Oxidative Stress in Applied Basic Research and Clinical Practice

Maria Jose Alcaraz
Oreste Gualillo
Olga Sánchez-Pernaute *Editors*

Studies on Arthritis and Joint Disorders

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

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Note from the Editor-in-Chief

All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

Donald Armstrong
Editor-in-Chief

Maria Jose Alcaraz • Oreste Gualillo
Olga Sánchez-Pernaute
Editors

Studies on Arthritis and Joint Disorders

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Editors
Maria Jose Alcaraz
Department of Pharmacology
University of Valencia
Burjassot, Valencia, Spain

Olga Sánchez-Pernaute
Rheumatology Division
Jimenez Díaz Foundation University Hospital
Madrid, Spain

Oreste Gualillo
NEIRID Lab
Institute of Medical Research (IDIS)
Santiago University Clinical Hospital
SERGAS, Santiago de Compostela
Spain

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Preface

Rheumatisms are complex diseases in which the incidence of exogenous factors is determinant. Searching for an individual pathogenic factor is often useless in this kind of diseases, whereas targeting general cell processes might be a better strategy. Rheumatic diseases are highly heterogeneous in both causative mechanisms and lesion type, but in most cases their development and progression are characterized by a failure of resident cells to preserve tissue homeostasis.

The physiology of joints and connective tissues makes them particularly exposed to oxidative stress. Joint tissues have a high vascular supply and offer defensive cells the possibility of establishing contact with environmental factors. They also can act as receptacles for systemic inflammatory mediators, and even behave in the fashion of secondary lymphoid organs, in the setting of systemic autoimmune diseases. All these features make oxidation derivates especially harmful to joints and connective tissues. Additionally, joints are a major source of oxidative mediators because of their lipid-rich composition, their capacity to produce cytokines, and the presence of catalytic metals, such as iron and copper.

Both mechanical forces and chemical reactions can lead to the production of radical oxygen species (ROS) in joints, and render oxidative damage to the cartilage and the synovial tissue. Most frequently oxidative damage alters cell survival and growth, not only through a caspase-dependent death process, but also promoting mechanisms of senescence. Not surprisingly, the increased generation of ROS—or a deficient red-ox capacity—has been claimed as a major pathogenic factor in degenerative diseases. Generally speaking, accumulation of ROS provokes alterations in the structure of lipids, nucleic acids, and proteins, depletes the mitochondrial buffering reserve, and promotes protein misfolding.

Rheumatologists are used to cope with a highly popular alternative medicine promising cures for rheumatic ailments, without conducting a single experiment or clinical trial. This traditional medicine can be traced back to the Middle Ages, when both physicians and sorcerers administered a number of herb-containing potions and beverages. One of the most famous healing potions coming from the Classical Period was the "panacea," whose components are acknowledged for their antioxidative capacity and continue to be used nowadays with different clinical indications [1].

Only in the last decades scientific evidence is accumulating, supporting that various components of plants and dietary supplements are useful remedies against rheumatic diseases and offer the additional benefit of low toxicity. Most probably, in the next few years, medicines resembling antique remedies will be made. Eventually, chemical therapies will leave way to biologics, and these perhaps to nutraceuticals. Being realistic, the latter look valuable as adjuvant therapies helping keep tissue homeostasis. As has already been shown in cancer, some dietary products act as cell-conditioning agents and improve their response to cytostatics.

Joints and connective tissues are a rich milieu of cells and matrix components, not only including fibroblasts and vessels, but also bone marrow precursors, immune-specific cells, and adipocytes. Altogether they provide a highly versatile structure, accessible to therapeutic intervention. It looks as a good scenario for antioxidant drugs, but unfortunately the development of these compounds is confronted with a dreaded lack of efficacy. The search for panacea is still going on and its perfect recipe is yet far to be deciphered.

Several hurdles need to be overcome in order to establish the therapeutic capacities of nutraceuticals in rheumatic diseases. A major pitfall is that their in vitro antioxidative capacity does not correspond to an in vivo effect. This could depend on the daily dose of the nutrient, but also digestion seems to play a role in avoiding a direct effect of most dietary components. On the other hand, the beneficial effect for the joints of changing our dietary habits is quite clear, and it has been suggested that the effects might rely on the production of endogenous intermediates. Some of these controversies will be solved with the help of the new high-throughput technology, which makes possible to track the route that follows the administration of molecules with a medical intention.

Another handicap is the laborious clinical trials needed to assess efficacy in these typically heterogeneous and slowly progressive diseases. Joint replacement, bone erosion, fracture, or stroke are long-term efficacy measures and only valid when large cohorts are evaluated. Epidemiologic studies are usually confronted with numerous confounding factors, and the clinical assessment is often based on the measurement of nonobjective variables. In this sense, molecular biomarkers are attracting much interest as they could help selection of candidates and assessment of response, after their validation in large population studies.

This book offers a state-of-the-art overview of how oxidative stress participates in the most prevalent joint diseases, as discussed by experts working in the field from different approaches. From autoimmunity to senescence, and from bench to bedside, their acknowledged contributions to the field are sure to shed light on the complexity of the subject.

Burjassot, Valencia, Spain — Maria Jose Alcaraz
Santiago de Compostela, Spain — Oreste Gualillo
Madrid, Spain — Olga Sánchez-Pernaute

Reference

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Contributors

Maria Jose Alcaraz Department of Pharmacology, University of Valencia, Burjassot, Valencia, Spain

Stuart J. Bennett School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, West Midlands, UK

Hans Konrad Biesalski Department of Biological Chemistry and Nutrition, Institute of Biological Chemistry and Nutrition, University of Hohenheim, Stuttgart, Germany

Marc J. Brouillette Ignacio Ponseti Orthopaedic Cell Biology Lab, Department of Orthopaedics and Rehabilitation, University of Iowa, Iowa City, IA, USA

F. David Carmona Instituto de Parasitología y Biomedicina López Neyra, Armilla, Granada, Spain

Victoria Clérigues Department of Pharmacology, University of Valencia, Burjassot, Valencia, Spain

Javier Conde NEIRID Lab (NeuroEndocrine Interaction in Rheumatology and Inflammatory Diseases), Institute of Medical Research (IDIS), Santiago University Clinical Hospital, SERGAS, Santiago de Compostela, Spain

Paul Eggleton Peninsula Medical School, University of Exeter, St Luke's Campus, Exeter, UK

Pedro Esbrit Laboratorio de Metabolismo Mineral y Óseo, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz, Madrid, Spain

Isabel García-Arnandis Department of Pharmacology, University of Valencia, Burjassot, Valencia, Spain

