Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

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Vinood B. Patel · Victor R. Preedy *Editors*

Biomarkers in Liver Disease

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Biomarkers in Disease: Methods, Discoveries and Applications

Series Editor
Victor R. Preedy
Diabetes and Nutritional Sciences Research Division
Faculty of Life Science and Medicine
King's College London
London, UK

In the past decade there has been a major sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies, such as microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of Biomarkers in Disease.The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

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Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Liver Disease

With 162 Figures and 124 Tables

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Editors
Vinood B. Patel
Department of Biomedical Science
Faculty of Science and Technology
University of Westminster
London, UK

Victor R. Preedy
Diabetes and Nutritional Sciences Research Division
Faculty of Life Science and Medicine
King's College London
London, UK

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Preface

In the present volume, *Biomarkers in Liver Disease*, we have sections on:

1. ***General Aspects and Introductory Material***
2. ***Body Fluids, Tissue, and Specific Biomarkers***
3. ***Genetic, Histological, Physical, and Imaging Methods***
4. ***Specific Diseases and Conditions***
5. ***Resources***

The editors recognize the difficulties in assigning particular chapters to particular sections, as some chapters can fit into more than one section. Nevertheless, the book has enormously wide coverage. Platforms and techniques include, for example, immunological, biochemical, histochemical methods, bioelectrical impedance analysis, and others. Conditions and biomedical areas encompass: adiposity, alcohol misuse, ascites, cirrhosis, diabetes, end-stage liver disease, extracellular matrix remodeling, hepatic fibrosis, hepatitis C virus infection, hepatocellular carcinoma, liver diseases in general, liver transplantation, nonalcoholic fatty liver disease, nutritional interventions, paracetamol-induced acute liver failure, portal hypertension, and tumor staging. Analytes and measures include alanine aminotransferase, alpha-fetoprotein, antioxidant response, AST-to-platelet ratio index, bilirubin, body fluids, CD133, CD163, cell-free DNA, cortisol, cytokines, EpCAM, extracellular vesicles, fibrinogen, hydroxyproline, inflammatory biomarkers, microRNAs, monocyte chemotactic protein-1, osteopontin, phosphatidylethanol, polymorphisms, PTX3, SCCA-IgM, scoring systems, sialic acids, sialidases, sialyltransferases, traditional markers, tumor staging, type VI collagen, VCAM-1, and YKL-40. There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts. This book is designed for clinical biochemists, hepatologists, gastroenterologists with sub-area interests, specialists working within the field of organ disease and treatments, health

scientists, epidemiologists, doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

The Editors

Series Preface

In the past decade, there has been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics. This has lead to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays, and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text that addresses these issues. Hence, the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Potential Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to

other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The series is expected to prove useful for clinicians, scientists, epidemiologists, doctors and nurses, and also academicians and students at an advanced level.

The Editors

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About the Editors

Vinood B. Patel
Department of Biomedical Science
Faculty of Science and Technology
University of Westminster
London, UK

Dr. Vinood B. Patel is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in tissue pathology particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in tissue pathology. Other areas of interest include identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimers disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy is a senior member of King's College London. He is also Director of the Genomics Centre and a member of the Faculty of Life Sciences and Medicine.

Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree for his outstanding contribution to protein metabolism in health and disease. Professor Preedy was elected as a Fellow to the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy has carried out research at the National Heart Hospital (part of Imperial College London), The School of Pharmacy (now part of University College London), and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on the science of health and has a long standing interest in biomarkers for over 30 years especially related to tissue pathology. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 600 articles, which include peer-reviewed manuscripts based on original research, abstracts and symposium presentations, reviews, and numerous books and volumes.

Editorial Advisor

Rajkumar Rajendram
Consultant in Internal and Perioperative Medicine
King Abdulaziz Medical City
Ministry of National Guard Health Affairs
Riyadh, Saudi Arabia

Contributors

Leon A. Adams School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia

Nancy E. Aguilar-Olivos Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico

Jawad Ahmad Division of Liver Diseases and Recanati-Miller Transplantation Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Krasimira Aleksandrova Nutrition, Immunity and Metabolism Start-up Lab, Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Ahmad H. Alghadir Department of Rehabilitation Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Riyadh, Saudi Arabia

A. Ata Alturfan Department of Sciences, Institute of Forensic Sciences, Istanbul University, Istanbul, Turkey

Agnieszka Bakula Department of Gastroenterology, Hepatology, Nutrition Disorders and Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland

Pedro Baptista Digestive Pathology Group, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Arpita Basu Department of Nutritional Sciences, 301 Human Sciences, Oklahoma State University, Stillwater, OK, USA

Paramita Basu Department of Biology, Texas Woman's University, Denton, TX, USA

Julia Beck Chronix Biomedical GmbH, Göttingen, Germany

A. Biasiolo Department of Medicine, University of Padua, Padua, Italy

Anna Blum Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Laura Boemeke Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

Radan Bruha General University Hospital, Charles University in Prague, Prague 2, Czech Republic

Anthony W. H. Chan Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Stephen L. Chan State Key Laboratory of Oncology in South China, Department of Clinical Oncology, Chinese University of Hong Kong, Shatin, NT, Hong Kong

Eun-Ju Chang Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Songpa-gu, Seoul, Republic of Korea

Bongkun Choi Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Songpa-gu, Seoul, Republic of Korea

Lech Chrostek Department of Biochemical Diagnostics, Medical University of Bialystok, Bialystok, Poland

Maxey Ching Ming Chung Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Miriam Cortes Liver Transplant Unit, Institute of Liver Studies, King's College Hospital, London, UK

Michael H. Court Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA, USA

Karina Ana da Silva Department of Physiological Sciences, Federal University of São Carlos (UFSCAR), São Carlos, São Paulo, Brazil

Maciej Dadalski Department of Gastroenterology, Hepatology, Nutrition Disorders and Paediatrics, The Children's Memorial Health Institute, Warsaw, Poland

Cynthia Aparecida de Castro Department of Physiological Sciences, Federal University of São Carlos (UFSCAR), São Carlos, São Paulo, Brazil

Jeroen de Jonge Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Letícia De Nadai Marcon Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Luis Fernando de Sousa Moraes Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Luis del Pozo Yauner Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Giuseppe Derosa Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for the Study of Endocrine-Metabolic Pathophysiology and Clinical Research, University of Pavia, Pavia, Italy

Laboratory of Molecular Medicine, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

Deepika Devuni Division of Liver Diseases and Recanati-Miller Transplantation Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Sandra Aparecida dos Reis Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Saad Elias Department of Internal Medicine A, Galilee Medical Center, Nahariya, Israel

Bar Ilan University, Safed, Israel

Walid Elmoghazy Hepatobiliary and Liver Transplant Surgery, Hamad Medical Corporation (HMC), Doha, Qatar

