccharide receptor antigen CD14 [93, 94], as well as the macrophage-associated phenotypes NSE, Mac-1, and Mac-2. In contrast, they are negative for osteoclast-specific markers, namely, tartrate-resistant acid phosphatase (TRAP), vitronectin, and calcitonin receptors [95, 96]. Osteoclast activity is directly and specifically inhibited by calcitonin [97], and therefore receptors that bind calcitonin are considered to be reliable and highly specific markers of mammalian osteoclasts [98]. However, only a small fraction (approximately 2–5 %) of the monocyte/macrophage phenotype cells will eventually differentiate to mature osteoclasts [94]. Under the

influence of the transcription factors PU-1 and MiTf, stem cells are committed into the myeloid lineage. In order to progress to the monocyte lineage and express the RANK receptor, M-CSF (macrophage colony-stimulating factor) is required. M-CSF is produced by mesenchymal/stromal cells, including osteoblasts, and is an absolute requirement for the proliferation and differentiation of osteoclast progenitors [99]. M-CSF acts via a tyrosine kinase receptor, named c-fms [100]. Precursors need the presence of RANKL to truly commit to the osteoclast lineage and begin the differentiation program. RANKL is a member of the tumor necrosis factors (TNF) family, which is expressed on the surface of osteoblasts/stromal cells and released by activated T-cells. It binds to RANK receptors on osteoclast precursors and induces their maturation through the nuclear factor-κB (NFκB) and Jun N-terminal kinase pathways [101, 102]. A member of the tumor necrosis factor receptors superfamily called *osteoprotegerin* is a decoy receptor for RANKL that inhibits the differentiation and function of osteoclasts [103, 104]. The transition from mononuclear precursor cell to mature osteoclast involves a stepwise loss of macrophage markers and gradual acquisition of phenotypic characteristics specific for osteoclasts. More specifically, since postmitotic osteoclast precursors begin to differentiate into committed osteoclast precursors, they express osteoclast-associated phenotypes, such as TRAP and calcitonin receptors [105]. In contrast, some of the macrophage-related markers, namely, NSE and Mac-1, disappear during osteoclast maturation. Furthermore, they respond to hormones, including 1,25-dihydroxyvitamin $D_{3,}$ parathyroid hormone, and certain cytokines such as IL-1, IL-6, prostaglandins, and colony-stimulating factors. When differentiation of the precursors into pre-osteoclasts is completed, these mononuclear cells begin to fuse, giving genesis to the multinucleated fully mature osteoclasts. Recent evidence suggests that mature osteoclasts undergo apoptosis after a cycle of resorption, a process augmented by estrogens [106].

8.5.2 Culture of Osteoclasts

Since osteoclasts originate from hemopoietic stem cells, bone marrow culture can be used for the study of osteoclast formation. Indeed, Testa and colleagues first demonstrated that multinucleated osteoclasts can be developed in long-term cultures of feline marrow cells [107]. Traditionally, osteoclasts have been generated in cocultures of osteoblasts or stromal cells and hematopoietic cells derived from spleen or bone marrow. For these studies, murine bone marrow cells can be aseptically extracted from long bones of 6–9-day-old mice, following removal of the adhering soft tissues [108]. Afterward, the ends of the bones are removed with scissors, and the bone marrow cells are extracted by slow injection of α-minimum essential medium (α-MEM) into one end of the bone. Bone marrow cells are collected and washed, suspended in α-MEM, and evaluated for viability. Approximately 1×10^7 bone marrow cells can be obtain from a tibia. Coculture methods rely upon the principle that osteoblasts secrete M-CSF and express RANKL after stimulation by 1,25-dihydroxyvitamin D_3 and dexamethasone. RANKL binds RANK receptors to monocytic osteoclast precursors, promoting their fusion and thus synthesis of

mature multinucleated osteoclasts [101]. Most of the coculture systems occupy UMR-106 rat osteosarcoma cell lines [109].