Rodolfo Gómez Division of Rheumatology, Fundación Jiménez Diaz, Madrid, Spain

Juan J. Gómez-Reino NEIRID Lab (NeuroEndocrine Interaction in Rheumatology and Inflammatory Diseases), Institute of Medical Research (IDIS), Santiago University Clinical Hospital, SERGAS, Santiago de Compostela, Spain

Helen R. Griffiths School of Life and Health Sciences, Aston University, Birmingham, West Midlands, UK

Oreste Gualillo NEIRID Lab (NeuroEndocrine Interaction in Rheumatology and Inflammatory Diseases), Institute of Medical Research (IDIS), Santiago University Clinical Hospital, SERGAS, Santiago de Compostela, Spain

Maria Isabel Guillén Department of Pharmacology and IDM, University of Valencia, Burjassot, Valencia, Spain

Department of Pharmacy, University CEU Cardenal Herrera, Moncada, Valencia, Spain

Jun Hashimoto Department of Immune Disease Center, National Hospital Organization, Osaka-Minami Medical Center, Osaka, Japan

Yves Henrotin Bone and Cartilage Research Unit, Institute of Pathology, University of Liege, Sart-Tilman, Liege, Belgium

Physical Therapy and Rehabilitation Department, Princess Paola Hospital, Vivalia, Marche-en-Famenne, Belgium

Gabriel Herrero-Beaumont Bone and Joint Research Unit, Rheumatology Service, IIS Fundacion Jimenez Diaz, Madrid, Spain

Universidad Autonoma de Madrid, Madrid, Spain

Makoto Hirao Department of Orthopaedics, National Hospital Organization, Osaka-Minami Medical Center, Osaka, Japan

Sergio A. Jimenez Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA, USA

James D. Katz Department of Medicine, The George Washington University, Washington, DC, USA

Eswar Krishnan Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

Diego Kyburz Center of Experimental Rheumatology, Department of Rheumatology, University Hospital, Zurich, Switzerland

Francisca Lago Research Laboratory 7 (Molecular and Cellular Cardiology), Institute of Medical Research (IDIS), Santiago University Clinical Hospital, SERGAS, Santiago de Compostela, Spain

María J. López-Armada Aging and Inflammation Research Lab, Instituto de Investigación Biomédica A Coruña (INIBIC), Complexo Hospitalario Universitario A Coruña (CHUAC), SERGAS, Coruña, Spain

Verónica López NEIRID Lab (NeuroEndocrine Interaction in Rheumatology and Inflammatory Diseases), Institute of Medical Research (IDIS), Santiago University Clinical Hospital, SERGAS, Santiago de Compostela, Spain

Laura Lugo Bone and Joint Research Unit, Rheumatology Service, IIS Fundacion Jimenez Diaz, Madrid, Spain

James A. Martin Ignacio Ponseti Orthopedic Cell Biology Lab, Department of Orthopedics and Rehabilitation, University of Iowa, Iowa City, IA, USA

Javier Martin Instituto de Parasitología y Biomedicina López Neyra, Armilla, Granada, Spain

Jose-Ezequiel Martin Instituto de Parasitología y Biomedicina López Neyra, Armilla, Granada, Spain

Giuseppina Mattace Raso Department of Experimental Pharmacology, University of Naples Federico II, Naples, Italy

Javier Megías Department of Pharmacology, University of Valencia, Burjassot, Valencia, Spain

Rosaria Meli Department of Experimental Pharmacology, University of Naples Federico II, Naples, Italy

Ali Mobasheri Musculoskeletal Research Group, Division of Veterinary Medicine, School of Veterinary Medicine and Science, Faculty of Medicine and Health Sciences, The University of Nottingham, Leicestershire, UK

György Nagy Departments of Genetics and Rheumatology, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary

Fabienne Niederer Center of Experimental Rheumatology, Department of Rheumatology, University Hospital, Zurich, Switzerland

Anja Niehoff Institute of Biomechanics and Orthopedics, German Sport University Cologne, Köln, Germany

Norihiro Nishimoto Department of Molecular Regulation for Intractable Diseases, Institute of Medical Science, Tokyo Medical University

Osaka Rheumatology Clinic, Osaka, Japan

María Pérez-Ferro Rheumatology Division, Jimenez Díaz Foundation University Hospital, Madrid, Spain

Sonsoles Piera-Velazquez Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA, USA

Sergio Portal-Núñez Laboratorio de Metabolismo Mineral y Óseo, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz, Madrid, Spain

Prem S. Ramakrishnan Ignacio Ponseti Orthopedic Cell Biology Lab, Department of Orthopedics and Rehabilitation, University of Iowa, Iowa City, IA, USA

Romina R. Riveiro-Naveira Aging and Inflammation Research Lab, Instituto de Investigación Biomédica A Coruña (INIBIC), Complexo Hospitalario Universitario A Coruña (CHUAC), SERGAS, Coruña, Spain

Fredeswinda I. Romero-Bueno Joint and Bone Research Unit, Fundación Jiménez Díaz, Madrid, Spain

Olga Sánchez-Pernaute Rheumatology Division, Jimenez Díaz Foundation University Hospital, Madrid, Spain

Morena Scotece NEIRID Lab (NeuroEndocrine Interaction in Rheumatology and Inflammatory Diseases), Institute of Medical Research (IDIS), Santiago University Clinical Hospital, SERGAS, Santiago de Compostela, Spain

Mehdi Shakibaei Musculoskeletal Research Group, Institute of Anatomy, Ludwig-Maximilian-University Munich, Munich, Germany

Katalin É. Szabó-Taylor Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary

Carlos Vaamonde-García Aging and Inflammation Research Lab, Instituto de Investigación Biomédica A Coruña (INIBIC), Complexo Hospitalario Universitario A Coruña (CHUAC), SERGAS, Coruña, Spain

Marta N. Valcárcel-Ares Aging and Inflammation Research Lab, Instituto de Investigación Biomédica A Coruña (INIBIC), Complexo Hospitalario Universitario A Coruña (CHUAC), SERGAS, Coruña, Spain

Manuel T. Velasquez Department of Medicine, The George Washington University, Washington, DC, USA

Paul G. Winyard Peninsula Medical School, University of Exeter, St Luke's Campus, Exeter, UK

Christoph Ziskoven Institute of Biomechanics and Orthopedics, German Sport University Cologne, Köln, Germany

Part I
Biology of Oxidative Stress in Joint Disorders

Chapter 1
Soluble Proteomic Biomakers in the Management of Arthritis

Yves Henrotin and Ali Mobasheri

Abstract This chapter introduces the readers of this volume to arthritic diseases including osteoarthritis (OA) and rheumatoid arthritis (RA) before focusing on collagenous and non-collagenous biomarkers of these joint diseases. The main objective of this chapter is to focus on reactive oxygen species and in vivo biomarkers of oxidative stress. Such biomarkers may be early indicators of oxidative stress-induced tissue damage and could be used to identify patients at increased risk of developing joint disease.