Ebru Emekli-Alturfan Department of Biochemistry, Faculty of Dentistry, Marmara University, Istanbul, Turkey

Guido Engelmann Department of Pediatrics, Lukas Hospital, Neuss, Germany

G. Fassina Xeptagen SpA, VEGA Park, Venice, Italy

Giuseppe Fede Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Sabrina Alves Fernandes Post Graduate Program in Bioscience and Rehabilitation; Post Graduate Program in Rehabilitation and Inclusion, IPA Methodist University, Porto Alegre, RS, Brazil

Sven Francque Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Wilrijk, Belgium

Sami A. Gabr Department of Rehabilitation Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Riyadh, Saudi Arabia

Department of Anatomy, Faculty of Medicine, Mansoura University, Mansoura, Egypt

A. Gallotta Xeptagen SpA, VEGA Park, Venice, Italy

Juan Carlos García-Cañaveras Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Ayman A. Ghfar Department of Chemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

Maria do Carmo Gouveia Peluzio Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Henning Grønbæk Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Ewa Gruszewska Department of Biochemical Diagnostics, Medical University of Bialystok, Bialystok, Poland

Alicja E. Grzegorzewska Department of Nephrology, Transplantology and Internal Diseases, Poznan University of Medical Sciences, Poznań, Poland

Yi Huang School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia

Hartmut Jaeschke Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Gary P. Jeffrey School of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia

Wladimiro Jimenez Biochemistry and Molecular Genetics Service, Biochemistry and Molecular Genetics Department, Hospital Clinic of Barcelona, Barcelona, Spain

Department of Biomedicine, University of Barcelona, Barcelona, Spain

Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Barcelona, Spain

Ewelina Kałużna Department of Molecular Pathology, Institute of Human Genetics, Polish Academy of Sciences, Poznań, Poland

Philipp Kanzow Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Department of Preventive Dentistry, Periodontology and Cariology, University Medical Center Göttingen, Göttingen, Germany

Morten A. Karsdal Nordic Bioscience, Biomarkers and Research, Herlev, Denmark

Samy Kashkoush Department of Hepatobiliary Surgery and Liver Transplantation, National Liver Institute, Minufiya University, Minufiya, Egypt

Organ Transplant Center, King Abdullah Specialist Children Hospital (KASCH) National Guard, Riyadh, Saudi Arabia

Konstantin Kazankov Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Norihiro Kokudo Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Olena Kolesnikova Department of Hepatology, Government Institution L.T. Malaya Therapy, National Institute of NAMS of Ukraine, Kharkiv, Ukraine

Otto Kollmar Helios Dr. Horst Schmidt Kliniken Wiesbaden, Wiesbaden, Germany

Aleksander Krag Department of Gastroenterology and Hepatology, University of Southern Denmark, Odense University Hospital, Odense C, Denmark

Agustín Lahoz Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Seow Chong Lee Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Diana J. Leeming Nordic Bioscience, Biomarkers and Research, Herlev, Denmark

Lisiane Lopes da Conceição Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Alberto Lue Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Philipp Lutz Department of Internal Medicine I, University of Bonn, Bonn, Germany

Timothy J. Lyons Centre for Experimental Medicine, Queen's University of Belfast, Northern Ireland, UK

Manoela Maciel dos Santos Dias Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Pamela Maffioli Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

PhD School in Experimental Medicine, University of Pavia, Pavia, Italy

Ki M. Mak Department of Medical Education/Center for Anatomy and Functional Morphology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Santiago Marfà Biochemistry and Molecular Genetics Service, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Cláudio Augusto Marroni Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

Elena Martínez-Crespo Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

A. Martini Department of Medicine, University of Padua, Padua, Italy

Barhoum Masad Bar Ilan University, Safed, Israel

Galilee Medical Center, Nahariya, Israel

Mitchell R. McGill Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA

Giovanni Meli Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Nahum Méndez-Sánchez Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico

Herold J. Metselaar Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Daniella Miranda Post Graduate Program in Medicine: Hepatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil

Søren Kragh Moestrup Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark

Holger Jon Møller Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Adrianna Mostowska Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Poznań, Poland

Salvatore Musumeci Department of Chemical Sciences, University of Catania and Institute of Biomolecular Chemistry, CNR, Catania, Italy

Valeriya Nemtsova Clinical Pharmacology Department, Kharkov National Medical University, Kharkiv, Ukraine

Marlene Christina Nielsen Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Mette J. Nielsen Nordic Bioscience, Biomarkers and Research, Herlev, Denmark

Assy Nimer Department of Internal Medicine A, Galilee Medical Center, Nahariya, Israel

Bar Ilan University, Safed, Israel

Hans Dieter Nischalke Department of Internal Medicine I, University of Bonn, Bonn, Germany

Michael Oellerich Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

Eugenia Pareja Unidad de Hepatología Experimental, Unidad Analítica, Instituto de Investigación Sanitaria, Fundación Hospital La Fe, Valencia, Spain

Unidad de Cirugía Hepato-Bilio-Pancreatica y Trasplante Hepático, Hospital Universitario y Politécnico La Fe de Valencia, Valencia, Spain

Vinood B. Patel Department of Biomedical Science, Faculty of Science and Technology, University of Westminster, London, UK

Julio Isael Pérez-Carreón Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Chien Yi M. Png Department of Medical Education, Icahn School of Medicine at Mount Sinai, New York, NY, USA

P. Pontisso Department of Medicine, University of Padua, Padua, Italy

Victor R. Preedy Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, King's College London, London, UK

Graziella Privitera Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Francesco Purrello Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Rajkumar Rajendram Diabetes and Nutritional Sciences Research Division, Faculty of Life Science and Medicine, School of Biomedical and Health Sciences, King's College London, London, UK

Department of Internal Medicine, King Abdulaziz Medical City, National Guard Hospital Affairs, Riyadh, Saudi Arabia

Sidsel Rødgaard-Hansen Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Ricardo Sánchez-Rodríguez Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Thomas D. Sandahl Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Sherif Saleh Department of Hepatobiliary Surgery and Liver Transplantation, National Liver Institute, Minufiya University, Minufiya, Egypt

Sabrina Schlesinger Institut für Epidemiologie, Christian-Albrechts-Universität zu Kiel, Kiel, Germany

Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, London, UK

Ekkehard Schütz Chronix Biomedical GmbH, Göttingen, Germany

M. Trinidad Serrano Liver Transplantation Unit, Gastroenterology and Hepatology Department, University Hospital Lozano Blesa, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Yousery E. Sherif Department of Chemistry, Faculty of Science and Arts, Ulla, Taibah University, Medina, Saudi Arabia

Clinical Pharmacology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

Qiang Shi Division of Systems Biology, National Center for Toxicological Research, The United States Food and Drug Administration, Jefferson, AR, USA