More recently, following the discovery of RANKL in 1998 [110], it has become possible to generate bone-resorbing osteoclasts without the requirement of osteoblasts in the culture. RANKL ligand and M-CSF can be added directly to osteoclast-precursor cultures, driving the formation of multinucleated, active bone-resorbing cells. This method is easier and more reliable than the coculture method since it employs cells from one single lineage. For osteoclastogenesis experiments that use bone marrow, the extracted cells are washed and cultured in α-MEM with fetal bovine serum (FBS) (10 %), M-CSF (5 ng/mL), and penicillin/streptomycin (1 %) for 48 h. Non-adherent hematopoietic stem cell precursors can be purified with Ficoll-Paque Plus (Amersham Biotech). The interfacial cell layer is isolated for culture in α-MEM with FBS (10 %), M-CSF (30–50 ng/mL), and RANKL (30–100 ng/mL). A TRAP kit can be used for osteoclast staining and counting. Other studies have described the development of osteoclasts from peripheral blood mononuclear cells (PBMNC) [105, 111, 112]. Briefly, 15 mL of blood is mixed with 15 mL of phosphate-buffered saline (PBS) (37°C), purified with 15 mL of Ficoll-Paque, and then centrifuged. Overlying cells are isolated, resuspended in 10 % PBS, diluted with 40 mL PBS, and centrifuged. Isolated PBMNC are placed in a 96-well plate and prewetted by soaking in 100 μL complete α-MEM containing 25 ng/mL M-CSF and 30 ng/mL recombinant RANKL at 37°C. The complete medium should be replaced every 2–3 days. The culture duration for both TRAP staining and pit assays is usually 2–3 weeks.

The multinucleated cells generated by cell cultures can be identified by the presence of certain osteoclast-specific markers: cathepsin K, calcitonin receptor, TRAP, type II carbonic anhydrase, and vitronectin receptor. However, the hallmark of osteoclast identification is the presence of resorption areas on calcified substrates, as defined by osteoclast-resorption lacunae (pit) assays [105, 109, 112].

8.5.3 Osteoblastic Cell Lineage

Osteoblasts arise from pluripotent mesenchymal stem cells, the colony-forming units—fibroblasts (CFU-Fs), which under appropriate stimulation can also give genesis to lipoblasts, chondroblasts, myoblasts, and fibroblasts [113, 114]. The bone morphogenic proteins (BMP) 2–7 and TGF-beta induce the upregulation of transcription factors that mediate the commitment of CFU-Fs toward the osteogenic lineage. The runt homology domain Runx2/Cbfa1 and the zinc finger protein osterix [54, 115, 116] are transcriptional regulators (Fig. 8.8) that facilitate the expression of genes (collagen type I, osteopontin, and alkaline phosphatase) that define the phenotypic features of bone-forming cells. Therefore, these proteins are referred as *master regulators* of osteoblast morphology. In vivo experiments have documented that knock-out mice lacking Runx2/Cbfa1 and osterix genes do not produce bone [116, 117]. Locally acting proteins, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and activator protein-1 (AP-1), as well as systemic, blood-circulating molecules, namely, corticosteroids, 1,25 dihydroxyvitamin

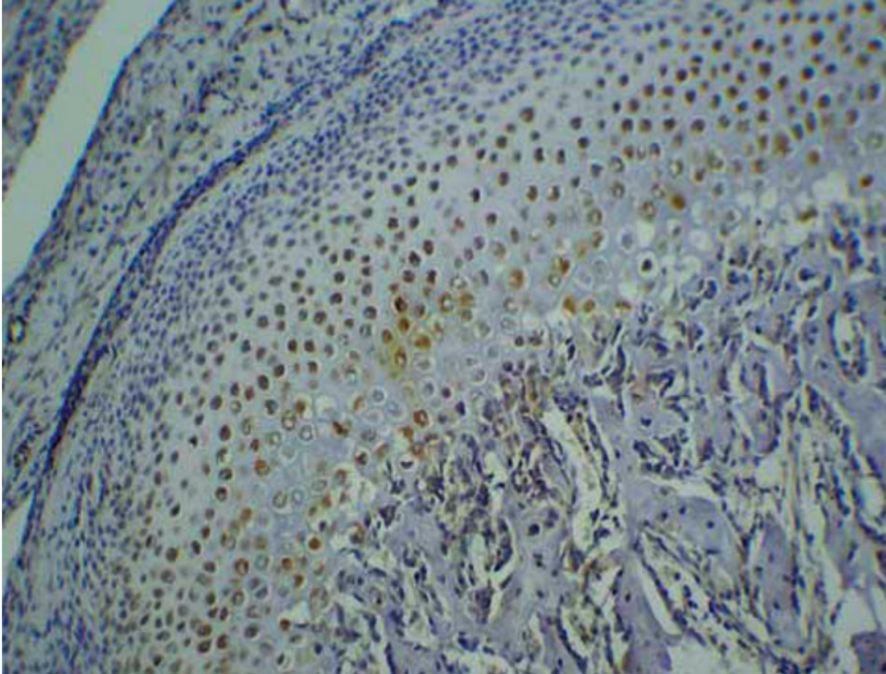

Fig. 8.8 Immunohistochemistry applied on section from mouse temporomandibular joint displaying strong reactivity for osteoblast-specific transcriptional regulator Runx2/Cbfa1 (original magnification ×20)

D_3, parathyroid hormone, prostaglandins, and cytokines, are also implicated in specific steps of osteoblastic proliferation and maturation [118].