Abbreviations

ACCP	Anti-cyclic citrullinated protein antibodies
ACPA	Anti-citrullinated protein antibodies
AGE	Advanced glycation end product
BMD	Bone mineral density
CDC	Centers for Disease Control and Prevention
CML	Carboxymethyl lysine
COMO	Cartilage oligomeric matrix protein
CRP	C-reactive protein
CTX-I	Carboxy-terminal cross-linked telopeptide of type I collagen

Y. Henrotin (✉)
Bone and Cartilage Research Unit, Institute of Pathology, Faculty of Medicine, University of Liege, Avenue de l'hôpital 3, CHU Sart-Tilman, 4000 Liège, Belgium

Physical Therapy and Rehabilitation Department, Princess Paola Hospital, Vivalia, Marche-en-Famenne, Belgium
e-mail: yhenrotin@ulg.ac.be

A. Mobasheri
Musculoskeletal Research Group, Division of Veterinary Medicine, School of Veterinary Medicine and Science, Faculty of Medicine and Health Sciences, The University of Nottingham, Leicestershire, UK

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CTX-II	Carboxy-terminal cross-linked telopeptide of type II collagen
DNA	Deoxyribonucleic acid
DTPA	Diethylene triamine penta-acetate
ECM	Extracellular matrix
EFSA	European Food Safety Authority
EGR-1	Early growth response protein 1
ELISA	Enzyme-linked immunosorbent assays
eNOS	Endothelial NOS
ESR	Erythrocyte sedimentation rate
ESR	Electron spin resonance
GC	Gas chromatography
GSH	Glutathione or gamma-L-glutamyl-L-cysteinylglycine
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HPLC	High-performance liquid chromatography
HRT	Hormone replacement therapy
IFN-γ	Interferon gamma
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
IL-1β	Interleukin 1 beta
IL-6	Interleukin-6
iNOS	Inducible NOS
JSW	Joint space width
LC	Liquid chromatography
MMP-13	Matrix metalloproteinase 13
MPO	Peroxynitrite
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	Pyrin-like protein containing a pyrin domain
NEN	Nonenzymatic nitrite
NF-κB	Nuclear factor kappa B
NIAMS	National Institute of Arthritis and Musculoskeletal and Skin Diseases
NO	Nitric oxide
NTX-I	Amino-terminal cross-linked telopeptide of type I collagen
OA	Osteoarthritis
PAS	Patient Activity Scale
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PMN	Polymorphonuclear leukocytes
PYCARD	Apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)
RA	Rheumatoid arthritis
RAGE	AGE receptor
RF	Rheumatoid factor
ROS	Reactive oxygen species
SOD	Superoxide dismutase

TBAR	Thiobarbituric acid reactants
TIINE	Collagen type II neoepitope
uPA	Urokinase or urokinase-type plasminogen activator
WHO	World Health Organization
WOMAC	Western Ontario and McMaster University Osteoarthritis Index
WT	Wild type
YKL-40	Cartilage glycoprotein-39

1.1 Introduction

Human life expectancy has consistently increased by a quarter of a year every year over the last 160 years [1]. It is predicted that life expectancy will continue to increase by 2.5 years each decade, meaning that the Western world's average life expectancy should reach and exceed 100 within the next 50 years [1]. This increase in life expectancy has been mainly due to the significant advances in medicine and healthcare. However, the increased life expectancy of human beings is accompanied by an increased prevalence of a range of arthritic, rheumatic, and musculoskeletal diseases.

Arthritic diseases of load-bearing synovial joints are leading causes of morbidity, disability, and loss of productivity throughout the world [2] (source: http://www.arthritis.org/).[1, 2] These are essentially "inflammatory" disorders. The term "arthritis" characterizes a group of conditions involving inflammatory damage to synovial joints [3]. According to the World Health Organization (WHO[3]), orthopedic, rheumatic, and musculoskeletal conditions comprise over 200 diseases and syndromes, which are usually progressive and associated with pain and disability. The most common form, osteoarthritis (OA), is one of the most prevalent and chronic diseases affecting the elderly [4]. The symptoms and signs characteristic of OA in the most frequently affected joints are heat, swelling, pain, stiffness, and limited mobility. OA is often a progressive and disabling disease, which occurs in the setting of a variety of risk factors, such as advancing age, obesity, and trauma, that conspire to incite a cascade of pathophysiological events within joint tissues [5]. Other sequelae include osteophyte formation and synovitis [6]. These manifestations are highly variable, depending on joint location and disease severity. Other forms of inflammatory arthritis include gouty arthritis, psoriatic arthritis, and rheumatoid arthritis (RA), an autoimmune disease in which the body's own immune system attacks synovial joints.

The aim of this chapter is to focus on biomarkers of joint disease by placing special emphasis on biomarkers of oxidative stress. We will briefly discuss the major forms of joint disease before discussing the biomarkers that can be used to diagnose them. This chapter also discusses the chemistry of reactive oxygen species (ROS) and antioxidants for targeting oxidative stress in joint diseases and attempts to link these back to biomarkers, focusing on biomarkers that may be early indicators of oxidative stress.

[1] http://www.who.int/healthinfo/statistics/bod_osteoarthritis.pdf

[2] http://whqlibdoc.who.int/bulletin/2003/Vol81-No9/bulletin_2003_81(9)_630.pdf

[3] http://www.who.int/en/

1.2 Osteoarthritis

OA is one of the most prevalent and chronic diseases affecting the elderly [4]. More than 20 million Americans are estimated to have OA.[4] Estimates from the Centers for Disease Control and Prevention (CDC[5]) suggest that OA is one of the top five causes of disability amongst nonhospitalized adults. The situation is similar in European countries. In 2006, it was estimated that around 35–40 million Europeans suffer from OA and nearly 25% of people aged 60 and above suffer from OA-induced disability. It is also anticipated that by the year 2030, 20% of adults will have developed OA in Western Europe and North America. Therefore, OA is expected to place a heavy economic burden on healthcare systems and community services throughout the world.

OA is the most common form of joint disease, with the majority of the population over 65 years of age demonstrating radiographic evidence of OA in at least one joint. Although it is rare in people under 40, it becomes much more common with age. The end-stage treatment for OA is surgery, either to modify or replace the joint. With increasing life expectancy, growth in the elderly population, and an alarming escalation of chronic, inflammatory, and age-related conditions (such as OA), there is increased demand for new treatments and preventative approaches. Although developing OA is a manifestation of aging, the disease may remain latent and asymptomatic, taking many years to reach clinical relevance. OA is not simply the common outcome of aging and joint injury; it is global, active, and inflammatory joint disease.