Estela Solanas Digestive Pathology Group, Aragon Institute for Health Research (IIS Aragon), Zaragoza, Spain

Peipei Song Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Luisa Spadaro Department of Clinical and Experimental Medicine, University of Catania- Garibaldi Hospital, Catania, Italy

Ulrich Spengler Department of Internal Medicine I, University of Bonn, Bonn, Germany

Marta Stelmach-Mardas Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poznan, Poland

Scott H. Stewart Department of Medicine, University at Buffalo, State University of New York, Buffalo, NY, USA

Hwee Tong Tan Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Wei Tang Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Ka-Fai To Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Julia Esperanza Torres-Mena Laboratorio de Bioquímica y Estructura de Proteínas, Instituto Nacional de Medicina Genómica, Mexico City, Mexico

Misael Uribe Liver Research Unit, Medica Sur Clinic & Foundation, Mexico City, Mexico

Luc J. W. van der Laan Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Cornelia J. Verhoeven Department of Surgery, Erasmus MC, University Medical Center Rotterdam, CA, Rotterdam, The Netherlands

Wim Verlinden Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Wilrijk, Belgium

Hendrik Vilstrup Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark

Libor Vítek 4th Department of Internal Medicine, and Institute of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Luisa Vonghia Department of Gastroenterology and Hepatology, Antwerp University Hospital, Edegem, Belgium

Philip D. Walson Department of Clinical Pharmacology, University Medical Center Göttingen, Göttingen, Germany

James L. Weemhoff Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Benjamin L. Woolbright Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Simon C. H. Yu Department of Imaging and Interventional Radiology, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Cihan Yurdaydin Department of Gastroenterology, University of Ankara Medical School, Ankara, Turkey

Part I

General Aspects and Introductory Material

Traditional Markers in Liver Disease

1

Giuseppe Derosa and Pamela Maffioli

Contents

G. Derosa (✉)
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for the Study of Endocrine-Metabolic Pathophysiology and Clinical Research, University of Pavia, Pavia, Italy

Laboratory of Molecular Medicine, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
e-mail: giuseppe.derosa@unipv.it

P. Maffioli
Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy

Center for Prevention, Surveillance, Diagnosis and Treatment of Rare Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

PhD School in Experimental Medicine, University of Pavia, Pavia, Italy
e-mail: pamelamaffioli@hotmail.it

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Abstract

Liver diseases are many and can often be difficult to diagnose, because symptoms can be vague and easily confused with other health problems. However, physicians can be helped by specific markers used to diagnose and follow up liver diseases. In fact some of the enzymes and the end products of the metabolic pathway occurring in the liver are very sensitive for the abnormality occurred and, for this reason, may be considered as biochemical markers of liver dysfunction. In this chapter, we will examine the main markers of liver diseases, dividing them as markers of hepatic necrosis, markers of hepatic obstruction, markers of liver's biosynthetic capacity, markers of hepatic steatosis, markers of hepatic fibrosis, and markers of hepatic tumor.

Keywords

Biosynthesis • Fibrosis • Liver diseases • Markers • Necrosis • Steatosis

List of Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactate dehydrogenase
γ-GT	Gamma glutamyl transferase
PT	Prothrombin time
INR	International normalized ratio
PICP	Procollagen type I carboxy-terminal peptide
PIIINP	Procollagen type III amino-terminal peptide
TGF-β1	Transforming growth factor-β1
AFP	α-Fetoprotein
NTP	5′ Nucleotidase
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis

Key Facts of Liver Diseases

- Liver diseases can be inherited or acquired, caused by a variety of factors including viral infections; illegal drug use; legal drug overuse, in particular paracetamol or acetaminophen; and alcohol abuse. Obesity is also associated with liver damage.
- There are over a hundred different forms of liver disease that affect both sexes at different ages.
- Liver disease can often be difficult to diagnose, because symptoms can be vague and easily confused with other health problems. All these conditions have specific markers used to diagnose and follow up them.
- The knowledge of biomarkers linked to liver disease is very important to promptly diagnose liver abnormalities and to guide physicians in the right direction to identify the causes.

Introduction

The liver is a vital organ, involved in several metabolic processes such as metabolism of fats, sugars, proteins, and vitamins and in the regulation of blood clotting. The liver plays the main role in the body's defenses, filtering toxins and microbes from the blood and regulating processes activated in responses to trauma, stress, or inflammation. The liver is also able to regenerate and repair itself. Given that the liver is such a complex organ, performing over 500 functions, it is not surprising that its function can be damaged in several ways. Liver diseases can be inherited or acquired, caused by a variety of factors including viral infections; illegal drug use; legal drug overuse, in particular paracetamol or acetaminophen; and alcohol abuse. Obesity is also associated with liver damage. Over time, damage to the liver results in cirrhosis, which can lead to liver failure, a life-threatening condition. There are over a hundred different forms of liver disease that affect both sexes at different ages. Liver diseases include: Alagille syndrome, alpha-1 antitrypsin deficiency, autoimmune hepatitis, biliary atresia, cirrhosis, cystic disease, fatty liver disease, galactosemia, gallstones, Gilbert's syndrome, hemochromatosis, liver cancer, lysosomal acid lipase deficiency, neonatal hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, porphyria, Reye's syndrome, sarcoidosis, toxic hepatitis, type 1 glycogen storage disease, tyrosinemia, Wilson's disease, and viral hepatitis A, B, and C.

Liver disease can often be difficult to diagnose, because symptoms can be vague and easily confused with other health problems. All these conditions have specific markers used to diagnose and follow up them. Some of the enzymes and the end products of the metabolic pathway occurring in the liver are very sensitive for the abnormality occurred and, for this reason, may be considered as biochemical markers of liver dysfunction. In particular, we chose to divide markers of liver disease in different groups, including:

- **Markers of hepatic necrosis**: aminotransferases including aspartate aminotransferase (AST), alanine amino transferase (ALT), and lactate dehydrogenase (LDH)
- **Markers of hepatic obstruction**: conjugated and unconjugated bilirubin, alkaline phosphatase, gamma glutamyl transferase (γ-GT)
- **Markers of liver's biosynthetic capacity**: albumin, ceruloplasmin, α-1 antitrypsin, prothrombin time (PT) and INR, pseudocholinesterase
- **Markers of hepatic steatosis**: alanine amino transferase (ALT), ferritin, ultrasound score of steatosis
- **Markers of hepatic fibrosis**: procollagen type I carboxy-terminal peptide (PICP) and procollagen type III amino-terminal peptide (PIIINP), AST/ALT ratio, constituents of extracellular matrix (hyaluronic acid, type IV collagen 7S domain, TGF-β1, metalloproteinase), FibroScan scoring
- **Markers of hepatic tumor**: α-fetoprotein (AFP), 5′ nucleotidase (NTP)

In this regard, the aim of this chapter will be to examine traditional markers in liver disease in order to give readers a guide about diagnosis and follow-up of this kind of disease.