8.5.4 Osteoblastic Cell Cultures

The vast majority of the osteoblastic cell culture protocols use two complementary approaches for isolation of bone-forming cells. The first one utilizes freshly isolated short-term cultures of tissue-derived cells (primary or early passage cultures), whereas the second (permanent cell cultures) uses permanent cell lines, either from osteosarcoma tumors [119, 120] or from osteoblastic cell clones selected from primary cultures [121, 122].

8.5.5 Primary and Early Passage Cultures

Short-term cultures are carried out on non-transformed cells that have not undergone genomic alterations mutations, and therefore they retain most of their native phenotypic characteristics [123]. In primary and early passage cultures, bone-forming cells

of different levels of differentiation can be encountered. Selective separation of these cells gives the investigators the opportunity to study the specific features of each subgroup, as well as the biological interactions between the bone-forming cells subpopulations [124]. Enzymatic digestion of bone matrix facilitated by proteases like collagenase or trypsin is the most commonly used method for the isolation of osteo-producing cells. Enzymatic digestion has been performed on fetal, neonatal, and adult calvariae, as well on long bones from mice [125], rats [126], chickens [127], cattle [128, 129], and humans [130, 131]. Osteoblastic cells are collected at different times as the digestion proceeds. Cells from the later digests (after 40–120 min) express the most "osteoblast-like" phenotype [126]. Nevertheless, primary culture systems have some disadvantages. Enzymatic isolation may have cytotoxic effects on the cells, whereas proteases may digest several cell surface proteins, affecting the phenotype of osteoblasts. Additionally, studies on mouse bone-forming cells have shown that in short-term cultures osteoblasts preserve their phenotypic features for a short period of time [125, 132].

Bone-forming cells can also be obtained from culture systems that use periosteum [133], bone marrow stroma [134], or periodontal ligaments [135–137]. These tissues contain mesenchymal/osteoblast precursors, which can lead to the genesis of cells that occupy bone-forming features. Dexamethasone [135], retinoic acid [138], and BMP-2 [134] have been shown to augment the aforementioned phenomena. This method provides significant information regarding the biochemical and molecular events that are implicated in the process of osteoblastic differentiation and maturation.

8.5.6 Permanent Cultures

Most of the permanent cell lines are derived from cells that have undergone malignant transformation and become immortalized. Immortalized osteoblastic cells have not acquired all the genetic and morphologic characteristics of fully transformed, osteosarcoma cells, and they maintain their osteoblastic phenotype on a continuing basis, providing large amounts of stable cell populations that are ideal for biochemical studies. The most popular osteosarcoma cell lines are UMR-6 (rat) and ROS 17–2 (rat). Each exhibits different features [124, 132] and serves different purposes. *Osteoblast-like* cell lines (namely, SaOS, TE-85, MG-63, OHS-4) have also originated from human osteosarcomas [119, 120]. The greatest disadvantage of osteosarcoma cell lines is that the process of immortalization-transformation may have affected the genotype and phenotype of the osteoblastic cells. In order to overcome this caveat, permanent osteoblastic cell lines, such as MC3T3-E1, have been developed from normal mouse calvarias [139]. Osteoblast characterization in cell cultures is based upon biochemical and morphological elements. Osteoblast differentiation/maturation occupies distinct phases, identified by the expression of different sets of genes.

More specifically, during the *proliferation phase*, osteoblasts express collagen type 1 and histone proteins, growth factors (TGF-beta), specific transcription

factors (c-Fos, c-Myc, Fra-1, c-Jun, JunD), and the osteoblastic *master regulato*r Cbfa1/Runx2 [140–143]. As the differentiation of the progenitor cells proceeds and extracellular matrix begins to mature, proteins such as collagen type 1, alkaline phosphatase, osteopontin, osteonectin, bone sialoproteins, and PTH/RTHrP are upregulated. During the period of matrix mineralization, mature osteoblasts are characterized by the expression of osteocalcin, osteopontin, osteocalcin, and collagenase. Apoptotic cells are observed during the mineralization phase associated with the formation of bone nodules and the expression of the apoptosis-related factors Bax and the cell cycle regulator/tumor suppressor gene P53 [106]. Both in vivo and in vitro studies have demonstrated that histologically similar osteoblasts in different proliferation and maturation stages display heterogeneous profiles in proteins and mRNA levels [140–142, 144]. Notably, a recent immunohistochemical in situ hybridization study conducted on fetal rat calvaria has shown that ALP and PTHrP-R are globally expressed by all osteoblasts irrespective of their maturation status [140]. Ultimately, osteoblasts are identified by their histological configuration, histochemical properties (i.e., ALP positive staining), and, most importantly, by their ability to synthesize bone matrix.