Although OA is primarily associated with aging, there are other important contributing factors [7]. These include obesity (which increases mechanical stress), underlying metabolic or endocrine disease, genetics and a history of joint trauma and instability, a history of joint trauma or repetitive use, genetics, heritable metabolic disorders, muscle weakness, underlying anatomical and orthopedic disorders (i.e., congenital hip dislocation), joint infection, crystal deposition, previous RA and various disorders of bone turnover, and blood clotting. The metabolic alterations that occur in obesity along with the pro-inflammatory factors produced by white adipose tissue in the chronically overweight are thought to be major factors in the progression of the disease [8].

Symptoms of OA in the most frequently affected joints include heat, swelling, pain, stiffness, and limited mobility. These manifestations are highly variable, depending on joint location and disease severity. OA can affect any synovial joint, but it primarily affects large load-bearing joints such as the hip and knee. The disease is essentially due to daily wear and tear of the joint. Its most prominent feature is the progressive destruction of articular cartilage [9]. It is generally accepted that OA begins in articular cartilage and eventually spreads to subchondral bone and other synovial tissues. However, there is the opposing view that suggests OA is a disease of subchondral bone and begins there. Despite the controversy regarding its initiation,

[4] http://www.niams.nih.gov/

[5] http://www.cdc.gov/

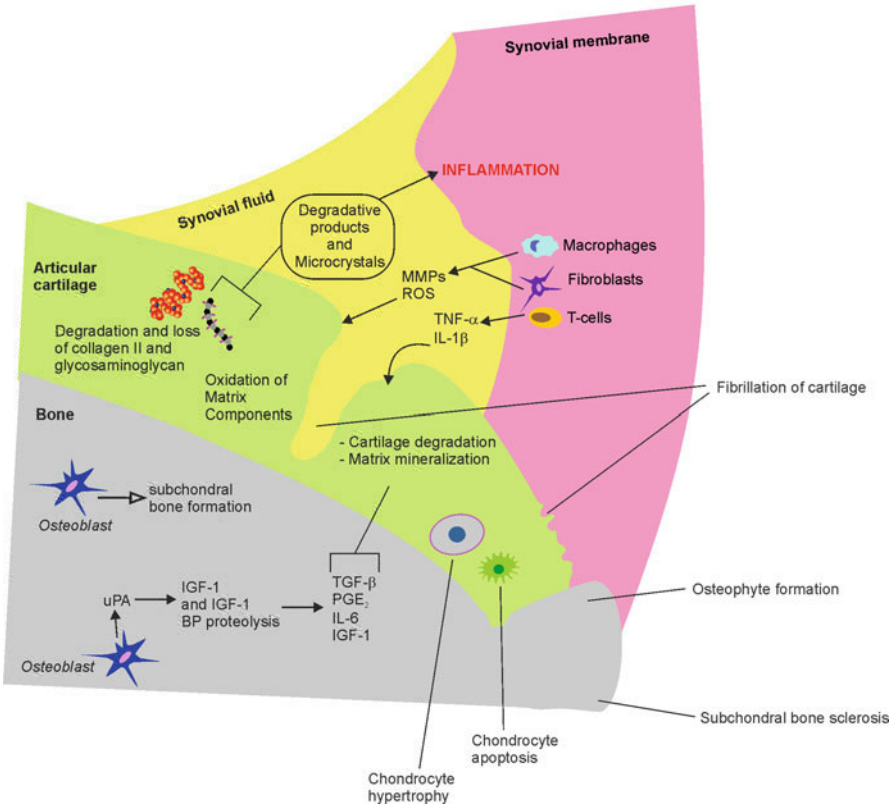

Fig. 1.1 The major molecular and cellular changes that occur in the synovial joint in OA. *MMPs* matrix metalloproteinases, *ROS* reactive oxygen species, *uPA* urokinase, *IGF-1* insulin-like growth factor-1, *IL-6* interleukin-6, PGE_2 prostaglandin E_2, *BP* binding protein

the current consensus is that OA is a disease involving not only articular cartilage but also the synovial membrane, subchondral bone, and periarticular soft tissues [10]. OA may occur following traumatic injury to the joint, subsequent to an infection of the joint, or simply as a result of aging and the mechanical stresses associated with daily life. The synovitis that occurs in both the early and late phases of OA is associated with alterations in the adjacent cartilage—these changes are highly similar to those seen in RA. Catabolic and pro-inflammatory mediators such as cytokines, nitric oxide (NO), prostaglandin E2 (PGE_2), and neuropeptides are produced by the inflamed synovium, which alter the balance of cartilage matrix degradation and repair. These events lead to excess production of the proteolytic enzymes responsible for cartilage breakdown [11]. Cartilage alterations induce further synovial inflammation, creating a vicious circle. The progressing synovitis will then exacerbate clinical symptoms and joint degradation in OA [11]. Figure 1.1 outlines the major molecular and cellular changes that occur in the synovial joint in OA.

1.3 Rheumatoid Arthritis

Rheumatoid arthritis[6, 7, 8] (RA) is an "autoimmune" disease in which the immune system attacks synovial joints and other tissues. Most of the damage occurs to the joint lining (synovium) and cartilage, which eventually results in erosion of two opposing bones. RA is a painful, chronically disabling, and progressive disease affecting 0.8–1% of the adult population. The symptoms of RA usually vary over time. Sometimes, symptoms only cause mild discomfort. At other times, they can be extremely painful, making it difficult to move around and perform everyday tasks. When symptoms worsen, this is known as a flare-up or flare. A flare-up is impossible to predict, making RA difficult to live with. It can cause severe disability, which varies between individuals and depends on the severity of the disease. It can significantly affect a person's ability to carry out even the simplest of everyday tasks. The disease can progress very rapidly (again the speed of progression varies widely between individuals), causing swelling and damaging cartilage and bone around the joints. Any joint may be affected, but it is commonly the hands, feet, and wrists. RA is a systemic disease, which means that it can affect the whole body and internal organs such as the lungs, heart, and eyes. Furthermore, RA is associated with an increased risk of coronary disease, infection, and lymphoma, as well as reduced life expectancy [12–16]. RA affects approximately three times more women than men, and onset is generally between 40 and 70 years of age, although it can occur at any age. There are studies that suggest RA is also associated with sex hormone production in the body. The peak incidence of RA in women coincides with the perimenopausal age, and the juvenile form occurs mainly during puberty, suggesting a connection of RA with hormonal alterations [17]. Although controversial, several studies have reported on ameliorating effects on clinical measures of disease activity and inflammation, improved bone mineral density (BMD), and presented results pointing towards retardation of joint damage by hormone replacement therapy (HRT) [16, 18, 19].