Markers of Hepatic Necrosis

Aminotransferases

The aminotransferases are the most frequently utilized and specific indicators of hepatocellular necrosis, because they are released into the bloodstream from damaged hepatocytes. They belong to a group of enzymes that catalyze the interconversion of amino acids and oxoacids by transfer of amino groups. Aminotransferases include aspartate aminotransferase (AST), also known as serum glutamic oxaloacetic transaminase (SGOT), and alanine aminotransferase (ALT), also known as serum glutamic pyruvic transaminase (SGPT). Alanine aminotransferase is primarily localized into the liver, while AST is present in a wide variety of tissues. Whereas the AST is present in both the mitochondria and cytosol of hepatocytes, ALT is localized to the cytosol (Sherlock 1997). The cytosolic and mitochondrial forms of AST are true isoenzymes and immunologically distinct. About 80% of AST activity in human liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme. Large increases in mitochondrial AST occur in serum after extensive tissue necrosis. Their activity in serum at any moment reflects the relative rate at which they enter and leave circulation. Of the numerous methods used for measuring their levels, the most specific method couples the formation of pyruvate and oxaloacetate – the products of the aminotransferase reactions to their enzymatic reduction to lactate and malate (Nalpas et al. 1986; Rej 1985). The primary clinical application of serum AST and ALT measurement is the detection and differential etiologic diagnosis of hepatic disease. Comparable elevations of both AST and ALT are typical of acute viral, toxic, or non ethanol drug-induced hepatitis. The similar

serum transaminase levels are due to cellular release of only cytoplasmic enzymes associated with reversible hepatic cell damage. In chronic hepatitis and cirrhosis, serum AST levels are higher than ALT, due to hepatic cell necrosis with release of mitochondrial AST. In alcohol hepatitis, AST is more significantly increased than ALT, while in hepatic steatosis, ALT is higher than AST. In this regard, ALT has been used as a surrogate marker for liver fat accumulation, as previously reported (Nanji et al. 1986). Previously reported biochemical studies suggested the existence of two isoforms of ALT in humans – a first isoform called ALT1 located on human chromosome 8q24.3 (Sohocki et al. 1997) and a second isoform, called ALT2, mapped to the human chromosome 16q12.1, which was mainly expressed in muscle and adipose tissues (Yang et al. 2002). An elevated ALT is considered a consequence of hepatocyte damage due to NAFLD. However, the measured plasma elevations of ALT may also be a consequence of high systemic ALT2 isoform levels that are largely derived from adipose tissue, as in obesity and insulin resistance in mice (Jadhao et al. 2004). These are due to insulin resistance, increased pro-inflammatory cytokine production, oxidative stress, and mitochondrial dysfunction leading to hepatocyte damage/destruction (Day 2002).

Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of lactate to pyruvate, an important step in energy production in cells. Many different types of cells contain LDH, including heart, kidney, liver, and muscle. Lactate dehydrogenase is a cytosolic enzyme which level increases in hepatocellular damage. However, estimation on total LDH activity is of no use as a part of liver function tests, because of the influence of extrahepatic diseases on the total LDH activity. LDH, in fact, is increased in several kinds of cancer, including testicular cancer, Ewing's sarcoma, non-Hodgkin's lymphoma, and some types of leukemia. Moreover, elevated LDH levels can be found in several noncancerous conditions, including heart failure, hypothyroidism, anemia, and lung or liver disease. There are five isoforms of LDH; the LDH_5 predominates in the liver; however, for the reasons reported above, this one is not routinely used in favor of more reliable markers of liver damage (Thapa and Anuj 2007).

Markers of Hepatic Obstruction

Bilirubin

Bilirubin is the catabolic product of hemoglobin produced within the reticuloendothelial system; red cell hemoglobin accounts for approximately 85% of all bilirubin. Bilirubin is released in unconjugated form (indirect bilirubin) which is bound to albumin in the plasma and transported to the liver. Bilirubin is conjugated with glucuronic acid in the hepatocytes; the conjugation is catalyzed by glucuronyl

transferase. Conjugated bilirubin (direct bilirubin) is secreted into the bile and enters the duodenum. In the small bowel, some of the bilirubin is hydrolyzed to yield unconjugated bilirubin and glucuronic acid, with the production of urobilinogen. Most urobilinogen is excreted in the stool, but some is reabsorbed and returned to the liver via the portal vein, enters circulation, and is excreted by the kidney (Nicoll 2007). Normal serum total bilirubin ranges from 2 to 21 μmol/L, unconjugated bilirubin level is less than 12 μmol/L, and conjugated bilirubin is less than 8 μmol/L. Serum bilirubin levels higher than 17 μmol/L suggest liver diseases, and levels higher than 24 μmol/L indicate abnormal laboratory liver tests (Thapa and Anuj 2007; Wong et al. 2004).

Jaundice occurs for bilirubin concentration of 40 μmol/L; at this level, bilirubin becomes visible within the sclera, skin, and mucous membranes (Beckingham and Ryder 2001). Unconjugated bilirubin is insoluble and is not excreted in the urine, but it is liposoluble and can accumulate in the brain and nerve tissues, causing damages; conjugated bilirubin, instead, is soluble and is excreted in the urine. Hyperbilirubinemia can be due to overproduction/impaired uptake, conjugation, or excretion/regurgitation of unconjugated or conjugated bilirubin from hepatocytes to bile ducts. On this basis, jaundice can be classified as prehepatic, hepatic, or posthepatic, and the prevalence of unconjugated or conjugated bilirubin can help to better define jaundice origin (Beckingham and Ryder 2001).

In particular, in prehepatic jaundice there is an excess of unconjugated hyperbilirubinemia. Prehepatic jaundice occurs in situations where unconjugated bilirubin is produced faster than the liver is able to conjugate it for excretion, as in increased hemolysis during spherocytosis, homozygous sickle cell disease, thalassemia major, and reabsorption of large hematomas.

In hepatic jaundice, usually both unconjugated and conjugated bilirubin rise, because necrosis of hepatocytes frees the already conjugated bilirubin and makes them unable to conjugate indirect bilirubin. The most common causes of hepatic jaundice are viral hepatitis, alcoholic cirrhosis, primary biliary cirrhosis, drug-induced jaundice, and alcoholic hepatitis. Also genetic defects can be responsible for hepatic jaundice (Tiribelli and Ostrow 1996). In particular in Gilbert's syndrome and Crigler-Najjar syndrome, there is a defect in the gene that encodes for glucuronyl transferase, which results in a reduction in the liver's ability to conjugate bilirubin; in this condition unconjugated bilirubin is high. In Dubin-Johnson syndrome, instead, there is a defect of ABCC2 gene, involved in the production of a protein called multidrug resistance protein 2 (MRP2) responsible to transport substances out of the liver. In this case, conjugated bilirubin is high.