8.5.7 Mechanical Stretching of Cell Cultures

Mechanical forces are essential physiological factors that regulate the structural properties of bone tissue. Mechanical loading stimulates the osteoblastic function and plays a fundamental role in bone remodeling and skeletal homoestasis [145–148]. In cell cultures, osteoblasts display significantly similar phenotypical and genotypic features with fibroblasts. Therefore, osteoblasts have been characterized as *sophisticated fibroblasts* [143]. Human periodontal ligament (hPDL) is connective tissue that lies between the tooth root and the alveolar bone [149]. PDL fibroblasts comprise an osteoblast-like population, which may undergo osteoblastic differentiation under the influence of a variety of extracellular stimuli, including mechanical loading in vivo and in vitro [136, 137, 150, 151]. This fact generated the notion that the development of a PDL fibroblast stretch application device might have considerable contribution toward understanding the molecular events that underlie mechanical sensing, biochemical coupling, and the response to mechanotransduction within the periodontal ligament tissue [152].

The stretch devices, similarly to those used for stretch application to other tissues [153], are mainly based on culturing cells in dishes with a flexible bottom. The culture surface can be stretched so that the cells attached to this surface are stretched also. HPDL fibroblasts are obtained from explant cultures of PDL tissues dissected from roots of healthy teeth [152, 154, 155]. The explant is cultured in Dulbecco's modified Eagle's medium (DMEM) enhanced with 10 % (volume per volume) FCS, nonessential amino acids, and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin). Cultures are maintained at 37°C in a 5 % CO_2 environment and fed every 2 or 3 days. Fibroblastic cells from the explants start outgrowing 8–10 days after the culture initiation. The cells are trypsinized with 0.15 %

trypsin/0.5 mM EDTA, harvested by centrifugation, washed in PBS, transferred into 75 cm^2 flasks containing the complete medium, and cultured to confluence (Passage 0). Following trypsin digestion, cells are subcultivated at a 1:4 split ratio on tissue culture dishes that carry a flexible, hydrophilic growth surface. PDL cells from third to sixth passages can be used for the experiments. The flexible bottom dishes can be altered from a flat position to a convex configuration by placing a Plexiglas template with a convex surface underneath and applying a weight on the top of the dish cover, thus forcing the membrane to adapt to the convex surface. The strain level can be measured by calculating the percentage of membrane expansion. The same effect can be also achieved by using more complicated systems with elaborate vacuums controlled by sophisticated software [156]. After continuous stretch application for the appropriate time intervals, the medium is removed, the cells are lysed, and proteins are extracted for further biochemical analyses [157, 158].

The future of orthodontics sounds bright and flourishing! In an era of cells, molecules, and targeted pharmaceutical intervention, orthodontics cannot stay behind. Basic research focusing on the tissue and cells reactions within the periodontal ligament and the surrounding alveolar bone slowly but steadily unravels the inside biological phenomena responsible for restructuring the architecture of the area and the occurring orthodontic tooth movement. Surely, it is a very specific research area that requires deep biological knowledge and dexterity with complex techniques far beyond the clinical interest of the everyday clinical orthodontist.

The techniques provided in this section focus on presenting the special tips and hinds required for those orthodontics who are involved in basic research, since general chapters do not covered such a specific material. Main histological and histochemical protocols, as well as specific osteoblast and osteoclast cell tissue techniques, are pivotal tools in order to understand in depth the remodeling alveolar background. More important, the external force application systems presented here show the variety of the parameters that should be taken into account when force application in biological systems is studied! One part is the cells and the molecules, but the second one, also decisive for the clinical outcome, is the force. And both parts up to date do not appear clear cut!

It is rather obvious that we, orthodontists, have reached our limits in terms of clinical intervention the classical way. It is the basic research that will give us that quantum leap that we need in optimizing our treatment: reduce the extent of treatment, abolish the cooperation, eliminate the invasive pin approaches, secure retention and treatment outcome, and last but not least teach how to use force magnitude and duration in a scientific way! Furthermore, this will provide information to the industry for the development of even more efficient orthodontic materials!

The time of pharmacological intervention within the periodontal ligament is not far. As targeted drugs develop in medicine for other conditions related to force application and bone remodeling such as in osteoporosis, orthodontics will follow. Studies appear more and more often examining the reaction of periodontal ligament tissue to external force application in the presence of injected drugs within the periodontal space. This is surely the future!

And since everything is in the periodontal ligament, its cells and fibers, and without this magnificent apparatus orthodontic tooth movement is not at all possible, we better be informed and well educated! The benefits are rather easy to imagine.

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