The pathogenesis of RA is poorly understood. Smoking is an important risk factor and makes the outlook much worse, but there is no mechanistic insight to explain why this is the case. There is no cure for RA, and more information is needed to help understand about the inflammatory processes that occur in the disease and how to manage it. Uncontrolled RA increases mortality through an increased risk of cardiovascular disease such as heart attacks and strokes; again the need for early treatment is imperative. Therefore, we need new and safer drugs for RA and better ways to monitor the disease and avoid prevent noxious stimuli that may cause inflammatory "flare-ups" in the most susceptible individuals.

[6] http://www.nras.org.uk/

[7] http://www.nras.org.uk/about_rheumatoid_arthritis/what_is_ra/what_is_ra.aspx

[8] http://www.arthritisresearchuk.org/arthritis_information/arthritis_types__symptoms/rheumatoid_arthritis.aspx

1.4 Biomarkers of OA

A major focus of clinical research in recent years has been the identification of new disease markers that can facilitate early diagnosis and optimize individualized treatments. Such markers can also facilitate the drug discovery process by reducing the high levels of attrition in clinical trials. A biomarker is a *characteristic* that is objectively *measured* and *evaluated* as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [20]. They are classified into "soluble" or "wet" biomarkers (i.e., proteins, peptides, metabolites) or as "dry" biomarkers including imaging (i.e., radiographs, magnetic resonance imaging (MRI), ultrasound), questionnaires, and visual analog scales. The National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) established the OA Biomarkers Network to develop and validate standardized, sensitive biomarker assays in blood and urine to facilitate the diagnosis of the preradiologic stage of OA in humans and animal models. The objective is to identify markers that can help us understand the biological processes involved in disease progression and allow us to monitor the effects of lifestyle (i.e., drastic weight loss), surgical or pharmacological treatment, thus accelerating the pace of drug discovery. Such biomarkers could also potentially be used to identify patients at increased risk of developing OA.

1.4.1 Collagenous Biomarkers

The dry weight of articular cartilage is predominantly composed of collagen [21], and the remainder is mainly proteoglycans and glycoproteins [22]. Type II collagen is the major fibrillar collagen component of articular cartilage. Type II collagen is possibly the ideal source of markers for studying cartilage remodeling [23]. First, this protein is relatively specific to articular cartilage (although it is also present in the vitreous humor of the eye, the nucleus pulposus of vertebral discs, the meniscus, respiratory tract, and insertion sites of tendons and ligaments into bone [24]). Second, it makes up most of the collagen network in the ECM and is the most abundant protein in cartilage, representing 25% of the wet weight, 50% of the dry weight, and 90–95% of the total collagen content. Third, type II collagen makes up only 1% of all collagen in the body, and the normal turnover is low, suggesting that pathological turnover from a single joint might be expected to raise the systemic level epitopes significantly [25]. The breakdown of type II collagen is a characteristic feature of OA, and products of cartilage collagen metabolism can be detected in the blood, synovial fluid, and urine. Since type II collagen is specific for hyaline cartilage and is in high abundance in this tissue, the major biomarkers of collagen turnover in cartilage are epitopes derived from type II collagen [26]. Type II collagen biomarkers may be classified in four groups, according to the localization of the epitope in the molecule and the process that leads to expression of the epitope. The first group, cleavage neoepitopes, is localized to the cleavage site and includes

the following: C2C, C1, 2C, collagen type II neoepitope (TIINE), CIIM, Coll2-1/4N1, and Coll2-1/4N2. The second group includes the denaturation epitopes, which are localized to the triple helical domain and made accessible by unwinding of the triple helix: Coll2-1 and its nitrated form Coll2-1NO2, CB11 peptide (COL2-3/4m), AH8, AH9, and AH12. Epitopes localized to the telopeptides of the molecule represent the third group: Col2CTx and carboxy-terminal cross-linked telopeptide of type II collagen (CTX-II). The final group includes epitopes of propeptide protein fragments that are released during collagen synthesis: carboxyl propeptide and type IIA N-propeptide of type II collagen.

1.4.2 Non-collagenous Biomarkers

In OA proteoglycans are also degraded, and their fragments are released from the ECM into the synovial fluid, and from there they may be filtered to the circulation and urine [27].

1.4.2.1 Cartilage Oligomeric Matrix Protein

Cartilage oligomeric matrix protein (COMP) has shown promise as a diagnostic and prognostic indicator and as a marker of the disease severity and the effect of treatment [28]. It seems to be a good prospect for detecting early-stage OA. It has shown promise as a diagnostic and prognostic indicator and as a marker of the disease severity and the effect of treatment [28, 29]. Enzyme-linked immunosorbent assays (ELISAs) for the detection of this protein and its fragments in synovial fluid and serum have been developed at tested in patients with knee and hip OA [30–32], RA [33], and other forms of inflammatory arthritis [34]. Persistently, high serum levels of COMP have been detected in patients with traumatic knee injury and posttraumatic OA [35, 36]. Large-scale population studies (the Johnston County Osteoarthritis Project) have confirmed that serum COMP protein reflects presence and severity of OA [37]. Several other mesenchyme-derived cells including synoviocytes and dermal fibroblasts produce substantial amounts of COMP. These findings raise important concerns regarding the utility of measurements of COMP levels in serum or in synovial fluid as markers of articular cartilage degradation because of the likelihood that a substantial proportion of COMP or COMP fragments present in serum or synovial fluid may be produced by cells other than articular chondrocytes [38].

1.4.2.2 Hyaluronic Acid

Hyaluronic acid also known as hyaluronan, hyaluronate, or HA has been found to be elevated in plasma from patients with OA and RA [39]. Serum HA levels have been suggested to predict disease outcome in knee OA [40]. The higher

concentrations found in serum from OA patients suggest that there is a relationship between increased levels of HA and the increased risk for OA. Therefore, HA levels may have predictive value for the progression of knee and hip OA [41–43]. HA has also been evaluated as a biomarker for equine OA [44] and canine hip dysplasia and canine cruciate disease [45, 46]. Assays for serum HA and methods for its quantitation in biological fluids have existed for several decades [47, 48]. The rationale for developing such assays was the realization that HA is a potential diagnostic marker for cartilage breakdown in RA and OA [49, 50]. Indeed, quantitative analysis of HA in the synovial tissues of patients with joint disorders has confirmed this, particularly in RA and following joint injury [51]. More recent studies in African Americans and Caucasians in the Johnston County Osteoarthritis Project support a role for serum HA as a biomarker of radiographic OA [52]. Also, HA was found to be significantly higher in hand OA in the CARRIAGE family study [53] and an independent study in the Czech Republic [54]. The major problem with HA as a biomarker is that its levels may also change diurnally [55], with physical activity [56], posture, and different diets [57]. HA is ubiquitous in the body and not only found in joint tissues but also in other connective tissues. Furthermore, it has not been validated for the early stages of OA. A more sensitive biomarker might be hyaluronidase, the enzyme responsible for HA degradation. Zymographic examination of synovial fluid and serum hyaluronidase activity in RA and OA patients has shown that the expression of this enzyme and its activity could be used as a marker of synovial inflammation [58].