Finally, posthepatic jaundice is characterized by an excess of conjugated hyperbilirubinemia. It is most often due to biliary obstruction by a stone in the common bile duct or by carcinoma of the pancreas. Pancreatic pseudocyst, chronic pancreatitis, sclerosing cholangitis, a bile duct stricture, or parasites in the bile duct are less common causes (Beckingham and Ryder 2001) (Table 2).

Alkaline Phosphatase

Alkaline phosphatase (ALP) is present in epithelial mucosa of the small intestine, proximal convoluted tubules of the kidney, bone, liver, and placenta. It is involved in lipid transportation in the intestine and calcification in the bones. The serum ALP activity is mainly from the liver, with 50% contributed by the bone (Mauro et al. 2006). Normal serum ALP ranges between 41 and 133 U/L (Nicoll 2007). In acute viral hepatitis, ALP usually remains normal or moderately increased. In the liver, epithelial cells lining the bile canaliculi produce alkaline phosphatase, and its serum activity is raised in patients with intrahepatic cholestasis, cholangitis, or extrahepatic obstruction; increased activity may also occur in patients with focal hepatic lesions in the absence of jaundice. Hepatic and bony metastasis can also be responsible for elevated levels of ALP. Other causes of elevated ALP include infiltrative liver diseases, abscesses, granulomatous liver disease, and amyloidosis. Finally, mildly elevated levels of ALP may be seen in cirrhosis, hepatitis, and congestive cardiac failure (Rosalki and Mcintyre 1999). Alkaline phosphatase is elevated in peripheral arterial disease, independent of other traditional cardiovascular risk factors (Cheung et al. 2009). On the other hand, low levels of ALP occur in hypothyroidism, pernicious anemia, zinc deficiency, and congenital hypophosphatasia (Simko 1991). In the presence of elevated ALP levels, clinicians need to differentiate among liver and bone disorders; in this case, levels of gamma glutamyl transferase (γ-GT) can be helpful, because they will be high in cholestatic disorders and normal in bone diseases (Mauro et al. 2006).

Gamma Glutamyl Transferase

Gamma glutamyl transferase is a microsomal enzyme present in hepatocytes and biliary epithelial cells, renal tubules, pancreas, and intestine. Serum γ-GT activity is mainly linked to hepatobiliary system, even though it is found in more concentration in the renal tissue (Mauro et al. 2006). The normal level of γ-GT is between 9 and 85 U/L (Nicoll 2007). Elevated levels of γ-GT can be found during acute viral hepatitis, the peak occurs in the second or third week of illness, and in some patients remain elevated for 6 weeks (Rosalki and Mcintyre 1999). Also in 30% of patients with chronic hepatitis C infection, γ-GT are elevated (Giannini et al. 2001). Also uncomplicated diabetes mellitus, acute pancreatitis, myocardial infarction, anorexia, Guillain-Barré syndrome, hyperthyroidism, obesity, and myotonic dystrophy can be responsible for elevated levels of γ-GT (Rosalki and Mcintyre 1999). In alcohol abuse, serum γ-GT levels can increase to more than ten times. This is partly due to structural liver damage, hepatic microsomal enzyme induction, or alcoholic pancreatic damage (Wu et al. 1976). Gamma glutamyl transferase can also be an early marker of oxidative stress, since serum antioxidant carotenoids including lycopene, α-carotene, β-carotene, and β-cryptoxanthin are

inversely associated with alcohol-induced increase of serum γ-GT as reported in moderate and heavy drinkers (Sugiura et al. 2005). Another condition responsible for γ-GT levels two to three times higher than the upper reference value in more than 50% of the patients is nonalcoholic fatty liver disease (McCullough 2002). Previously published papers showed a significant positive correlation between serum γ-GT and triglyceride levels in diabetic patients. As reported above, the main use of γ-GT is to help discriminate hepatic diseases from bone diseases when ALP is elevated.

Markers of Liver's Biosynthetic Capacity

Albumin

The liver is responsible for the production of several serum proteins. In particular, parenchymal cells are responsible for synthesis of albumin, fibrinogen, and other coagulation factors and most of the alpha and beta globulins. Albumin is quantitatively the most important plasma protein synthesized by the liver and, for this reason, can be a useful indicator of hepatic function. Albumin half-life in serum is as long as 20 days, and it takes at least 10 days for the concentration to fall below the normal range despite impaired liver function; for this reason, serum albumin level is not a reliable indicator of hepatic protein synthesis in acute liver disease. Even if the liver is the only site of synthesis of albumin, albumin synthesis can be affected also by nutritional status, hormonal balance, and osmotic pressure. Serum albumin levels are typically depressed in patients with cirrhosis and ascites. In patients with or without ascites, the serum albumin level correlates with prognosis, and are included in the Child-Pugh score, a scoring system used to quantify the severity of chronic liver disease inclusive of cirrhosis. The score is composed of five categories, including total bilirubin, serum albumin, INR, the presence of ascites, and the presence of hepatic encephalopathy. The higher the score, the worse the severity of cirrhosis (Table 1).

Normally serum albumin levels range between 3.5 and 4.5 g/dL. The average adult has approximately 300–500 g of albumin. The serum levels at any time reflect its rate of synthesis, degradation, and volume of distribution (Nicoll 2007).

Ceruloplasmin

Ceruloplasmin is synthesized in the liver and is an acute phase protein. It binds with copper and serves as a major carrier for copper in the blood. Normal plasma level of ceruloplasmin is 200–600 mg/L (Nicoll 2007). High levels of ceruloplasmin have been found in infections, rheumatoid arthritis, pregnancy, non-Wilson liver disease, and obstructive jaundice. Low levels, instead, have been reported in neonates, Menkes disease, kwashiorkor, marasmus, protein losing enteropathy,

Table 1 Child-Pugh score

Parameter	Value	Score
Total bilirubin	<34 μmol/L	1 point
	34–50 μmol/L	2 points
	>50 μmol/L	3 points
Serum albumin	>3.5 g/dL	1 point
	2.8–3.5 g/dL	2 points
	<2.8 g/dL	3 points
INR	<1.7	1 point
	1.7–2.3	2 points
	>2.3	3 points
Ascites	None	1 point
	Mild	2 points
	Moderate	3 points
Hepatic encephalopathy	None	1 point
	Grades I–II	2 points
	Grades III–IV	3 points
Child-Pugh total score	Class A	5–6 points
	Class B	7–9 points
	Class C	10–15 points

Table 2 Differential diagnosis of jaundice

	Urine		Blood			
	Urobilinogen	Bilirubin	Urobilinogen	Bilirubin	ALT and AST	γ−GT and ALP
Normality	Trace	Absent	Normal	Normal	Normal	Normal
Prehepatic jaundice	Increased	Absent	Increased	Normal	Normal	Normal
Posthepatic jaundice	Decreased or absent	Present	Normal	Increased	Normal or mild increase	Marked increase
Intrahepatic jaundice	Decreased or absent	Present	Increased	Increased	Marked increased	Normal or mild increase

copper deficiency, and aceruloplasminemia (Mauro et al. 2006). In Wilson's disease, a rare inherited disorder that causes too much copper to accumulate in your liver, brain, and other vital organs, ceruloplasmin level is depressed. Decreased rate of synthesis of the ceruloplasmin is responsible for copper accumulation in the liver, because of copper transport defect in Golgi apparatus, since ATP7B is affected (Rosalki and Mcintyre 1999). Serum ceruloplasmin levels were high in the chronic active liver disease (CALD), but low in the Wilson's disease; for this reason, ceruloplasmin levels are the most reliable screening test to differentiate between chronic active liver disease and Wilson's disease (LaRusso et al. 1976).

α-1 Antitrypsin

Alpha-1 antitrypsin (α-1 antitrypsin) is a glycoprotein synthesized by the liver and is an inhibitor of serine proteinases, especially elastase. After being synthesized in the liver, α-1 antitrypsin is normally released in the bloodstream and reaches the lungs. If there are some mutations in the coding sequence of α-1 antitrypsin, its export from the hepatocyte is blocked and it gets stuck in the liver cells. Alpha-1 antitrypsin has many important roles in the lung, including removing bacteria and fighting infections; it is needed to control enzyme activity to prevent healthy lung tissue from being damaged. So a deficiency in the concentration of circulating α-1 antitrypsin predisposes to early onset panlobular emphysema, even in nonsmokers. Moreover, the abnormal accumulation of the glycoprotein in hepatocytes results in programmed cell death, hepatic inflammation, fibrosis, and cirrhosis (Fairbanks, and Tavill 2008). Its normal concentration is 1–1.6 g/L.

Prothrombin Time and INR

Clotting is the final step of a complex series of enzymatic reactions that involve at least 13 factors. The liver is the major site of synthesis of several coagulation factors: I (fibrinogen), II (prothrombin), IV, V, VI, VII, IX, X, and XI. Most of these are present in excess, and abnormalities of coagulation happen when there is substantial impairment in the ability of the liver to synthesize them. This occurs in both biliary obstruction and parenchymal liver disease, because of a combination of poor absorption of fat soluble vitamin K, due to the absence of bile in the gut, and a reduced ability of damaged hepatocytes to produce clotting factors. Abnormal clotting can be measured using prothrombin time of Quick (PT), which evaluates the extrinsic coagulation pathway. It can be expressed in seconds or as a ratio of the plasma prothrombin time to control plasma time (prolonged international normalized ratio or INR), which is also used, as already reported above, to calculate Child-Pugh score (Table 1). Normal value of PT is between 9 and 11 s, a prolongation of more than 2 s is considered abnormal. In acute and chronic hepatocellular disease, the PT may serve as a prognostic indicator (Friedman et al. 2003). However, a prolonged PT is not specific for liver diseases; it can be found in various deficiencies of coagulation factors, disseminated intravascular coagulation, and ingestion of certain drugs.

Pseudocholinesterase

Pseudocholinesterase is primarily synthesized in the liver. When liver function is impaired, pseudocholinesterase synthesis is also impaired. Serum cholinesterase activity can determine if there is a quantitative defect in enzyme function. Pseudocholinesterase deficiency impairs the metabolism of succinylcholine, mivacurium, or

ester local anesthetics. Normal range is between 3,200 and 6,600 IU/L. This number varies by different laboratory standards and is subject to much interindividual variability. Decreased serum activities have been shown in many liver diseases, such as cirrhosis, end-stage liver disease, hepatitis, and liver abscesses. In patients with end-stage liver disease, normal serum pseudocholinesterase levels were again seen after liver transplant, with the transplanted liver assuming the role of production immediately. Additionally, serum cholinesterase activity may drop 30–50% in acute hepatitis, with a 50% decrease in cirrhosis and chronic malignancies being perhaps among some of the most substantial decreases of the acquired conditions. However, as the half-life of serum cholinesterase is approximately 10–14 days, it is considered an unreliable source for tracking liver disease (Soliday et al. 2010).

Markers of Hepatic Steatosis

Liver steatosis or nonalcoholic fatty liver disease (NAFLD) is characterized by a wide spectrum of conditions. NAFLD covers a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis. Simple steatosis without fibrosis or inflammation has a benign clinical course in most cases without excess mortality. NASH, instead, may have a more progressive course that can lead to cirrhosis in 10–15% of patients, affecting survival (Ekstedt et al. 2006). It develops in subjects who are not heavy alcohol consumers and have negative tests for viral and autoimmune liver diseases (Angulo 2002; Matteoni et al. 1999; Brunt 2001). Recently, NAFLD has been linked to insulin resistance and type 2 diabetes mellitus, and metabolic syndrome (Cortez-Pinto et al. 1999; Marchesini et al. 2001).

Alanine Aminotransferase

We have already described ALT role as a marker of hepatic steatosis in the paragraph about index of hepatic necrosis.

Ferritin

Increased ferritin, but normal transferrin saturation, is frequently found in patients with hepatic steatosis. The simultaneous disorder of iron and glucose and/or lipid metabolism, in most of the cases associated with insulin resistance, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH (Fargion et al. 2001). Indeed, serum ferritin level was significantly higher in the NASH patients than those with simple steatosis, according to a Japanese study (Yoneda et al. 2010). In that study, the serum ferritin level was related with insulin resistance.

Ultrasound Score of Steatosis

Percutaneous liver biopsy is the current standard means of diagnosing and grading steatosis, but it is an invasive procedure with potentially serious complications including hemorrhage, infection, bile leak, and a mortality of up to 0.3% (Bravo et al. 2001). For this reason, noninvasive methods such as computed tomography (CT), magnetic resonance imaging (MRI), and sonography are more commonly applied in clinical practice and in population-based studies (Joy et al. 2003; Saadeh et al. 2002; Siegelman and Rosen 2001). The most diffuse method is ultrasonography, because it is cost-effective and widely available, even if it is limited by interobserver and intra-observer variability (Strauss et al. 2007).