1.4.2.3 YKL-40 (Cartilage Glycoprotein-39)

YKL-40 or chitinase 3-like 1 (cartilage glycoprotein-39) is a biomarker that provides a snapshot of inflammatory events in joint tissues, potentially allowing rapid assessment of pharmacotherapy [59, 60]. Its presence in synovial fluid and serum may reflect articular cartilage degradation and the degree of synovial inflammation in the knee joint [61, 62]. This protein and related proteins may participate in cartilage remodeling and degradative processes in OA joints [63–66]. Furthermore, plasma levels of YKL-40 are raised in patients with RA and other inflammatory conditions [67]. Finally, serum or urinary determinations of these molecules are difficult to interpret adequately due to their complex metabolism in the body [59].

1.5 Biomarkers of RA

RA can be difficult to diagnose, and there are no blood tests that can definitively rule in or rule out the disease. A number of laboratory tests and biomarker assays have been developed and clinically validated to help to confirm the diagnosis of RA in human patients [68]. The rational use of laboratory testing and biomarkers for investigating early, undifferentiated joint pain also requires a detailed history and careful

physical examination of the patient [69]. Full blood cell count, serum uric acid, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), anti-cyclic citrullinated protein antibodies (ACCP), and antinuclear (ANA) antibody titers form a reasonable screening panel when RA is suspected based on the clinical manifestations.

The American College of Rheumatology has recently published recommendations for the use of RA disease activity measures in clinical practice [70]. They recommend the Clinical Disease Activity Index, Disease Activity Score with 28-joint counts (erythrocyte sedimentation rate or C-reactive protein), Patient Activity Scale (PAS), PAS-II, Routine Assessment of Patient Index Data with three measures, and Simplified Disease Activity Index because they are accurate reflections of disease activity; are sensitive to change; discriminate well between low, moderate, and high disease activity states; have remission criteria; and are feasible to perform in clinical settings [70].

1.6 Reactive Oxygen Species and Endogenous Antioxidants

Oxidation reactions in living cells produce free radicals, reactive oxygen species (ROS), and their derivatives. These dangerous and harmful chemical products can accumulate over time, causing extensive structural damage or even cell death. The cytotoxic effects of ROS can cause a variety of health problems including inflammatory disease, tissue necrosis, organ failure, atherosclerosis, infertility, birth defects, premature aging, mutations, and malignancy [71]. ROS production initiates an "inflammatory state" which unless quenched may result in chronic inflammatory disease states, e.g., arthritis, hepatitis, nephritis, myositis, scleroderma, lupus erythematosus, and multiple system organ failure [71]. ROS are involved in the initiation of inflammatory responses [72]. For example, ROS such as H_2O_2 can stimulate the transcription factor NF-κB, which is crucial for cellular processes such as inflammation, immunity, cell proliferation, and apoptosis [73]. Therefore, ROS-mediated upregulation of NF-κB can cause dysregulation of many inflammatory responses.

Living cells maintain a complex and interrelated protective system of endogenous antioxidant vitamins, minerals such as selenium and manganese as cofactors, and glutathione to protect themselves from the harmful effects of ROS [74, 75]. Cells also use a variety of antioxidant enzymes such as catalase, superoxide dismutase, and various peroxidases to quench and control cellular levels of ROS. Deficiency in antioxidants or inhibition of the antioxidant enzyme systems may cause oxidative stress and may damage or kill cells. Oxidative stress is an important component of many diseases. Therefore, the biology of ROS and antioxidants is widely investigated in the context of understanding the role of these chemicals in chronic diseases characterized by oxidative stress. The next two sections will discuss examples of endogenous antioxidants.

1.6.1 Catalase

Hydrogen peroxide (H_2O_2) is a harmful by-product of many normal metabolic processes. In the synovial joint, hydrogen peroxide is an important mediator of tissue damage [76], especially since its concentration is enhanced by pro-inflammatory cytokines [77]. In order to prevent damage to cells and tissues, hydrogen peroxide must be rapidly converted into other, less dangerous substances. Catalase is the ubiquitous enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen. During acute inflammation, the production of hydrogen peroxide by polymorphonuclear cells is a suspected cause of cellular damage in the joint [78]. Hydrogen peroxide from polymorphonuclear cells plays an important role in cartilage degradation through direct damage to cartilage during inflammatory processes in the joint [79]. Chondrocytes within cartilage are sensitive to toxic oxygen metabolites [80] and have been shown to contain catalase and the glutathione peroxidase/reductase systems. These enzyme systems appear to be involved in the removal of hydrogen peroxide in these cells. Immunohistochemical studies in the rat have confirmed the presence of catalase within articular chondrocytes [81]. Interruption of either of these peroxide-metabolizing systems sensitizes cartilage to a greater inhibition of matrix synthesis and oxidative damage by hydrogen peroxide. Hydrogen peroxide suppresses chondrocyte proteoglycan synthesis as measured by ^{35}S sulfate incorporation assays and [82]. Inhibition of catalase with 3-amino 1,2,4 triazole or azide further inhibits matrix synthesis, possibly because of exposure to higher steady state levels of hydrogen peroxide [78].

1.6.2 Glutathione

Glutathione or gamma-L-glutamyl-L-cysteinylglycine (GSH) is one of the main endogenous antioxidants in cells and is involved in diverse functions including apoptosis, disulfide bond formation, detoxification, antioxidant defense, maintenance of thiol status, and modulation of cell proliferation. Increased oxidative stress with aging reduces chondrocyte survival, and this correlates with intracellular GSH levels [83]. Increased oxidative stress makes chondrocytes much more susceptible to oxidant-mediated cell death. This occurs through the dysregulation of the GSH antioxidant system [83]. The reduction in the capacity of antioxidant buffering systems such as GSH may represent an important contributing factor to the development of OA in older adults [83]. There have been a number of in vitro studies on GSH in chondrocytes. *N*-acetylcysteine, a precursor of GSH, has been shown to protect growth plate chondrocytes and temporomandibular joint chondrocytes from the effects of oxidative stress in vitro [84, 85]. *N*-acetylcysteine has been shown to prevent NO-induced chondrocyte apoptosis and cartilage degeneration in an experimental model of rabbit OA [86]. *N*-acetylcysteine also activates extracellular signal-regulated kinase signaling pathway in articular chondrocytes, which may provide a