For an approximative estimation of hepatic steatosis, hepatic parenchyma can be compared to kidney parenchyma during ultrasound examination (Fig 2): in normal conditions, the liver and renal cortex are of a similar echogenicity; in steatosis, instead, renal cortex appears hypoechoic compared to the liver parenchyma. The brighter the hepatic parenchyma compared to the kidney parenchyma, the higher the steatosis degree. A better grading of severity of hepatic steatosis is possible with an ultrasound score, according to this score:

- Level 0 was defined as a normal hepatic echo pattern.
- Level 1 was defined as a slight increase in echo pattern with normal visualization of vessels and diaphragm.
- Level 2 was defined as a moderate increase in echogenicity with reduced visibility of portal veins and diaphragm.
- Level 3 was defined as a pronounced increase in hepatic echo pattern with poor visibility of intrahepatic vessels and posterior right lobe of the liver.

This score derives from the evaluation of different aspects of the liver during ultrasound examination that considers liver echotexture, echo penetration and visibility of diaphragm, and clarity of liver blood vessel structures (Chan et al. 2004).

Markers of Hepatic Fibrosis

Procollagen Type I Carboxy-Terminal Peptide (PICP) and Procollagen Type III Amino-Terminal Peptide (PIIINP)

In the healthy human liver, the most abundant collagens are the fibril-forming types I and III. Collagen types I and III are synthesized as procollagens with a small amino-terminal and a larger carboxy-terminal propeptide. Once secreted into the extracellular space, the propeptides are removed by specific endopeptidases, thus allowing integration of the rigid collagen triple helix into the growing fibril. After this process type I carboxy-terminal peptide (PICP) is cleaved off procollagen type I during synthesis of the fibril-forming collagen type I, while the three-amino acid

procollagen type III amino-terminal peptide (PIIINP) is cleaved off procollagen type III forming collagen type III (Nimni 1993). Both are released in serum, and for this reason their serum concentrations have been proposed as a useful marker of collagen type I and III synthesis (Veidal et al. 2010). This is supported by a diversity of clinical observations demonstrating that high serum levels of these peptides reflect ongoing tissue fibrosis. During fibrogenesis, type I collagen levels increase up to eightfold. Additionally, the ratio of the type I/III also changes from 1:1 in the healthy liver to 1:2 in the cirrhotic liver (Sakugawa et al. 2005).

PICP levels are normal in patients with mild chronic hepatitis C and elevated in 50% of patients with moderately advanced or advanced chronic hepatitis C, including patients with liver cirrhosis of this etiology. PIIINP relative concentration in the basement membrane is higher in hepatic fibrogenesis (Lieber et al. 2008). In acute hepatitis, levels of serum PIIINP correlate with aminotransferase levels. In chronic liver disease, serum PIIINP reflects the stage of liver fibrosis (Giboney 2005). Unfortunately, PIIINP is not specific for the fibrosis of the liver as it is also elevated in acromegaly, lung fibrosis, chronic pancreatitis, and rheumatologic disease (Sakugawa et al. 2005).

AST/ALT Ratio

The predictive value of the AST/ALT ratio has been validated in nonalcoholic liver disease, chronic viral hepatitis, primary sclerosing cholangitis, and primary biliary cirrhosis (Lieber et al. 2008). In many forms of acute and chronic liver injury or steatosis (fatty infiltration of the liver), this ratio is less than or equal to 1, while in alcoholic hepatitis, an AST/ALT ratio is often greater than 2.

Constituents of Extracellular Matrix

The constituents of extracellular matrix are expected to be released into circulation during turnover of fibrosis in the liver. For this reason, they are reliable markers to differentiate NASH from simple steatosis, especially those with significant liver fibrosis. Marked elevation of serum hyaluronic acid and type IV collagen 7S domain, both extracellular matrix components, occurred in NASH patients with advanced fibrosis compared to those with mild fibrosis. Serum hyaluronic acid levels were also markedly elevated in patients with NASH than with steatosis only. Hyaluronic acid is a high molecular weight glycosaminoglycan, which is an essential component of extracellular matrix in virtually every tissue in the body. In the liver, it is synthesized by the hepatic stellate cells and degraded by the sinusoidal endothelial cells (Lindqvist, and Laurent 1992). The best cutoff value using ROC analysis was $\geq$43 ng/mL to detect NASH, and $\geq$50 ng/mL to detect severe fibrosis (Yoneda et al. 2007). Regarding type IV collagen 7S domain, it is involved in connective tissue metabolism, and has been identified as a biochemical marker for assessing fibrogenesis and the severity of fibrosis in patients with cirrhosis (Yoneda et al. 2007).

The best cutoff value using ROC analysis was ≥5 ng/mL to detect NASH and ≥5 ng/mL to detect severe fibrosis for type IV collagen 7S domain. The positive predictive value (PPV) for detecting NASH can be as high as 97.1% when both markers are greater than the cutoffs.

Another marker of hepatic fibrosis is transforming growth factor-β1 (TGF-β1), a cytokine involved in tissue growth, differentiation, extracellular matrix production, and immune response. This cytokine has three isoforms (β1, β2, and β3), but only TGF-β1 has been linked to liver fibrogenesis. A correlation between TGF-β1 levels and the rate of fibrosis progression has been reported (Kanzler et al. 2001).

Other constituents of extracellular matrix are metalloproteinases (MMPs); they belong to a family of structurally related proteolytic enzymes that mediate the degradation of the extracellular matrix and the basal membranes (Sun 2010). The three most commonly studied human metalloproteinases are MMP-2 (gelatinase-A), MMP-3 (stromelysin), and MMP-9 (gelatinase-B). MMP-2 is secreted by activated hematopoietic stem cells; elevated levels of MMP-2 and its proenzyme have been observed in various liver diseases (Takahara et al. 1997). During hepatic fibrogenesis, the expression of MMP-2 is markedly increased. The potential for MMP-2 for predicting liver fibrosis remains unclear as some contradictory data have been reported by previous studies (Walsh et al. 1999; Hayasaka et al. 1996). In contrast to MMP-2, MMP-9 levels show their value mainly in the diagnosis of hepatocellular carcinoma (Badra et al. 2010): MMP-9 levels were negatively correlated to the histological severity of the liver disease in patients with chronic hepatitis C. Metalloproteinases activity is strictly controlled by tissue inhibitors of matrix metalloproteinases (TIMPs); TIMPs are secreted proteins that interact with MMPs and modulate their activation and functioning. TIMP-1 controls activity of most MMPs, whereas TIMP-2 specifically inhibits MMP-2. TIMPs-dependent inhibition of extracellular matrix degradation may promote liver fibrosis; elevation of TIMPs' levels has been observed in chronic liver disease.