mechanism for the promotion of chondrocyte survival by this thiol antioxidant [87]. When over-expressed, the enzyme glutathione-S-transferase can protect chondrocytes from the effects of oxidative stress [88]. GSH depletion and NO both decrease insulin-like growth factor I (IGF-I) receptor function chondrocytes in vitro [89]. Insulin-like growth factor I (IGF) helps maintain healthy articular cartilage; however, arthritic cartilage becomes less responsive to the anabolic actions of IGF-I. Thus, it is interesting that GSH depletion can reduce the responsiveness of chondrocytes to this important anabolic growth factor. ROS such as superoxide, hydrogen peroxide, and hydroxyl radical are typically produced in mitochondria as electrons leak from the electron transport chain and react with oxygen to form superoxide. It is estimated that 1–3% of oxygen reduced in cells may form superoxide in this way [90]. Hydrogen peroxide is formed from the dismutation of superoxide and by oxidases. These three reactive species are controlled via multiple-enzyme systems like superoxide dismutase (SOD), catalase, glutathione-S-transferase, and thioredoxin. SOD converts superoxide to hydrogen peroxide, which is then removed by glutathione peroxidase or catalase and therefore has the capacity to prevent the formation of highly aggressive ROS, such as peroxynitrite or the hydroxyl radical [91]. The production of hydrogen peroxide (H_2O_2) by inflammatory and synovial cells is an important cause of cellular damage during joint inflammation. Effective H_2O_2-metabolizing systems are important in the maintenance of normal biosynthetic rates in cartilage during inflammation. In addition to the antioxidant vitamins and GSH, chondrocyte antioxidant defenses include catalase, glutathione-S-transferase, and glutathione peroxidase. These enzymes afford protection against H_2O_2-dependent inhibition of proteoglycan biosynthesis [78]. Immunohistochemical studies have identified superoxide dismutases, catalase, and glutathione-S-transferases in rat joints [81]. Interestingly, there were no major age-related changes in antioxidant enzyme distribution in rat joints [81]. Microinjection of antibodies against superoxide dismutase and glutathione peroxidase has been shown to decrease their viability, whereas injection of control (nonimmune) has no effect [92]. These findings highlighted the importance of glutathione peroxidase as antioxidant and the relative efficiency of SOD according to the balance between the radical production and the activity of the other antioxidant systems in chondrocytes.

1.7 ROS and the Inflammasome

ROS are also linked to mitochondria and the inflammasome [93]. The inflammasome is a protein complex that stimulates caspase-1 activation to promote the processing and secretion of pro-inflammatory cytokines [94]. This multiprotein oligomer consists of caspase 1, PYCARD, NALP, and sometimes caspase 5 (also known as caspase 11 or ICH-3). Inflammasome-dependent inflammatory responses are triggered by a variety of stimuli including infection, tissue damage, and metabolic dysregulation [95]. Recent work suggests that mitochondria are involved in integrating distinct signals and relaying information to the inflammasome. Dysfunctional

mitochondria generate ROS, which is required for inflammasome activation. Interestingly, mitochondrial dysfunction has been linked to OA [96, 97]. Analyses of mitochondrial electron transport chain activity in cells from OA-affected cartilage show decreased activity of complexes I, II, and III compared to normal chondrocytes [98]. Therefore, it is possible that mitochondrial dysfunction in arthritis is exacerbated by ROS and catabolic processes that alter cellular metabolism. The inflammasome is negatively regulated by autophagy, which is a catabolic process that removes damaged or otherwise dysfunctional organelles, including mitochondria [95]. Autophagy has been shown to be a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and OA [99]. These studies suggest that the connections between mitochondria, metabolism, and inflammation are important for cell function and malfunctioning of this network is associated with many chronic inflammatory diseases. ROS generation and inflammasome activation are linked with mitochondrial dysfunction and may explain the frequent association of mitochondrial damage with inflammatory diseases.

1.8 Oxidative Stress in Joint Diseases

Oxidative stress, defined as an imbalance between oxidative processes and reduction equivalents (antioxidants), is involved in the development of degenerative joint diseases. There is a substantial body of published research that suggests that arthritic diseases are characterized by inflammation and oxidative stress. Oxidative stress produces reactive oxygen species (ROS) that play key roles in the development of OA. ROS are also involved in RA. In both diseases, metabolic reactions in chondrocytes and synoviocytes produce free radicals, ROS, and their derivatives. These dangerous chemicals accumulate in the synovial joint, causing extensive structural damage cell death and inflammation. For example, in OA, oxidative damage contributes to chronic inflammation and promotes age-related diseases [100]. This results in senescence-associated secretory phenotype, which has many of the characteristics of an "osteoarthritic chondrocyte" in terms of the cytokines, chemokines, and proteases produced [100].

1.9 In Vivo Markers of Oxidative Stress

Research on biomarkers of oxidative stress has the potential to develop a "fingerprint" or "chemical signature" for measuring and monitoring oxidative stress in health and disease. Many analytical techniques are available for the measurement of oxidative stress status in culture systems, animal models, and human subjects. The techniques are as diverse as blood tests for oxidized lipids and proteins indicative of oxidative damage, volatile hydrocarbons in exhaled breath, and oxidized DNA bases excreted in urine. The real challenge is to identify and validate measurable, sensitive, and

specific biomarkers for oxidative damage resulting from different types of oxidative insults and to understand the interrelationships among the markers identified in order to determine which of the available biomarkers of oxidative stress are the most specific, sensitive, and selective.

Free radicals can be measured directly using electron spin resonance (ESR), most often coupled to spin trapping to increase the sensibility of the method. However, ESR is difficult to use in human subjects in vivo [101]. Many indirect methods of RNOS measurements have been proposed, based on the use of antioxidants and enzyme inhibitors or on the measurement of stable compounds derived from ROS activity and considered as "markers of oxidant stress." Among the possible markers of oxidant stress, there are isoprostanes [102] hydroxynonenal and lipid peroxides, nitrated and oxidized proteins, chlorinated compounds, protein carbonyl, oxidized glutathione, and malondialdehyde (detected as thiobarbituric acid reactants; TBARs) [103]. These techniques, however, have limitations. They are not all specific for oxidative stress and are at risk of artifacts. For example, isoprostanes can be produced by platelets independently of oxidant stress [104, 105], and the chemical reaction of malondialdehyde detection is influenced by the presence of iron in the sample [106].

Moreover, these markers are not specific of one particular RNOS, and all RNOS do not generate one particular by-product. Therefore, it is speculated that probably more than one marker is needed to assess overall "oxidative stress." For example, nitrated proteins indicate that the "oxidative stress" involves peroxynitrite or MPO; nitrotyrosine shows that $^{\bullet}NO$ is involved and malondialdehyde that lipid radicals have been generated in the cell membrane. Further, these markers are not specific for the tissue in which oxidative stress occurs. For example, if the oxidative stress is located in the joint, biomarkers should be measured ideally in synovial fluid, but not in blood or urine.