FibroScan Scoring Card

Hepatic fibrosis can be quantified throughout assessment of stiffness, using an ultrasound-based technology introduced in the latest years. This technique called transient ultrasound elastography or FibroScan measures the stiffness of the hepatic parenchyma using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves produced by a specialized ultrasound vibrator applied to the body wall and coupled with 1D ultrasound imaging that measures the propagation speed of a wave using a pulse-echo ultrasound. Since fibrotic tissue is harder than healthy liver tissue, the shear wave measurement provides immediate quantitative assessment of the degree of stiffness. FibroScan was reported to be a reliable method for the diagnosis of significant fibrosis (AUC = 0.84), severe fibrosis (AUC = 0.89), and cirrhosis (AUC = 0.94) accompanying various liver diseases including hepatitis B and C, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) (Ziol et al. 2005; Friedrich-Rust et al. 2008). However, previously

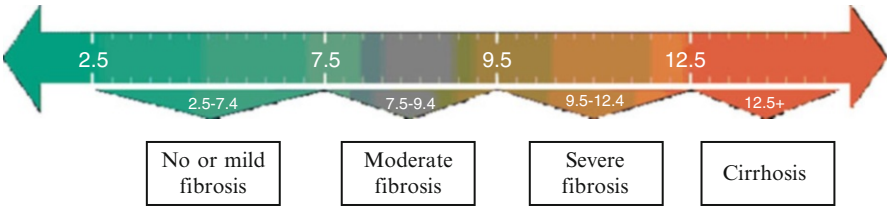

Fig. 1 FibroScan score and fibrosis degree

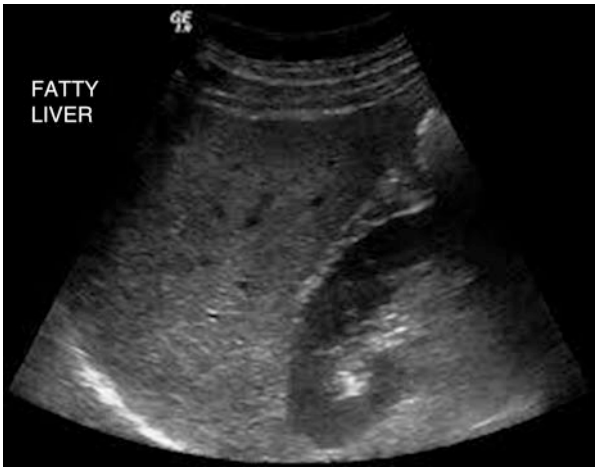

Fig. 2 Ultrasound image of hepatic steatosis

reported papers showed that FibroScan accuracy in assessing lower degrees of liver fibrosis is not as reliable compared to diagnosing advanced fibrosis and cirrhosis (Ziol et al. 2005).

Stiffness assessed by FibroScan is expressed in kPa, using a score between 2.5 and 75 kPa. Between 90 and 95% of healthy people without liver disease will have a liver scarring measurement less than 7.0 kPa; patients with chronic hepatitis C and a liver stiffness more than 14 kPa has approximately a 90% probability of having cirrhosis, while patients with liver stiffness more than 7 kPa have around an 85% probability of at least significant fibrosis (Fig. 1).

Markers of Hepatic Tumor

α-Fetoprotein

The α-fetoprotein (AFP) gene is highly activated in the fetal liver, but is significantly repressed shortly after birth. The normal level of AFP is 0–15 μg/L (Nicoll 2007). The finding of elevated AFP levels in response to liver injury and during the early

stages of chemical hepatocarcinogenesis led to the conclusion that maturation arrest of liver-determined tissue stem cells gives rise to hepatocellular carcinomas. An AFP value above 400–500 μg/L has been considered to be diagnostic for hepatocellular carcinoma (HCC) in patients with cirrhosis. A higher AFP concentration, ≥400 μg/L in HCC patients, is associated with greater tumor size, bilobar involvement, portal vein invasion, and a lower median survival rate (Gowda et al. 2009). It has also been reported that higher serum AFP levels independently predict a lower sustained virological response rate among patients with chronic hepatitis C. There are three different AFP variants, differing in their sugar chains (AFP-L1, AFP-L2, AFP-L3). AFP-L1 is the main glycoform of AFP in the serum of patients with nonmalignant chronic liver disease, while AFP-L3 is the main glycoform of AFP in the serum of HCC patients. α-Fetoprotein-L3 can be detected in one-third of patients with small HCC (<3 cm); it acts as a marker for clearance of HCC after treatment: an AFP-L3 level of 15% or more is correlated with HCC portal vein invasion (Hagiwara et al. 2006). Estimating the AFP-L3/AFP ratio can be helpful in diagnosis and prognosis of HCC (Asmaa et al. 2009).

5′ Nucleotidase

5′ Nucleotidase is a glycoprotein generally disseminated throughout the tissues of the body; it is localized in cytoplasmic membrane, and it catalyzes the release of inorganic phosphate from nucleoside-5′-phosphates. The normal range established is 0–15 U/L (Nicoll 2007). Raised levels of NTP activity were found in patients with obstructive jaundice, parenchymal liver disease, hepatic metastases, and bone disease (Daniel and Marshal 2007). Elevation of NTP can be found in acute infective hepatitis and in chronic hepatitis (Pratibha et al. 2004). However, NTP is almost a marker of early hepatic primary or secondary tumors. The increase of both ALP and NTP suggests intra- or extrahepatic obstruction due to malignancy (Smith et al. 1966).

Potential Applications to Prognosis, Other Diseases, or Conditions

Liver diseases have specific markers that can be used to diagnose and follow up them. Some of the enzymes and the end products of the metabolic pathway occurring in the liver are very sensitive for the abnormality occurred and, for this reason, may be considered as biochemical markers of liver dysfunction. The knowledge of biomarkers linked to liver disease is very important to promptly diagnose liver abnormalities and to guide physicians in the right direction to identify the causes. This can help to reduce the costs linked to liver diseases, their diagnosis and treatment.

Summary Points

- This chapter focuses on biomarkers relevant to liver diseases.
- Biomarkers include measurable indicators of some biological state and are useful to diagnose or follow up a specific condition or risk factor.
- Biomarkers relevant to liver diseases include markers of hepatic necrosis, hepatic obstruction, liver's biosynthetic capacity, hepatic steatosis, hepatic fibrosis, and hepatic tumor.
- The knowledge of biomarkers linked to liver disease is very important to promptly diagnose liver abnormalities and to guide physicians in the right direction to identify the causes.

Mini Dictionary

Biomarker: the term refers to a measurable indicator of some biological state or condition that can be used for diagnosis or follow-up of a particular disease.

Hepatic necrosis: the term refers to death of hepatic parenchyma which may involve single cell, or multicell in piecemeal, focal, periacinar, midzonal, periportal, or paracentral locations.

Biosynthetic capacity: the term refers to the production of a complex chemical compound from simpler precursors in a living organism.

Hepatic steatosis: the term refers to excessive amounts of triglycerides and other fats inside liver cells.

Hepatic fibrosis: the term refers to a reaction to chronic injury to the liver; it includes biliary fibrosis, postnecrotic scarring, diffuse hepatic fibrosis, and periacinar fibrosis.

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