There are two other popular ways to identify oxidant stress: the first is to measure the changes in the "antioxidant status" by estimating the consumption of the endogenous antioxidants or the changes in the activity or the expression of the antioxidant enzymes. The second is to measure the "total antioxidant capacity" of a biological sample (plasma, tissue extract) by testing the capacity of this sample to inhibit the transformation of a selected substrate by an in vitro generated free radical. These methods have encountered a great success, not only in severe diseases to identify oxidant stress, but also to evaluate the capacity of healthy humans to fight against a potential oxidative stress [107]. However, this method has some limitations. It does not allow the identification of the molecule(s) involved in the antioxidant status failure. The global antioxidant status is largely influenced by nutrient intake, physical activity, and other life conditions. There is no norm, and a longitudinal investigation is needed to research an individual variation. The RNOS-generating system used in this technique (hypoxanthine/xanthine oxidase) does not reproduce the complex RNOS-generating system involved in "oxidant stress" in vivo. Therefore, the capacity of a biological sample to scavenge $O_2^{\bullet-}$ does not necessarily reflect its capacity to scavenge RNOS in vivo. Another major problem is its application to plasma samples which naturally contain a high concentration of albumin, an excellent "antioxidant" barrier to RNOS activity. An increase in total antioxidant capacity is considered to be a poor marker for evaluating oxidative stress [108].

Table 1.1 Biomarkers of bone, cartilage, and synovial turnover

Tissue	Molecule	Markers of synthesis	Markers of degradation
Bone	Type I collagen		N-telopeptide (NTX-I) (s, u)
			C-telopeptide (CTX-I) (s, u)
	Non-collagenous proteins	Osteocalcin (s)	
Cartilage	Type II collagen	PIIANP (s)	
		PIINP (s)	
		NPII (p)	
		PIICP (s)	
			CTX-II (u, sf)
			Col2CTX (u)
			TIINE 45 mer or NET2C (u)
			Coll 2-1 (s, u)
			Coll 2-1 NO_2 (s, u)
			C2C (s, u)
			C1,2C (u)
			CIIM (u, s)
	Aggrecan	846 (cartilage matrix)	
			G1-G2 (s)
			342-FFGVG (s)
			374-ARGSV (u)
	Non-aggrecan and non-collagenous proteins		Pentosidine (s)
			COMP (s, sf)
	Proteases and inhibitors		MMP-13 (u)
Synovium	Type III collagen		III-Nys (synovial tissue)
	Non-collagenous proteins	Hyaluronic acid (HA) (p, s)	

s serum, *p* plasma, *u* urine, *sf* synovial fluid

Usually, OA progression is monitored by measurement of changes in joint space width on plain radiographs with a graduated magnifying lens or with a computer after digitization of the radiograph. This must be considered a rather indirect measure of cartilage integrity, as articular cartilage itself is invisible on the radiographs and thus has to be assessed indirectly from the spacing between the subchondral bone ends of the joint. Furthermore, joint space width does not allow detection of early structural damage, remains difficult to use in daily practice, and is poorly reproducible. It fails to measure a dynamic metabolic process and is confounded by the presence of meniscal lesions or extrusion. Its change overtime is very small, occurs in only a subset of patients (the progressors), and is not correlated with joint function and pain. Magnetic resonance imaging (MRI) is a promising noninvasive tool for evaluation of cartilage, but access to this technique is confined and very expensive. Further, MRI and radiographs provide a static picture of the cartilage lesion. These imaging techniques fail to explore the metabolic changes occurring in OA cartilage. Biochemical factors of bone synovium or cartilage turnover have been proposed as alternative diagnostic and prognostic tools for monitoring treatment efficacy (Table 1.1) [109–112]. The challenge is to identify tissue and disease-specific markers of oxidative stress. In order to address this aim, our group and others have developed a new generation of biomarkers useful for measuring the oxidative stress occurring in hyaline cartilage. These biomarkers have been validated in RA and OA.

1.9.1 Oxidant-Induced Changes in Collagens

1.9.1.1 Oxidative Cleavage

Collagen is the only protein susceptible to fragmentation by $O_2^{\bullet -}$ [113]. In comparison, proteins such as serum albumin or various enzymes are not degraded by $O_2^{\bullet -}$. This $O_2^{\bullet -}$-induced collagen degradation was characterized by the release of small 4-hydroproline-containing peptides, suggesting scissions in the triple helical part of the collagen molecule [114, 115]. This collagen oxidative degradation was inhibited by SOD but not by catalase or chelating agents such deferroxxamine or diethylene triamine penta-acetate (DTPA), confirming the key role that is played by $O_2^{\bullet -}$ in the process. Nevertheless, the action of $^{\bullet}OH$ on collagen remains questionable since it was demonstrated that its action is quite different in the absence or in the presence of O_2 [116]. In the presence of O_2, $^{\bullet}OH$ generated by gamma radiolysis released pattern of peptides different from that generated by $O_2^{\bullet -}$. OH-generated peptides are characterized by an increase of aspartic and glutamic acid residues and a decrease in the amount or 4-hydroproline and proline residues. In contrast, when irradiations of collagen are performed in the absence of oxygen, no collagen cleavage is observed but a polymerization of collagen.

Hypochlorite (HOCL/OCL−) within the predicted range generated by PMNs or monocytes at sites of inflammation (10–50 mM) does not cause fragmentation of collagen I or II [117]. Only the supra-physiological concentrations of 1–5 mM cause extensive fragmentation of collagen [118]. *N*-chloramine (5–50 mM) does not cause fragmentation but greatly increases the degradation of collagen by collagenase and elastase. The mechanism by which *N*-chloramines, and probably other oxidants, increase the proteolytic susceptibility of collagen is not clearly determined, although it is assumed that *N*-chloramines react with amino groups and disrupt the secondary and tertiary structures of collagen molecules [119]. Disruption of the tertiary structure of collagen by oxidation exposes hydrophobic regions and promotes the degradation of fibrillar collagens by proteases. Another explanation would be that oxidation and disruption of pyridinoline cross-links could result in the loss of functional interactions of collagen fibrils and consequently an increase in the susceptibility of collagen to proteolytic degradation.

Finally, exposure of collagen to ROS results in modification of the primary structure of collagen. Exposure of proline peptides to a Fenton system (Cu(II)/peroxide) results in conversion of some proline residues into hydroxyproline, along with formation of γ-aminobutyric acid [120].

Exposure of purified type II collagen to $FeSO_4$-EDTA ($^{\bullet}OH$ source) or xanthine oxidase-hypoxanthine system ($O_2^{\bullet -}$ source) also induced a cleavage of the proline producing more terminal glutamate residues, which are Ca^{2+} affinity ligand. These oxidative-induced changes promote crystal formation which is an important feature in some rheumatic diseases including OA, gut, or Kashin–Beck's disease [121].